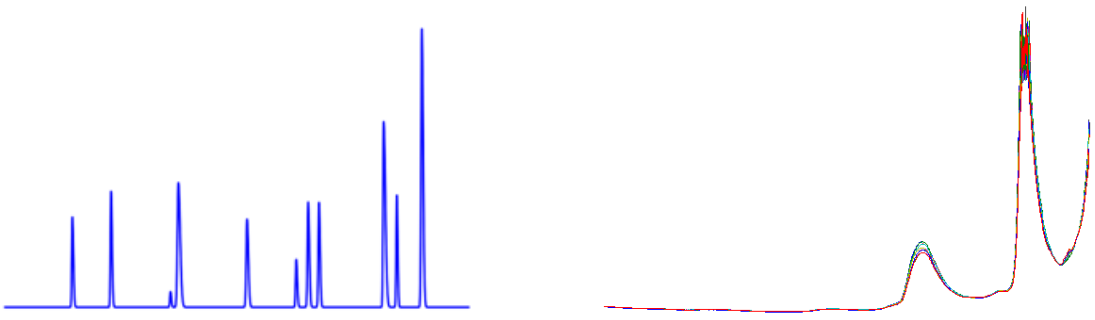
Faculté de Médecine

Laboratoire de Chimie Analytique

Faculté des Sciences Pharmaceutiques

Laboratoire d'Analyse de médicaments

Développement de méthodes génériques pour le dosage des médicaments commercialisés sur le marché congolais : Utilisation de méthodes séparatives et non séparatives



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Diplômé d'études approfondies en Sciences Pharmaceutiques

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Chapitre I

Introduction

1. Etat de lieu

Le trafic des médicaments de qualité inférieure explose dans toutes les régions du globe [1,2]. L'Organisation Mondiale de la Santé (OMS) estime que 10% du marché mondial des médicaments est contrefait [3-7]. Ce problème de santé publique est plus inquiétant dans les pays en voie de développement où la contrefaçon touche particulièrement des médicaments de première nécessité tels que les antibiotiques, les antipaludéens, les anti-inflammatoires non stéroïdiens, les antirétroviraux [8-12]. Des nombreuses études montrent que plus de 25% des médicaments seraient contrefaits ou de qualité inférieure en Afrique centrale et jusqu'à 80% dans certaines régions notamment en République Démocratique du Congo (RDC) [13]. Malheureusement dans ce pays, les informations relatives à la problématique de la circulation des médicaments de qualité inférieure sont très souvent indisponibles à cause de l'insuffisance d'études réalisées à grande échelle. Cependant, quelques cas des médicaments contrefaits ont été signalés par le Ministère Congolais de la Santé Publique en 2009 et en 2012, notamment celui de la quinine goutte orale et de l'amoxicilline suspension. L'action menée par INTERPOL en République du Congo du 04 au 05 juin 2013 et en RDC du 10 au 11 juin 2013 avait permis de retirer du marché plus de 70 tonnes des médicaments contrefaits [14].

1.1. Catégorie des médicaments de qualité inférieure

Selon Paul Newton la terminologie « médicaments de qualité inférieure » est considérée comme un ensemble de trois grandes catégories de produits comme illustré dans la figure 1 [15]. Lors de la 63ème assemblée générale de l'OMS tenue au mois de mai de l'année 2010, la terminologie « produits médicaux hors normes/altérés/falsifiés/faussement étiquetés/contrefaits » (en Anglais SSFFC pour « Substandard/Spurious/Falselly-Labelled/Falsified/Counterfeit ») fut adoptée par l'ensemble des membres participants dont la Belgique et la RDC [16]. D'autres chercheurs préfèrent utiliser la terminologie «la criminalité pharmaceutique » qui englobe les produits contrefaits, les malfaçons (sous-standards) et autres produits de santé illicites [17,18]. De manière générale, les trois terminologies mentionnées ci-dessus convergent vers les mêmes catégories des médicaments. Dans le cadre de notre travail, nous ferons allusion à la terminologie « médicaments de qualité inférieure » qui est très utilisée dans plusieurs publications scientifiques et apparait simple ou moins lourde par rapport à celle adoptée par les pays membres de l'OMS [19-27].

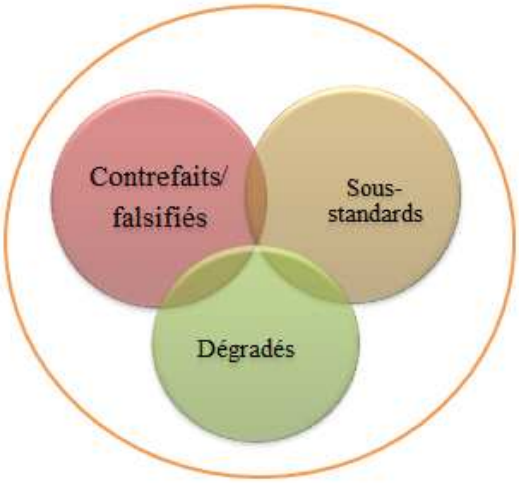

Fig. 1. Catégories des médicaments de qualité inférieure selon Paul Newton

Pour bien comprendre les points communs et les différences entre ces trois groupes des médicaments de qualité inférieure, il nous semble utile de définir les différents termes.

1.1.1. Médicaments contrefaits et/ou falsifiés

L'OMS définit un médicament contrefait comme étant celui qui est délibérément et frauduleusement muni d'une étiquette n'indiquant pas son identité et/ou sa source véritable. Il peut s'agir d'une spécialité ou d'un produit générique. Parmi les produits contrefaits, il y a ceux qui contiennent les bons excipients ou de mauvais excipients. D'autres ne contiennent pas de principe actif où en contiennent mais en quantité insuffisante et d'autres sont caractérisés par un conditionnement falsifié [18,28].

Nous pouvons distinguer deux types de contrefaçon, celle portant atteinte au brevet et dont les produits présentent une conformité acceptable notamment la présence de principes actifs. Ce sont des produits fabriqués par des laboratoires souvent localisés dans des pays en voie de développement comme la Chine, l'Inde, le Pakistan, le Brésil, le Mexique, le Nigéria, la Tanzanie, le Kenya [29]. L'autre contrefaçon est dite totale. Dans ce cas le produit ne contient pas le principe actif. L'objectif du contrefacteur est de tromper l'acheteur par le fait d'imiter le produit original. D'ailleurs le sens même du mot « contrefaçon » du latin « *contrefacere* » signifie imiter.

Il est important de mentionner que la définition de médicaments contrefaits telle que donnée par l'OMS ne prend pas en compte les normes de la traçabilité que doivent respecter les produits pharmaceutiques [18]. C'est ainsi que pour mettre en évidence les dangers sanitaires des faux médicaments, la directive Européenne de 2011 a défini un médicament falsifié comme étant tout médicament comportant une fausse présentation à trois niveaux, d'abord (*i*) au niveau de son identité c'est-à-dire de son emballage et de son étiquetage, de sa dénomination ou de sa composition s'agissant de n'importe lequel de ses composants, y compris les excipients et du dosage de ces composants ; ensuite (*ii*) au niveau de sa source, de son pays de fabrication, de son pays d'origine ou du titulaire de son autorisation de mise sur le marché ; et enfin (*iii*) au niveau de son histoire, y compris des enregistrements et des documents relatifs aux circuits de distribution utilisés [30].

1.1.2. Médicaments sous-standards

Ce sont des médicaments qui ne sont pas conformes aux normes et aux spécifications de la qualité suite à des conditions inadéquates de fabrication ou à un certain laxisme qui affecte la qualité du produit fini. La négligence dans la fabrication peut être intentionnelle mais très souvent non car certains fabricants ne sont pas conscients de la mauvaise qualité de leur produit [15]. Ce genre de situation est pour la plupart des cas rencontrée dans les pays en voie de développement où les capacités de régulation, de l'assurance et de contrôle qualité sont faibles. Les médicaments sous-standard sont parfois préparés sans respecter les principes des Bonnes Pratiques de Fabrication comme par exemple le fait de travailler dans un environnement de préparation inadéquat, avec des équipements de fabrication mal nettoyés ou non qualifiés, avec des méthodes de fabrication non validées, avec une main d'œuvre peu habilitée et des sources de matière première non pré-qualifiées.

Dans cette catégorie on peut retrouver les impuretés résiduelles provenant de la chaîne de fabrication ou consécutives à une purification insuffisante des matières premières (principes actifs et excipients). Les impuretés résiduelles comprennent les traces de métaux lourds, les catalyseurs et les solvants résiduels provenant des processus de synthèse des principes actifs, des excipients et des produits pharmaceutiques eux-mêmes. En fonction de la teneur ces impuretés modifient la qualité des médicaments.

1.1.3. Médicaments dégradés

La dégradation des médicaments est très souvent consécutive aux conditions de conservation inappropriées, situation particulièrement critique sous les climats tropicaux notammen en RDC [15]. Dans ce contexte, les médicaments dégradés contiennent le bon principe actif ainsi que les impuretés de dégradation à des teneurs dépassant les limites [15].

Tenant compte de toutes ces définitions, notre constat est que la présence du principe actif demeure le point commun à ces trois catégories de médicaments de qualité inférieure. Par ailleurs, un médicament contrefait pourrait présenter de bons principes actifs et de bons excipients. De ce fait, il peut être déclaré de bonne qualité et sortir du cercle des médicaments de qualité inférieure proposé par P. Newton [15]. Ces considérations nous permettent d'apporter une modification au diagramme initial qui devient comme présenté à la figure 2 avec une zone d'intersection entre les trois catégories et une zone de médicament contrefait de bonne qualité (figure 2).

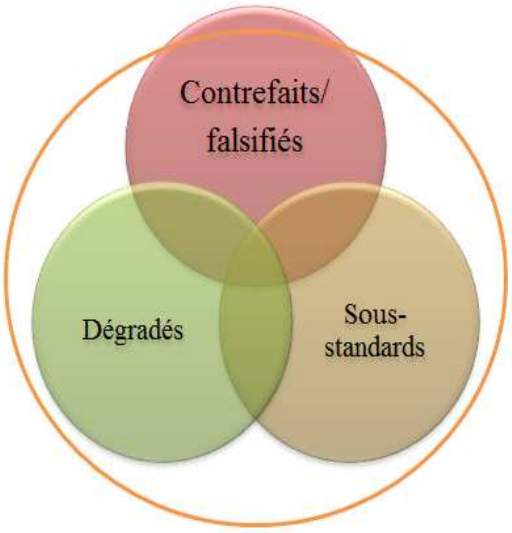

Fig. 2. Nouvelle représentation du diagramme de Paul Newton

1.2. Conséquences de la commercialisation des médicaments de qualité inférieure

Le trafic des médicaments de qualité inférieure présente des conséquences néfastes à plusieurs niveaux. Au niveau de la santé publique on peut observer notamment les échecs thérapeutiques qui peuvent être mortels, l'apparition de résistances aux traitements par exemple avec les antibiotiques, les antipaludéens et les antirétroviraux, la crise de confiance

entre les prestataires des soins de santé et le malade, la croissance de la mortalité et de la morbidité. Au niveau socio-économique nous pouvons mentionner la baisse de chiffre d'affaire des industries pharmaceutiques pouvant aller jusqu'à une perte totale de leur crédibilité, l'aggravation de la pauvreté de la population à cause de la déperdition financière socio-familiale et la germination des conflits sociaux en référence à la sorcellerie ou aux mauvais esprits ou encore au mauvais sort en cas d'un décès provoqué par ces médicaments.

1.3. Présentation générale de la République Démocratique du Congo (RDC)

1.3.1. Aperçu géographique et climatique

La RDC est le deuxième pays le plus vaste d'Afrique après l'Algérie et le premier en Afrique centrale avec une superficie de 2.345.409 Km^2 [31,32]. Signalons que ses frontières sont parmi les plus longues du continent avec une délimitation au Nord par la République Centrafricaine et le Sud Soudan ; à l'Est par l'Ouganda, le Rwanda, le Burundi et la Tanzanie ; au Sud par la Zambie et l'Angola et à l'Ouest par la République du Congo, l'enclave de Cabinda et l'Océan Atlantique [31,32]. Jusqu'en 2014 la RDC comptait 11 provinces, 45 districts et 225 territoires administratifs. La nouvelle loi sur la réorganisation territoriale et administrative promulguée le 2 mars 2015 prévoit la mise en place de 26 provinces telles qu'illustrées dans la figure 3.

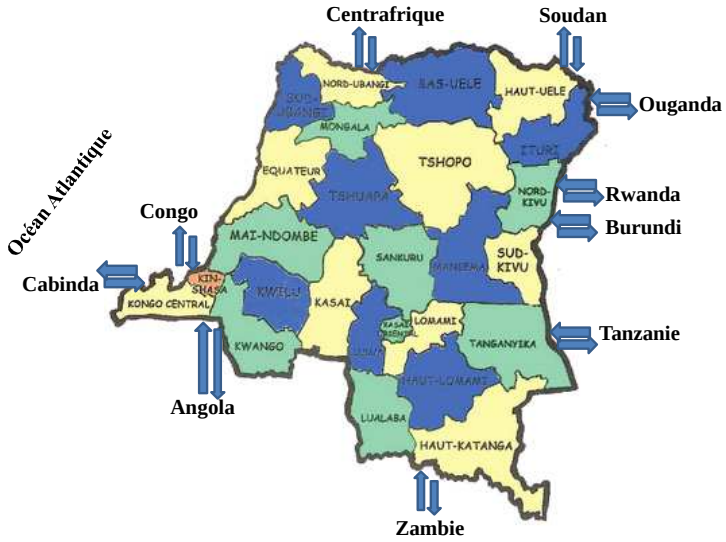

Fig. 3. Carte administrative des 26 provinces de la RDC prévues par la loi sur la réorganisation territoriale et administrative de 2015

Etant située de part et d'autre de l'équateur, la RDC connait toute la gamme des climats caractéristiques de la zone tropicale humide mais aussi celui des montagnes à l'Est du pays. La température varie de 15 à 40 °C suivant les saisons et les localisations géographiques. On note parfois des températures plus extrêmes. Il existe une saison sèche caractérisée par les faibles températures avec des précipitations quasiment nulle et une saison humide caractérisée par des hautes températures et de très fortes précipitations. L'humidité atmosphérique est constamment élevée (70 à 85%) dans certaines provinces comme par exemple la province de l'Equateur et la province Orientale [32]. La température et l'humidité relative constituent des facteurs importants à surveiller mais surtout à tenir compte dans le circuit de distribution des médicaments car elles sont à la base de la dégradation de plusieurs médicaments.

1.3.2. Aperçu démographique

La RDC est le pays le plus peuplé de l'Afrique centrale. En 2007, l'Institut National de la Statistique de la RDC a estimé la population congolaise à 65,8 millions d'habitants dont près de 7,9 millions vivait dans la seule ville de Kinshasa qui est la capitale du pays [31]. Actuellement, on estime cette population à plus de 70 millions d'habitants [33], avec une jeunesse de moins de 15 ans comptant pour 48% de la population et les adultes de plus 60 ans pour 4% [31].

L'enquête démographique et de santé de 2007 renseigne qu'en 1984, 70% de la population vivait en milieu rural contre près de 30% dans les villes. Avec les multiples mouvements de population occasionnés par l'exode rurale mais surtout par les conflits armés de ces dernières décennies, la proportion de la population vivant en milieu urbain s'est vue augmentée à 43% [31]. Ce dernier chiffre fait l'objet d'une doit actuellement être revu à la hausse pour les mêmes raisons d'exode rurale forcée.

1.3.3. Le secteur Pharmaceutique

Le secteur pharmaceutique en RDC est sous le contrôle du Ministère de la Santé Publique. Pour améliorer la politique sanitaire, ce dernier avait élaboré et adopté en 1997 la politique pharmaceutique nationale (PPN) qui était révisée en 2005. La mission essentielle assignée à cette politique était de définir, d'orienter, d'encadrer et de coordonner toutes les

activités pharmaceutiques de la production à l'utilisation des médicaments, en passant par l'importation, la distribution, la dispensation et la prescription, afin de rendre disponibles partout et accessibles les médicaments essentiels de bonne qualité, efficaces, tout en favorisant leur usage rationnel [34].

En plus de cette politique pharmaceutique nationale, il existe au sein du Ministère de la Santé Publique la Direction de la Pharmacie, Médicaments et Plantes Médicinale (DPM) créée par l'ordonnance 82-027 du 19 mars 1982. Cette direction est l'autorité de règlementation pharmaceutique. La DPM a comme rôle de coordonner et de contrôler le secteur pharmaceutique national afin de préserver et contribuer à l'amélioration de la santé de la population en veillant à la qualité et à la disponibilité des produits pharmaceutiques, parapharmaceutiques et vétérinaires [31]. La DPM comprend 6 divisions subdivisées en 16 bureaux représentés dans la figure 4.

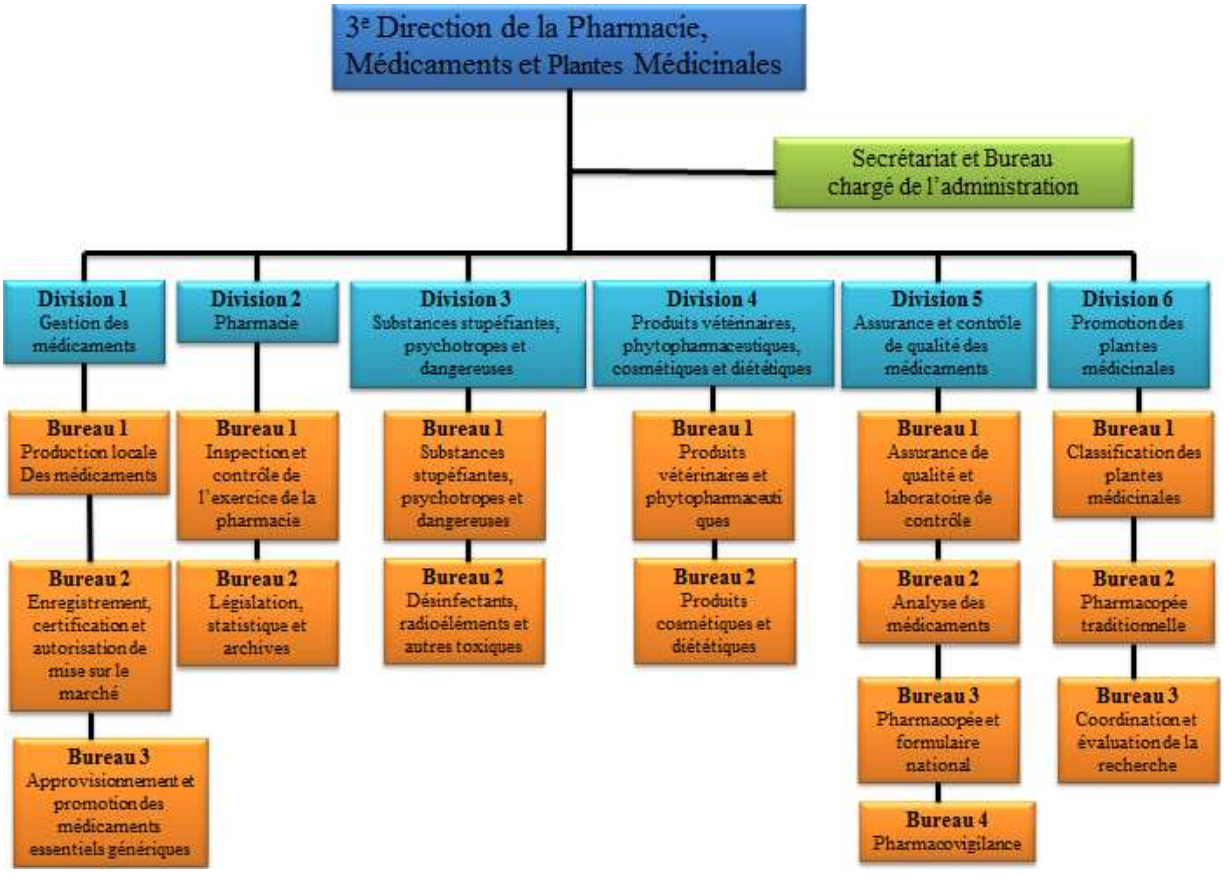

Fig. 4. Organigramme de la Direction de la Pharmacie, Médicaments et Plantes Médicinales [31]

La Division 5 qui s'occupe de l'assurance et contrôle qualité des médicaments commercialisés en RDC nous intéresse dans le cadre de ce travail. Sa mission principale est de protéger la population Congolaise en veillant sur la qualité des médicaments. Cependant, la complexité du circuit d'approvisionnement des médicaments est l'un des facteurs qui ne permet pas à cette direction de mettre en place une traçabilité correcte et un suivi du médicament de l'étape d'importation ou de production à celle d'utilisation par le patient. En 2002, le Ministère de la Santé Publique a créé le Système National d'Approvisionnement en Médicaments Essentiels (SNAME) grâce à l'appui des partenaires en vue de mettre en œuvre les stratégies de la PPN [30]. Ce système est coordonné par le Programme National d'Approvisionnement en Médicaments Essentiels (PNAM) qui, en théorie, est soumis au contrôle de la DPM.

La coexistence de plusieurs systèmes d'approvisionnement des médicaments et autres produits de santé à côté du Système National d'Approvisionnement en Médicaments Essentiels et Génériques rend complexe le circuit d'approvisionnement des médicaments. Une étude réalisée par l'OMS en 2009 a mis à jour un système d'approvisionnement et de distribution des médicaments en RDC extrêmement complexe avec plusieurs agences d'approvisionnement et des circuits de distribution des médicaments.

Signalons qu'à côté de SNAME, il existe le secteur privé constitué par les importateurs et grossistes répartiteurs, les fabricants et les détaillants qui se caractérisent par la prolifération des établissements pharmaceutiques à travers tout le pays. Pour se faire une idée, la ville de Kinshasa compte à elle seule plus de 5000 pharmacies ouvertes au public pour la plupart sans autorisation du Ministère de la Santé Publique. A côté de ces officines, il existe le marché illicite des médicaments qui freine le développement socio-économique du pays.

La complexité des systèmes d'approvisionnement des produits pharmaceutiques telle qu'illustrée dans la figure 5 ne favorise pas le développement contrôlé de l'ensemble du secteur pharmaceutique.

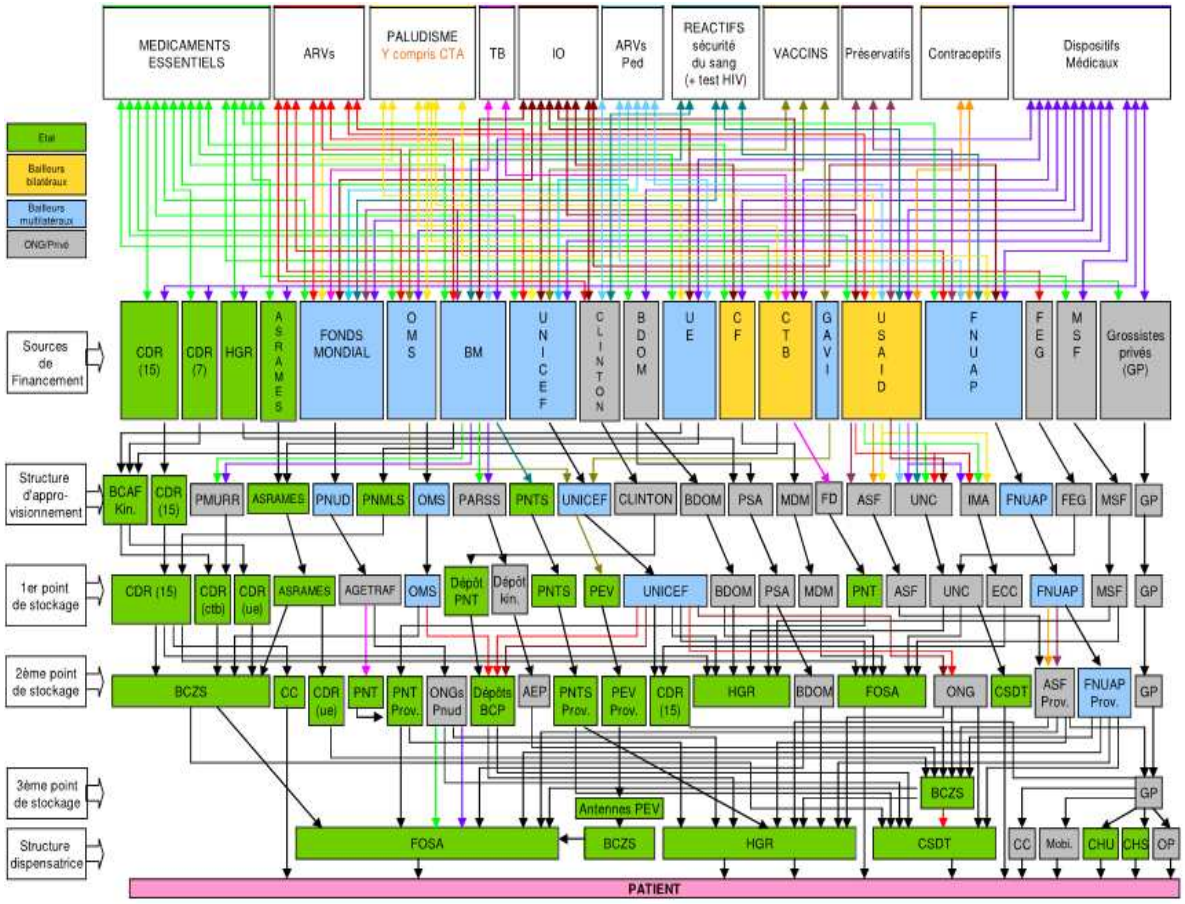

Fig. 5. Système d'approvisionnement des produits pharmaceutiques en RDC publié en mai 2009 [31]

D'une manière globale, l'état de lieu du secteur pharmaceutique en RDC est chaotique. On peut en extraire les difficultés majeures suivantes :

- la faible capacité institutionnelle et gestionnaire de la DPM et du PNAM ;

- l'absence d'une loi pharmaceutique et des réglementations adaptées, efficaces et bien appliquées ;

- la couverture insuffisante des formations sanitaires en médicaments et autres consommables essentiels;

- la mauvaise gestion des médicaments dans les structures sanitaires et pharmaceutiques ;

- la vente des médicaments dans des endroits inappropriés et insalubres;

- l’absence d’un cadre de concertation, de coordination et de suivi des projets de coopération en matière de santé;

- le faible financement des médicaments par l’Etat ;

- la corruption et les conflits d’intérêts ;

- le mauvais état des routes et des voies de communication pour la distribution des médicaments ;

- le coût élevé des médicaments ainsi que l’absence d’une politique de tarification des produits pharmaceutiques ;

- l’ouverture anarchique et exagérée des établissements pharmaceutiques et leur répartition inéquitable sur le territoire national ;

- l’insuffisance du personnel en qualité et en quantité ;

- la complexité et l’existence des systèmes parallèles d’approvisionnement en médicament ;

- l’insuffisance du système d’assurance de qualité (Bonnes Pratiques de Fabrication, Bonnes Pratiques de Distribution et Bonnes Pratiques Officinales) et du système OMS de certification de la qualité des produits entrant dans le commerce international ;

- l’absence d’un laboratoire national de contrôle de qualité des médicaments bien équipé [32,34].

Toutes ces faiblesses auxquelles s’ajoute la perméabilité des frontières partagées avec les 9 pays voisins favorisent la circulation des médicaments de qualité inférieure y compris l’importation non contrôlée des médicaments qui vont se retrouver dans le marché illicite. Celui-ci sera amplifié à cause des ruptures fréquentes de stock de médicament au niveau du marché légal. Il faut également signaler que les détournements des dons humanitaires sont parmi les sources d’approvisionnement des médicaments (surtout des spécialités pharmaceutiques) dans le marché illicite qui va présenter les avantages de disponibilité et de faible coût afin de concurrencer le marché légal.

1.4. Lutte contre la commercialisation des médicaments de qualité inférieure en RDC

Conscient du phénomène de la circulation des médicaments de qualité inférieure dans son territoire, le Ministère de la Santé Publique de la RDC multiplie des efforts en vue de chercher les voies et les moyens pour combattre ce fléau. Mentionnons le cas du IV^{ème} Salon International de la Pharmacie du Congo (SIPHACO) qui a été organisé à Kinshasa du 18 au 20 décembre 2013 autour du thème «Tous contre la criminalité pharmaceutique » [14]. La stratégie proposée s'articule autour de plusieurs points à savoir :

- l'éducation de masse et la sensibilisation qui doivent revêtir d'une importance particulière car la population n'est pas suffisamment informée sur le phénomène de la circulation des médicaments de qualité inférieure. Cette action peut être réalisée grâce à des campagnes de lutte contre les faux médicaments en se servant des supports de communication comme des messages audio-vidéo et à travers la vulgarisation des résultats de recherches scientifiques.

- La mise en place des mécanismes efficaces de coopération et de collaboration au niveau national, régional plus spécialement aux frontières avec les pays voisins de la RDC et international, notamment en renforçant les réseaux de règlementation et en encourageant les échanges d'informations.

- La mise en place d'une structure nationale de lutte contre la contrefaçon qui doit élaborer les stratégies et travailler en franche collaboration avec le Ministère de la Justice et des Droits Humains, celui de l'Intérieur et Sécurité (Police), celui de la Santé Publique (DPM), celui de l'Economie et du Commerce (Douane), celui de la Communication et des Médias (Radio et Télévision). Sur le plan judiciaire, il a été proposé d'introduire la pénalisation du délit de contrefaçon des médicaments.

- Le renforcement des capacités du personnel impliqué dans l'assurance qualité des médicaments pour une bonne traçabilité des médicaments (de la production ou importation à la dispensation au malade).

- La stratégie analytique qui consiste au renforcement des laboratoires de contrôle de qualité en équipements modernes et renforcer les capacités du personnel commis au contrôle de qualité. Ce dernier aspect constitue un point en rapport avec notre thèse.

1.5. Stratégie analytique en RDC

La grande majorité des laboratoires de contrôle de qualité en RDC ne dispose pas d'équipement utilisant les techniques analytiques de pointe comme la chromatographie liquide, l'électrophorèse capillaire largement utilisés en Europe et en Amérique du Nord. Très souvent ces laboratoires recourent aux réactions en solution et à la chromatographie sur couche mince (CCM) en vue d'identifier les principes dans les médicaments tandis que la titrimétrie à l'aide d'une burette est utilisée pour le dosage de ces principes actifs. Depuis moins de deux ans, la plupart des laboratoires de contrôle de qualité ce sont vu équipés des spectrophotomètres Ultraviolet-Visible pour le dosage des principes actifs dans des échantillons médicamenteux suivant une prise en charge analytique présentée à la figure 6.

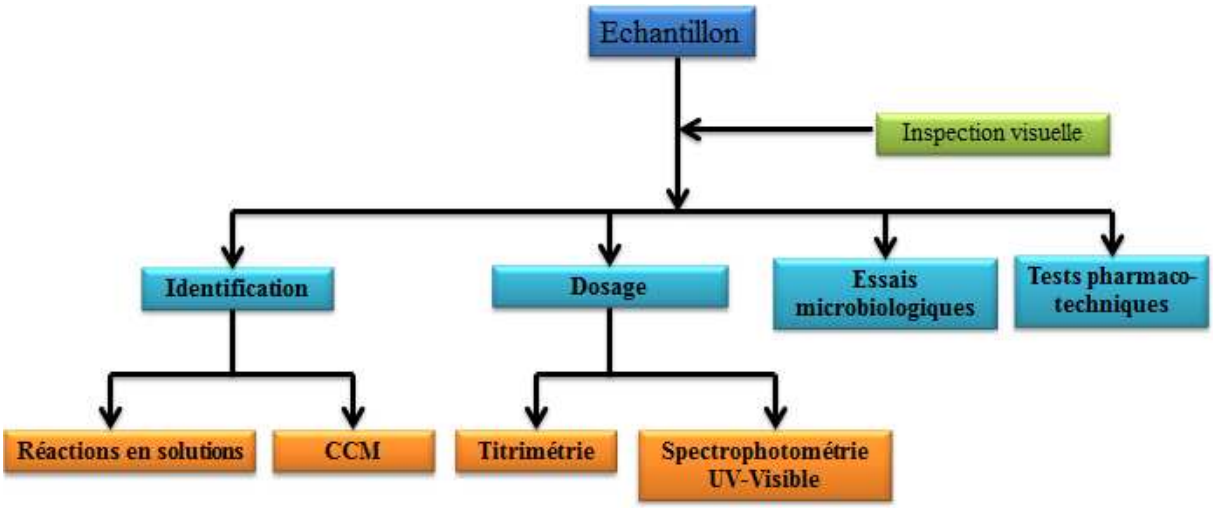

Fig. 6. Logigramme de la stratégie analytique utilisée dans les laboratoires de contrôle de qualité en RDC

Les méthodes analytiques utilisées avec les techniques précitées (titrimétrie, CCM, spectrophotométrie UV-Vis) sont souvent adaptées en s'inspirant des méthodes pharmacopées destinées à l'analyse des matières premières. Elles présentent les avantages d'être simples, rapides et moins coûteuses. La titrimétrie par exemple n'exige pas d'étalonnage au préalable car une seule manipulation est suffisante pour doser un produit. Par contre, elles présentent plusieurs faiblesses telles qu'énumérées ci-après.

a) Interférences dans le milieu complexe du médicament

A titre d'exemple, lorsqu'une suspension orale ou un sirop renferme un excipient ou un adjuvant contenant l'azote (base faible) comme par exemple l'aspartame, ce dernier va interférer lors du dosage du principe actif à caractère basique faible (par exemple la luméfantrine) par l'acide perchlorique en protométrie en milieu non aqueux. De ce fait, le résultat de dosage obtenu sera assorti d'une erreur systématique importante mais non attribuée à la méthode.

b) Problème de quantification dans les associations pharmaceutiques

Dans le cas des associations pharmaceutiques, n'ayant pas la possibilité de déterminer la teneur de plusieurs principes actifs en mélange, les laboratoires de contrôle de qualité se limitent à quantifier uniquement le produit majoritaire ou facile à quantifier afin de décider de la qualité du produit. Cette situation favorise la circulation des médicaments sous-standard commercialisés en association.

c) Problème de quantification des impuretés de dégradation dans les produits finis

A titre d'exemple, l'acide salicylique et le 4-aminophénol qui sont des impuretés de dégradation respectivement de l'acide acétylsalicylique et du paracétamol ne peuvent être analysés en présence de leur produits parents, alors que cela constitue un requis compte tenu des conditions climatiques de la RDC mais aussi des risques néfastes sur les patients ou les consommateurs (cas d'hépato-toxicité avec le 4-aminophénol).

d) Problème de choix de spécifications pour le teste de dosage des principes actifs

Chaque laboratoire de contrôle de qualité choisi les spécifications parfois en fonction des résultats obtenus pour décider de la conformité du produit. Cette pratique de juge et partie est source de risque de circulation des médicaments hors spécifications.

e) Problème de validation des méthodes

Les méthodes titrimétriques utilisées par la plupart des laboratoires de contrôle de qualité ne sont pas validées par manque de formation ou d'information des analystes sur cette thématique. L'application de méthodes analytiques non validées augmente le risque au niveau

du producteur que des consommateurs (patients). Cette situation favoriserait la circulation des médicaments sous-standards en RDC.

2. Validation de méthodes analytiques

La mise en œuvre d'une méthode analytique se décompose en quatre grandes phases successives telles qu'illustrées dans la figure 7 [35].

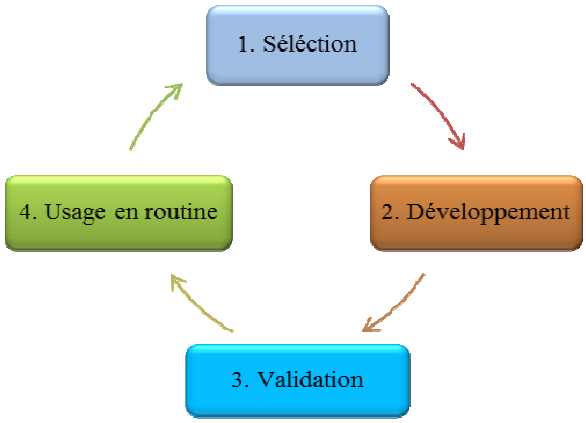

Fig. 7. Cycle de vie d'une méthode analytique

Comme point de départ d'une méthode analytique, la sélection permet d'en définir clairement les objectifs pour proposer des solutions appropriées, matérialisées en termes de conditions opératoires initiales. Les objectifs de la méthode étant clairs, il est indispensable d'effectuer des expériences complémentaires (phase de développement) en vue de s'assurer de la capacité de la mise en œuvre de la méthode par rapport à l'usage requis.

Le développement de la méthode peut se faire au moyen ou non d'un plan d'expériences selon la problématique définie lors de la phase de la sélection. Il consiste à optimiser les paramètres instrumentaux et les conditions opératoires.

Après la phase de développement, il devient de plus en plus évident et essentiel de démontrer au moyen de la validation qu'une méthode optimisée correspond à l'usage attendu tout en fournissant par ailleurs des résultats fiables.

Une fois, la méthode est validée, elle peut être appliquée en routine avec confiance. Cette phase inclus le plus souvent une validation en routine et parfois une validation partielle.

La validation d'une méthode analytique constitue l'étape la plus importante d'un cycle de vie d'une méthode car elle permet de donner aux laboratoires ainsi qu'aux autorités compétentes les garanties que chaque mesure qui sera réalisée ultérieurement en routine, sera

suffisamment proche de la « vraie valeur » inconnue de l'échantillon ou du moins comprise dans la limite acceptable, en fonction de la finalité de la procédure [36-42].

La stratégie de l'erreur totale qui utilise le profil d'exactitude comme outil de décision est très utilisée pour valider une méthode d'analyse [36-42]. Le profil d'exactitude tel qu'illustré dans la figure 8 est construit à partir des intervalles de tolérance d'espérance β de mesures attendues à chaque niveau de concentration. Il est intégré dans la limite d'acceptation ($\pm\lambda$) pour permettre à l'analyste de prendre des décisions basées sur l'objectif de la méthode analytique. Généralement, la limite d'acceptation est de 1% à 3 % pour le dosage des principes actifs dans une matière première, de 5 % ou 10% pour les formes pharmaceutiques et de 15 % ou 20% pour les analyses dans les matrices biologiques ou environnementales [42,43].

Le profil d'exactitude tient compte du risque associé à la méthode. Il permet en outre d'accepter ou de rejeter une méthode analytique suivant l'usage attendu. Une méthode est valide si les intervalles de tolérances d'espérance β sont compris dans les limites d'acceptation fixées.

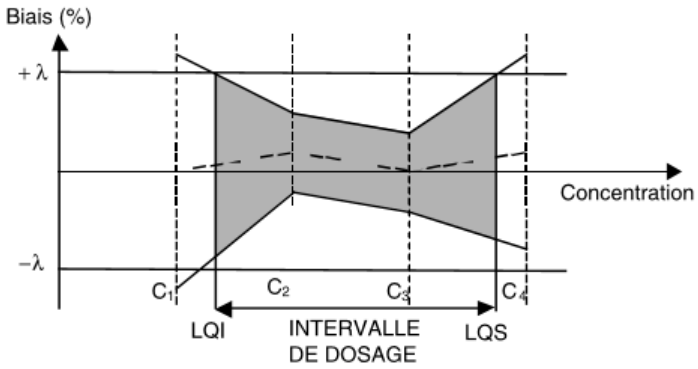

Fig. 8. Illustration de profil d'exactitude. LQI : limite de quantification inférieure. LQS : limite de quantification supérieure. C : concentration. λ : limite d'acceptation

La zone grise montre l'intervalle de dosage dans lequel la procédure est capable de quantifier avec une exactitude connue et un risque fixé par l'analyste.

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Chapitre II

Objectifs du travail

Chapitre II. Objectifs du travail

Un médicament est défini comme étant toute substance ou composition présentée comme possédant des propriétés curatives ou préventives à l'égard des maladies humaines ou animales, ainsi que toute substance ou composition pouvant être utilisée chez l'homme ou chez l'animal ou pouvant être administrée en vue d'établir un diagnostic médical, de restaurer, corriger ou modifier une fonction physiologique en exerçant une action pharmacologique, immunologique ou métabolique. A partir du moment où il est de qualité inférieure, il y a un risque que le médicament ne réponde pas à ce qu'on attend de lui, par conséquent sa présentation ou son utilisation constitue un problème majeur de Santé Publique.

En RDC, les antipaludéens, les antibiotiques et les anti-inflammatoires non stéroïdiens (AINS) font partie des classes pharmacologiques les plus visées par le problème de qualité inférieure en raison de leur forte prescription et de leur large utilisation. Pour faire face à ce problème, le Ministère de la Santé Publique *via* la DPM a mis en place une série de stratégies parmi lesquelles l'assurance qualité et le contrôle de qualité au moyen de méthodes analytiques robustes et fiables. Celles-ci devront permettre la prise de décisions rapides et scientifiquement appuyées en vue de préserver la qualité des médicaments et aussi de sécuriser les patients et les consommateurs.

Dans ce contexte, l'objectif principal de ce travail est de répondre à la préoccupation des autorités du Ministère Congolais de la Santé Publique par la mise à disposition d'outils analytiques adéquats dans le but de contribuer au renforcement de la stratégie analytique. Les trois classes pharmacologiques citées ci-haut et qui font partie du «package médical » en RDC feront l'objet de notre étude.

La première partie de notre travail sera consacrée au développement des méthodes génériques en utilisant la technique séparative de la chromatographie liquide. Il sera question de tracer simultanément plusieurs principes actifs dans un premier temps dans le but de les détecter et ainsi de mettre en évidence des falsifications ou des contrefaçons et dans un second temps de quantifier les principes actifs qui auront été détectés. La technique séparative sera particulièrement utile dans le cas de la stratégie analytique pour examiner la qualité des médicaments en associations (polythérapie).

Chapitre II. Objectifs du travail

La deuxième partie de notre travail se focalisera sur le développement des méthodes simples et rapides en utilisant les techniques non séparatives qui conviennent pour l'analyse des produits présentés en monothérapie. Nous nous intéresserons d'abord à la spectrophotométrie UV-Visible compte tenu de la présence croissante ces deux dernières années des spectrophotomètres UV-Visible dans plusieurs laboratoires de contrôle qualité en RDC. Ensuite, nous porterons un intérêt particulier à la spectroscopie vibrationnelle Raman mais surtout à celle de Proche Infrarouge (PIR) étant donné la récente acquisition *via* le projet interuniversitaire ciblé (PIC) d'un tout premier spectrophotomètre PIR à Kinshasa au Laboratoire d'Analyse des Médicaments de l'Université de Kinshasa. Les avantages en termes de simplicité, de rapidité et surtout de la non ou peu d'utilisation de solvant organique ne pourront que renforcer davantage et de manière pérenne la stratégie analytique.

Dans les deux cas (techniques séparatives et techniques non séparatives) et avant toute application en routine, nous éprouverons les méthodes développées en se servant de la stratégie de validation basée sur l'erreur totale qui utilise le profil d'exactitude comme outil de décision.

Enfin, en considérant les résultats qui seront obtenus tout au long du cycle de vie de chaque méthode, nous nous efforcerons de proposer une stratégie analytique sous forme de logigramme qui sera présenté aux autorités du Ministère Congolais de la Santé Publique.

Chapitre III

Technique séparative

Chapitre III. Technique séparative

Dans ce chapitre, nous avons choisi la chromatographie liquide comme technique séparative afin de réaliser un criblage permettant la détection rapide et simultanée de plusieurs molécules actives appartenant à une même classe pharmacologique. Les classes mentionnées dans les objectifs de ce travail seront étudiées. Etant donné l'importance du nombre des principes actifs à détecter et pour bien circonscrire le développement des méthodes, nous avons opté pour la stratégie basée sur la planification expérimentale par approche multivariée et en utilisant l'espace de conception en anglais « *Design Space* ». Cette stratégie sera d'une grande utilité pour permettre de repérer des zones correspondant à des conditions chromatographiques idéales en vue de séparer un maximum de composés.

Mentionnons que dans ce genre de stratégie, les données analytiques obtenues au cours d'une planification expérimentale offrent la possibilité de développer des méthodes pour l'analyse des associations pharmaceutiques connues et des impuretés de dégradation ciblées dans des temps d'analyse plus réduits. Elles permettent également de développer d'autres méthodes en cas de substitution ou d'introduction d'une nouvelle molécule active dans le groupe de molécules initiales. Enfin, les données analytiques sont d'une grande utilité en cas de transfert géométrique vers des techniques analytiques plus rapides telle que la chromatographie liquide ultra haute performance.

Lorsque l'on débute un développement de méthode chromatographique, une étape importante est la construction d'un espace de conception. Ce dernier peut être défini comme la combinaison multidimensionnelle où l'interaction des variables d'entrée et les paramètres du procédé ayant permis de donner l'assurance de la qualité. En d'autres termes, l'espace de conception est un sous-espace du domaine expérimental à l'intérieur duquel les objectifs ont été atteints en considérant l'incertitude des procédés concernés.

De ce fait, la première étape consistera à sélectionner les facteurs ou paramètres chromatographiques tels que le pH, le débit de la phase mobile, le pourcentage du modificateur organique et le temps de gradient caractérisés par un effet marqué sur la réponse qui sera modélisée. Afin de maximiser les chances d'identifier une séparation optimale observée entre les pics chromatographiques, le domaine expérimental sera élargi au maximum tout en tenant compte des contraintes liées aux analytes, par exemple leur instabilité à des pH

extrêmes et à des températures variées. Concernant ce dernier paramètre analytique, il sera utile d'évaluer son effet sur les réponses à travers la robustesse de la méthode étant donné que celle-ci est destinée à être utilisée dans des laboratoires où le contrôle de la température n'est pas très aisé compte tenu des conditions climatiques (cas de la RDC souligné dans l'état de lieu).

La deuxième étape concernera l'exécution des expériences suivant un plan qui sera sélectionné selon l'objectif poursuivi. En effet, un plan factoriel complet permet d'étudier les effets des différents facteurs avec comme conséquence un nombre très élevé d'expériences à réaliser tandis que les plans pour surface de réponse (plan composite centré, D-optimal,…) permettent d'optimiser un procédé où une méthode *via* un nombre d'expériences limité.

Dans la troisième étape, les temps de rétention au début, à l'apex et à la fin de chaque pic de molécules étudiées seront sélectionnés comme réponses à modéliser et conservés dans une base de données analytiques. Pour une paire critique de pics donnée, la séparation « S » utilisée comme attribut critique de qualité (CQA) est la différence entre le temps de rétention au début du second pic et le temps de rétention à la fin du premier pic. L'erreur obtenue sur la prédiction des temps de rétention est propagée au CQA. Ensuite, les simulations de Monte-Carlo sont utilisées pour obtenir la distribution des S à partir de la prédiction jointe de la distribution des temps de rétention d'une paire critique, pour une condition chromatographique expérimentale donnée. Considérant la distribution de S et des temps de rétention, la probabilité que S soit supérieure à 0 sera utilisée afin de définir l'espace de conception. *In fine*, l'espace de conception définissant la zone du domaine expérimentale où la probabilité prédite que $S > 0$ est supérieure à un niveau de qualité prédéfini sera le critère visuel et statistique qui nous servira pour la sélection des conditions chromatographiques optimales.

Section III. 1.

Développement de méthodes d'analyse des anti-Inflammatoires non stéroïdiens par chromatographie liquide

Cette section se réfère à l'article «**Application of an innovative design space optimization strategy to the development of liquid chromatographic methods to combat potentially counterfeit nonsteroidal anti-inflammatory drugs**» publié dans le Journal of Chromatography A 1263 (2012) 113-124.

Section III.1. Technique séparative – AINS

Contexte

Les anti-inflammatoires non stéroïdiens (AINS) font partie des groupes des médicaments ayant des propriétés analgésiques, antipyrétiques et anti-inflammatoires. Certains possèdent également des propriétés antiagrégantes. Ils font partie des médicaments les plus consommés en RDC et se retrouvent aussi bien dans des établissements pharmaceutiques autorisés c'est-à-dire dans le circuit légal que dans ceux ouverts anarchiquement ou circuit illicite. Ils sont vendus soit en monothérapie soit en association pharmaceutique. Cependant, la perméabilité du circuit légal du médicament, la liberté du choix du site d'achat avec ou sans ordonnance et l'insuffisance du contrôle de qualité des médicaments sont des facteurs favorisant la circulation de faux médicaments dont les AINS et par conséquent exposent le malade à des risques très élevés relatifs à leur consommation. D'autre part, pour favoriser la production pharmaceutique locale, le Ministère Congolais de la Santé Publique avait publié en date du 06 décembre 2010 l'Arrêté N°1250/CAB/MIN/SP/069/CJ/OMK/2010 interdisant l'importation de 15 médicaments les plus consommés entre autre le paracétamol. C'est ainsi que dans un souci de préserver la qualité des soins par la mise à disposition de médicaments de bonne qualité, nous avons ciblé dans notre travail ce premier groupe de médicaments.

Grâce à la stratégie d'optimisation basée sur la combinaison du plan composite centré et l'espace de conception, nous avons développé trois méthodes analytiques génériques pour détecter 27 molécules d'intérêt. La base des données analytiques obtenues lors des expériences a permis de développer deux méthodes analytiques de courte durée en vue de quantifier les associations pharmaceutiques respectivement dans les formes solides et dans les formes liquides. Pour s'affranchir de la fiabilité de ces méthodes, nous avons validé l'une d'entre elles notamment celle dédiée au dosage du paracétamol, de l'ibuprofène et de la caféine en association dans des gélules. Signalons qu'à notre meilleure connaissance, cette association ne se retrouve qu'en RDC. De plus, la forme pharmaceutique gélule étant particulièrement facile à trafiquer, il nous est paru important de connaitre la teneur de ces trois principes actifs dans plusieurs échantillons collectés à Kinshasa d'une manière aléatoire. Les résultats sont très interpellants.

Section III.1. Technique séparative – AINS

Summary

In the context of the battle against counterfeit medicines, an innovative methodology has been used to develop rapid and specific high performance liquid chromatographic methods to detect and determine 18 non-steroidal anti-inflammatory drugs, 5 pharmaceutical conservatives, paracetamol, chlorzoxazone, caffeine and salicylic acid. These molecules are commonly encountered alone or in combination on the market. Regrettably, a significant proportion of these consumed medicines are counterfeit or substandard, with a strong negative impact in countries of Central Africa. In this context, an innovative design space optimization strategy was successfully applied to the development of LC screening methods allowing the detection of substandard or counterfeit medicines. Using the results of a unique experimental design, the design spaces of 5 potentially relevant HPLC methods have been developed, and transferred to an ultra high performance liquid chromatographic system to evaluate the robustness of the predicted DS while providing rapid methods of analysis. Moreover, one of the methods has been fully validated using the accuracy profile as decision tool, and was then used for the quantitative determination of three active ingredients and one impurity in a common and widely used pharmaceutical formulation. The method was applied to 5 pharmaceuticals sold in the Democratic Republic of Congo. None of these pharmaceuticals was found compliant to the European Medicines Agency specifications.

Section III.1. Technique séparative – AINS

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used against pain, fevers of various origins and inflammation [1–3]. Although they are widely prescribed throughout the world, many of them are associated with side effects. These drugs are often used in self-medication, as their purchase is also unrestricted over the Internet. The risk of administration of uncontrolled medicines is thus naturally greater.

Furthermore, NSAIDs are often subject to the practice of counterfeit medicines, which is gaining increasing momentum in the world and particularly in low-income populations. This has adverse consequences for public health [4]. The World Health organization (WHO) reported 6% of drugs worldwide are counterfeit and the Food and Drug Administration (FDA, USA) estimated this proportion to be 10% [5]. This proportion varies from one country to another. In some African countries, Marini et al. estimated that up to 80% of medical products are counterfeit [6,7]. To ensure the quality of drugs and to help battle counterfeit medicines, the development of screening methods that can simultaneously trace many of the most commonly used molecules is an important effort. In this context, separative techniques are the usual option when planning to explore a substantial number of molecules. Conversely, while techniques such as near infrared or Raman spectroscopy are simpler and quicker procedures and require no sample preparation [8], their potential still remains limited when dealing with the sample analysis of complex mixtures of active ingredients or their impurities at low concentration levels [9].

Several liquid chromatographic (LC) methods are described in the literature for analyzing NSAIDs [9–20]. However, none of these includes an exhaustive list of the molecules present in NSAIDs. Thus they are of limited use in the context of complex or unknown mixtures screening. For instance, Iuliani et al. optimized a LC method for separating only seven NSAIDs regardless of major pharmaceuticals products such as naproxen, diclofenac, etc., or other major molecules often associated to NSAIDs such as paracetamol, caffeine, etc. [21].

Section III.1. Technique séparative – AINS

In the present study, several HPLC separation conditions were optimized for targeted subsets of 27 molecules used alone or in combination. The first objective was the optimization of the separation conditions for these 27 molecules among which were 18 NSAIDs: ibuprofen (IBU), diclofenac (DIC), mefenamic acid (MA), ketoprofen (KTO), nimesulide (NIM), dextropropoxyphene (DEX), niflumic acid (NA), tenoxicam (TE), piroxicam (PI), sulindac (SUL), phenylbutazone (PHE), flurbiprofen (FU), suprofen (SUF), naproxen (NAP), tiaprofenic acid (TA), phenoprofen (i.e. fenoprofen, PF), indomethacin (IDO) and acetylsalicylic acid (AA). Molecules often associated with NSAIDs were added to the list: chlorzoxazone (CHL), caffeine (CAF), paracetamol (i.e. acetaminophen, PAR) and salicylic acid (SAL), a degradation product of AA. Finally, five pharmaceutical conservatives found in syrups or suspensions were concurrently analyzed with the rest: nipagine (i.e. methylparaben, NIP), nipasol (i.e. propylparaben, NIS), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and sodium benzoate (BEN).

To achieve this objective, design of experiments (DoE) was utilized to establish a design space (DS) [22], based on a predictive statistical model of the retention times. DoE with predictive probability is a very innovative framework to simultaneously optimize the separation and estimate the method robustness over the experimental domain.

According to ICH Q8 (R2) [23], the DS is “the multidimensional combination and interaction of input variables (e.g. material attributes) and process parameters that have been demonstrated to provide assurance of quality”. Thus, the DS is a subspace of the experimental domain where assurance of quality has been proven. In the present study, the DS will define the combinations of HPLC parameter ranges in such a way as to yield maximum robustness of the chromatographic separation [24]. The analytical DS is also called “method operable design region” (MODR; [25–28]) in order to distinguish it from the process DS. The minimal expected robustness is described by acceptance criteria (Λ) that apply on some performance attributes defined in the analytical target profile (ATP) of the method [25–28]. The ATP defines the intended purpose of the analytical method. In particular, the ATP will be a set of values or indices that provides some indications about the overall achievement of the analytical method as well as their corresponding acceptance criteria. These performance indices can be called critical quality attributes (CQA) of the analytical method to keep a

nomenclature close to the one used in pharmaceutical processes development [25–29]. In chromatographic terms, CQAs may be the resolution ($R_{s,crit}$) or the separation (S_{crit}) of a critical pair of peaks, and the run time of the method (t_{tot}), while the acceptance criteria Λ may be $S_{crit} > 0$ and $t_{to\ t} < 45$ min considered concurrently.

In this context, a result given as a predictive probability that the CQAs will fall within acceptance criteria allows the quantification of the reliability of the method robustness. This leads to a risk-based definition of the DS that may be expressed as

$$DS = \{x_0 \in \chi : P(CQAs \in \Lambda / x_0, data) \geq \pi\}$$

In other words, the DS is a region of an experimental domain χ (often called knowledge space) where the posterior probability that the CQAs are within acceptance criteria Λ, is higher than a specified quality level л, conditionally on the available data. By the use of the posterior predictive distribution of the CQAs, the posterior probability accounts for the parameter uncertainties and interactions estimated by the statistical model, as well as residual variability [30].

Note that if a large and high quality DS is identified within the experimental HPLC parameter ranges, the corresponding optimized method may be considered robust, as deliberate changes in the operating conditions (included in the defined DS) will not negatively impact the quality of the output.

In order to provide faster analysis, a transfer to ultra high performance liquid chromatography (UHPLC) was the second part of the planned study. This leads to a shortened run time and reduced solvent consumption, which results in reduced time and cost to identify substandard or counterfeit medicines in laboratories where UHPLC systems are available [31]. For that purpose, the robustness of the methods firstly developed with conventional HPLC system was found mandatory to permit the geometric transfer. Indeed, the induced variability when changing from one LC system to another may lead to small changes in the retention times of the analytes. Logically, this occurrence could be more pronounced when moving from a conventional LC system to a UHPLC system and even more if this latter one

may suffer from a small loss in peak efficiency due to the drastic reduction of the analysis time. Consequently, the objective in this context was also to demonstrate that the knowledge coming from the built DS (i.e. robustness area) could facilitate the geometric transfer even if these facts might potentially impact on the separation quality of the transferred analytical methods.

The third objective was to validate one of the developed HPLC methods, using the accuracy profile as decision tool for the determination of four compounds [32,33]. A common NSAIDs combination marketed in some African countries was used. It consisted of capsules containing paracetamol, ibuprofen, caffeine and potentially 4-aminophenol, a well-known impurity of paracetamol allowing to obtain information on the storage conditions.

Finally, the quantitative method was applied to analyze five drugs marketed in the Democratic Republic of Congo (DRC).

2. Experimental

2.1. Materials

Ketopofen (99.7%), diclofenac (99.7%), naproxen (>98%), piroxicam (>99%), nimesulide (100.0%), sulindac (98%), suprofen (99.1%), sodium benzoate (99.9%), 4-aminophenol (98%), flurbiprofen (batch F8514-5G), phenoprofen (batch 029K1043), tenoxicam (batch T0909-5G) and mefenamic acid (batch 36H0945) were purchased from Sigma–Aldrich (Antwerp, Belgium). Caffeine (100.1%), paracetamol (99.5%), ibuprofen (99.6%), indomethacin (99.2%), nipagin (100.1%), nipasol (101.9%), chlorzoxazon (99.1%), acetylsalicylic acid (batch 08G31-B28-232951), salicylic acid (batch 06K14-B09-216351), and butylated hydroxytoluene (batch 05E31-B05) were purchased from Fagron N.V. (Waregem, Belgium). Dextropropoxyphen (batch 203100), phenylbutazone (batch 00951QA) and butylated hydroxyanisole (batch 511527) were purchased from Federa (Brussels, Belgium). Tiaprofenic acid was purchased from Erfa S.A (Brussels, Belgium). Niflumic acid (batch 0411545-2) was purchased from Cayman Chemical Company (Lansing, Michigan, USA). Lactose (batch 70756355176) was purchased from DMV Fronterra Excipients (Goch, Germany). Methanol (HPLC gradient grade), hydrochloric acid (37%), ammonium hydroxide (32%) and ammonium hydrogen carbonate (99%) were purchased from Merck (Darmstadt,

Germany). Ammonium formate (99%) was provided by Alfa Aesar (Karlsruhe, Germany). Trifluoroacetic acid (batch 1001007) was purchased from Fisher Scientific Bioblock (Tournai, Belgium). Ultrapure water was obtained from a Milli-Q Plus 185 water purification system (Millipore, Billerica, MA, USA). For the preparation of validation standards, a matrix formulation of capsules containing 200 mg of paracetamol, 400 mg of ibuprofen, 40 mg of caffeine and 10 mg of lactose was used.

The 27 materials were divided into 5 groups as presented in Table 1. These groups were based on the pharmaceutical form of the NSAIDs and were intended to expedite the determination of the method DS.

Table 1 Groups of compounds studied in this work. (Bold) submixture used for the validation experiments

Groups	Subgroup	Molecules
Group 1 (Compounds often presented in combination in tablet or capsule)	-	PAR, AA, IBU, DIC, CHL, DEX, NIM, KTO, MA, SAL and CAF
Group 2 (Compounds presented in combination in syrup and suspension)	-	PAR, IBU, NIM, MA, NIP, NIS, BEN, BHA and BHT
Group 3 (NSAIDs found alone in tablet or capsule)	-	IDO, TE, PI, FU, TA, NAP, SUF, PHE, PF and NA
Group 4 (Pharmaceutical combinations presented in tablet or capsule)	1	PAR, AA and CAF
	2	PAR and IBU
	3	PAR and DIC
	4	PAR, DIC and CHL
	5	**PAR, IBU and CAF**
	6	PAR and MA
	7	PAR and DEX
	8	PAR, DEX and CAF
Group 5 (Compounds presented in syrup and suspension)	1	PAR, NIP, NIS, BEN, BHA and BHT
	2	PAR, IBU, NIP, NIS, BEN, BHA and BHT
	3	IBU, NIP, NIS, BEN, BHA and BHT
	4	NIM, NIP, NIS, BEN, BHA and BHT
	5	MA, NIP, NIS, BEN, BHA and BHT

2.2. Standard sample preparation

2.2.1. Mixture preparation groups

1 mg/mL stock solutions of each of the 27 studied materials were prepared in methanol. Mixture solutions were obtained by diluting stock solutions in methanol–water (50:50, v/v) in such a way as to obtain a working concentration of 50 µg/mL for HPLC analyses. Solutions injected into the UHPLC were 10 µg/mL for each material. Aliquots of

these solutions were filtered with 0.20 µm PTFE syringe filtration disks into vials for injection in the HPLC and UHPLC systems.

2.2.2. Solution used for calibration and validation

A stock solution of PAR, IBU, CAF and 4-aminophenol was prepared by dissolving 100 mg of each material in 100 mL methanol. A stock solution of lactose was prepared by dissolving 100 mg of lactose in 100 mL of water (1 mg/mL). Heating and ultrasonic bath were necessary to ensure a complete dissolution.

For the calibration standards (CS), dilutions were performed in methanol–water (50:50, v/v) in order to obtain solutions at concentration levels of 200 µg/mL, 400 µg/mL and 600 µg/mL, except for 4-aminophenol where a dilution was made to obtain a concentration of 0.5 µg/mL (i.e. 0.1% of 500 µg/mL of paracetamol, being the reference concentration of 100%). For PAR, IBU and CAF, three concentration levels were sufficient to generate different regression models for the calibration, while for 4-aminophenol, a one-level calibration was made as advised in the European Pharmacopoeia monograph of paracetamol for the determination of impurities [34].

For validation standards (VS), independent stock solutions of PAR, IBU, CAF, 4-aminophenol were prepared in the same way as described for the CS. For the matrix, the same lactose solution was added into each working solution to obtain an amount of lactose of 4% relative to the amount of IBU. Subsequent dilutions in methanol–water (50:50, v/v) were carried out in order to obtain 5 solutions at different concentration levels (200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL and 600 µg/mL) of PAR, IBU and CAF. For the 4-aminophenol, only one concentration level of 0.5 µg/mL was tested as described previously. The VS were independently prepared in the matrix, simulating as much as possible the formulation and its future routine analysis.

2.3. Instrumentation and chromatographic conditions

The optimization, validation and routine analysis were performed on a HPLC system comprised of a Waters 2695 separation module coupled to a Waters selector valve 7678 and a Waters 996 Photodiode array (PDA) detector (Waters, Eschborn, Germany). The analytical

column was an XBridge C18 (250 mm × 4.6 mm i.d., particle size 5 µm), preceded by a guard column XBridge guard C18 (20 mm × 4.6 mm i.d., particle size 5 µm), both from Waters. The HPLC method was transferred to a UHPLC system Acquity ultra performance liquid chromatography (UPLCTM) system from Waters, comprised of a binary solvent manager, an autosampler with a 10 µL loop, operating in the partial loop with needle overfill injection mode, and a PDA detector. The UHPLC system was equipped with an Acquity BEH C18 column (50 mm × 2.1 mm i.d., particle size 1.7 µm) from Waters. XBridge and Acquity BEH columns are made with same stationary phase chemistry, providing an equivalent selectivity allowing for a geometrical transfer. The analytes were monitored photometrically at 220 nm while chromatographic data were recorded from 210 to 400 nm for all the studied conditions. For the HPLC system, the injection volume was 10 µL and the mobile phase flow rate was 1 mL/min. For the UHPLC system, the injection volume and the mobile phase flow rate were reduced geometrically to 2 µL and 613 µL/min, respectively. After each injection, the HPLC system was reconditioned for 30 min and the UHPLC system for 2 min.

The buffer solutions consisted of 20 mM ammonium formate, except for pH higher than 5 for which 20 mM ammonium hydrogen carbonate was used. The pH was adjusted with hydrochloric acid and ammonium hydroxide except for pH 1.85 where a solution of 0.1% trifluoroacetic acid was used.

2.4. Software

Empower 2.0 for Windows was used to control the HPLC and the Acquity UPLC^{TM} systems, and to record and interpret the chromatograms.

An algorithm was set up to model and to compute the DS. The algorithm was written in R 2.13, which is available as free-ware for most operating systems [35].

HPLC calculator v3.0 was used to carry out the necessary computations to identify the UHPLC conditions from the HPLC conditions using gradient geometric transfer methodology [36,37].

The accuracy profiles as well as the statistical calculations including the validation results and uncertainty estimates were obtained using e-noval® V3.0 software (Arlenda, Belgium).

3. Results and discussions

3.1. Design of experiments

For the optimization of HPLC conditions, an augmented central composite design was generated using the following factors: the Ph of the aqueous part of mobile phase (pH), the gradient time needed to linearly modify the proportion of methanol from 15% to 95% of methanol (TG), and the column temperature (Temp). Experiment at the center of the experimental domain (i.e. at pH = 4.43, TG = 40 min and Temp = 27.5 ◦C) was repeated trice, including the preparation of new buffer solutions. Factors values are presented in Table 2. The data augmentation consisted of the addition of 8 vertices of the cuboid domain and of 4 × 2 intermediate support points to obtain better estimates of the pH effect (red) at the central levels of TG (green) and Temp (blue), leading to 32 experimental conditions.

An isocratic elution step with 95% methanol for 10 min was applied after the gradient to ensure the elution of all the tested molecules. For each experimental run, the three retention times of each peak (apex, begin and end) were recorded. When coelutions prevented the data treatment to be carried out properly, an independent component analysis (ICA) was used to accurately determine the retention times of coeluted peaks [38]. Furthermore, individual injections were made when strong coelutions of many compounds with too similar UV spectra did not allow peak identification and tracking.

Table 2 Factors and corresponding levels of the augmented central composite design.

Factors	**Levels**						
pH	1.85	2.42	3.14	4.42	5.71	6.42	7
Gradient time (TG, min)	-	20	24.5	40	55.5	60	-
Temperature (Temp, °C)	-	20	21.7	27.5	33.3	35	-

3.2. Model

The retention times at the beginning ($\mathbf{t}_{B,p}$), at the apex ($\mathbf{t}_{R,p}$) and at the end ($\mathbf{t}_{B,p}$) of every pth peak (p = 1,. . .,P) result in 3P vectors of N data for the N chromatograms resulting from the designed experiment. Next, the logarithms of defined normalized retention times are taken as modeled responses:

$$k_{B,P} = \log\left(\frac{t_{B,P} - t_0}{t_0}\right),\ k_{R,P} = \log\left(\frac{t_{R,P} - t_0}{t_0}\right),\ k_{E,P} = \log\left(\frac{t_{E,P} - t_0}{t_0}\right)$$

where t_0 is the dead time of the LC system.

This defines $\mathbf{Y} = (\mathbf{k}_{B,1}, \mathbf{k}_{R,1}, \mathbf{k}_{E,1},. . .,\mathbf{k}_{B,P}, \mathbf{k}_{R,P}, \mathbf{k}_{E,P})$, the ($N \times 3P$) matrix containing the responses that are modeled jointly using a multivariate regression model:

$$Y = XB + E$$

where the vector $\boldsymbol{\varepsilon}_n$, the nth line of $\mathbf{E}$ is assumed independent and identically distributed as multivariate Normal, i.e. $\boldsymbol{\varepsilon}_n \sim N(0,\Sigma)$, n = 1,. . .,$N$. $\mathbf{X}$ is the ($N \times F$) centered and reduced design matrix containing the F effects to be included in the model (see later) and B is the ($N \times 3P$) matrix containing the F parameters for each of the 3P responses. The modeled effects in $\mathbf{X}$ are then similar for every response and should be chosen according to the particular experimental design. $\boldsymbol{\Sigma}$ is the covariance matrix of the residuals. Responses from the experimental data were modeled using the following multivariate linear model

$$Y = XB + E\ ,$$

$$\boldsymbol{y}\mathrm{i} = \beta_{0j} + \beta_{1j}.\mathrm{pH} + \beta_{2j}.\mathrm{pH}^2 + \beta_{3j}.\mathrm{pH}^3 + \beta_{4j}.\mathrm{pH}^4 + \beta_{5j}.\mathrm{TG} + \beta_{6J}.\mathrm{TG}^2 + \beta_{7j}.\mathrm{Temp} + \beta_{8j}.\mathrm{Temp}^2 + \beta_{9j}.\mathrm{pH.TG} + \beta_{10j}.\mathrm{pH.Temp} + \beta_{11j}.\mathrm{Temp.TG} + \beta_{12j}.\mathrm{pH.Temp.TG} + \mathrm{Ɛ}_j$$

where y_j is the j^{th} column of $\mathbf{Y}$ (j = 1,…., 3P). As commonly practiced, quantitative factors were centered and scaled to [−1, 1] before being included in $\mathbf{X}$.

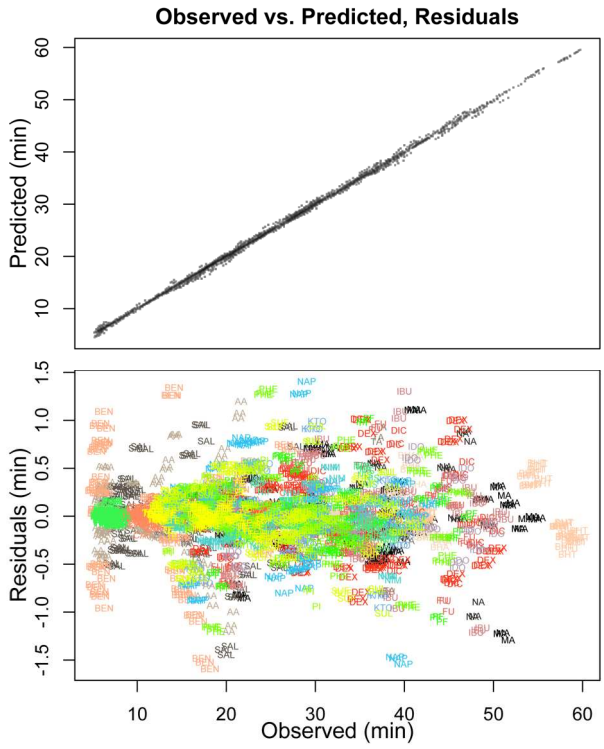

Fig. 1. Modeling results: (top) observed vs. predicted responses and (bottom) residuals

3.3. Model quality

Goodness of fit is evidenced by the relationship between observed and predicted responses, and the corresponding residuals in Fig. 1. As seen on the graph (bottom), the majority of the residuals are distributed in the [−1, 1] min interval and the p-values of the Shapiro–Wilk normality test of residuals are all above 0.05 except for the responses of Phenylbutazone. Given the satisfactory fit of the model, it can be used to predict the chromatographic behavior of each compound and to compute DS.

3.4. Design space

The optimization process was repeated for each of the five groups of materials. For each group, the model was simplified in order to account only for the included materials, with the effect of reducing the size of the response matrix **Y** [39]. The computed Monte-Carlo probability surfaces for $P(S_{crit} > 0)$ for groups 1–3 are presented in Figs. 2–4, respectively. In Fig. 5 showing each subgroup of group 4, the DS consists in the intersection of the individual DSs with the acceptance criteria $P(S_{cri}\ t > 0, t_{tot} < 25)$. Thus a unique optimal condition can be

identified for all the subgroups. Similar computation was depicted in Fig. 6 (group 5) using the joint probability $P(S_{crit} > 0, t < 40$ min) for optimization.

The DS shapes depicted in Figs. 2–6 show broad regions with respect to variations in TG ([40, 60] min for group 1 and 2, [57, 60] min for group 3, [20, 30] min for group 4 and [34, 36] for group 5), in Temp ([20, 35] ◦C for group 1, 2 and 4, [20, 27] ◦C for group 3 and from [23, 30] ◦C for group 5) and pH (from [3.90, 4.30] for group 2 and [1.83, 3.5] for group 4). These broad regions are key results as they represent robustness w.r.t. changes of the operating conditions if quality level is high. Robustness across a wide range of temperature is particularly important in the case of using these methods in laboratories where the control of temperature is difficult. This can be the case for some developing countries. Conversely, three of the developed methods are far less robust with respect to pH ([2.80, 3.10] for group 1, [6.90, 7.00] for group 3 and [6.05, 6.20] for group 5). Fortunately, the relatively poor method robustness with respect to pH should not be problematic since this is easier to control, and care can be taken during the buffer preparation and subsequent pH measurements.

The results of optimal conditions and operating ranges are summarized in Table 3 for each of the 5 groups. For the three factors, the operating range is obtained as the interval in which the probability to achieve a satisfactory quality is higher than the specified quality level л. л is selected for each method in order to allow the identification of a risk-based DS. Except for groups 3, this quality level is generally high, indicating guarantees of quality for future use of the methods.

To support the ability of the DS to predict analytical conditions that permit chromatographic separation for the 5 groups, the different optimal conditions were tested twice (involving the preparation of new buffer solutions) to assess repeatability. Results are presented in Figs. 7–11.

Figs. 7 and 9 illustrate the quality of the predicted optimal condition for the screening methods of groups 1 and 3, respectively. By the same way, Fig. 10 illustrates the prediction quality of a unique method to identify and quantify eight combinations of NSAIDs in tablet. Fig. 11 shows the results of a unique method to analyze five NSAIDs in suspension or syrup,

in the presence of several adjuvants. As can be seen in Figs. 7–11, the chromatograms generated from materials tested under optimal conditions (bottom) are in close agreement with the corresponding predicted chromatograms (top) since the chromatographic peaks are accurately predicted by the uncertainty distribution.

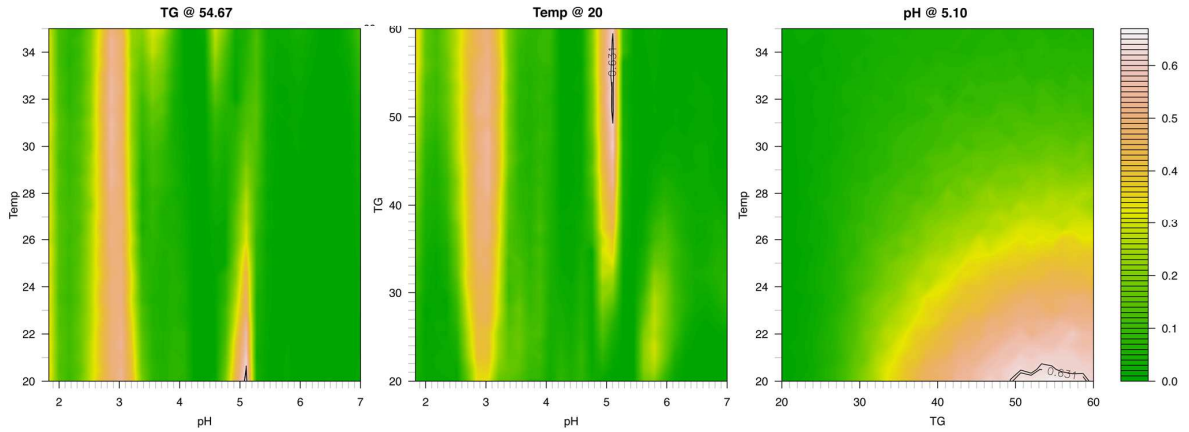

Fig. 2. Probability surfaces $P(S_{crit} > 0)$ for group 1. The DS is located in the white region with minimum quality level of л = 63%. The different colors are related to the probability to reach a separation of 0 min as shown in the legend on right panel. The green color represents the worst design region: the probability to obtain a separation of the critical pair of at least 0 min is the smallest. The white color depicts the best design region where the probability to obtain a separation of the critical pair of at least 0 min is the highest.

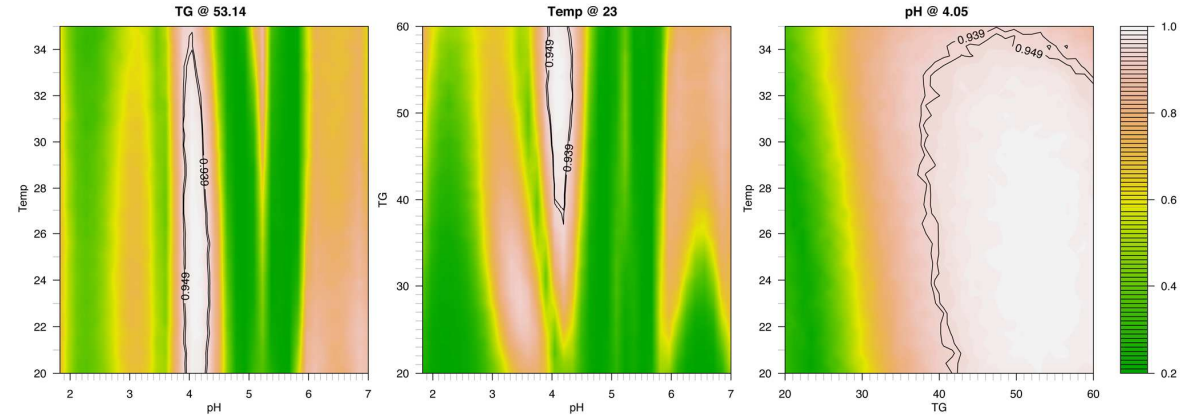

Fig. 3. Probability surfaces $P(S_{crit} > 0)$ for group 2. The DS is located in the white region with minimum quality level of л = 95%. The different colors are related to the probability to reach a separation of 0 min as shown in the legend on right panel. The green color represents the worst design region: the probability to obtain a separation of the critical pair of at least 0 min is the smallest. The white color depicts the best design region where the probability to obtain a separation of the critical pair of at least 0 min is the highest.

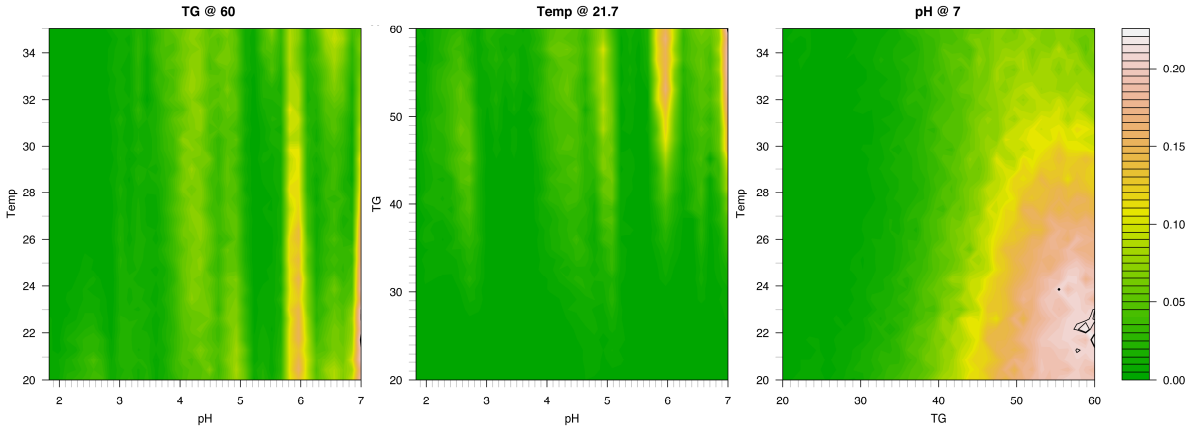

Fig. 4. Probability surfaces $P(S_{crit} > 0)$ for group 3. The DS is located in the white region with minimum quality level of л = 22%. The different colors are related to the probability to reach a separation of 0 min as shown in the legend on right panel. The green color represents the worst design region: the probability to obtain a separation of the critical pair of at least 0 min is the smallest. The white color depicts the best design region where the probability to obtain a separation of the critical pair of at least 0 min is the highest.

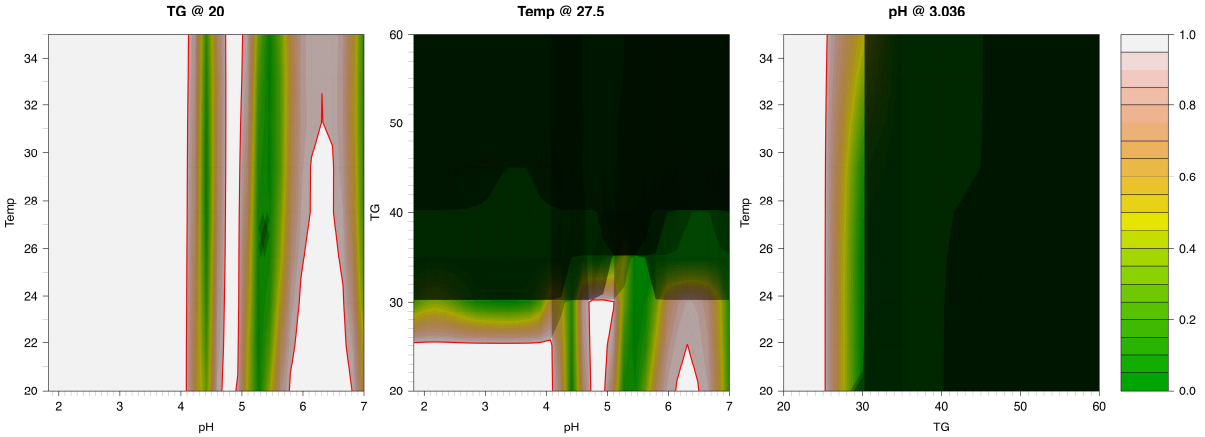

Fig. 5. Design space identification $P(S_{crit} > 0, T_{tot} < 25)$ for group 4. The DS (located in the white region, л = 95%) consists in the intersection of the DS for every submixture. The different colors are related to the probability to simultaneously reach a separation of 0 min and maximum run time of 25 min as shown in the legend on right panel. The green color represents the worst design region: the probability to obtain a separation of the critical pair of at least 0 min and to have a maximum run time of 25 min is the smallest. The white color depicts the best design region where the probability to obtain a separation of the critical pair of at least 0 min and to have a maximum run time of 25 min is the highest.

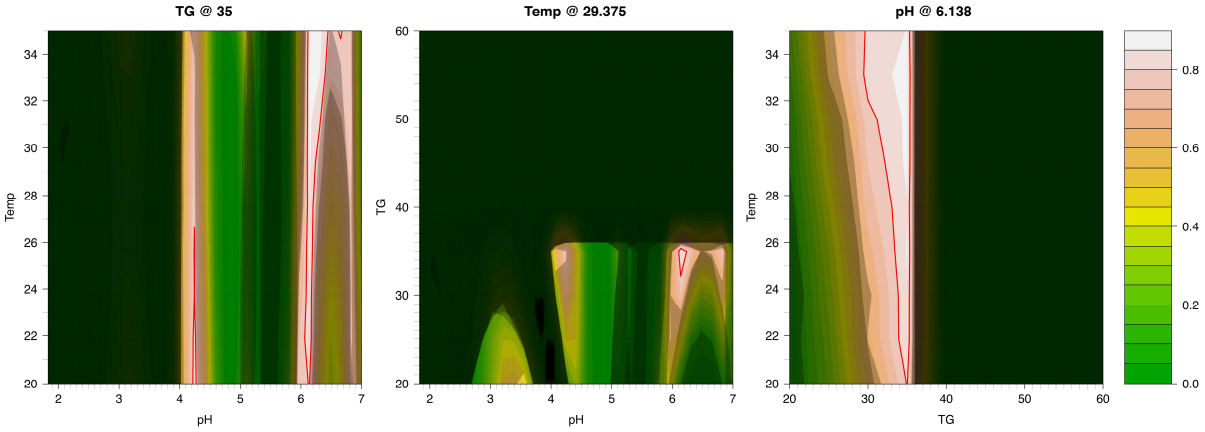

Fig. 6. Design space identification $P(S_{crit} > 0, T_{tot} < 45)$ for group 5. The DS (located in the white region, л = 95%) consists in the intersection of the DS for every submixture. The different colors are related to the probability to simultaneously reach a separation of 0 min and maximum run time of 25 min as shown in the legend on right panel. The green color represents the worst design region: the probability to obtain a separation of the critical pair of at least 0 min and to have a maximum run time of 25 min is the smallest.
The white color depicts the best design region where the probability to obtain a separation of the critical pair of at least 0 min and to have a maximum run time of 25 min is the highest.

Table 3 Optimal conditions and operating range within DS for the separation of the 5 groups of tested molecules

Optimal conditions	**Optimal *P*(S>0)**	**Π**	**pH**	**Gradient time (min)**	**Temperature (° C)**
Group 1	67.0%	0.63	3.05 (2.80-3.10)	49.30 (40.00-60.00)	34.5 (20.0-35.0)
Group 2	~100.0%	0.95	4.05 (3.90-4.30)	53.14 (40.00-60.00)	23.0 (20.0-35.0)
Group 3	23.0%	0.20	7.00 (6.90-7.00)	60.00 (57.00-60.00)	21.7 (20.0-27.0)
Group 4	~100.0%	0.95	3.00 (1.83-3.50)	20.00 (20.00-30.00)	27.0 (20.0-35.0)
Group 5	~100.0%	0.95	6.14 (6.05-6.20)	35.00 (34.00-36.00)	29.4 (23.0-30.0)

These results are corroborated in Table 4. The difference between the predicted and the observed critical separation was always negligible (less than 1 min). Notably, this is consistent with the pattern of residuals (Fig. 1).

4-Aminophenol was not part of the experimental plan. It always elutes close to the column dead time. However, it was tested under conditions of optimal separation for all groups containing paracetamol, and was well separated from other compounds in every mixture and submixture. For instance, its retention time was 3.4 min at the optimal condition of group 4.

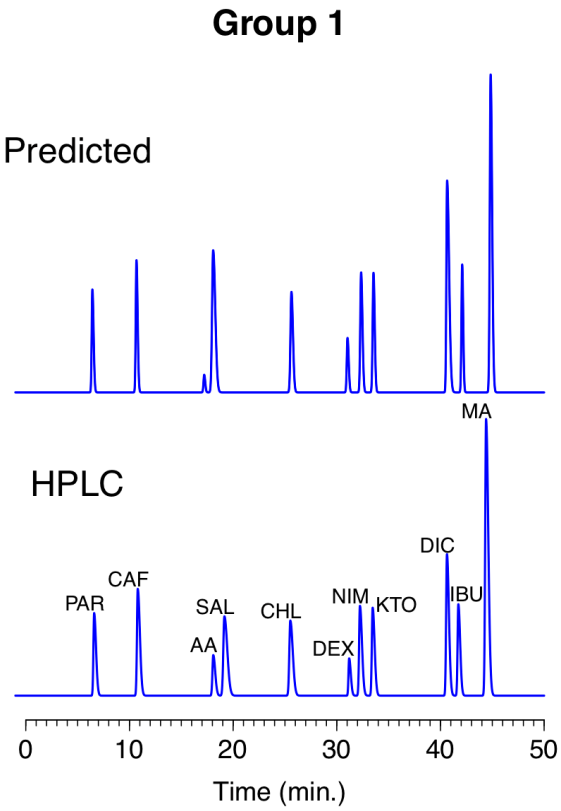

Fig. 7. Optimal condition for group 1: (top) predicted chromatogram and (bottom) observed chromatogram

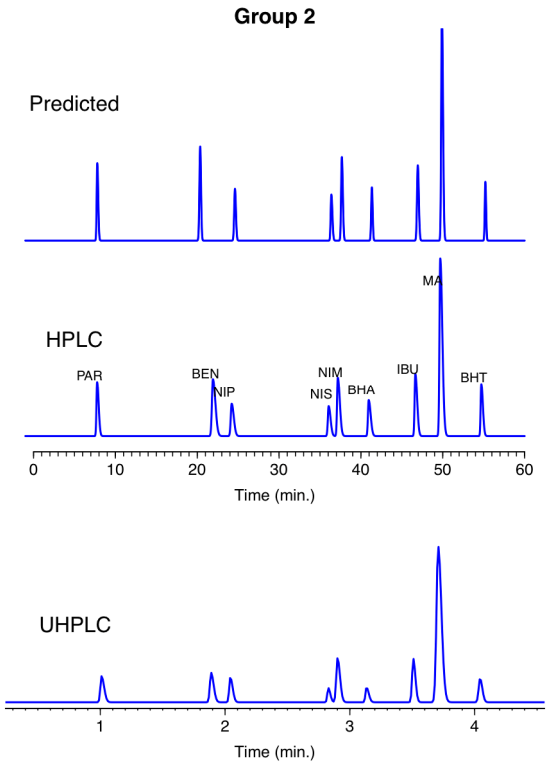

Fig. 8. Optimal condition for group 2: (top) predicted chromatogram, (middle) observed chromatogram (HPLC) and (bottom) observed chromatogram resulting of the transfer to UHPLC.

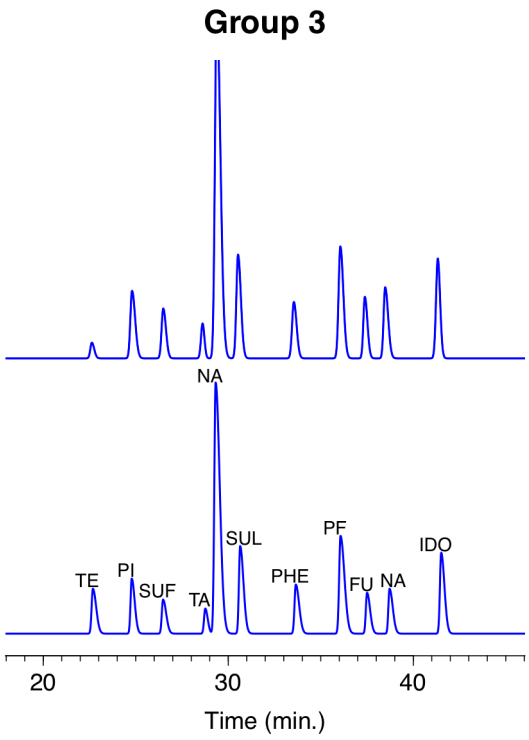

Fig. 9. Optimal condition for group 3: (top) predicted chromatogram and (bottom) observed chromatogram.

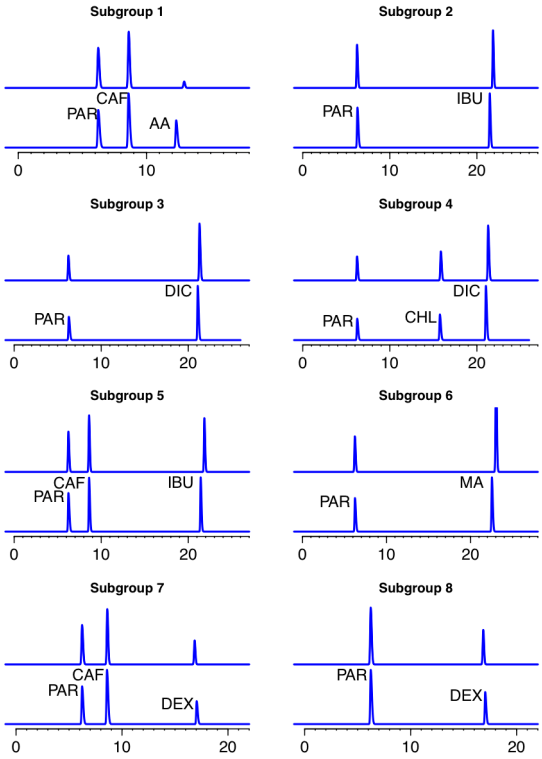

Fig. 10. Optimal condition for the height submixtures of group 4. For each subfigure: (top) predicted chromatogram and (bottom) observed chromatogram.

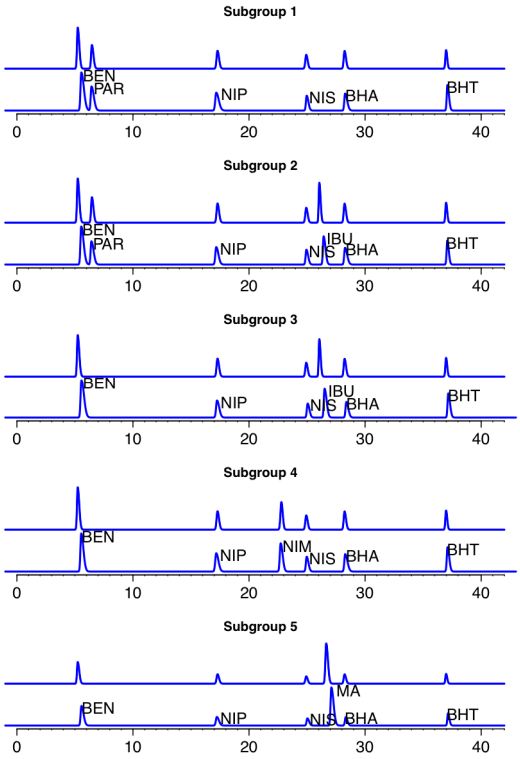

Fig. 11. Optimal condition for the five submixtures of group 5. For each subfigure: (top) predicted chromatogram and (bottom) observed chromatogram.

Table 4 Predicted and observed critical chromatographic separations.

Groups	Subgroup	Predicted Separations (min)	Observed Separations (min)	Error (min)
1	-	0.259	0.516	-0.257
2	-	0.894	0.317	0.577
3	-	0.115	0.016	0.099
4	1	1.946	2.034	-0.088
	2	15.193	15.827	-0.634
	3	14.636	14.475	0.161
	4	9.168	9.175	-0.007
	5	1.946	2.041	-0.095
	6	16.350	15.975	0.375
	7	1.946	2.033	-0.087
	8	10.186	10.483	-0.297
5	1	0.616	0.225	0.391
	2	0.616	0.267	0.349
	3	0.597	1.000	-0.403
	4	1.558	1.734	-0.176
	5	0.923	0.675	0.248

3.5. Transfer

One of the other objectives of the present study was the reduction of analysis time. This has important implications in the identification of substandard or counterfeit medicines since rapid analytical results provide fast decisions about suspected medicines. For this reason the screening method using HPLC (Fig. 8(b)) was transferred to UHPLC (Fig. 8(c)) following geometric transfer methodology [36, 37].

Analytical conditions were similar on HPLC and UHPLC, except those described in Section 3.3 and the gradient time, which was set to 3.52 min. As illustrated in Fig. 8, the

methods yielded very similar optimal separation. This is associated with a 15-fold reduction in analysis time from screening method, with 25 times less consumption of mobile phase. Because the UHPLC column geometry has been chosen to maximize the reduction of analysis time, a slight loss in peak capacity was observed as predicted by the chromatographic theory. When transferring from HPLC to UHPLC, peak capacity reduced by a factor of 0.62, 0.68 and 0.87 for group 1, group 2 and group 3, respectively. The peak capacity for group 3 is overestimated due to the coelution of NAP and TA leading to an underestimation of peaks width. Detailed results are presented in Table 5. Relative retention times were used to compare elution performance of the two LC systems. This was achieved by dividing every retention time by t_{tot}. Relative predicted and observed retention times were close. The difference obtained between the HPLC relative retention times and the UHPLC ones never exceeded 0.01 (Table 5). Similar results were obtained for the other groups of molecules, confirming the adequate geometric transfer of the methods from HPLC to UHPLC. Moreover, these latter results clearly show that the variability induce by the transfer has not overly degraded the chromatographic separation. Thus, it also permitted the demonstration of the high robustness of the developed methods by means of the proposed DS optimization strategy.

Table 5 Results of the transfer from HPLC to UHPLC. (Columns 2–5) predicted retention times (Pred Rt), predictive intervals (Pred Int) and observed (Obs) retention times for HPLC experiments. (Columns 6–9) relative (rel) predicted and observed retention times from HPLC experiments. (Columns 10–11) observed and relative observed retention times for UHPLC experiments. (Bold) comparison of relative retention times.

Compound	Pred Rt	Lower Pred Int	Upper Pred Int	Obs Rt	Pred Rt (rel)	Lower Pred Int (rel)	Upper Pred Int (rel)	Obs Rt (rel)	Obs Rt UPLC	Obs Rt UPLC (rel)	Obs Rt (rel) Error
PAR	7.79	7.67	7.91	8.01	**0.141**	0.135	0.139	**0.146**	0.54	**0.150**	**-0.004**
BEN	20.36	17.91	23.01	22.28	**0.369**	0.318	0.410	**0.406**	1.42	**0.396**	**0.010**
NIP	24.61	24.26	24.98	24.54	**0.446**	0.434	0.447	**0.447**	1.57	**0.438**	**0.009**
NIS	36.39	36.06	36.71	36.36	**0.659**	0.649	0.661	**0.662**	2.35	**0.657**	**0.005**
NIM	37.67	37.15	38.21	37.45	**0.682**	0.668	0.687	**0.682**	2.43	**0.680**	**0.002**
BHA	41.34	40.98	41.70	41.23	**0.749**	0.737	0.751	**0.750**	2.66	**0.743**	**0.007**
IBU	46.95	45.74	48.13	46.92	**0.850**	0.824	0.867	**0.854**	3.04	**0.849**	**0.006**
MA	49.90	48.51	51.11	49.98	**0.904**	0.876	0.923	**0.910**	3.25	**0.908**	**0.002**
BHT	55.21	54.76	55.64	54.95	**1.000**	0.988	1.004	**1.000**	3.58	**1.000**	**0.000**

3.6. Method validation

After the optimization process, it is necessary to demonstrate that an analytical method provides accurate quantification results. This is carried out through a method validation. In this study, a quantitative method for capsules containing PAR, IBU and CAF (HPLC method for group 4, subgroup 5) was validated by applying the concept of total error represented by an accuracy profile [40, 41]. As the capsules contain PAR, a quantitative method for 4-aminophenol impurity was developed concurrently. According to the European Medicines Agency, a formulation is declared compliant if its active molecules are within 5% of the nominal content [42]. The accuracy profiles were used to assess the ability of the analytical methods to accurately quantify these three active ingredients, with acceptance limits that were set at 5% of the targeted concentration of the analytes (i.e. 5% relative total error is tolerated). The objective is thus to establish the dosing range in which the method is providing accurate results. To adequately estimate the total error of the quantitative methods under investigation and to mimic routine use of the method, three independent replicates were made for each

concentration level. The process was also repeated independently during three days to estimate intermediate precision.

When a method is validated using the total error approach, and following the accuracy profile methodology, the validation parameters designated in ICH Q2 (precision, accuracy, and linearity) are simultaneously combined to define a concentration range over which there is high probability to obtain future analytical results within the predefined acceptance limits [43–45]. Accuracy profiles are presented in Fig. 12. For PAR, IBU and CAF, a simple linear regression model was determined to be suitable for calibration. For 4-aminophenol, a one-level calibration was found appropriate. Individual validation parameters are presented in Table 6.

Table 6 Summary of the validation criteria for PAR, IBU, CAF and 4-aminophenol.

Validation criteria	**Conc. (µg/mL)**	**PAR**	**IBU**	**CAF**	**4-aminophenol**
Trueness:	0.50				<0.01 (0.21)
Absolute bias (µg/mL)	200	1.72 (0.86)	2.06 (1.03)	-0.71 (-0.36)	
(Relative bias (%))	300	1.30 (0.44)	-0.29 (-0.09)	-3.01 (-1.01)	
	400	-0.71 (-0.18)	-1.15 (-0.29)	-3.64 (-0.91)	
	500	3.10 (0.62)	1.46 (0.29)	-0.44 (-0.09)	
	600	1.27 (0.21)	-1.32 (-0.22)	-3.65 (-0.61)	
Precision:	0.50				1.19 / 1.35
Repeatability (%) /	200	0.20 / 0.36	0.18 / 0.67	0.36 / 0.59	
Intermediate precision (%)	300	0.16 / 0.33	0.35 / 0.35	0.75 / 0.75	
	400	0.17 / 0.40	0.38 / 0.55	1.08 / 1.08	
	500	0.16 / 0.55	0.32 / 0.63	1.21 /1.21	
	600	0.26 / 0.48	0.50 / 0.56	1.16 / 1.16	
Accuracy:	0.50				0.47-0.51
95% β-expect. tol. int. (µg/mL)					(-3.34-3.75)
(Rel. 95% β-expect. tol. int (%))	200	198.3−203.2 (-0.37−2.09)	195.0−207.1 (-2.02−4.09)	194.4−202.2 (-2.32−1.62)	
	300	296.2−303.4 (-0.77−1.64)	295.7−300.8 (-0.95−0.76)	290.0−301.0 (-2.84−0.83)	
	400	391.2−403.4 (-1.72−1.37)	390.1−403.6 (-1.98−1.40)	383.9−404.8 (-3.55−1.72)	
	500	488.4−512.8 (-1.83−3.08)	487.2−510.7 (-2.07−2.65)	482.4−511.8 (-3.04−2.87)	
	600	587.9−608.7 (-1.53−1.95)	587.1−604.3 (-1.66−1.22)	576.4−610.3 (-3.45−2.22)	
Linearity :	Slope	1.001	1.001	1.002	
	Intercept	0.979	-1.555	-4.071	
	R^2	0.9998	0.9997	0.999	

3.6.1. Trueness

Trueness is reported as the mean bias observed between the series of measurements and the targeted concentrations. Using the calibration curve of each molecule, the concentrations of the VS were back-calculated and expressed in terms of absolute bias (µg/mL) and relative bias (%) [32,45]. The trueness of the developed methods was satisfactory, while the relative biases were close to 0 and were less than or equal to 1.03%.

3.6.2. Precision

Precision refers to the ability of the method to provide proximate results from multiple measurements of the same samples, under the same analytical conditions. Precision is expressed as relative standard deviation (RSD%), and is reported for repeatability and intermediate precision at each targeted concentration. As shown in Table 6, precision was acceptable. The RSD% values never exceeded 1.21%.

3.6.3. Accuracy

Accuracy was assessed using the 95% β-expectation tolerance interval in order to analyze the closeness of agreement of individual measurements, and the assumed true value of the associated measurement [45]. This combines the uncertainties associated with trueness and precision and is expressed as measured values and as a percentage of the targeted concentration (Table 6). The methods was found to provide accurate results, as the lower and upper tolerance bounds are included within the acceptance limits for all the targeted concentration levels (Fig. 12), thus assuring that each future result will fall within the acceptance range with a probability of at least 95% [33]. Moreover, the relative 95% β-expectation tolerance intervals are generally within a range of [−3, +3]%.

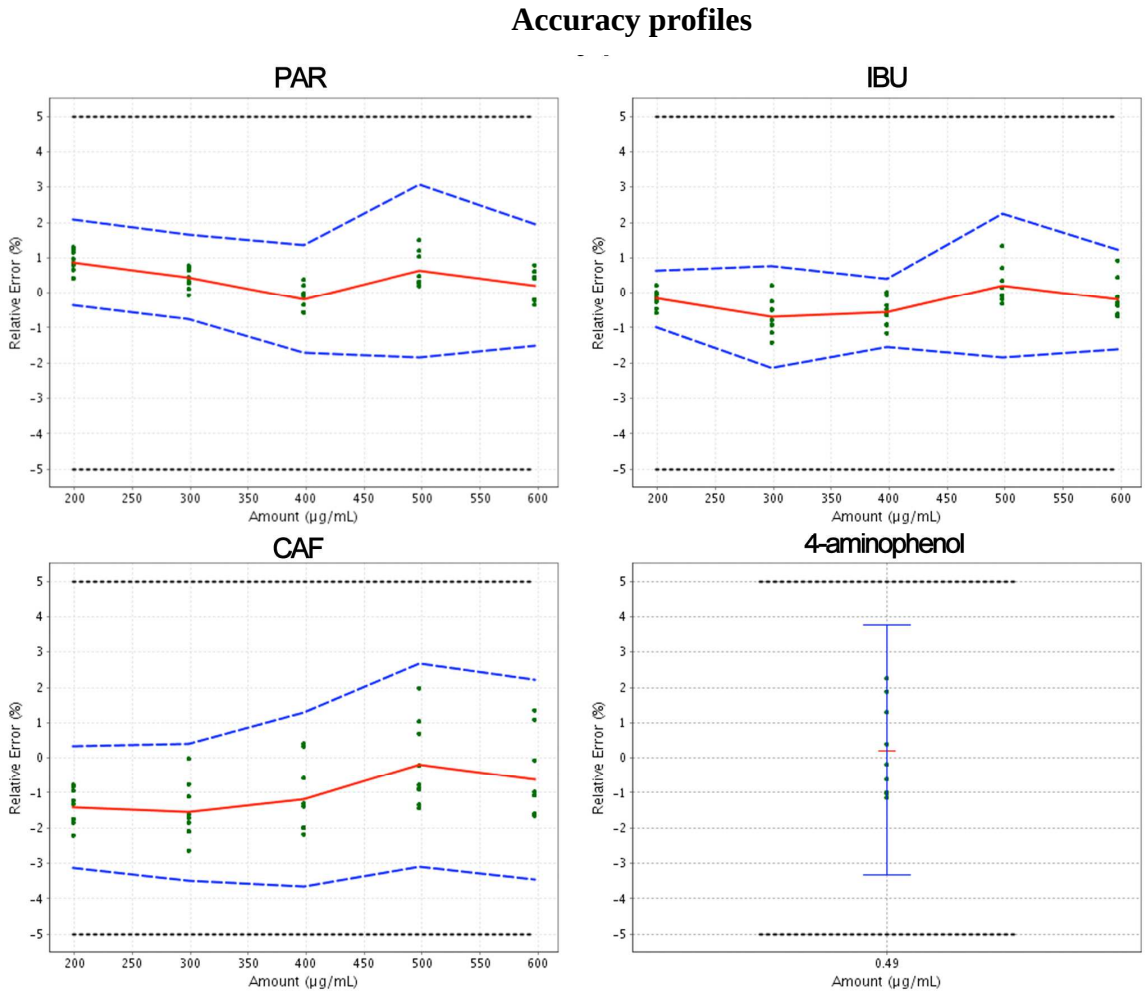

Fig. 12. Accuracy profiles for quantitative methods validation (PAR = paracetamol, IBU = ibuprofen and CAF = caffeine). Continuous line (red) bias (%). Dotted lines (black) acceptance limits (±5%). Dashed lines (blue) 95% β-expectation tolerance intervals. Dots (green) individual measures. For the 4-aminophenol, a one-level calibration is used.

3.6.4. Linearity

The linearity of the results expresses the ability of the methods to produce results directly proportional to the concentrations. A simple regression model was adjusted to the observed vs. targeted concentration results to measure the linearity of the results.

The coefficient of determination (R^2) obtained for the three compounds were all higher than 0.999 thus supporting the adequacy of the linear model adjusted. In addition, the linearity of the results was illustrated by the slopes of these regression models that are close to 1, ranging from 1.001 to 1.002 for all three compounds. This demonstrates the linearity of the

results for the developed method. Finally, for each concentration level of the VS, the 95% β-expectation tolerance intervals were all within ± 5% of the targeted concentration of the analytes studied.

3.7. Application

The validated method was applied to the identification and assay of the three active ingredients (PAR, IBU, and CAF). As an example, five different brands of pharmaceutical drugs coded A, B, C, D and E were tested. These were purchased in capsule form in the DRC and are mainly of Indian origin. Dilutions of the drugs were adapted so that the concentrations fell within the assay ranges.

The five drugs contained the three active ingredients but, as shown in Table 7, most of the products were in one way or another non-compliant. For example, product A had a declared nominal amount of caffeine equal to 30 mg, while only 90% of this amount was measured. For product B, the measured amount of caffeine was about 95% of the claimed nominal amount of 40 mg. From the low number of experiments carried out, this product is however considered non compliant. Finally, products C, D and E were not compliant since the contents of the 3 active ingredients were below the acceptance criteria.

The determination of the impurity of PAR (i.e. 4-aminophenol) was also established because of its potential toxicity. The European Pharmacopoeia places a limit of no more than 0.005% of 4-aminophenol in 100% paracetamol raw material [34]. With pharmaceutical formulations, slightly higher concentrations might be tolerated due to the possible natural degradation of PAR during the manufacturing process. However, to our knowledge, no precise specification exists. The method for 4-aminophenol was determined to be valid to quantify as low as 0.1% of 4-aminophenol (0.5 µg/mL) in 100% paracetamol (500 µg/mL); however further experiments have shown that the limit of detection for 4-aminophenol is about 0.1 µg/mL. This would represent 0.02% of 4-aminophenol in 100% paracetamol, equivalent to four times the acceptance criterion of the Eur. Pharm.

The presence of 4-aminophenol was investigated in the five tested drugs and was not detected. Therefore, this latter observation seems to show that the low levels of active

ingredients are linked to an insufficient dosing of these medicines rather than poor storage conditions.

Table 7

Assay results of five pharmaceuticals marketed in DRC. Results consist in the mean percentage of claimed nominal content and the standard deviation computed on 3 independent samples. Specifications are set to 95–105% of the claimed nominal content (mg). (Bold) non-compliant results for the tested tablets.

Drug	PAR Content	CAF Content	IBU Content
A	325 mg 98.4 ± 0.41 %	30 mg **90.7 ± 1.49 %**	200 mg 103.7 ± 0.74 %
B	325 mg 100.0 ± 0.35 %	40 mg **94.7 ± 0.63 %**	200 mg 103.0 ± 0.58 %
C	200 mg **90.4 ± 0.22 %**	40 mg **85.2 ± 0.79 %**	400 mg **91.1 ± 0.73 %**
D	325 mg **78.2 ± 0.39 %**	40 mg **74.5 ± 0.44 %**	400 mg **77.9 ± 0.15 %**
E	325 mg **78.9 ± 0.28 %**	40 mg **75.9 ± 0.31 %**	400 mg **80.6 ± 0.35 %**

4. Conclusions

The main objective of this work was to develop generic methods able to trace, screen and determine multiple non-steroidal anti-inflammatory molecules and common associated molecules, in order to help detect the potential counterfeiting of these drugs.

Using an experimental design based on three analytical factors (temperature, pH and gradient time), HPLC methods for five groups of NSAIDs and molecules of interest were developed in an innovative predictive risk-based framework. As an outcome of this original methodology, DSs were identified. This approach had proved to be very helpful to optimize the separations of the tested molecules, allowing, for instance, their further quantification.

The experiments showed that only the pH and gradient time had significant effects on peak separations within the explored experimental domain. The effect of temperature on quality was assessed and found to be limited. This may be due to the narrow range of temperatures investigated, but may suggest that using these methods in laboratories with no or insufficient temperature control is acceptable.

In order to concurrently support the robustness demonstrated using the computed DS and to provide faster analytical methods with less solvent consumption, geometric transfer was used to adapt the optimized HPLC method to a UHPLC system. Faster methods are critical for laboratories involved in the control of drugs and counterfeits due to the increasing demands of analysis by legal authorities.

As an example of validation and application of their use in routine testing, a selected method was used for the determination of paracetamol, ibuprofen, caffeine and one impurity of paracetamol (4-aminophenol). The method was successfully validated using the total error approach and accuracy profile methodology. Finally, the method was effectively applied to analyze 5 brands pharmaceuticals marketed in the Democratic Republic of Congo. On the basis of the dramatic results obtained, it was confirmed that substandard and counterfeit medicines remain a crucial problem on public health in low-income countries.

Acknowledgments

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Section III. 2

Développement de méthodes de criblage des antibiotiques par chromatographie liquide

Cette section se rapporte à l'article «**Application of an innovative design space optimization strategy to the development of LC methods for the simultaneous screening of antibiotics to combat poor quality medicines**» publié dans le Journal of Pharmaceutical and Biomedical Analysis 85 (2013) 83-92.

Section III.2. Technique séparative - Antibiotiques

Contexte

Les antibiotiques sont des médicaments utilisés pour lutter contre les infections bactériennes. Ces dernières sont au cœur de la santé et sont parmi les causes les plus importantes de mortalité en RDC. Les antibiotiques sont des médicaments vitaux et sont en tête de liste des médicaments essentiels. Par conséquent, le Ministère de la Santé Publique devrait favoriser l'accès à ces produits et ce dans toute l'étendue du pays. Signalons que dans les faits ce n'est pas souvent le cas. La difficulté voir l'impossibilité d'accès, le coût élevé et le manque d'ordre dans le secteur pharmaceutique en générale sont à la base d'automédication. A cela s'ajoutent la prescription médicale par des personnels non formés ou peu formés et la commercialisation des antibiotiques de qualité inférieure. Ces dysfonctionnements amoindrissent davantage le succès d'antibiothérapie tout en favorisant l'émergence des souches résistantes. Les antibiotiques notamment l'amoxicilline sont également concernés par l'Arrêté Ministériel mentionné dans la section III.1.

Il est dès lors important d'appuyer les autorités de régulation du Ministère Congolais de la Santé Publique dans leur mission de garantir la qualité des médicaments. Dans ce contexte, une méthode analytique générique a pu être développée dans cette partie de notre travail pour détecter 19 molécules d'intérêt. La méthode a été optimisée au moyen du plan D-optimal associé à un espace de conception. La même méthode a permis d'analyser 7 associations pharmaceutiques d'antibiotiques commercialisées en RDC. La méthode de dosage de l'association céftriaxone et sulbactam (poudre pour injection) a été validée et ensuite appliquée pour connaitre la teneur de ces 2 molécules dans 3 médicaments commercialisés à Kinshasa. Comme pour les anti-inflammatoires non stéroïdiens, les résultats de dosage sont très interpellants.

Section III.2. Technique séparative - Antibiotiques

Summary

The poor quality of medicines is a crucial problem of public health. Therefore, it is important to have analytical tools to attend decisions of the legal authorities while combating this offense. In this context, the main objective of this study was to develop generic methods able to trace, screen and determine several antibiotics and common associated molecules by mean of liquid chromatographic techniques. For that purpose, an innovative Design Space optimization strategy was applied, targeting 16 antibiotics and 3 beta-lactamase inhibitors. The robustness of the developed method allowed using its use in an environment where operational factors such as temperature are not easy to control and eased its transfer to Ultra High Performance Liquid Chromatography. To demonstrate its ability to quantify the targeted molecules, the developed and transferred method was fully validated for two active ingredients commonly used in association, sulbactam and ceftriaxone, using the accuracy profile as decision tool. Based on this successful step, the method was then used for the quantitative determination of these two active ingredients in three pharmaceutical brands marketed in the Democratic Republic of Congo. Two out of the three pharmaceutical products did not comply with the specifications.

Section III.2. Technique séparative - Antibiotiques

1. Introduction

The manufacture and the sale of poor quality medicines increase worldwide, causing serious consequences to the public health and to the socio-economy. Although, precise and detailed data on such medicines is not easy to obtain, one can find information of their trade that is ranging from 1% in the developed countries to over 10% in the developing countries, depending on the geographical area and on the period of survey [1,2]. Poor quality medicines can be classified into three main categories: counterfeit (falsifying with an intention to avoid the right of intellectual property), substandard (poor quality control during manufacture due mainly to neglecting without any intention) and degraded (chemical and biological instabilities especially in tropical climates) [3].

Counterfeiters are very active in developing countries where medicines are largely used such as antibiotics and antiparasitics [4]. According to the literature, a wide range of antibacterial agents have been found to be substandard or counterfeit [4]. Although nopart of the world is exempted, Southeast Asia and Africa seem to be particularly plagued by poor quality antibacterial agents [4–12].

Some causes of the large diffusion of pharmaceutical counterfeiting in developing countries are lack of controls at importation and insufficient quality control of medicinal products at different levels of the distribution chain including import, wholesalers, official and informal vendors [1]. To ensure the quality of medicines and contribute in fighting against poor quality medicines, the development of screening analytical methods that can simultaneously trace several of the most commonly used molecules is an essential analytical strategy [13]. In this context, the separative technique stays as one of the best options to analyze simultaneously several molecules. Over the last decade, several liquid chromatographic (LC) methods were developed and published for the concurrent screening of potentially counterfeit medicines [1,13–17]. However, none of these includes an exhaustive list of the antibiotics molecules, limiting their use when screening complex or unknown mixture of this pharmacological group. For instance, M.C. Gaudiano et al. (2008), optimized a LC method for the separation of six antibiotics regardless the major pharmaceutical products such as ceftriaxone, ciprofloxacin, etc., or other main molecules often associated to

betalactams such as sulbactam, tazobactam, etc. [1]. In the present study, we focused on LC techniques targeting a subset of 19 molecules that are marketed as single or combined antibiotics. Thus, our objective was to optimize the separation conditions for 19 of these molecules among which 16 are antibiotics: amoxicillin (AMO), ampicillin (AMP), cefadroxil (CFA), cefotaxime(CFO), ceftriaxone (CFT), chloramphenicol (CHL), ciprofloxacin(CIP), clindamycin (CLI), doxycycline (DOX), levofloxacin (LEV),metronidazole (MET), norfloxacin (NOR), phenoxymethylpenicillium (PENI-V), sulfamethoxazole (SLF), tetracycline (TET) andtrimethoprim (TRI). The remaining molecules, clavulanic acid(CLA), sulbactam (SUL) and tazobactam (TAZ) are beta-lactamase inhibitors, often associated with β-lactams antibiotics.

Nowadays, LC method development can be achieved using different methodologies. In this study, a distinct and innovative methodology combining design of experiments (DoE) and design space (DS) as suggested in ICH Q8(R2) [18,19] was exploited to simultaneously optimize the separation and evaluate the method robustness over the examined experimental domain (i.e. the knowledge space).

As a second objective, the HPLC method developed was transferred to Ultra High Performance Liquid Chromatography (UHPLC) by means of a geometric transfer in order to verify that the DoE-DS strategy can ease the development of robust fast analytical methods.

The third objective was to validate the transferred UHPLC method using the accuracy profile as decision tool for the determination of the tested compounds [20,21]. For that purpose, an antibiotic association containing ceftriaxone and sulbactam powders for injection (intramuscular and intravenous) and marketed in some African countries was used.

Finally, the validated method was used to analyze several drugs often targeted by counterfeit marketed in the Democratic Republic of Congo (DRC).

2. Experimental

2.1. Materials

Cefotaxime (94.3%), ceftriaxone (93.9%), clavulanic acid (43.3%, a mixture of potassium clavulanate and microcrystalline cellulose as excipient (1:1)), levofloxacin (99.0%), norfloxacin (99.1%), sulbactam (91.5%) and tazobactam (99.2%) were purchased from Molekula Limited (Dorset, UK). Amoxicilline (99.1%), ciprofloxacin (>98%), clindamycine (95.8%), doxycycline (97.6%), metronidazole (99.9%), penicillin-V (100.2%), sulfamethoxazole (99.9%), tetracycline (96.6%) and trimethoprime (99.2%) were purchased from Fagron N.V. (Waregem, Belgium). Ammonium acetate (98.0%), ammonium hydroxide (32%), hydrochloric acid (37%), methanol (HPLC gradient grade) and sodium chloride (>99.5%) were purchased from Merck (Darmstadt, Germany). Ammonium formate (98.1%) and ammonium hydrogen carbonate (97.5%) were purchased from BDH Prolabo (Almere, The Netherlands). Ampicillin (98.0%) was purchased from Applichem Biochemica (Darmstadt, Germany). Cefadroxil (97.0%) was purchased from DR. Ehrenstorfer Gmbh (Augsbourg, Germany). Chloramphenicol (99.2%) was purchased from N.V LEPETIT BELGILA S.A (Brussels, Belgium). Ultrapure water was obtained from a Milli-Q Plus 185 water purification system (Millipore, Billerica, MA, USA). For the preparation of validation standards validations tandards, a matrix formulation of powder for injection containing 1000 mg of ceftriaxone, 500 mg of sulbactam and 170 mg of sodium chloride was provided by an Indian manufacturing laboratory legally authorized in the DRC.

2.2. Standard sample preparation

2.2.1. Mixture preparation groups

Different mixtures of antibiotics were prepared as following: Group 1 (see section 3.1): In a first step, 10 mg of AMO, CFT,MET, SLF, TRI, PENI-V and LEV, 40 mg of CIP, CHL and DOX, 30 mg of SUL and AMP, were dissolved in a 10.0 mL volumetric flask with methanol. This solution was annotated S1. In a second step, 70 mg of CLI were dissolved in a 10.0 mL volumetric flask with 1 mL of solution S1 and with methanol that was used to complete to volume. This last antibiotic was used at higher concentration level due to its weak absorptivity in the UV range. The final solution obtained was diluted twice (2.5 mL/5.0 mL) in a mixture of water and methanol (92%/8%, v/v) prior to injection at the HPLC system, and

was diluted tenth (1.0 mL/10.0 mL) in the same solvent, prior to injection at the UHPLC system. Before analysis, an aliquot of each solution was filtered with 0.20 µm PTFE syringe filtration disks into a vial for injection in the HPLC and UHPLC systems.

Group 2 (see section 3.1): In a first step, 10 mg of TAZ, CFT, MET,CFO, NOR, TET, PENI-V and LEV, 40 mg of CHL and DOX, 30 mg of SUL and AMP, were dissolved in a 10.0 mL volumetric flask with methanol. This solution was annotated S2. In the second step, 70 mg of CLI and 40 mg of CLA were dissolved in a 10.0 mL volumetric flask with 1 mL of solution S2 and completed to volume with methanol. Prior to their use to the HPLC and UHPLC systems, the finals solutions were prepared as for group 1, dilution twice and dilution tenth, respectively, followed by filtration with 0.20 µm PTFE syringe filtration disks

Group 3 (see section 3.1) was prepared as group 1, replacing amoxicillin by cefadroxil.

All these solutions were prepared sheltered from light to avoid degradation of light-sensitive antibiotics. The ultrasonic bath was necessary to ensure a complete dissolution.

2.2.2. Solution used for calibration and validation

A stock solution containing CFT and SUL was prepared by dissolving 100 mg and 50 mg, respectively, in 100 mL water. Another stock solution was prepared by dissolving 100 mg of sodium chloride in 100 mL of water.

For the calibration standards (CS), dilutions were performed in water in order to obtain solutions at three concentration levels of 160 µg/mL, 320 µg/mL and 480 µg/mL for CFT and the corresponding concentration levels of 80 µg/mL, 160 µg/mL and 240 µg/mL for SUL.

For validation standards (VS), independent stock solutions of CFT and SUL were prepared in the same way as described for the CS. For the matrix, the same sodium chloride solution was added into each working solution to obtain an amount of sodium chloride of 17% relative to the amount of CFT. Subsequent dilutions in water were carried out in order to obtain solutions at five different concentration levels namely 160 µg/mL, 240 µg/mL, 320 µg/mL, 400 µg/mL and 480 µg/mL of CFT, and 80 µg/mL, 120 µg/mL,160 µg/mL, 200

µg/mL and 240 µg/mL of SUL. The VS were independently prepared in the matrix, in such a way to simulate as much as possible the corresponding antibiotic formulation and its routine analysis.

2.3. Instrumentation and chromatographic conditions

The optimization was performed on a HPLC system comprised of a Waters 2695 separation module coupled to a Waters selector valve 7678 and a Waters 996 Photodiode array (PDA) detector (Waters, Eschborn, Germany). The analytical column was an XBridge C18 (250 mm × 4.6 mm i.d., particle size 5 µm), preceded by a guard column XBridge guard C18 (20 mm × 4.6 mm i.d., particle size 5 µm), both from Waters. The transfer of HPLC method, validation and routine analysis were performed on an UHPLC system Acquity ultra performance liquid chromatography™ (UPLC™) system from Waters, comprised of a binary solvent manager, an autosampler with a 10 µL loop operating in the partial loop with needle overfill injection mode, and a PDA detector. The UHPLC system was equipped with an Acquity BEH C18 column (50 mm × 2.1 mm i.d., particle size 1.7 µm) from Waters. XBridge and Acquity BEH columns are made with the same stationary phase chemistry, providing an identical selectivity and allowing the geometric transfer of method.

The analytes were monitored photometrically at a wavelength of 220 nm while chromatographic data were recorded from 210 to 400 nm for all the studied experimental conditions. For the HPLC system, the injection volume was 10 µL and the mobile phase flow rate was 1 mL/min. For the UHPLC system, these instrumentation parameters were reduced geometrically to 2 µL and 613 µL/min, respectively. After each injection, the HPLC system was reconditioned for 30 min and the UHPLC system for 2 min. HPLC column dead time was estimated at 3.05 min.

Measurements of pH were performed with a SevenEasy S20 pHmeter (Mettler Toledo, Columbus, OH, USA). The tested buffer solutions of the mobile phase consisted of 10 mM ammonium formate (pKa 3.8), 10 mM ammonium acetate (pKa 4.8) or 10 mM ammonium hydrogen carbonate (pKa 6.4), according to pH value at the buffer. The pH was adjusted with hydrochloric acid or ammonium hydroxide depending on the targeted value.

2.4. Software

Empower 2.0 for Windows was used to control the HPLC and the Acquity UPLC™ systems, to record the signals from the detector and interpret the generated chromatograms.

An algorithm was set up to develop a Bayesian model and to compute the DS using Monte-Carlo simulations. The algorithm was written in R 2.13, which is available as free-ware for most operating systems [22].

HPLC calculator v3.0 (University of Geneva, Switzerland) was used to carry out the necessary computations to identify the UHPLC conditions from the HPLC conditions using geometric transfer methodology [23,24].

The accuracy profiles as well as the statistical calculations including the validation results and uncertainty estimates were obtained using e-noval®V3.0 software (Arlenda, Belgium).

3. Results and discussions

3.1. Design of experiments

The experimental conditions for modeling each peak chromatographic behavior were set up by a D-optimal design comprising three factors: pH of the aqueous part of the mobile phase, gradient time (t_G) (to linearly modify the methanol proportion in the mobile phase from 8% to 95%) and the temperature of chromatographic column (T°). To ensure that all the tested molecules will be eluted after an experimental run, an isocratic elution step with 95% methanol and 5% of aqueous buffer solution was applied after the gradient for 10 min. Considering the levels of these factors presented in Table 1, 19 experimental conditions were generated in order to simultaneously optimize the method, estimate its robustness and evaluate the adequacy between theoretical predicted chromatographic results and the obtained mathematical model. To identify an optimal separation zone of the chromatographic peak analytes, the gradient time was tested over a quite large range where as the temperature of the column was varied only between 23°C and 30°C and the pH values only between 2.7 and 7.0 due to the instability of certain antibiotics at high temperature and basic pH value.

Table 1a Factors and corresponding levels of D-optimal design

Factors	**Levels**					
pH	2.7	3.8	4.6	4.9	5.9	7.0
Gradient time (t_G, min)	20		40		60	
Temperature (T°, °C)	23.0		26.5		30.0	

3.2. Modeling and optimization methodology

For each experimental run or experimental condition, the three retention times (apex, begin and end at baseline) of each peak were recorded. The logarithms of normalized retention time termed k (i.e. log (k_{tR}) with $k_{tR}= (t_R - t_0)/t_0$, where t_0 is the column dead time) were used as modeled responses. For the first D-optimal design generated, the responses from the experimental data were modeled by the following multivariate multiple linear model,

$$Y=\beta_o + \beta_1.\text{pH} + \beta_2.\text{pH}^2 + \beta_3.\text{pH}^3 + \beta_4.t_G + \beta_5.t_G^2 + \beta_6.\text{T°} + \beta_7.\text{T°}^2 + \beta_8.\text{pH}.t_G + \beta_9.\text{pH}.T° + \beta_{10}.T°.t_G + \beta_{11}.\text{pH}.T°.t_G + E$$

$$\mathbf{Y= XB + E} \quad (1)$$

with $\boldsymbol{\varepsilon}_n$, the nth line of **E**, assumed to follow a multivariate Normal distribution, $\boldsymbol{\varepsilon}_n \sim N(\mathbf{O}, \boldsymbol{\Sigma})$, n = 1, . . ., *N*, with *N* the number of experiments. **X** is then the ($N \times F$) centered and reduced design matrix and **B** is the ($F \times M$) matrix containing the *F* effects for each of the *M* responses. Regressions with stepwise selection of model parameters have been useful to identify a multivariate model that has the best fitting properties for every response, jointly. **Σ** is the covariance matrix of the residuals. For the second D-optimal design generated, the responses from the experimental data were modeled by the same multivariate multiple linear model used in the case of the first D-optimal but without the term pH^3. These models were estimated in the Bayesian framework using a slightly informative prior distribution for the covariance of responses belonging to the same peak [19]. As commonly practiced, quantitative factors were centered and scaled to [−1, 1] before being included in the model. The goodness of fit is evidenced by the relationship between observed and predicted responses, and corresponding residuals as shown in Fig. 1. In addition, all models were significant (p-value < 0.05) and the adjusted R^2 values were higher than 0.98, thus confirming the adequacy of fit of the models.

As seen on the graph (bottom), the majority of residuals are distributed in [−5,5] min interval which will influence the uncertainty over the prediction of the modeled responses. The residuals also show no systematic trend that confirms the absence of bias of the models fitted

to each response and hence there adequate fit. There was a good relationship between the predicted retention times and those obtained, thus validating the multivariate multiple linear regression model. Given the satisfactory fitness of the model, it can be used to predict the chromatographic behavior of each compound and to compute DS.

The separation between the peaks of the critical pair has been chosen as a critical quality attribute (CQA) for the evaluation of quality chromatogram [13]. Lebrun et al. [13,25] proposed to use the separation criterion (S) defined as the difference between the beginning of the second eluting peak and the end of the first eluting peak of the critical pair. The separation criterion (S_{crit}) is clearly easy to compute and to interpret. If $S_{crit} \geq 0$, the critical peaks pair is baseline-resolved. The probability for S to be higher than a selected specification λ was used to determine the DS [13,25]. According to ICH Q8 (R2), the DS is "the multidimensional combination and interaction of input variables (e.g. material attributes) and process parameters that have been demonstrated to provide assurance of quality" [18]. In mathematical terms, the DS can be defined by Eq. (2):

$$DS = \{x_0 \in \chi : P(CQA > \lambda / x_0, data) \geq \pi\}, \quad (2)$$

where x_0 is a point of the experimental domain χ. λ is set to 0 min, л is the quality level and CQA are some critical quality attributes which is limited in our case to the separation criterion S_{crit} solely. The quality level л has to be understood as the minimum required probability that the S_{crit} is at least 0 min for all compounds simultaneously. This probability depends on the data obtained as well as on the uncertainty of the residuals and of the estimated parameters of the models.

Over the experimental domain, the probability of peak separation as given by Design Space remains low, only 10%, due to the very similar chromatographic behavior of some molecules in the tested experimental domain, which did not allow the separation of all 19 molecules. This was the case of AMO, CFA and TAZ which led us to split each of them in a separate group, as shown in Table 2.

Focusing on the area of DS corresponding to the 10% probability, an alternative DoE was generated to maximize the possibility of optimizing the separation conditions of the three groups of compounds and estimate the method robustness. The new design with the corresponding factors levels that targeted a reduced area of the levels to be tested (pH: 5–7, t_G: 40–60 min and T°: 25–30◦C) are presented in Table 3.

Table 1b Experimental matrix of D-optimal design for the investigation of pH, t_G and T° respectively

Trial	Experimental Design			Experimental set up		
	X_1	X_2	X_3	pH	t_G	$T°$
1	-0.5	0	1	3.8	40	30.0
2	0	0	0	4.9	40	26.5
3	1	-1	1	7.0	20	30.0
4	1	-1	-1	7.0	20	23.0
5	0.5	1	-1	5.9	60	23.0
6	-1	-1	-1	2.7	20	23.0
7	-1	0	0	2.7	40	26.5
8	1	1	0	7.0	60	26.5
9	1	1	1	7.0	60	30.0
10	0.5	0	1	5.9	40	30.0
11	-1	-1	1	2.7	20	30.0
12	-1	1	-1	2.7	60	23.0
13	1	0	-1	7.0	40	23.0
14	-0.1234355	-1	-1	4.6	20	23.0
15	-0.5	1	0	3.8	60	26.5
16	0.5	-1	0	5.9	20	26.5
17	0	0	0	4.9	40	26.5
18	-1	1	1	2.7	60	30.0
19	0	0	0	4.9	40	23.0

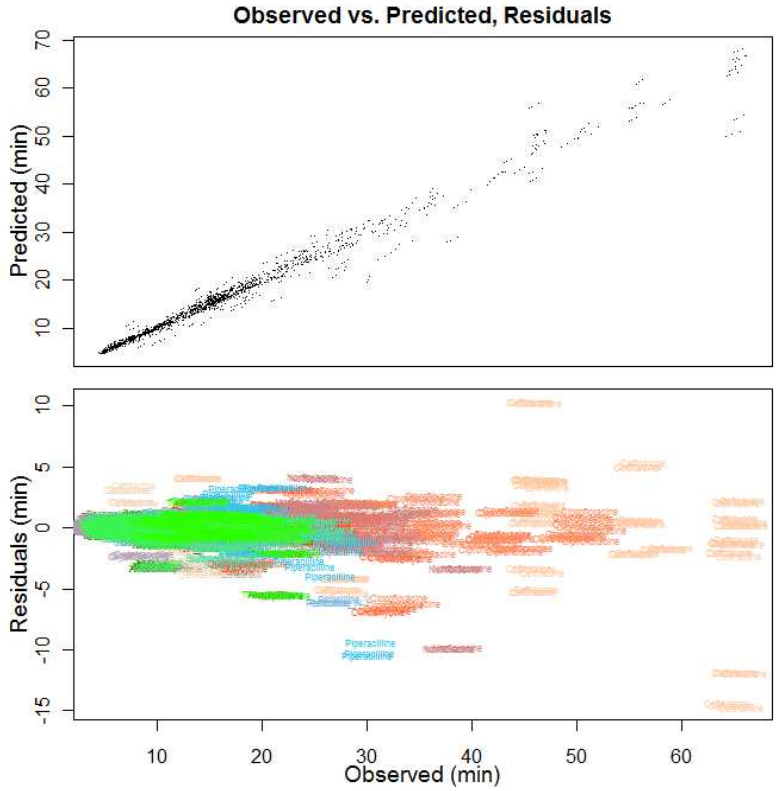

Fig. 1. Modeling results: observed vs predicted responses (Top) and residuals (Bottom). A = Clavulanic acid, B = Amoxicillin, C = Ampicillin, D = Cefadroxil,E = Cefotaxime, F = Ceftriaxone, G = Chloramphenicol, H = Ciprofloxacin, I = Clindamycin, J = Doxycycline, K = Levofloxacin, L = Metronidazole, M = Norfloxacin, N = Tetracycline,O = Phenoxymethylpenicillium, P = Sulbactam, Q = Sulfamethoxazole, R = Tetracycline, S = Tozobactam, T = Trimethoprim.

Table 2 Groups of compounds studied in this work

Groups	**Molecules**
Group 1	AMO, AMP, CFT, CHL, CIP , CLI, DOX, LEV, MET, PENI-V, SLF, SUL and TRI
Group 2	CFT, CFO, CHL, CLA, CLI, DOX, LEV, MET, NOR, PENI-V, SUL, TAZ and TET
Group 3	AMP, CFA, CFT, CHL, CIP, CLI, DOX, LEV, MET, PENI-V, SLF, SUL and TRI

Table 3a Factors and corresponding levels of the new D-optimal design

Factors	**Levels**			
pH	5.0	6.0		7.0
Gradient time (t_G, min)	40.0	50.0	53.2	60.0
Temperature (T°, °C)	25	27.5		30

Table 3b Experimental matrix of the new D-optimal design for the investigation of pH, t_G and T° respectively

Trial	Experimental Design			Experimental set up		
	X_1	X_2	X_3	pH	t_G	$T°$
1	1	1	-1	7.0	60	25.0
2	-1	0	-1	5.0	50	25.0
3	0	-1	0	6.0	40	27.5
4	0	0	0	6.0	50	27.5
5	-1	1	-1	5.0	60	25.0
6	0	0	0	6.0	50	27.5
7	1	0	1	7.0	50	30.0
8	0	0	0	6.0	50	27.5
9	0	0.3	1	6.0	53.2	30.0
10	-1	1	1	5.0	60	30.0
11	1	-1	1	7.0	40	30.0

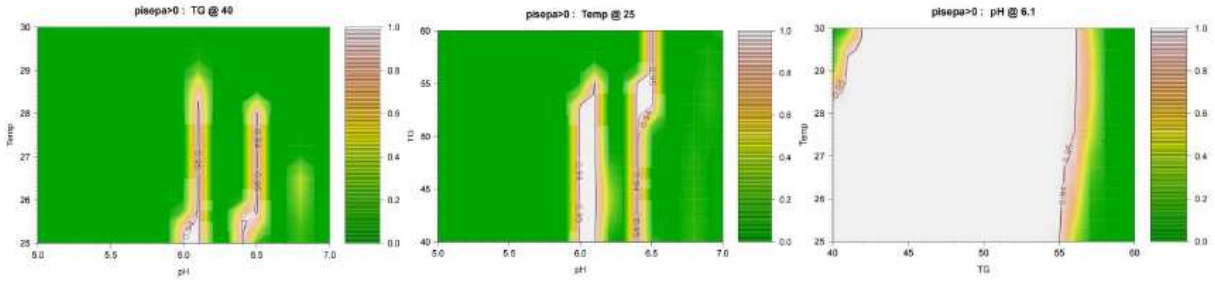

Fig. 2. Probability surfaces P (S_{crit}> 0) or group 1 and 3. The DS is located in the white region with minimum quality level of л = 95%.

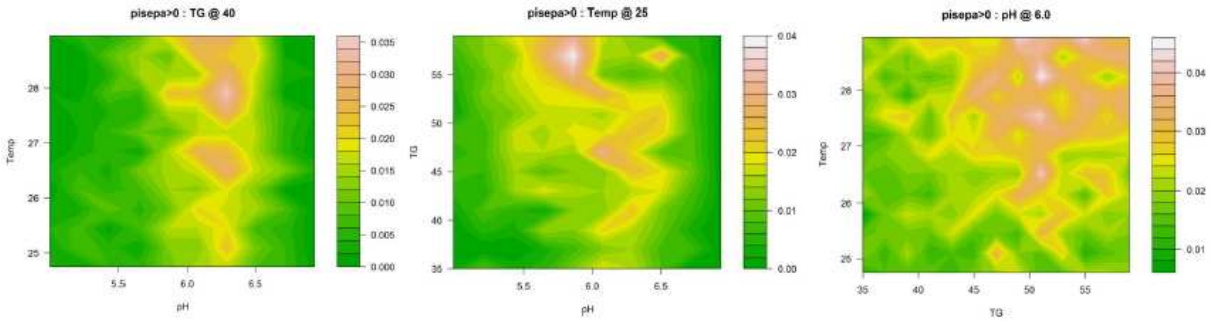

Fig. 3. Probability surfaces P (S_{crit}> 0) for group 2. The DS is located in the white region with minimum quality level of л = 4%.

3.3. Design Space

The computed Monte-Carlo predictive probability surface for $P(S_{crit}> 0|$ data) for groups 1 and 3 are presented in Fig. 2. Fig. 3 shows the probability results for the group 2, at the same optimal conditions obtained for groups 1 and 3.

The high quality DS shape depicted in Fig. 2 shows broad regions with respect to variations in t_G [40,56] min, in T° [25,28]°C and pH [5.90, 6.10], while the DS shown in Fig. 3, provides no greater region for these three factors. The white color depicts the best design region where the probability to obtain a separation of the critical pair of at least 0 min is maximum and the green color represents the worst design region with a low probability of separation. Broad white regions are key results as they represent robustness area with regards to deliberate modifications of the operating conditions. In these white regions, the predicted quality level is high, and hence the modifications of the operational parameters inside them do not influence the responses. These white regions shown in Fig. 2 are the DS and imply robustness of the method. A large temperature robustness area is important for using methods in laboratories without an efficient temperature control, especially in developing countries. The optimal conditions for groups 1 and 3 have high quality level (л = 95%) guaranteeing the reliable future use of the method in routine analyses. Group 2 was also tested in the same optimal conditions; however, the predicted quality level was low (л = 4%), due to the impossibility to found operational conditions to separate CFT and MET. The final optimal conditions are defined by the operating conditions where the probability of having a separation of 0 min is maximum. They are: pH 6.0, t_G 40 min and T° 25°C.

To support the ability of DS to accurately predict analytical conditions that permit chromatographic separation for the analytes of the three groups, the optimal conditions were tested twice, involving the preparation of new buffer solutions. The results presented in Figs. 4–6, illustrate the quality of the predicted optimal conditions for the screening methods of groups 1, 2 and 3, respectively.

Indeed, the retention times of the majority of the peak analytes estimated in the predicted chromatograms were obtained under the optimal conditions in practice. On the other hand, the peak elution order was also as predicted. As observed in Fig. 3, the DS area of group 2 was narrower compared to that in Fig. 2 for groups 1 and 3, confirming the low probability for separation of the molecules of that group, under the optimal conditions. In addition, negative value was reported for the mean predicted separation (see Table 4) mainly due to no separation of CFT and MET (see the predicted chromatogram in Fig. 5).

Nonetheless as mentioned before, the experimental chromatogram of group 2 obtained under the optimal conditions of groups 1 and 3 gives acceptable separation value with observed separation $S \geq 0$ (see Table 5), allowing to screen the compounds of group 2. Even the critical peak pair (CFT and MET) which were not separated in the predicted chromatogram of group 2 are found to be separated in the predicted chromatograms of groups 1 and 3 under the same optimal conditions. This can be explained by the relatively large range of the graph of residuals (see Section 3.2, Fig. 1) that influences the uncertainty of the separation prediction. Other aspects (e.g. chemical structure) may also contribute to influence the uncertainty of the separation prediction, such as chromatographic behavior of specific molecules such as CFT that has several pKa values (3.0; 3.2; 4.1). Indeed, CFT was not well predicted as visualized in Fig. 1.

Table 4 Predicted and observed critical chromatographic separations in optimal conditions

Groups	Predicted Critical Separations (min.)	Observed Separations (min.)	Error (min.)
1	0.320	0.009	0.311
2	-0.686	0.033	-0.719
3	0.070	0.008	0.062

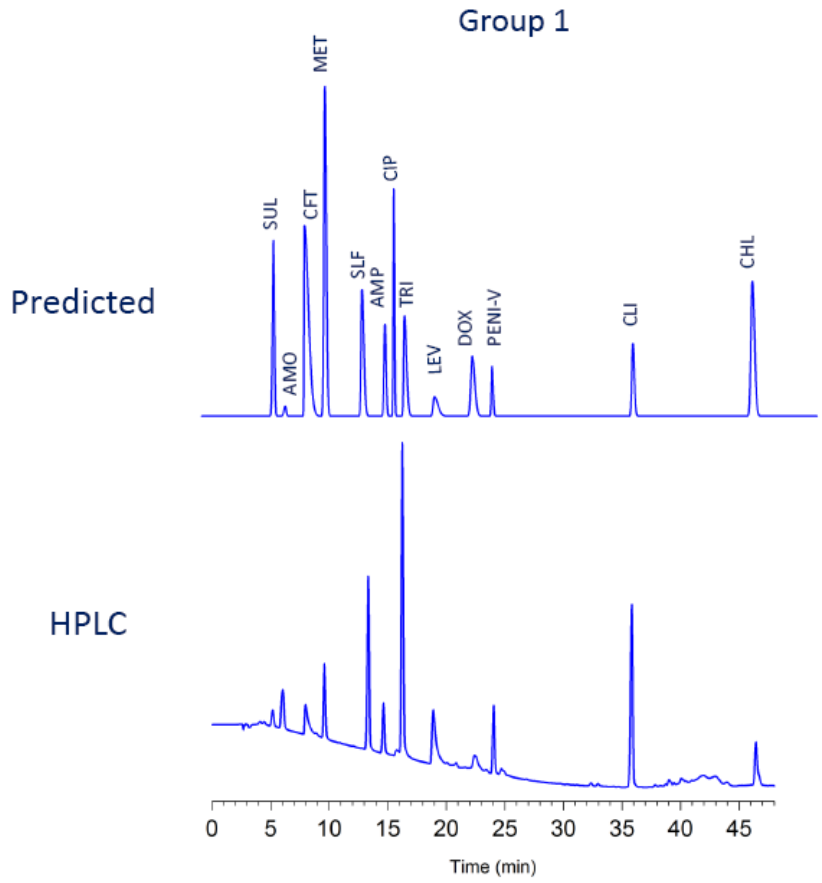

Fig. 4. Optimal conditions for group 1 with predicted chromatogram (Top) and observed chromatogram (Bottom).

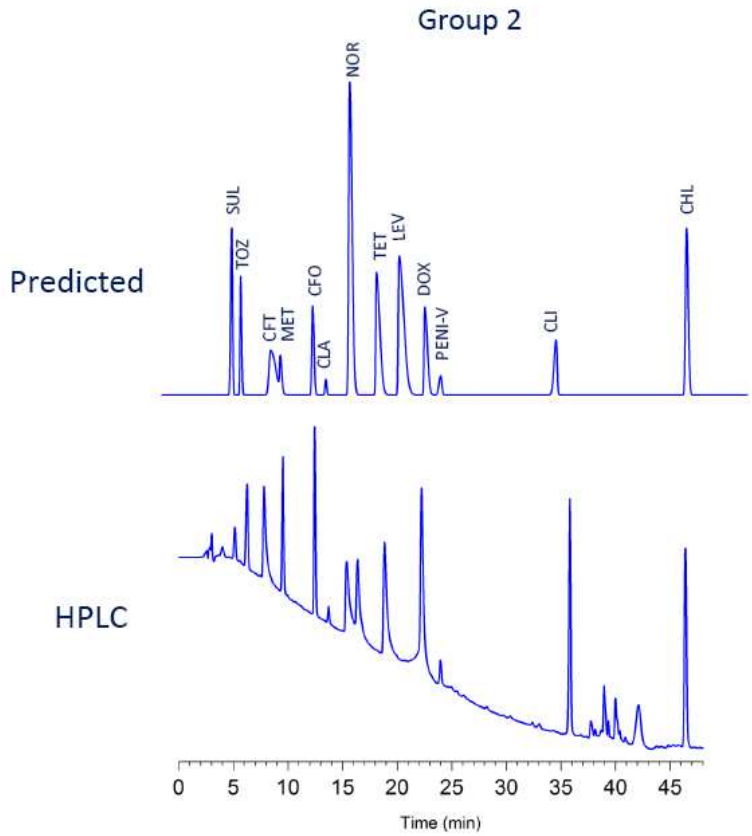

Fig. 5. Optimal conditions for group 2 with predicted chromatogram (Top) and observed chromatogram (Bottom).

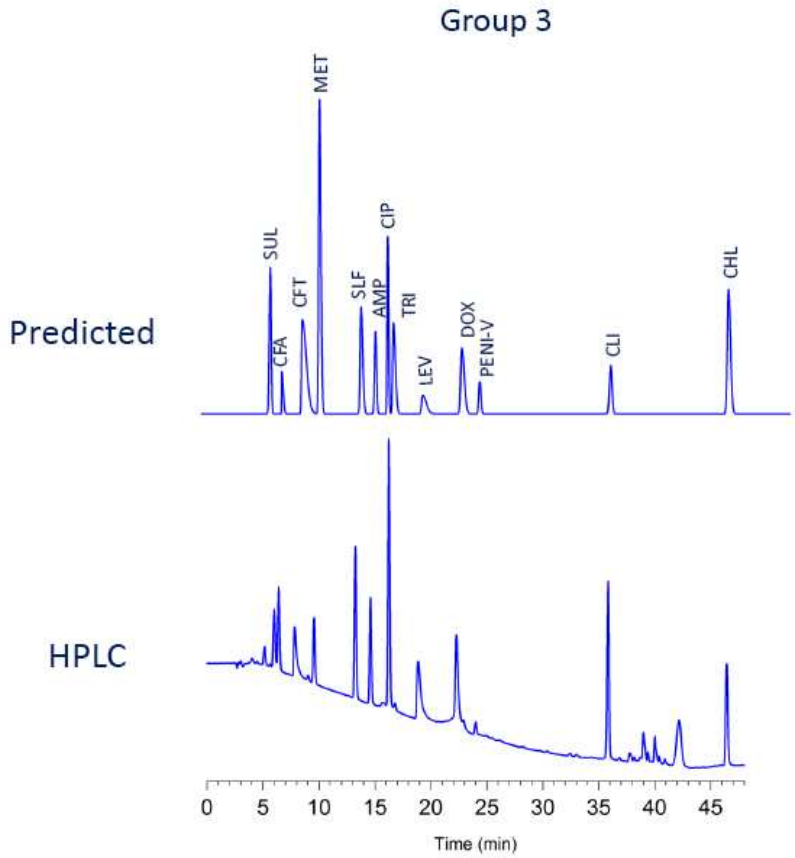

Fig. 6. Optimal conditions for group 3 with predicted chromatogram (Top) and observed chromatogram (Bottom).

Table 5 UHPLC gradient conditions

Time (min)	Flow rate (ml/min)	Methanol (%)	Buffer pH6.00 (%)
0.00	0.613	8	92
2.63	0.613	95	5
2.97	0.613	95	5
3.04	0.613	8	92
4.74	0.613	8	92

Finally a single method for screening the 19 antibiotics and associated compounds split in three groups was developed. One can remark that the negative prediction of separation for group 2 does not prevent the use of this method for screening of antibiotics.

The method not only allows the simultaneous screening of the studied antibiotics but it offers the possibility to analyze seven most used associated antibiotics marketed in the emerging countries. These associations and the corresponding retentions times of their molecules

(HPLC) are: amoxicillin (6.04 min) –clavulanic acid (13.73 min), sulbactam (5.18 min) – ampicillin (14.64 min), tazobactam (6.27 min) – ceftriaxone (7.99 min), sul-bactam (5.18 min) – ceftriaxone (7.99 min), sulbactam (5.18 min) –cefotaxime (12.47 min), sulfamethoxazole (13.34 min) – trimethoprim (16.26 min) and metronidazole (9.61 min) – norfloxacin (15.39 min). Beside the possibility of analyzing several molecules in association, another advantage of the developed method is the relatively short analysis time, useful in the context of drug quality control laboratories especially those dealing with various imported or exported pharmaceutical products. However, the developed method does not separate pair products such as trimethoprim (16.26 min) – tetracycline (16.39 min) and cefadroxil (6.02 min) –amoxicillin (6.04 min), but they are not marketed in association.This does not prevent the use of the developed generic analytical method because even if the retention times are close, the UVspectra of these pair of products are different enough to perform qualitative analysis.

3.4. Geometrical transfer of the optimized method

In case of custom seizures, the deadline for a response is only three days. To reduce the analysis time and solvent consumption, required for a rapid decision making on the analyzed samples, a geometric transfer to UHPLC was applied. Such a method transfer is often accompanied by a slight loss of peak efficiency, which will also challenge the HPLC method robustness [23,24]. In this study, we maintained the same analytical conditions for HPLC and UHPLC, except those described in Section 2.3 and the gradient time, which was set to 2.63 min (Table 5). Hence, Figs. 7–9 yielded very similar optimal separations.

Detailed results of the transfer are presented in Table 6. The relative retention times (i.e. retention time/retention time of the last peak eluted) were used to assess the method transfer success. Relative predicted and observed retention times were very close, confirming the adequacy of the geometric transfer from HPLC to UHPLC.

Moreover the method transfer did not affect the chromatographic selectivity and separation of peaks. The transfer permitted to further confirm the high robustness of the developed method by means of DS optimization strategy.

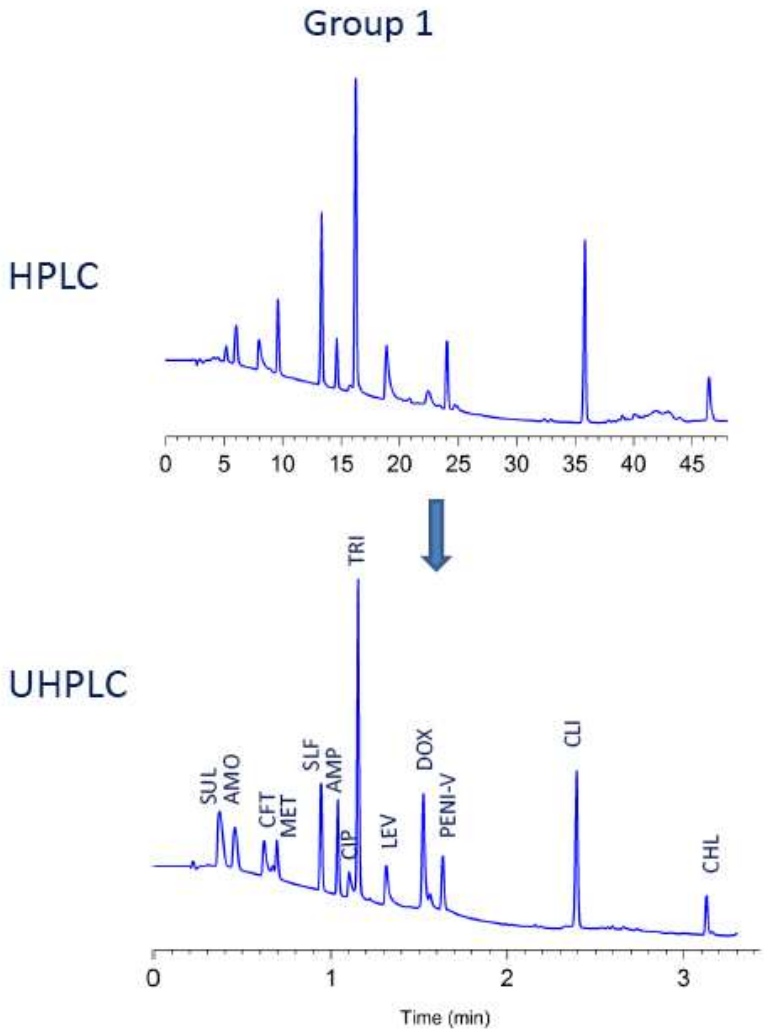

Fig. 7. Observed chromatogram with HPLC equipment (Top) and observed chromatogram resulting of the transfer to UHPLC (Bottom) for Group 1.

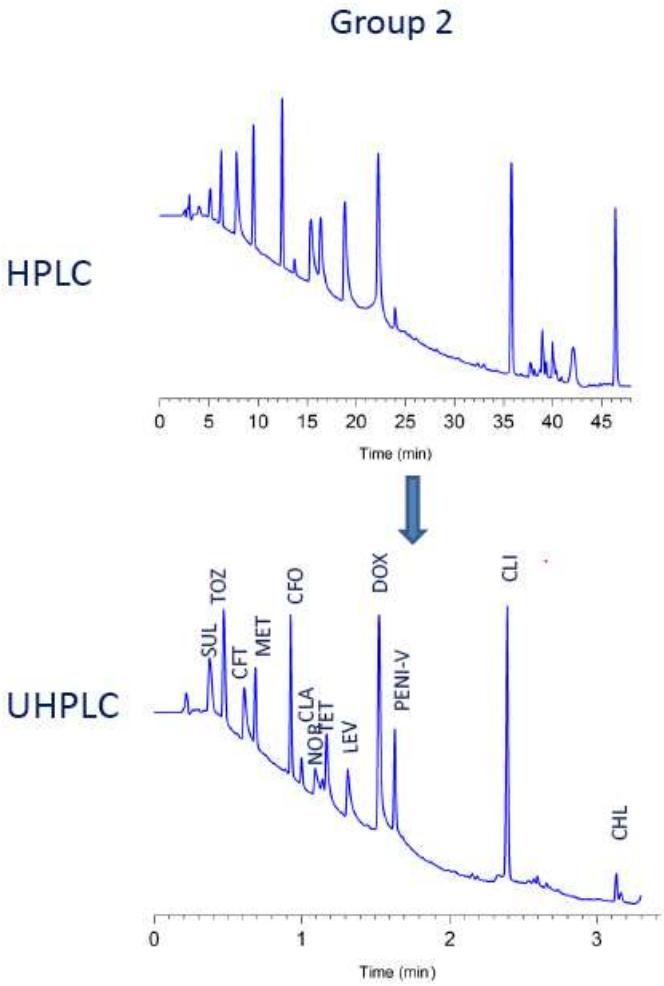

Fig. 8. Observed chromatogram with HPLC equipment (Top) and observed chromatogram resulting of the transfer to UHPLC (Bottom) for Group 2.

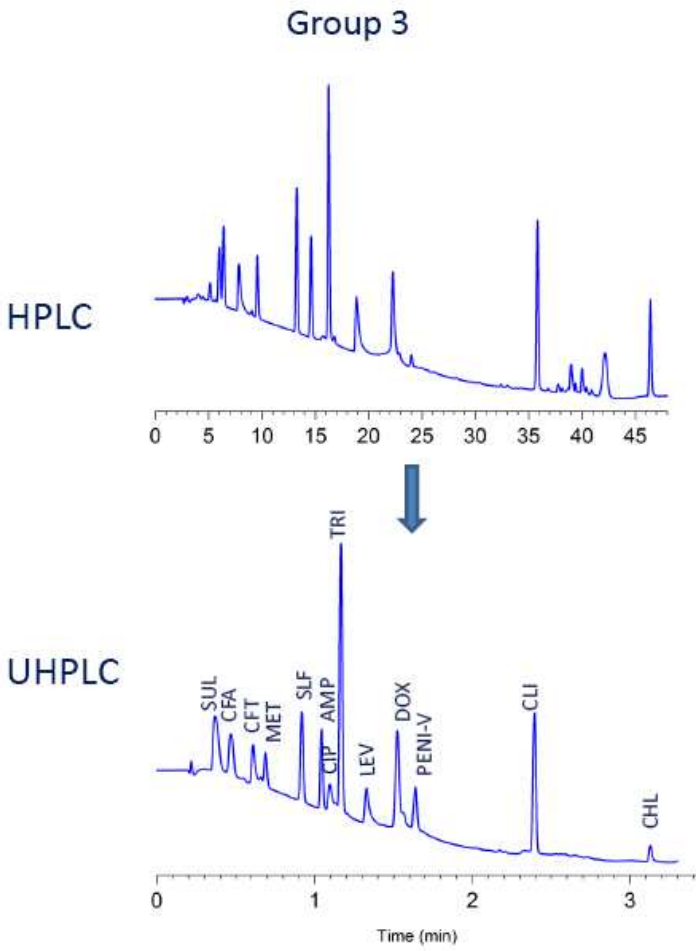

Fig. 9. Observed chromatogram with HPLC equipment (Top) and observed chromatogram resulting of the transfer to UHPLC (Bottom) for Group 3.

Table 6 Results of the transfer from HPLC to UHPLC. The columns 2 and 3 are the predicted retention times (Pred Rt) and the observed (Obs Rt) retention times for HPLC experiments. The columns 4 and 5 are the relative (rel) predicted retention times and the observed retention times from HPLC experiments. The columns 6 and 7 are the observed (obs) and the relative observed retention times for UHPLC experiments.

Compound	HPLC				UHPLC	
	Pred Rt	Obs Rt	Pred Rt (rel)	Obs Rt (rel)	Obs Rt	Obs Rt (rel)
AMO	6.20	6.04	0.133	0.130	0.46	0.146
AMP	16.66	14.64	0.316	0.315	1.04	0.332
CHL	46.45	46.45	1.000	1.000	3.13	1.000
CFT	8.05	7.99	0.173	0.172	0.62	0.199
CFO	12.58	12.47	0.271	0.268	0.93	0.297
CHL	46.45	46.45	1.000	1.000	3.13	1.000
CIP	15.77	15.76	0.340	0.339	1.10	0.353
CLA	13.77	13.73	0.296	0.296	1.00	0.319
CLI	35.83	35.84	0.771	0.772	2.39	0.765
DOX	22.43	22.41	0.483	0.483	1.53	0.487
LEV	18.91	18.90	0.407	0.407	1.31	0.420
MET	9.62	9.61	0.207	0.207	0.69	0.222
NOR	15.94	15.39	0.343	0.331	1.10	0.351
PENI-V	24.05	24.04	0.518	0.518	1.64	0.523
SLF	13.36	13.34	0.288	0.287	0.95	0.302
SUL	5.19	5.18	0.112	0.111	0.37	0.118
TAZ	6.04	6.27	0.130	0.135	0.47	0.150
TET	20.42	16.39	0.440	0.353	1.32	0.422
TRI	16.26	16.26	0.350	0.350	1.16	0.369

3.5. Method validation

After the optimization process, it was necessary to demonstrate the ability of developed method to be used for its intended purpose through the process of the validation. The UHPLC conditions given in Table 5 were validated. In this study, a quantitative method applied to powder for injection containing SUL and CFT was validated using the total error strategy, represented by an accuracy profile [26,27]. The acceptance limits were set at ±5% according to European Medicines Agency (EMA) for marketed pharmaceuticals brands [28]. The accuracy profile methodology is fully compliant to the ICH Q2 requirements [29,30].

Validation of the developed method was carried out during three days with three replicates for each concentration level. The selected calibration model is a linear regression. The concentration results were backcalculated using the calibration curves. These concentrations were used to determine the relative bias, the repeatability, the intermediate precision, the β-expectation tolerance intervals at 95% probability level, and the linearity.

The accuracy profiles for both compounds are given in Fig. 10 and the validation criteria are listed in Table 7.

As shown in Table 7, the relative standard deviation (RSD %) values of repeatability and intermediate precision were below 0.75 and 0.87, respectively. This indicates a good precision of the method. The trueness of the developed method is also good with the relative and absolute biases less than 2.39% and 9.62 µg/mL, respectively. The relative 95% β-expectation tolerance intervals are generally within a range of [−4.81, +3.03]% indicating the ability of the method to provide accurate results. As the lower and upper tolerance bounds are included within the acceptance limits for all the targeted concentration levels (Fig. 10), one can ensure that each future result will fall within the acceptance range with a probability of at least 95% [27,28]. In addition, the very good linearity of the results is illustrated by the slopes close to 1 of the regression models obtained between the reference concentration of the validation standards of each active substance and the results obtained by the analytical procedure.

Since the β-expectation tolerance intervals are included within the acceptance limits, one can conclude that the method is suitable for its quantification purpose.

Table 7 Summary of the validation criteria for CFT and SUL

Validation criteria	**Conc. (µg/mL)**	**CFT**	**Conc. (µg/mL)**	**SUL**
Trueness: Absolute bias (µg/mL) (Relative bias (%))	160	-1.38 (-0.85)	80	0.61 (0.76)
	240	-0.25 (-0.10)	120	1.93 (1.61)
	320	-2.30 (-0.71)	160	1.82 (1.14)
	400	-9.61(-2.38)	200	1.75 (0.87)
	480	-4.06 (-0.84)	240	1.03 (0.43)
Precision: Repeatability (%) /Intermediate precision (%)	160	0.41 / 0.87	80	0.19 / 0.27
	240	0.48 / 0.54	120	0.52 / 0.52
	320	0.75 / 0.77	160	0.68 / 0.74
	400	0.44 / 0.73	200	0.14 / 0.14
	480	0.26 / 0.56	480	0.24 / 0.32
Accuracy: 95% □-expect. tol. int. (µg/mL) (Rel. 95% □-expect. tol. int (%))	160	154.6−165.3 (-4.17−2.46)	80	80.0−81.3 (-0.05−1.58)
	240	238.3−245.2 (-1.53−1.32)	120	120.4−123.5 (0.34−2.88)
	320	314.2−326.5 (-2.62−1.20)	160	158.8−164.8 (-0.75−3.03)
	400	384.0−403.5 (-4.81−0.04)	200	199.5−204.0 (-0.25−1.99)
	480	469.7−490.3 (-2.97−1.29)	240	238.8−243.3 (-0.50−1.36)
Linearity :	Slope	0.982	Slope	1.002
	Intercept	2.371	Intercept	1.161
	R^2	0.999	R^2	0.999

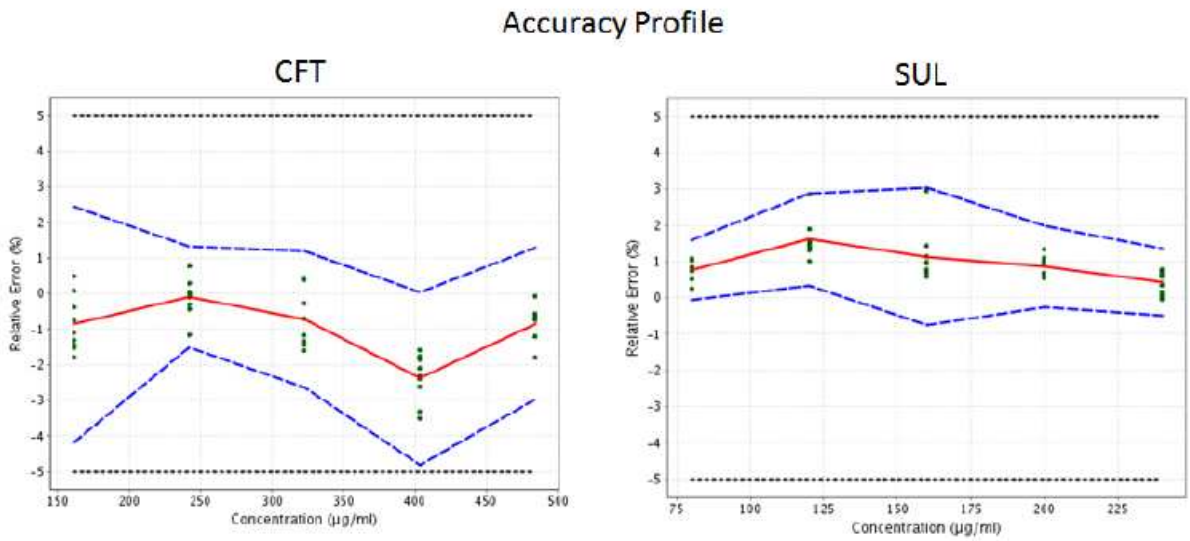

Fig. 10. Accuracy profiles for quantitative methods validation for ceftriaxone and sulbactam. Legend: CFT = Ceftriaxone, SUL = Sulbactam; Red = bias (%). Black = acceptance limit at ±5%. Blue = 95% β-expectation tolerance interval. Green = individual measurements.

3.6. Application

The developed and validated method was applied to the identification and assay of the two active ingredients (CFT and SUL) in powder for injection. For that purpose, three different brands of pharmaceutical drugs manufactured in India were tested as a real case. They were purchased from different retail pharmacies in Kinshasa (DRC). The three drugs contained the two active ingredients, but as shown in Table 8, two products were non-compliant to the specifications of EMA (95.0–105.0%) [28]. They were overdosed in CFT, and drug overdosing is harmful for the public health as it can lead to serious poisoning of vital organs [31].

Table 8 Assay results of three pharmaceutical medicines coded A, B and C, marketed in DRC. Results consist in the mean percentage of claimed nominal content and their 95% confidence interval computed on 3 independent samples. Specifications are set to 95–105% of the claimed nominal content (mg). Non-compliant results for the tested powder for injection are in bold.

Drug	CFT Content	SUL Content
A	1000 mg	500 mg
	96.7 ± 0.89 %	97.2 ± 1.32 %
B	1000 mg	500 mg
	105.0 ± 2.73 %	98.0 ± 2.06 %
C	1000 mg	500 mg
	115.1 ± 1.76 %	99.2 ± 1.81%

4. Conclusion

The main objective of this work was to develop generic methods able to trace, screen and determine various antibiotic molecules and common associated molecules, in order to help detecting the potential poor quality medicines.

To achieve this objective, Design Space strategy was successfully applied to optimize simultaneously three selected factors, the temperature, the gradient time and the pH. This led to the development of HPLC method able to screen 19 antibiotics and associated compounds.

The Design Space strategy enabled to develop a robust method, confirmed by a successful method transfer from HPLC to UHPLC. A fast and low solvent consumption method is a real advantage to perform efficient analysis in quality control laboratories.

The method was successfully validated using the total error approach and accuracy profile methodology for the determination of ceftriaxone and sulbactam. Finally, the method was effectively applied to analysis of three brand pharmaceuticals marketed in the Democratic Republic of Congo for which results led to two out of specifications results.

Acknowledgments

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Section III. 3.

Méthodes génériques par CLHP pour l'analyse de deux combinaisons fixes sous forme de comprimé recommandées par L'OMS: Développement, transfert et validation

Cette section se réfère à l'article «**Fighting Poor Quality Medicines: Development, Transfer and Validation of Generic HPLC Methods for Analyzing two WHO Recommended Antimalarial Tablets**» publié dans American Journal of Analytical Chemistry 6 (2015) 127-144.

Section III.3. Technique séparative – Antipaludéens

Contexte

Le paludisme est un problème majeur de santé publique et une des causes de mortalité infantile en RDC mais aussi dans d'autres pays en voie de développement. Déjà en 2012 la RDC faisait partir des 6 pays les plus touchés de la région africaine de l'OMS à côté du Nigéria, de la Tanzanie, de l'Ouganda, du Mozambique et de la côte d'Ivoire. La RDC et le Nigeria représentent à eux seuls plus de 40% du total mondial des décès dus au paludisme. La thérapeutique de la forme simple du paludisme repose principalement sur l'usage des antipaludéens classiques et ce sous diverses associations dans le but de remédier à la résistance de plus en plus observée avec la thérapie mono-médicamenteuse. Pour répondre à la préoccupation de la population Congolaise et des prestataires de santé dans la thérapeutique antipaludéenne, plusieurs ateliers ont été organisés à Kinshasa en 2012 et en 2013, sous la coordination du Programme National de Lutte contre le Paludisme en RDC en présence des experts internationaux, nationaux et des autorités de santé. En matière de traitement contre le paludisme, les associations Luméfantrine-Artémether et Amodiaquine-Artésunate recommandées par l'OMS ont été retenues pour le paludisme simple et la quinine pour le paludisme compliqué.

Dans ce contexte, il nous est paru pertinent de développer des méthodes analytiques pour contrôler la qualité de ces associations et contribuer au rehaussement du succès thérapeutique. Lors de nos études en rapport avec cette section, nous avons optimisé une méthode analytique pour détecter 8 molécules souvent retrouvées dans les associations antipaludéennes. A cet effet, nous nous sommes servis d'un plan d'expériences factoriel complet et d'un espace de conception. En utilisant les données analytiques obtenues lors de ces expériences, nous avons affiné la méthode initiale pour aboutir à une seule méthode plus courte et dédiée aux deux associations antipaludéennes recommandées par l'OMS. A l'issue de la validation, la méthode a été appliquée pour évaluer la qualité ses échantillons provenant de Kinshasa mais aussi du Bénin et du Rwanda. Ceci conforte aussi l'intérêt d'un transfert géographique Nord-Sud de la méthode.

Section III.3. Technique séparative – Antipaludéens

Summary

As serious but neglected public health problems, poor quality medicines, i.e. for antimalarial medicines, urged to be fought. One of the approaches is to consider the analytical chemistry and separative techniques. In this study, a generic liquid chromatographic method was firstly developed for the purpose of screening 8 antimalarial active ingredients, namely amodiaquine (AQ), piperaquine (PPQ), sulfalene (SL), pyrimethamine (PM), lumefantrine (LF), artesunate (AS), artemether (AM) and dihydroartemisinine (DHA) by applying DoE/DS optimization strategy. Since the method was not totally satisfying in terms of peak separation, further experiments were undergone applying the same development strategy while splitting the 8 ingredients into five groups. Excellent prediction was observed prior to correlation between retention times of predicted and observed separation conditions. Then, a successful geometric transfer was realized to reduce the analysis time focusing on the simultaneous quantification of two WHO's recommended ACTs in anti-malarial fixed-dose combination (AM-LF and AS-AQ) in tablets. The optimal separation was achieved using an isocratic elution of methanol-ammonium formate buffer (pH 2.8; 10mM) (82.5: 17.5, v/v) at 0.6ml/min through a C18 column (100mm×3.5mm, 3.5 µm) thermostated at 25°C. After a successful validation stage based on the total error approach, the method was applied to determine the content of AM/LF or AS/AQ in seven brands of antimalarial tablets currently marketed in West, Central and East Africa. Satisfying results were obtained compared to the claimed contents.

Section III.3. Technique séparative – Antipaludéens

1. Introduction

Poor quality medicines are serious but neglected public health problems. Anti-infective medicines are particularly afflicted [1]. Poor-quality antimalarials that contain sub-therapeutic amounts of active ingredient increase the risk of malaria drug resistance, thus undoing the significant gains in malaria control seen in the last decade [2]. In 2012, WHO estimated 207 million malaria cases worldwide [2]. The successful control of this disease depends mainly on treatment with efficacious anti-malarial drugs. Most of the countries do have a National Malaria Treatment Policy that specifies medicines for treatment of both uncomplicated and severe malaria as well as malaria in case of pregnancy and in case of first line treatment fails. As resistance develops to known medicines, it is necessary to commercialize new ones or to use the existing medicines in combination for example in case of malaria infection with *Plasmodium falciparum*. Indeed, the use of two or more drugs with different action mechanism is now recommended to provide adequate cure rate and delay any development of resistance [3]. WHO recommends that all persons of all ages in all epidemiological settings with suspected malaria should receive a parasitological confirmation of diagnosis by either microscopy or rapid diagnostic test (RDT), and that uncomplicated *Plasmodium falciparum* malaria should be treated with an artemisinin-based combination therapy (ACT) [2].

Fast acting artemisinin-based compounds are combined with a drug from a different class. Companion drugs include lumefantrine, mefloquine, amodiaquine, sulfadoxine / pyrimethamine, piperaquine and chlorproguanil / dapsone. The artemisinin derivatives usually used include dihydroartemisinin, artesunate and artemether. Implementation of the recommendation to use ACTs is limited by the small number of available and affordable co-formulated anti-malarial drugs, but most countries are now starting to implement this regimen. A co-formulated drug is one in which two different drugs are combined in one tablet; this is important to ensure both drugs are used.

Artemether/lumefantrine was the first fixed-dose artemisinin-based combination therapy recommended and pre-qualified by WHO for the treatment of uncomplicated malaria caused by *P*. falciparum. It has been shown to be effective both in sub-Saharan Africa and in areas with multi-drug resistant *P*. falciparum in Southeast Asia. It is currently recommended as first-line treatment for uncomplicated malaria in several countries. However, its complex treatment regimen of two doses daily for three days could affect patient adherence to

treatment. A fixed-dose combination of amodiaquine-artesunate was launched in February 2007 [3]. The benefits of ACTs are their high efficacy, fast action and the reduced likelihood of resistance developing. In order to make best use of them, it is critical to address issues of quality.

According to WHO 200,000 deaths over one million that occur from malaria annually would be avoidable if the available medicines were effective, of good quality and used correctly [4]. A recent study published in “The Lancet” concluded that up to 40% of artesunate products (the best medicine to combat resistant malaria today) contain no active ingredients and therefore have no therapeutic benefits. At best, the regular use of substandard or counterfeit medicines leads to therapeutic failure or drug resistance; in many cases it can lead to death [4].

In this context, analytical chemistry and especially separative screening methods such as liquid chromatography (LC) methods are suitable to help fighting against such medicines and therefore can be used [5-7].

Recently, Debrus *et al.* published interesting work on an innovative HPLC method development for the screening of 19 antimalarial drugs based on a generic approach, using design of experiments, independent component analysis and design space. That method was found somewhat time consuming due to the gradient mode [8].

In the present study, several HPLC separations considering isocratic mode (short run time) were optimized for targeted subsets of 8 antimalarial active ingredients (AAI) used alone or in combination.

The first objective was the optimization of the separation conditions (screening method) for these 8 AAI among which were 4 companion drugs (amodiaquine (AQ), piperaquine (PPQ), sulfalene (SL), pyrimethamine (PM) lumefantrine (LF)) and Artemisinin derivatives include dihydroartemisinin (DHA), artesunate (AS) and artemether (AM). Their chemical structures are presented in Figure 1.

The second objective was the simultaneous determination of artemether, lumefantrine, artesunate, amodiaquine in fixed dose combination tablets as recommended by WHO. As suggested in ICH Q8 (R2) and previously successfully tested by Debrus *et al* [8, 9], a

combining design of experiments (DoE) and Design Space (DS) was exploited to simultaneously optimize the separation based on predictive modeling technique using retention time-based responses [10]. Thereafter, a geometric transfer was performed for the HPLC developed methods in order to evaluate the robustness and improved gain of analysis time that is a challenge in the framework of fighting against counterfeit medicines.

The third objective was to validate the transferred method using the accuracy profile as decision tool for the simultaneous quantitation of artemether and lumefantrine; artesunate and amodiaquine in fixed dose combination (FDC) tablets.

Finally, the validated method was used to analyze several antimalarial drugs marketed in Benin (West Africa), DRC (Central Africa) and Rwanda (East Africa).

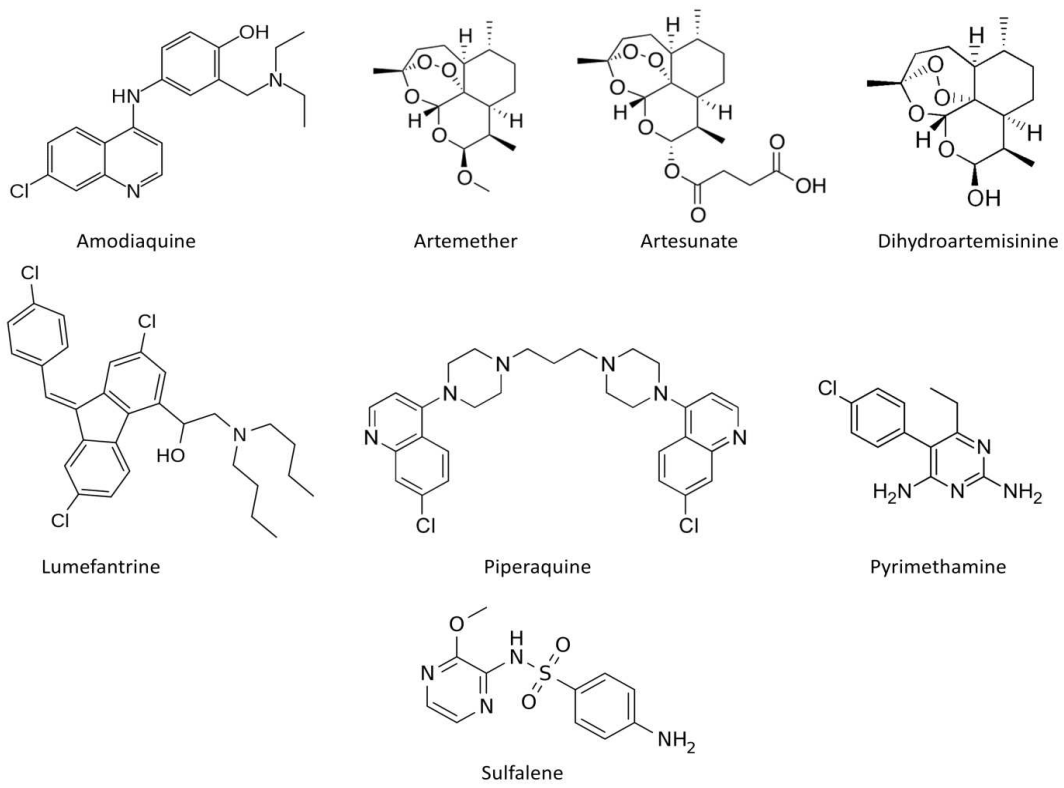

Figure 1. Chemical structures of the 8 studied antimalarial drugs.

2. Experimental

2.1. Chemical and reagents

Methanol (HPLC gradient grade), formic acid (98%-100%) and orthophosphoric acid Eur Ph. grade (85%) were purchased from Merck (Darmstadt, Germany). Ammonium formate (99%) was provided by BDH Prolabo (Almere, Netherlands). Ultrapure water was obtained from a Milli-Q Plus 185 water purification system from Millipore (Billerica, MA, USA). Artesunate (99.8%) and dihydroartemisinin alpha and beta (100.0%) were purchased from Apoteket AB (Stockholm, Sweden). Lumefantrine (99.4%) and artemether (99.5%) were kindly donated by Fourrts laboratories (Chennai, India) and Meridian Pharmacare Pvt Ltd (Bangalore, Inde). Amodiaquine hydrochloride (99.0%), Piperaquine tetraphosphate (99.2%) and Pyrimethamine (99.0%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Sulfalene (100.0%) was purchased from Fagron NV/SA (Waregem, Belgium).

For the preparation of validation standards, a matrix formulation of tablets containing 20 mg of AM and 120 mg of LF was provided by Fourrts laboratories (Kanchipuram, Inde). Mefanther® 20/120 mg tablet were kindly donated by the same laboratories. Antimalarial drugs containing AS and AQ 50/150 were purchased in drugstore located in DRC (Kinshasa). Antimalarial drugs containing AM (20, 40 or 80 mg) and LF (120, 240 or 480 mg) were purchased in drugstore located in Benin (Cotonou), DRC (Kinshasa) and Rwanda.

2.2. Sample preparation

2.2.1 Mixture preparation groups

Individual stock solutions of AM, AS and DHA at 5mg/ml and of AQ, PPQ, PM 1mg/ml were prepared in methanol. A stock solution of LF at 100 µg/mL was prepared in methanol acidified by phosphoric acid (0.1% acid phosphoric in methanol (w/v)). Mixture solutions were prepared by diluting stock solutions in methanol-water (50:50, v/v) to achieve the following concentrations: 2.5 mg/ml for AM, AS, DHA; 50µg/ml for LF, SL and 25µg/mL for PPQ, PM and AQ.

2.2.1 Solutions used for calibration and validation

A stock solution of calibration standards (CS) of AM (240 µg/ml) and LF (1440

µg/ml) was prepared in methanol acidified by acid orthophosphoric. A stock solution of AS (240µg/mL) and AQ (720µg/mL) was prepared in methanol. Dilutions were performed in methanol-water (50:50) in order to obtain solutions at 3 different concentration levels:

Level 1(40%): 80 µg/ml (AM) – 480 µg/ml (LF) and 80 µg/ml (AS) -240 µg/ml (AQ);

Level 3 (80%): 160 µg/ml (AM) - 960 µg/ml (LF) and 160 µg/ml (AS)- 480 µg/ml (AQ);

Level 5 (120%): 240 µg/ml (AM) - 1440 µg/ml (LF) and 240 µg/ml (AS) - 720 µg/ml (AQ).

The levels of the concentration were chosen in order to allow construction of different regression models that will determine back-calculated concentrations of validation standards.

For each concentration level three replications were run for three days corresponding to three series (p=3).

The validation standards were prepared in matrices, here tablets, obtained by the manufacturers of the corresponding medicines in order to better simulate the sample preparation in routine analysis. Stock solutions were obtained as in the case of calibration standards to which is added a corresponding amount of the matrix. Dilutions were performed in methanol-water (50:50) in the same way as described for the CS in order to obtain solutions at 5 different concentration levels

Level 1 (40%): 80 µg/ml (AM) - 480 µg/mL (LF) and 80 µg/ml (AS) -240 µg/mL (AQ)

Level 2 (60%): 120 µg/ml (AM) - 720 µg/mL (LF) and 120 µg/ml (AS)- 360 µg/mL (AQ)

Level 3 (80%): 160 µg/ml (AM) - 960 µg/mL (LF) and 160 µg/ml (AS)- 480 µg/mL (AQ)

Level 4 (100%): 200 µg/ml (AM) - 1200 µg/mL (LF) and 200 µg/ml (AS) - 600 µg/mL (AQ)

Level 5 (120%): 240 µg/ml (AM) - 1440 µg/mL (LF) and 240 µg/ml (AS) - 720 µg/mL (AQ).

Three independent preparations (n=3) were carried out per each of the five concentration levels (m=5). All these preparations were repeated for three days corresponding also to three series (p=3).

For routines analyses, the concentrations of reference standards were 200 µg/mL of AM and 1200 µg/mL of LF in a mixture, 200 µg/mL of AS and 600 µg/mL of AQ in another mixture. For the sample tablets, powdered portions were taken and treated in the same way as reference solutions to give final expected concentrations of 200 µg/ml (AM) - 1200 µg/mL (LF) for AM-LF combination and 200 µg/mL (AS) - 600µg/mL (AQ) for AS-AQ combination. The solutions were freshly prepared and protected from light. They were filtered through 0.45 µm PTFE syringe filtration disks prior to their analysis onto the liquid chromatographic system.

2.3. Instrumentation and Chromatographic conditions

The experiments for optimization of the LC conditions, for the validation work and for the routine analysis were carried out on a LC system from Waters 2695 (Waters, Milford, USA) composed of a Waters selector 7678, autosampler, photodiode array detector (PDA) Waters 2996 and Empower 2.0 software. The analytical column for optimization was an XBridge C18 (250 × 4.6 mm i.d.; 5 µm particle size) preceded by a guard column XBridge guard C18 (20 × 4.6mm i.d.; 5 µm particle size) both from Waters. The optimized conditions were transferred to an XBridge C18 (100 × 4.6 mm i.d.; 3.5 µm particle size), 4 µl for injection volume. Peak analytes were monitored at 230 nm during optimization and at 210 nm during validation and routine application. However, the UV spectra were recorded online from 210 nm to 400 nm to allow the peak identification at all the experiments. The injection volume was 10µl for all tested experimental conditions. The buffer solution of the isocratic mobile phase consisted of 10 mM ammonium formate (pKa = 3.8) adjusted to pH of 2.8 with formic acid.

2.4. Design of Experiments

Design of experiments (DoE) was used to define the Design of Space (DS). Flow of mobile phase (F), column temperature (T°C) and proportion of methanol in the mobile phase (%OM) were selected as the factors to investigate (see Table 1a). As those HPLC methods were developed for their suitability for routine use in resource-restraint environments, the choice of the methanol as organic modifier was justified by its low cost compare to acetonitrile.

Because of the temperature control problem that might be encountered in that kind of

environment we decided to include that factor in the study and to extend the range for test. A total of 29 experimental conditions were defined as shown in Table 1b. In the present case, a full factorial design was used to allow simultaneous optimization of the method, estimate its robustness and evaluate the adequacy between chromatographic behaviors as predicted by the liquid chromatography theory and those obtained by the mathematical models.

2.5. Software

Empower 2.0 for Windows was used to control the HPLC and to record the signals from the detector and interpret the chromatograms. An algorithm was set up to develop a Bayesian model and to compute the DS.

The algorithm was written in R2.13, which is available as free-ware from: http://www.rproject.com.

HPLC calculator V 3.0 (University of Geneva, Switzerland) was used to carry out the necessary computations for the geometric transfer methodology.

The accuracy profiles as well as the statistical calculations including the validation results and uncertainty estimates were obtained using e-noval® V3.0 software (Arlenda, Belgium).

3. Results and Discussion

3.1. Modeling and Optimization methodologies

3.1.1. Influence of the Factors on the Peak Separations

Due to acidic and alkaline comportments of the AAI to test, and considering literature data we choose to perform the experiments in acidic media. Preliminary tests allowed setting the pH to 2.8 as well as setting up the range and the levels of each factor (see Table 1a). They indicated that the retention time of AM was too long (>60 min) with 75% of methanol in the mobile phase, pH 2.5. To prevent a possible thermal degradation of the different analytes, the maximum temperature for the column oven was limited to 35°C while the minimum temperature to 25°C, the average ambient temperature in tropical countries where further analyses are intended to be pursued.

The influence of the critical factors on the separation of the chromatographic peaks was then assessed by means of full factorial design. As can be noticed in Figure 2, the flow rate and the percentage of organic modifier considerably influence somehow the retention times of AAI: the increase of the mobile phase flow rate as of the organic modifier percentage significantly decreases the retention times of antimalarial drugs, but often at the expense of peak separation. There were also peak coelutions of certain antimalarial compounds and even a reversal of the peak elution order for some others. However, by decreasing the level of these two factors, an increase of the retention times of the tested compounds was observed with improved peak separations. Based on this observation, the ideal would be to work at low level of the flow rate and low percentage of methanol to achieve separation of these antimalarials in this experimental domain.

3.1.2. Modeling

For better reliable prediction of the chromatographic conditions of each AAI, modeling was performed using the retention time of each strategic part of the chromatographic peak, i.e. the beginning, the apex and the end [10-13].

Table 1. (a) Factors and corresponding levels selected for the full factorial design; (b) Experimental matrix of full factorial design for the investigation of organic modifier, flow rate and temperature

(a)

Factors	**Levels**		
Organic modifier (%)	80	85	90
Flow rate (mL/min)	0.3	0.5	0.7
Temperature of the column oven (°C)	25.0	30.0	35.0

(b)

Trial	Experimental Design			Experimental set up		
	X1	X2	X3	Organic modifier (%)	Flow rate (mL/min)	Temperature (°C)
1	-1	-1	0	80	0.3	30
2	1	0	1	90	0.5	35
3	0	0	0	85	0.5	30
4	1	-1	1	90	0.3	35
5	0	-1	1	85	0.3	35
6	-1	1	1	80	0.7	35
7	-1	1	-1	80	0.7	25
8	1	-1	0	90	0.3	30
9	0	0	0	85	0.5	30
10	0	0	0	85	0.5	30
11	-1	-1	-1	80	0.3	25
12	1	1	-1	90	0.7	25
13	-1	1	0	80	0.7	30
14	-1	0	1	80	0.5	35
15	1	0	-1	90	0.5	25
16	0	0	-1	85	0.5	25
17	0	0	1	85	0.5	35
18	0	-1	1	85	0.3	35
19	0	-1	-1	85	0.3	25
20	0	1	1	85	0.7	35
21	-1	0	0	80	0.5	30
22	1	0	0	90	0.5	30
23	1	1	1	90	0.7	35
24	0	-1	0	85	0.3	30
25	1	-1	-1	90	0.3	25
26	0	1	0	80	0.7	30
27	-1	-1	1	85	0.3	35
28	1	1	0	90	0.7	30
29	-1	0	-1	80	0.5	25

The quality of the obtained linear regressions was assessed by the adjusted coefficient of determination (R^2 ajusted), the graph residues and the adequacy between the retention times predicted by the model and those observed. As shown in Figure 3a, an excellent relationship was observed between the predicted versus the experimental values of the retention times (R^2 ajusted values close to 1). In addition, most of the residuals (Figure 3b) were located within the [−1.5 min, +1.5 min] interval, confirming the fitness of the model and its suitability for the optimization of the separation.

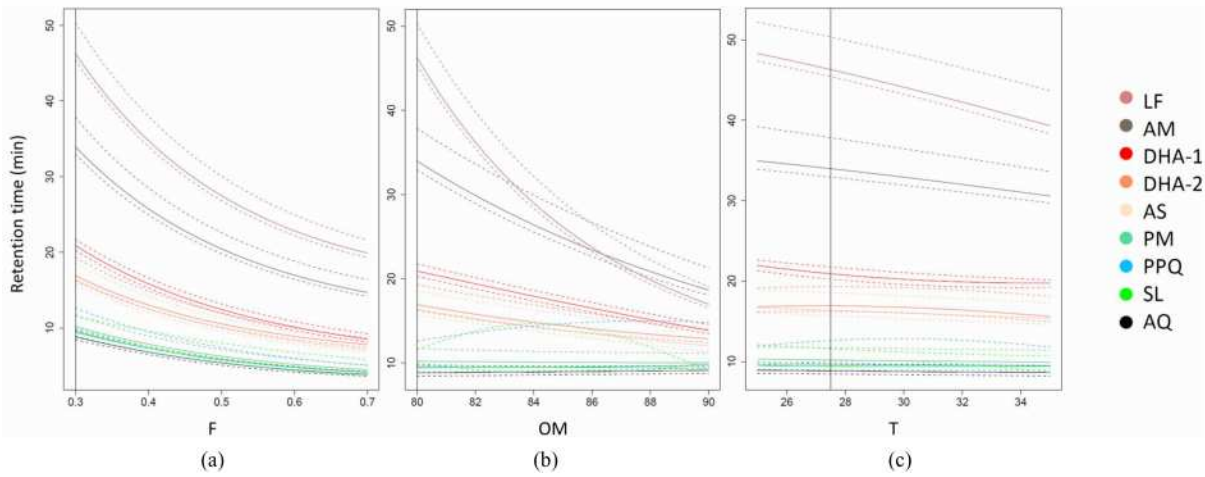

Figure 2. Predicted retention times (min) of different compounds versus to flow rate (F) (a), to organic modifier (OM) (b) and to temperature (T) (c).

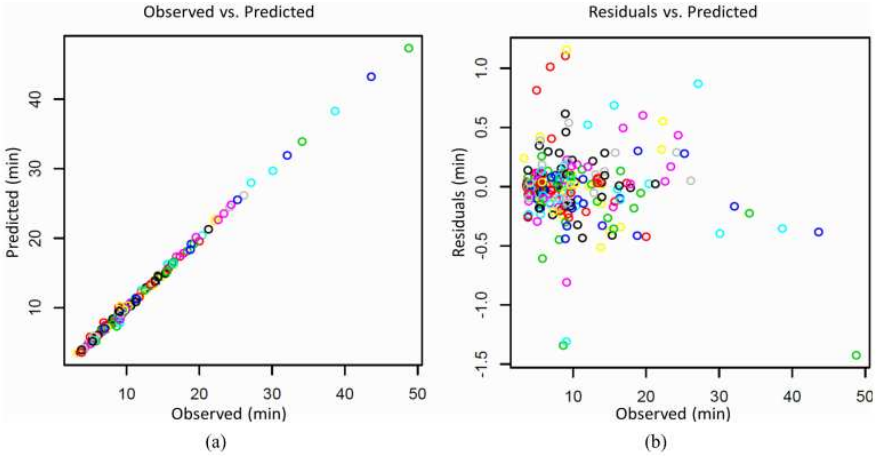

Figure 3. Modelling results (a) Predicted versus experimental values for retention times; (b) Corresponding residuals plots.

3.1.3. Prediction of Optimal Separations

The good relationship between the predicted retention times and those obtained allowed validating the linear regression model and optimizing selected criteria. The separation between the peaks of the critical pair has been chosen as a critical quality attribute (CQA) for the evaluation of quality chromatogram [8]. As proposed by Lebrun *et al.* [8, 10] we used in this work the separation criterion (*S*) defined as the difference between the beginning of the second eluting peak (t_{RB}) and the end of the first eluting peak (t_{RE}) of the critical peak pair.

Over the experimental domain, as shown on Figure 4, the probability of peak separation P(*S*>0) was low: 0.4%. Due to the very similar chromatographic behavior of some AAI, the tested experimental domain (F= 0.3mL/min, T= 27.5°C, % OM = 80%) did not allow a simultaneous separation of all AAI peaks. This was the case of AQ, SL PM, DHA, AS, PPQ as shown in Figure 5.

This low probability of peak separation led us to split molecules with similar chromatographic behavior in 4 separated groups (Table 2) while the group 5 was constituted by WHO's recommended ACTs drugs marketed in Africa

These five groups were experimented with the same design tested before applying the same corresponding factors levels as mentioned in Table 1. The optimal conditions for each group and quality level are given in Table 3 including the quite large operating range within DS that indicates the robustness of the method for each group.

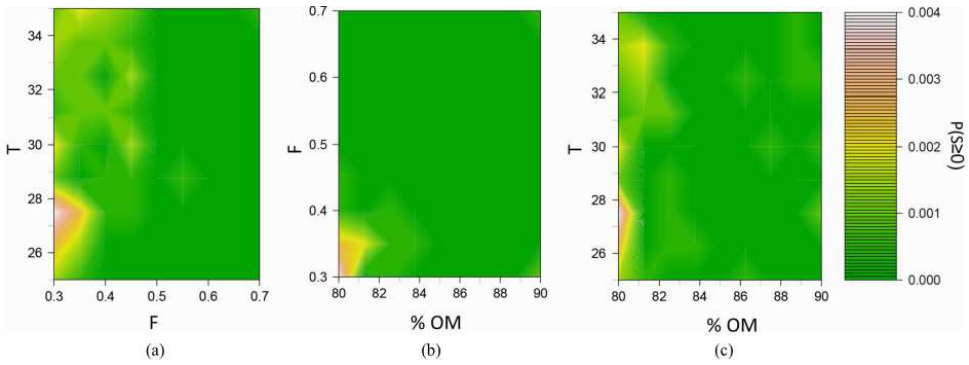

Figure 4. Probability surfaces to reach S ≥ 0. (a) Temperature (˚C) versus Flow rate (ml/min); (b) Flow rate (ml/min) versus Organic modifier (%); (c) Temperature (˚C) versus organic modifier (%).

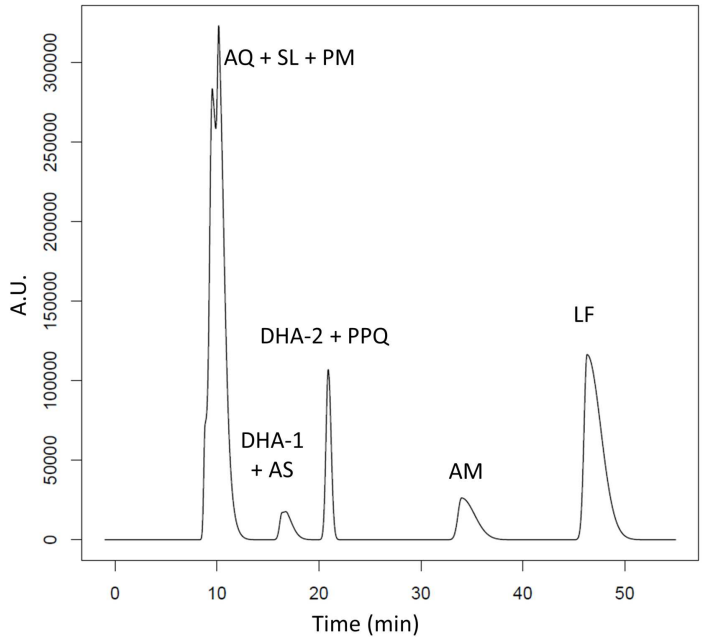

Figure 5. Predicted chromatogram at optimal conditions for 8 antimalarials.

Table 2. Groups of compounds studied in this work.

Groups	Subgroups	Molecules
Group 1	-	AM, LF, DHA-1, DHA-2 and PPQ
Group 2	-	AM, LF, AS and AQ
Group 3	-	AM, LF, DHA-1, DHA-2 and PM
Group 4	-	AM, LF, AS and SL
Group 5	1	AM and LF
	2	AS and AQ

Legend: AM = Artemether, LF = Lumefantrine, DHA = Dihydroartemisinin, PPQ = Piperaquine, AS = Artesunate, AQ = Amodiaquine, PM = Pyrimethamine, SL = Sulfalene.

Table 3. Optimal conditions and operating range within DS for the separation of the 5 groups of antimalarial.

Optimal condition by group						Final optimal conditions
Optimal conditions		Optimal	Flow rate	Organic modifier (OM in %)	Temperature	
Groups	Subgroups	P (S>0)	(F in mL/min)		(T in °C)	
1	-	68.0%	0.45 (0.45-0.61)	81.3 (80.0-82.1)	25.0 (25.0-35.0)	**OM:** 80.0% **F:** 0.5 mL/min **T:** 25°C
2	-	99.5%	0.70 (0.55-0.70)	80.0 (80.0-81.8)	32.5 (25.0-34.5)	
3	-	73.0%	0.45 (0.41-6.50)	81.3 (81.0-82.1)	25.0 (25.0-27.0)	
4	-	92.9%	0.65 (0.48-7.00)	80.0 (80.0-81.5)	32.5 (25.0-35.0)	
5	1	98.5%	0.70 (0.61-0.70)	81.3 (80.5-82.0)	26.3 (25.0-35.0)	**OM:** 82.5% **F:** 0.6 mL/min **T:** 25°C

One can say that a large temperature robust range (25°C to 35°C, except for group 3 (25°C to 27°C)) is important for applying easily the methods in the laboratories without an efficient temperature control system that is often met in resource-restraint environments.

In order to facilitate the screening of AAI in groups 1 to 4, a single method was generated by computing DS obtained only for these groups. One single method was also generated for groups 5.1 and 5.2. The optimal conditions are given in Table 3.

To support the ability of DS to predict analytical conditions that permit chromatographic separation for the AAI in the 5 groups, we tested the mixture of these AAI in each optimal condition using an XBridge C18 (250×4.6mm i.d.; 5µm particle size), preceded by a guard column XBridge guard C18 (20×4.6mm i.d.; 5µm particle size).

The experimental and the predicted chromatograms are given in the figures 6-11 where it can be noticed a close agreement between the different predicted chromatograms and the

corresponded experimental ones.

The correlation between the predicted retention times and observed for the chromatograms recorded at the optimal condition was very good. Indeed, in all cases, the linear correlation coefficient was very close to the unit, validating the accuracy of the prediction. Concerning the two WHO's recommended ACTs, the liquid chromatography method developed for the simultaneous quantification offered the advantage of being used in isocratic mode, unlike the methods of the American pharmacopoeia and international pharmacopoeia offering the gradient mode and are time consuming [14, 15].

In order to reduce the analysis time and thus the solvent consumption, the geometric transfer was performed for each developed method following geometric transfer methodology while checking their robustness [16]. The corresponding analytical conditions were: 4µl for injection volume, 0.6mL/min for the flow rate, 82.5 % for the organic modifier, 17.5% for the buffer. The buffer solution of the isocratic mobile phase consisted of 10 mM ammonium formate (pKa 3.8) adjusted at pH of 2.8 with formic acid and 25°C for the oven temperature of the column whose characteristics are described at section 3.2.

The chromatograms in Figures 6-11 and the results in Table 4 demonstrated the adequate geometric transfer. Indeed, both relative predicted retention times and observed ones were closer for AM, LF, AS, AQ applying the separation conditions before and after geometric transfer. The transferred methods were reduced of about half the run time and obviously a half reduction of the solvent consumption.

It was found important to highlight that the same optimal condition can be used to analyze dihydroartemisinin-piperaquine because of the very good separation observed (data not shown). By cons, the optimized method cannot be used to analyze the associations such as sulfalene-pyrimethamine-dihydroartemisinin and artesunate-sulfalene-pyrimethamine, due to the co-elution of the chromatographic peaks corresponding to sulfalene and pyrimethamine. These associations of antimalarial drugs are also marketed in certain African countries.

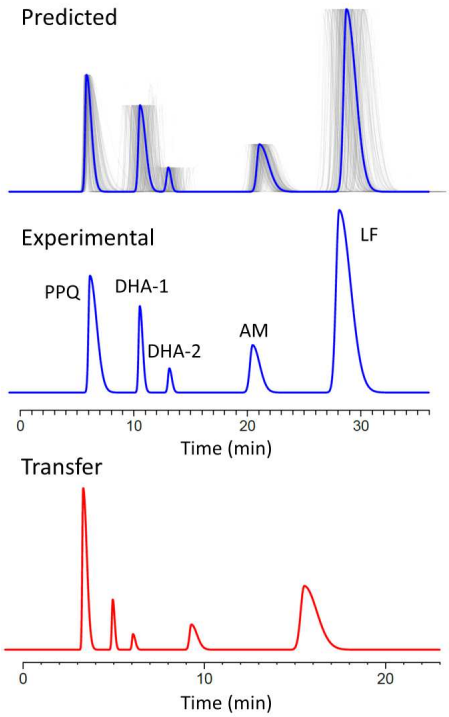

Figure 6. Optimal condition for Group 1: (top) predicted chromatogram, Grey: simulations showing the uncertainty of prediction; (middle) observed chromatogram and (bottom) observed chromatogram resulting of the transfer.

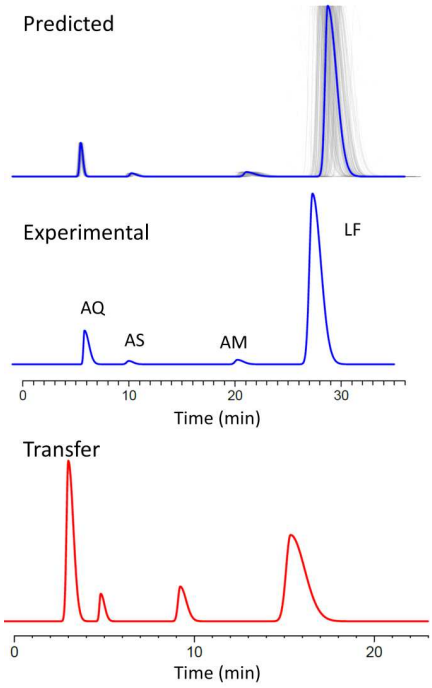

Figure 7. Optimal condition for Group 2: (top) predicted chromatogram, Grey: simulations showing the uncertainty of prediction; (middle) observed chromatogram and (bottom) observed chromatogram resulting of the transfer.

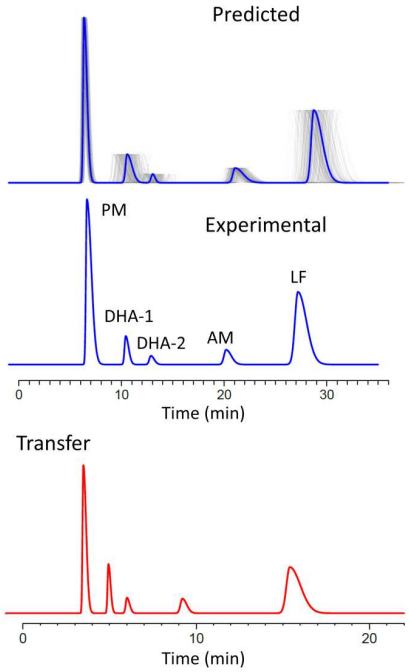

Figure 8. Optimal condition for Group 3: (top) predicted chromatogram, Grey: simulations showing the uncertainty of prediction; (middle) observed chromatogram and (bottom) observed chromatogram resulting of the transfer.

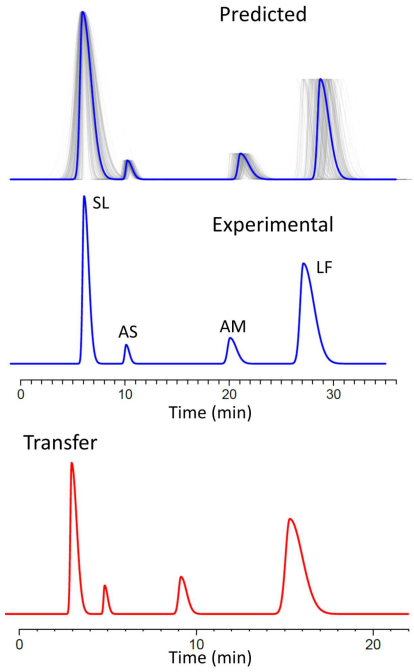

Figure 9. Optimal condition for Group 4: (top) predicted chromatogram, Grey: simulations showing the uncertainty of prediction; (middle) observed chromatogram and (bottom) observed chromatogram resulting of the transfer.

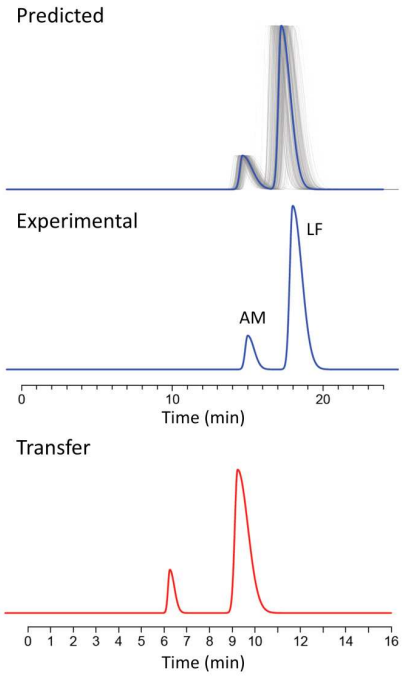

Figure 10. Optimal condition for Group 5.1: (top) predicted chromatogram, Grey: simulations showing the uncertainty of prediction; (middle) observed chromatogram and (bottom) observed chromatogram resulting of the transfer.

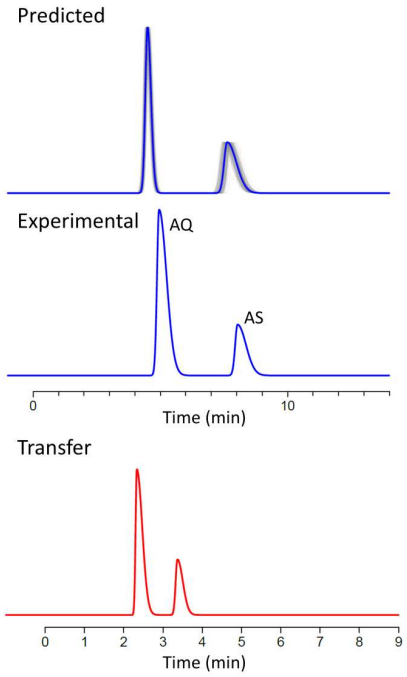

Figure 11. Optimal condition for Group 5.2: (top) predicted chromatogram, Grey: simulations showing the uncertainty of prediction; (middle) observed chromatogram and (bottom) observed chromatogram resulting of the transfer.

Table 4. Results of the method geometric transfer.

Compounds	HPLC optimal				HPLC Transfer		Relative observed retention times Error
	Predicted retention times	Observed retention times	Relative predicted retention times	Relative observed retention times	Observed retention times	Relative observed retention times	
AM	21.529	20.335	0.791	0.746	8.572	0.572	0.184
AQ	5.505	5.270	0.202	0.193	2.297	0.153	0.040
AS	10.142	9.731	0.373	0.357	4.006	0.268	0.089
DHA-1	11.016	10.032	0.405	0.368	4.100	0.274	0.094
DHA-2	13.946	12.662	0.513	0.464	5.226	0.349	0.115
LF	27.195	27.274	1.000	1.000	14.976	1.000	0.000
PM	6.724	6.087	0.247	0.223	2.621	0.175	0.048
PPQ	6.486	5.680	0.238	0.208	2.461	0.164	0.044
SL	5.966	5.686	0.219	0.208	2.159	0.144	0.064

3.2. Method Validation

In current practice, after the optimization step, it becomes increasingly obvious and essential to demonstrate through a method validation that optimized method provides reliable results. In this work, the transferred method was also validated using the accuracy profile as decision tool and for the simultaneous quantitation of the couples artemether/lumefantrine and artesunate/amodiaquine in fixed dose combination (FDC) tablets [17, 18].

We considered the validation criteria commonly used in analytical procedures set out in document Q2A of the International Conference on Harmonization (ICH) [19] namely: selectivity/ specificity, trueness, precision (repeatability and intermediate precision), accuracy, linearity, limit of detection (LOD) and limit of quantitation (LOQ).

An analytical method is specific if it guarantees that the measured signal is only related to the substance intended to be analyzed (targeted compound) and if it allows quantitation of a physicochemical parameter or a chemical group from a single or several substance(s) in the sample [20]. The non-interference of the ingredients present in the matrix was assessed by injecting matrix solutions of each formulation provided by manufacturer and solution containing mixture of targeted compound (AM, LF, AS, AQ). Absence of any interference was noted.

Secondly, we investigated the response function of the method. It is the existing relationship between the response (signal) and the concentration (quantity) of the analyte sample within the range of concentrations tested. The calibration curve was the most appropriate response function. Table 5 presents the most appropriate selected regression models that have been sorted according to the accuracy index.

The selected calibration model is linear regression due to his high level of accuracy index. The concentrations results were back-calculated using the calibration curves. These concentrations were used to determine the relative bias, the precision (repeatability and intermediate precision), the ß-expectation tolerance intervals at 95% probability level, and the linearity. The accuracy profiles for the four compounds are given in Figure 12 while the validation criteria are summarized in Table 6.

The acceptance limits have been set at ±10% according to the International Pharmacopeia and the intended use of the analytical procedure [15].

Trueness refers to the closeness of agreement between a conventionally accepted value or reference value and a mean experimental one. It gives information on systematic error. As shown in Table 6, trueness was expressed in terms of absolute bias (in µg/mL) or relative bias (%) at each concentration level of the validation standards. The trueness of the developed method is good with the absolutes biases and relative biases less than 6% (Table 6).

Precision is the closeness of agreement among measurements from multiple sampling of a homogeneous sample under the recommended conditions. It gives some information on random errors and it can be evaluated at two levels: repeatability and intermediate precision. As can be seen in Table 6, precision was expressed in terms of relative standard deviation values for repeatability and for intermediate precision that were below 3.3%. This indicates a good precision of the developed method.

Table 5. Evaluation of quality of fit for the selected regression model for artemether, lumefantrine, artesunate and amodiaquine.

Active ingredient	Model	Indexes for:			
		Precision	Trueness	Dosing range	Accuracy
Artemether	Linear regression through 0 fitted using the highest level only	0.830	0.746	0.414	0.635
	Linear regression through 0 fitted using the level 1.0 only	0.662	0.866	0.569	0.688
	Weighted (1/X) linear regression	0.461	0.970	1.000	0.765
	Linear regression	0.485	0.962	0.982	0.771
Lumefantrine	Linear regression through 0 fitted using the highest level only.	0.763	0.864	0.498	0.690
	Linear regression through 0 fitted using the level 1.0 only	0.657	0.845	1.000	0.828
	Weighted (1/X) linear regression	0.674	0.868	0.645	0.723
	Linear regression	0.648	0.864	1.000	0.824
Artesunate	Linear regression through 0 fitted using the highest level only.	0.725	0.957	0.944	0.869
	Linear regression through 0 fitted using the level 1.0 only	0.713	0.952	0.999	0.878
	Weighted (1/X) linear regression	0.689	0.974	0.997	0.874
	Linear regression	0.713	0.952	1.000	0.877
Amodiaquine	Linear regression through 0 fitted using the highest level only.	0.884	0.001	0.521	0.001
	Linear regression through 0 fitted using the level 1.0 only	0.931	0.001	0.157	0.001
	Weighted (1/X) linear regression	0.662	0.996	1.000	0.870
	Linear regression	0.660	0.996	1.000	0.870

Table 6. Summary of the validation criteria for artemether, lumefantrine, artesunate and amodiaquine.

Validation criteria	Level	Artemether-Lumefantrine		Artesunate-Amodiaquine	
		Artemether	Lumefantrine	Artesunate	Amodiaquine
Trueness: Absolute bias (µg/mL) (Relative bias (%)	1	1.76 (2.20)	-9.83 (-2.05)	1.40 (1.74)	0.79 (0.33)
	2	0.83 (0.69)	-42.54 (-5.91)	-0.97 (-0.81)	0.33 (0.08)
	3	-4.34 (-2.71)	36.58 (3.81)	-0.04 (-0.03)	3.78 (0.79)
	4	-4.34 (-2.17)	25.12 (2.09)	-4.76 (-2.38)	-2.71 (-0.45)
	5	3.22 (1.34)	-44.56 (-3.18)	1.77 (0.74)	8.21 (-0.33)
Precision: Repeatability (RSD in %) / Intermediate precision (RSD in %)	1	3.28 / 3.28	0.56 / 0.77	3.29 / 3.29	3.24 / 3.24
	2	2.04 / 2.37	0.44 / 0.67	0.80 / 0.80	1.28 / 1.28
	3	2.18 / 2.18	2.11 / 2.11	0.53 / 0.53	1.01 / 1.01
	4	1.24 / 1.24	1.81 / 1.81	1.19 / 1.24	1.08 / 1.08
	5	1.63 / 1.89	0.97 / 0.99	1.03 / 1.41	1.13 / 1.33
Accuracy: β-expectation tolerance interval (in µg/mL) (Relative β-expectation tolerance interval (in %)	1	75.33−88.19	459.30−481.10	74.96-87.85	221.80-259.80
	2	113.30−128.40	662.20−692.70	116.70−121.40	349.10-371.50
	3	147.10−164.2	947.00−1046.00	157.90−162.00	471.90-495.60
	4	189.60−201.70	1172.00−1278.00	189.00−201.40	581.40-613.10
	5	231.2−255.2	1321.00−1390.00	231.00−251.30	708.30-748.20
Uncertainty : Relative expanded uncertainty (%)	1	6.92	1.69	6.94	6.83
	2	5.12	1.49	1.68	2.69
	3	4.60	4.45	1.12	2.13
	4	2.61	3.81	2.64	2.28
	5	4.09	2.10	3.10	2.39
Linearity :	Slope	0.994	1.002	0.992	1.010
	Intercept	0.329	-8.938	0.703	-2.659
	R^2	0.994	0.988	0.997	0.998

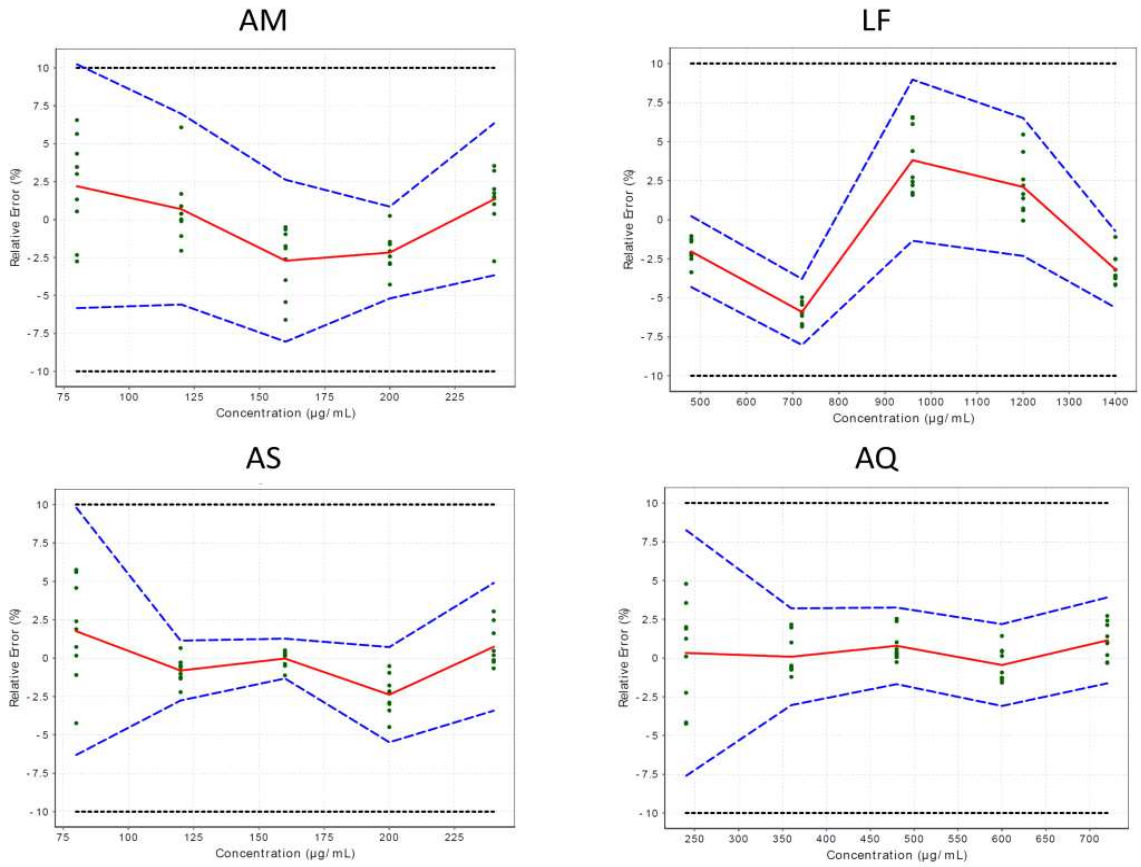

Figure 12. Accuracy profiles for quantitative methods validation of artemether (AM) and lumefantrine (LF) in tablet and of artesunate (AS) and amodiaquine (AQ) in tablet. The plain red line represents the relative bias, the dashed lines the 95% β-expectation tolerance limits and the dotted lines the 10% acceptance limits. The dots express the relative error of the backcalculated concentrations plotted with respect to their targeted concentration.

The linearity of an analytical method is the ability within a definitive range to obtain results directly proportional to the concentration (quantity) of the analyte in the sample. A linear regression model is fitted on the back-calculated concentrations as a function of the introduced concentrations. The good linearity of the results was illustrated (Table 6) by the slopes close to 1 of the regression models obtained between the introduced and the back-calculated concentrations.

Accuracy refers to the closeness of agreement between the test result and the accepted reference value, namely the conventionally true value. The accuracy takes into account the total error, i.e. systematic and random errors, related to the test result. It is assessed from

the accuracy profile illustrated in Figure 12. An accuracy profile is obtained by linking on one hand the lower bounds and on the other hand the upper bounds of the ß- expectation tolerance intervals calculated at each concentration level. As shown in Table 6, the relative ß- expectation tolerance intervals are generally within a range of [-0.73, 9.81 %] excepted level 1 for AM. As the lower and upper tolerance bounds are included within the acceptance limits for all the targeted concentration levels (excepted level 1 for AM), one can ensure that each future result will fall within the acceptance range with a probability of at least 95% [21, 22].

The limit of detection (LOD) is the smallest quantity of the targeted substance that can be detected, but not accurately quantified in the sample. Reported values were: 24.05, 75.17, 3.285 and 11.47 µg/ml for AM, LF, AS and AQ, respectively.

The lower limit of quantification (LOQ) is the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well defined accuracy. The definition can also be applicable to the upper limit of quantitation which is the highest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well defined accuracy. The limits of quantitation were obtained by calculating the smallest and highest concentrations beyond which the accuracy limits or ß-expectation limits go outside the acceptance limits. The dosing range is the interval between the lower and the upper limits where the procedure achieves adequate accuracy. Dosing ranges were 82.90 to 240 µg/mL for AM, 480 to 1440 µg/mL for LF, 80 to 240 µg/mL for AS and 240 to 720 µg/mL for AQ, respectively.

The uncertainty is a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to measurand. As shown in Table 6, relative expanded uncertainly (%) have been found less than 7 %.

3.3. Method Application

The validated method was then used to determine the content of the four targeted compounds found in two sets of tablets samples with fixed dosage combinations. The first set consisted to five different brands coded A1, A2, A3, A4, A5, respectively, and claimed to contain artemether and lumefantrine while the second set coded B1, B2 was claimed to

contain artesunate and amodiaquine. The results obtained for the analyses are presented in Table 7. They consisted in the mean percentage of claimed nominal content and the standard deviation computed on 3 independent samples. Specifications were set to 90.0%-110.0% of the claimed nominal content (mg). All the batches presented artemether and lumefantrine or artesunate and amodiaquine contents very close to the labeled amount and within the specifications. The artemether contents in the tablet samples were within 99.1% to 100.6%, while those of lumefantrine within 94.6% to 99.9%. The artesunate contents in the tablet samples were within 99.3% to 100.7% and those of amodiaquine within 93.4% to 104.4%.

Table 7. Content of seven samples marketed in DRC, Rwanda and Benin.

Drug	Artemether (AM) - Lumefantrine (LF)		Artesunate(AS)–Amodiaquine (AM)		Country of sampling
	Artemether	Lumefantrine	Artesunate	Amodiaquine	
A1	20 mg 100.6 ± 1.8 %	120 mg 99.9 ± 0.6 %	-	-	Benin
A2	20 mg 100.1 ± 0.9 %	120 mg 98.0 ± 0.4 %	-	-	Benin
A3	20 mg 100.2 ± 1.2 %	120 mg 98.2 ± 0.8 %	-	-	Benin
A4	20 mg 99.1 ± 1.5 %	120 mg 94.8 ± 0.9 %	-	-	Rwanda
A5	80 mg 100.5 ± 0.5 %	480 mg 94.6 ± 0.3 %	-	-	DRC
B2	-	-	50 mg 100.7 ± 0.7%	153 mg 93.4 ± 0.2 %	DRC
B3	-	-	100 mg 99.3 ± 0.3 %	270 mg 104.4 ± 0.5 %	DRC

4. Conclusion

In the perspective of fighting against poor quality antimalarials, we undertake the development and validation of one generic procedure of dosage (HPLC-UV/ Isocratic mode) for the simultaneous quantification of two WHO's recommended ACTs in anti-malarial fixed-dose combination (artemether-lumefantrine and artesunate-amodiaquine) tablets by using the DoE/DS optimization strategy.

Three Analytical factors were selected for the experimental design namely: Flow rate of mobile phase (F), column temperature (T°C) and proportion of methanol in the mobile phase (%OM). The experiments showed that only the Flow rate of mobile phase (F) and proportion of methanol in the mobile phase (%OM) had significant effects on peak separations within the explored experimental domain. Design space strategy led to the development of one fast HPLC method able to screen 9 AAI and one for the simultaneous quantitation of two WHO's recommended ACTs in anti-malarial FDC (AM-LF and AS-AQ) tablets.

The LC method developed for the simultaneous quantitation offers the advantage of being used in isocratic mode, unlike the methods of the American and international pharmacopoeias offering the gradient mode and are time consuming. This method was then successfully validated prior to selectivity, linearity, accuracy, trueness and precision, for simultaneous quantitation of AM, LF, AS and AQ using the approach based on total error and accuracy profile as decision tool.

This method can be applied in the routine regulatory quality control of β-artemether and lumefantrine, artesunate and amodiaquine containing FDC drug products. Application to 7 commercial antimalarial formulations marketed in Benin (West Africa), DRC (Central Africa) Rwanda (East Africa) and containing AM/LF or AS/AQ per tablet gave a content in good agreement with the declared content.

This study was the first report of simultaneous determination of artemether lumefantrine artesunate and amodiaquine in fixed dose combination tablets.

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Chapitre V

Techniques non séparatives

Chapitre IV. Techniques non séparatives

Les techniques non séparatives de spectroscopie constituent un outil précieux dans l'identification et le dosage des molécules actives suite à leur simplicité, à la rapidité de leur mise en œuvre et à leur coût relativement faible comparativement aux méthodes chromatographiques. Le domaine de la spectroscopie s'étend sur tout le spectre électromagnétique. Les ondes électromagnétiques Ultraviolet - Visibles ont une gamme de longueurs d'onde qui s'étendent de 200 à 780 nm tandis que les ondes Proche Infarouge s'étendent de 780 à 2500 nm.

Par rapport à la chromatographie liquide, ces techniques présentent cependant l'inconvénient de ne pas s'apprêter à l'analyse des composés à l'état de trace et des impuretés. En effet, l'abscence de préparation d'échantillon permettant une concentration de l'analyte limite l'analyse à des échantillons ayant une concentration suffisante (supérieure au pourcentage dans le cas de la spectroscopie PIR). Le développement de méthode en spectrophotométrie Ultraviolet-Visible consiste à fixer certains facteurs comme la longeur d'onde d'absorption, le milieu de dissolution et les niveaux de concentration.

La spectroscopie vibrationnelle (Proche Infrarouge et Raman) se présente de plus en plus comme technique de choix pour le contrôle de la qualité des médicaments. Cet enthousiasme résulte des avantages mentionnés ci-dessus liés à leur utilisation. De plus, elle s'inscrit parfaitement dans le concept de "Chimie Verte" par la non utilisation des solvants organiques réduisant la pollution associée. Les deux techniques vibrationnelles sont complémentaires. Dans la spectroscopie Proche-Infrarouge, l'absorption est fonction de la modification de moment dipôlaire de la molécule tandis que dans la spectroscopie Raman elle est fonction de changement de polarisabilité de la molécule suite au champ électrique de la lumière excitante. Dès lors, une molécule active en spectroscopie PIR aura une faible activité Raman et inversément.

Le développement de méthodes d'analyse en spectroscopie vibrationnelle est long et complexe tant au niveau de l'échantillonnage que de la sélection du modèle en fonction de la région spectrale traitée, du prétraitement mathématique et de l'outil chimiométrique retenu.

Cependant, une fois développé et validé, le modèle peut être utilisé en routine permettant une analyse rapide des échantillons.

Plusieurs outils statistiques sont utilisés à l'heure actuelle pour améliorer l'extraction des informations obtenues à partir des données analytiques (Spectres Proche Infrarouge ou Raman). Parmi ces outils l'on peut citer l'analyse en composante principale (ACP) et la régression des moindres carrés partiels (PLS). L'ACP consiste à transformer des variables liées entre elles (dites "corrélées") en nouvelles variables indépendantes les unes des autres "non corrélées" de telle sorte qu'elles expliquent le maximum de variabilité des données (approche qualitative). La régression des moindres carrés partiels (PLS) quant à elle, permet d'établi un modèle linéaire en construisant de nouvelles variables maximisant la covariance entre une variable à prédire (variable dépendante) et les données spectrales recueillies dans une matrice (variable indépendante) (approche quantitative).

L'évaluation des modèles se fait en pratique sur une combinaison des indicateurs. Le meilleur modèle sera celui présentant à la fois les plus faibles erreurs d'étalonnage et de prédiction, le plus petit nombre de facteurs et la plus faible somme du carré des résidus. Cependant, l'approche de l'erreur totale est nécessaire afin d'attester de la validité des modèles développés.

En rapport avec notre travail, ce chapitre sera consacré à la spectroscopie Ultraviolet-Visible pour le contrôle qualité des antibiotiques (cas d'amoxicilline et du métronidazole) et des antipaludéens (cas de la quinine), ensuite à la spectroscopie vibrationnelle (Proche Infrarouge ou Raman) pour le contrôle des antipaludéens (cas de la quinine à nouveau) et le contrôle qualité des antiinflammatoires non stéroïdiens (cas du paracétamol). Ceci permettra de prendre également en compte les techniques non séparatives étudiées dans ce chapitre pour contrôler la qualité du « package médical » en RDC.

Section IV.1.

Application de la stratégie de l'erreur totale pour la validation des méthodes spectroscopiques Uv-Visible

Cette section se rapporte à l'article «**Application of Total Error Strategy in Validation of Affordable and Accessible UV-Visible Spectrophotometric Methods for Quality Control of Poor Medicines**» publié dans American Journal of Analytical Chemistry 6 (2015) 106-117.

Section IV.1. Spectrophotométrie UV-Visible – antibiotiques - antipaludéen

Contexte

L'amoxicilline poudre en capsule, le métronidazole en suspension orale et la quinine en comprimé et comme goutte orale sont parmi les médicaments très utilisés pour soigner respectivement les infections bactériennes et la malaria aigue, qui pour rappel, sont des pathologies mortelles. En RDC, ils sont vendus dans les circuits pharmaceutiques formels et informels, avec ou sans ordonnance médicale. Ils sont susceptible d'être contrefaits vue leur forte utilisation, leur prix assez élevé et surtout la facilité de manipuler les formes pharmaceutiques concernées.

Ainsi, pour appuyer les laboratoires locaux de contrôle de qualité, nous avons développé et validé quatre méthodes spectroscopiques Ultraviolet-Visible en vue d'analyser les médicaments précités. Signalons que ce travail a été réalisé au sein du laboratoire d'analyse des médicaments de l'université de Kinshasa constituant ainsi un premier transfert de compétence analytique dans le domaine de la validation de méthodes par application de la stratégie de l'erreur totale.

Les quatre méthodes ont été appliquées avec succès pour le dosage de plusieurs échantillons prélevés à Kinshasa.

Section IV.1. Spectrophotométrie UV-Visible – antibiotiques - antipaludéen

Summary

In the framework of fighting against the poor quality medicines sold in developing countries using classical analytical methods easily accessible in those countries, four UV-Visible spectrophotometric methods for one antimalarial (quinine) and two antibiotics (amoxicillin and metronidazole) have been developed and validated according to the total error strategy using the accuracy profiles as a decision tool. The dosing range was 2 - 10 µg/mL (for quinine sulfate in tablet), 4 - 12µg/mL (for quinine bichlorhydrate in oral drop-metronidazole benzaote in oral suspension) and 15 - 35 µg/mL (for amoxicillin trihydrate in capsule). The validated methods were then applied in determining the content of some analogous medicines sold in the Democratic Republic of Congo. Thus, the proposed UV-Visible spectrophotometric methods are simple and suitable to quantify quinine, amoxicillin and metronidazole in different pharmaceutical forms.

Section IV.1. Spectrophotométrie UV-Visible – antibiotiques - antipaludéen

1. Introduction

Nowadays, malaria remains the most dramatic tropical parasitic disease in the world. It is responsible of more than one million deaths annually. Nearly 60% of the world population lives in areas at risk, namely in Africa, Asia, Central America and South America [1].

Used a longtime ago in the treatment of uncomplicated malaria, monotherapy drug treatment have shown increasing cases of resistance mainly of Plasmodium falciparum [2]. To avoid this phenomenon towards recent antimalarial drugs, World Health Organization (WHO) recommended the use of combination therapies such as the Artemisinin-based combination [3]-[6]. Hence, quinine is still recommended alone in the treatment of severe malaria attacks as well as for chloroquine-resistant falciparum malaria [7].

Malaria disease is often accompanied with symptoms such as fever, diarrhea, vomiting and some infections, which justifies the medical prescription of other drugs that can relieve these symptoms and treat these infections besides of antimalarial medicine. Amoxicillin and metronidazole are among the most used antibiotics and antiparasitic to treat certain infections. These kinds of medicines are very often bought in self-medication in many developing countries.

Besides the high risk of failure treatment due to plasmodial resistance to monotherapy, the spread of illicit distribution and the marketing of poor quality medicines worldwide and particularly in developing countries are seriously affecting the success of this treatment. Several other consequences towards the public health can be noticed such as adverse reactions, increase of morbidity and of mortality as well as loss of public confidence and waste of scarce resources, etc. [8]. Poor quality medicines can be classified into three main categories: Counterfeit, substandard and degraded medicines [9]. WHO reported that 6% of drugs worldwide are counterfeit while Food and Drug Administration (FDA, USA) estimated this proportion to 10% [10]. In fact, the true proportion cannot be estimated since values vary from one region to another and are related to a certain period. In 2003, P.Newton et al. published the results of a survey conducted in Southeast Asia on the medicines containing

artesunate that is used against malaria chemo-resistant. In 40% of cases, these medicines were found to be falsified [11].

Therefore, to guarantee an access of populations to safe and sure medicines, it is important to set up appropriate measures that will allow evaluating and preserving the quality of those medicines, for example: having appropriate analytical method, strong quality assurance and regulatory, etc.

However, several laboratories in the developing countries do not yet dispose new analytical technology capable of satisfying to these requirements (HPLC, GC etc.). They still apply the classic methods such as titration or those described in pharmacopoeia for raw material analysis that they adapt and the UV-Visible spectrophotometric methods. Hence, these methods are affordable, available and technically feasible. Therefore, it is mandatory to verify if this adaptation fits well with the new purpose namely the assay of the active ingredient in new pharmaceutical formulations usually not described qualitatively of quantitatively.

In this study, we were interested to verify by means of validation four UV-Visible spectrophotometric methods currently used in several quality control laboratories in Kinshasa in Democratic Republic of Congo (DRC): method for the determination of quinine 20% in oral drops and of quinine tablets 500 mg, method for the determination of amoxicillin trihydrate ($3H_2O$) 500 mg in capsule and method for the determination of metronidazole 125 mg/5mL in oral suspension. The validation of analytical methods is an obligation concept that is relatively recent in main sub-Saharan countries however that is increasing in requirement by legal health authorities. For the preparation of validation standards, four matrix formulations were provided by the Indian manufacturing laboratories legally authorized in the DRC. Finally, the validated method was used to analyze drugs marketed in DRC. The chemical structures of the concerned active ingredients are shown in Figure 1.

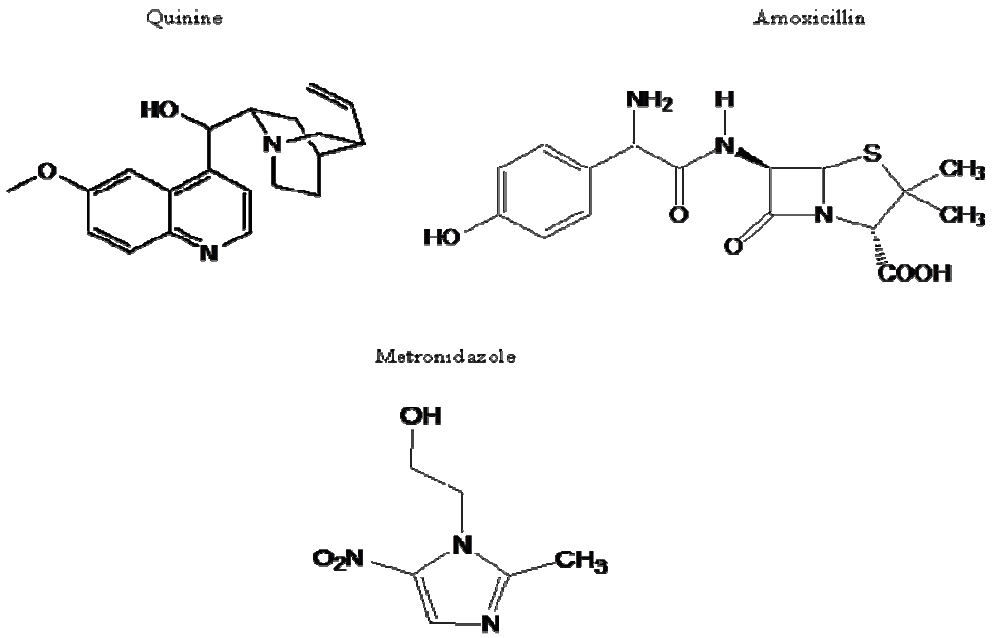


Figure 1. The chemical structures of quinine, amoxicillin and metronidazole.

2. Method Validation Strategy

Validation of an assay is a set of operations carried out to demonstrate that a procedure is sufficiently accurate and reliable to have confidence in the results provided for the intended use (of the assay) [12]-[16]. Several strategies have been developed for this purpose [12] [17]-[22]. From a statistical point of view, they have certain insufficiency including the lack of consideration of decision making based on acceptance limits defined a priori, lack of use of the risk related to the future use of the method. A new validation strategy based on the accuracy profile was described [23] [24]. It is in perfect agreement with the objective of an analytical method, namely its ability to quantify as accurately as possible each of the unknown quantities a laboratory will determine in the future [24]. This new validation strategy combines two basic and fundamental criteria, bias and precision to the final result of a measurement, and therefore reflect the total measurement error, systematic error and random error, respectively [23]. The principle of this validation strategy can be translated by Equation (1) which states that the difference between a value of measurement (x) and its true value (μ) must be less than an acceptance limit (λ).

$$-\lambda < x - \mu < \lambda \Leftrightarrow |x - \mu| < \lambda \quad (1)$$

From Equation (1), one can notice that the consideration of acceptance limits introduces a first concept for the analyst to take decisions based on the objective of the

analytical method and the limitation or control of error propagation. Commonly, the acceptance limit is fixed at 1% or at 2% for the assay of active ingredients in a raw material, at 5% for dosage forms [23] [24]. In other referential, it is set at 10% for pharmaceutical dosage forms [25]. Always from the Equation (1), one can notice a second concept the accuracy profile that is constructed from estimates of the tolerance interval of β-expectation measurements at each concentration level. Another important concept derived from that Equation (1) is the set of "good analytical procedure" with a known risk which may result in the following equation:

$$Pr[x - \mu > \lambda] \leq \beta \quad (2)$$

With β the proportion of measurements within the limits of acceptance, and λ the quantity defining the acceptance limits fixed a priori according to the constraints of the industry. The risk of a procedure is evaluated by the proportion of measurements (Pr) that could be out of the acceptance limits [23] [26]. Thus, the accuracy profile can be considered as a decision tool based on the risk associated with the method. The concept of risk is related to the guarantee for the future analysis of unknown samples while applying the validated method. As a decision tool, the accuracy profile can be used to accept or reject an analytical method according to the expected usage. Thus decision tool can be exploited as a diagnostic tool, for example, to select the most suitable regression model for calibration, to determine the limits of upper and lower quantification that will define the dosing interval [27].

3. Experimentation

3.1. Chemical and Reagents

Amoxicillin trihydrate (99.1%) and metronidazole benzoate (99.9%) were purchased from Fagron N. V. (Waregem, Belgium), quinine sulfate (96.9%) from Sigma Aldrich (Antwerp, Belgium) and quinine bichlorhydrate (100.8%) from Molekula Limited (Dorset, UK). Sodium hydroxide was supplied from Merck (Darmstadt, Germany) while the following excipients aerosil, avicel, benzoic acid, gelatin, lactose, magnesium stearate, propylene glycol, talc, starch, sodium benzoate, sorbitol, sodium saccharin, sodium carboxymethyl cellulose (CMC), tween 80, xanthine gum were obtained at New Cesamex Laboratory (Kinshasa, DRC).

3.2. Materials

The spectrophotometer used was brand HP/Agilent 8453 with Chemstation software from Agilent Technologies (Gyeonggi-do, South Korea) and an electronic analytical balance SHIMADZU AUW 220 D (Kyoto, Japan). Prior to their use, the material were qualified according to an internal qualification procedure. The wavelengths used were 229 nm for amoxicillin, 235 nm for quinine and for metronidazole. Ultrapure water was obtained from Aqua MaxTM—Basic 360 series, purification system from YL Instruments (Gyeonggi-do, South Korea). For statistical data treatment, validation treatment and graph drawing, the Enoval® software v3.0 (Arlenda, Liege, Belgium) was used.

3.3. Standard Sample Preparations

Four different dissolutions solvent were used for the dissolution of the active ingredients: ultra pure water for quinine dihydrochloride, methanol for quinine sulfate and metronidazole benzoate, 0.1 N sodium hydroxide for amoxicillin trihydrate. The same solvent was used as blank. The stock solutions were prepared by dissolving 10mg of each of the active ingredients in 100 mL of solvent corresponding to dissolution media.

For the calibration standards (CS), dilutions were performed in different dissolutions solvent in order to obtain solutions at three concentration levels (m = 3), as mentioned in Table 1.

For validation standards (VS), independent stock solutions of the active ingredients were prepared in the same way as described for the CS. The excipients of each pharmaceutical formulation were added in stock solutions. Subsequent dilutions were carried out in dissolutions solvent to obtain solutions at five different concentration levels (m = 5), as mentioned in Table 2.

The VS were independently prepared in the matrix, in such a way to simulate as much as possible the corresponding formulation and its routine analysis. For each concentration level and each standard, three independent repetitions (n = 3) were performed daily for three days (p = 3).

Table 1. Content of each targeted active ingredient for the calibration standards and the validation standards according to pharmaceutical formulations.

Quinine sulfate in tablet		**Quinine bichlorhydrate in oral drop**		**Metronidazole benzoate in oral suspension**		**Amoxicillin trihydrate in capsule**	
Level of content	Concentration	Level of content	Concentration	Level of content	Concentration	Level of content	Concentration
1	2µg/mL	1	4µg/mL	1	4µg/mL	1	15µg/mL
2	4µg/mL	2	6µg/mL	2	6µg/mL	2	20µg/mL
3	6µg/mL	3	8µg/mL	3	8µg/mL	3	25µg/mL
4	8µg/mL	4	10µg/mL	4	10µg/mL	4	30µg/mL
5	10µg/mL	5	12µg/mL	5	12µg/mL	5	35µg/mL
Total = 15 samples/day		Total = 15 samples/day		Total = 15 samples/day		Total = 15 samples/day	

Table 2. Qualitative and quantitative composition of the different matrices.

Drug 1 : Tablet of quinine sulfate (500 mg)				
Starch and aerosil	Avicel	Gelatin	Talc	Magnesium Stearate
0.125 mg and 0.125mg	60mg	1 mg	25mg	30mg
Drug 2 : Oral drop of quinine bichlorhydrate (3g/15mL)				
propylene glycol		Benzoic acid		
0.22g		0.01g		
Drug 3 : Capsule of amoxicillin trihydrate (500 mg)				
Talc		Magnesium stearate		
90 mg		55 mg		
Drug 4 : Suspension of metronidazol benzoate (2.5g/100mL)				
Sugar	propylene glycol	Na-CMC and tween 80	Sorbitol	Sodium benzoate
4.7 g	2.5g	312.5 mg and 250 mg	3.125g	93.8 mg

4. Results and Discussion

4.1. Validation

4.1.1. Consideration of Different Limits

As a first step in the validation of the four UV-Visible spectrophotometric methods evaluated in this study, the acceptance limit was settled at 10% that corresponds to the assay specification of 90.0% - 110.0% for the pharmaceutical formulations concerned. Unless specified, we have considered a risk of 5%, meaning that the assay method should give guarantee of having 95% of future measurements within the 10% acceptance limits.

4.1.2. Evaluation of Quality of Results

After having run the practical experiences of validation according to the experimental design, several tools were used to evaluate the quality of the results collected for each analytical method.

At first, by means of calibration standards data several regression models were computed and used to evaluate the fitting of the validation standards. For each regression model, several indexes were computed, namely, the precision, the trueness and the dosing range. An index value closer to 1 indicated a good suitability of the validation parameters, e.g., good precision, good trueness according to the 10% acceptance limits, whereas a dosing range index closer to 1 relates to the ability of the assay method to cover the maximum of the tested range for quantification. In Table 3 we reported only the most adequate and appropriate regression models to easy use in routine. The accuracy index values were also computed as the geometric mean of the three other indexes. As can be seen, the accuracy indexes for quinine sulfate in tablet, quinine bichlorhydrate in oral drop, metronidazole benzoate in oral suspension and amoxicillin trihydrate in capsule were all above 0.82 indicating very good method accuracy with the ability to quantifiy quinine sulfate, quinine bichlorhydrate, metronidazole benzoate and amoxicillin trihydrate all over the tested dosing range (index of 1).

4.2. Validation Parameters

The following models were selected to evaluate the other validation parameters: linear regression for quinine bichlorhydrate in oral drop and for metronidazole benzoate in oral

suspension, linear regression through 0 fitted using the highest concentration level only for quinine sulfate in tablet and for amoxicillin trihydrate in suspension. The risks were set at 5% in all cases.

Table 3. Evaluation of quality of fit for the selected regression model for quinine sulfate, Quinine bichlorhydrate, amoxicillin trihydrate, and metronidazole benzoate.

Active ingredient	Regression models	Indexes for			
		Precision	Trueness	Dosing range	Accuracy
Quinine sulfate in tablet	Linear Regression Through 0 Fitted using the highest level only.	0.704	0.993	1.000	0.888
	Linear regression through 0 fitted using the level 1.0 only	0.669	0.998	1.000	0.874
	Weighted (1/X) Linear Regression	0.688	0.998	1.000	0.882
	Linear regression	0.702	0.996	1.000	0.888
Quinine bichlorhydrate in oral drop	Linear Regression Through 0 Fitted using the highest level only.	0.632	0.954	1.000	0.832
	Linear regression through 0 fitted using the level 1.0 only	0.584	0.988	1.000	0.845
	Weighted (1/X) Linear Regression	0.693	0.993	1.000	0.883
	Linear regression	0.702	0.993	1.000	0.887
Amoxicillin trihydrate in capsule	Linear Regression Through 0 Fitted using the highest level only.	0.738	0.998	1.000	0.903
	Linear regression through 0 fitted using the level 1.0 only	0.719	0.995	1.000	0.895
	Weighted (1/X) Linear Regression	0.653	0.991	1.000	0.865
	Linear regression	0.662	0.990	1.000	0.869
Metronidazole benzoate in suspension	Linear Regression Through 0 Fitted using the highest level only.	0.641	0.901	1.000	0.822
	Linear regression through 0 fitted using the level 1.0 only	0.653	0.953	1.000	0.871
	Weighted (1/X) Linear Regression	0.697	0.994	1.000	0.879
	Linear regression	0.703	0.993	1.000	0.887

4.2.1. Method Selectivity

As a next step, the selectivity criteria was evaluated for each UV-Visible spectrophotometric method using the matrix blank containing only the excipients of drug as mentioned in Table 2 with the compound not targeted by the method. No interference was observed as analytical response.

4.2.2. Trueness

The results of trueness illustrate the mean of bias observed between the series of measurements and the reference concentrations. Using the calibration curve of each analyte and the concentrations of the validation standards (VS) was back-calculated and expressed in terms of absolute bias (µg/mL) and relative bias (%). As shown in Table 4, the trueness of the developed methods was found acceptable since the relative biases were below 2.78%.

4.2.3. Precision

Precision refers to the ability of the methods to provide proximate results obtained from multiple measurements of the same samples, under the same conditions. It was expressed in terms of relative standard deviation (RSD, %) for repeatability (intraday variations) and intermediate precision (inter-day variations) at each concentration level. As shown in Table 4, precision was found acceptable, as the RSD values for repeatability and intermediate precision were below 2.37% and 2.62%, respectively.

Table 4. Summary of the validation criteria for quinine sulfate, quinine bichlorhydrate, amoxicillin trihydrate, and metronidazole benzoate

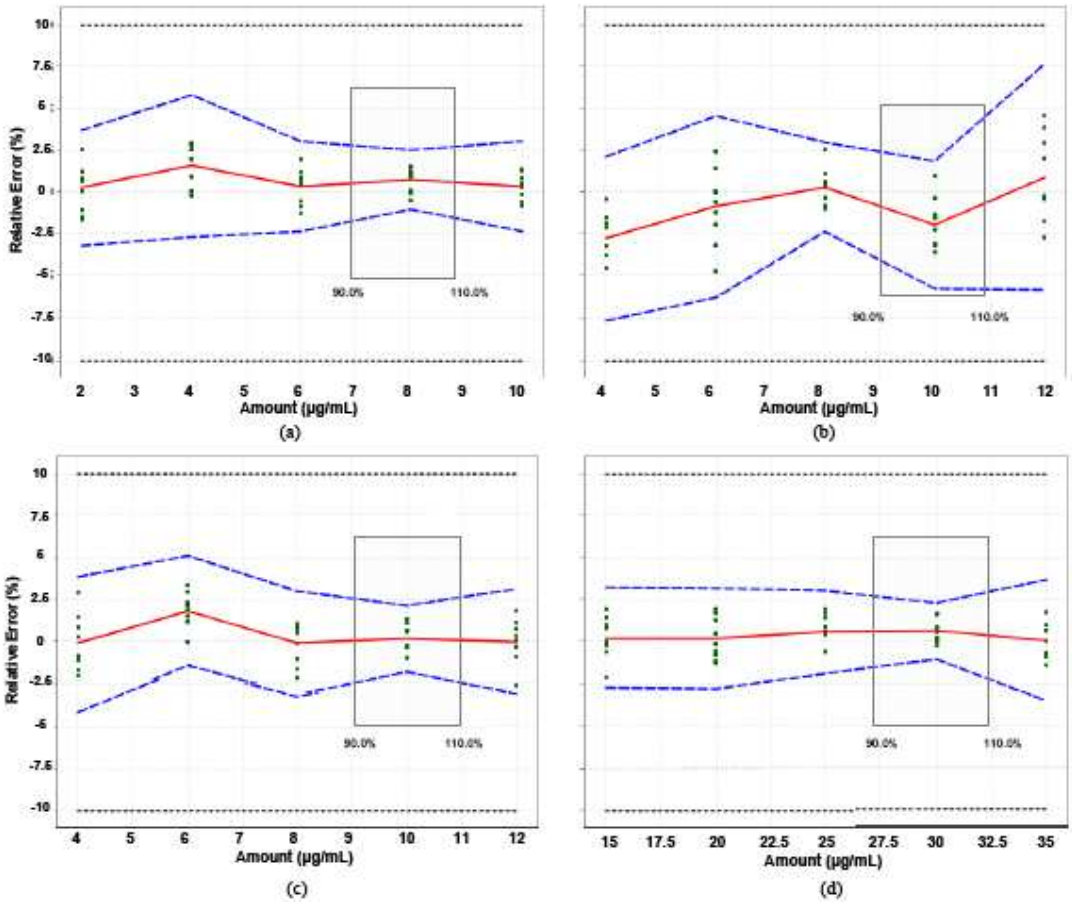
Validation criteria	level	Quinine sulfate of the tablet	Quinine bichlorhydrate of the oral drop	Amoxicillin $3H_2O$ of the capsule	Metronidazole benzoate of the suspension
Trueness: Absolute bias (µg/mL) (Relative bias (%))	1	0.01 (0.26)	-0.11 (-2.77)	0.04 (0.24)	0.01 (-0.15)
	2	0.06 (1.58)	-0.05 (-0.87)	0.04 (0.18)	0.11 (1.81)
	3	0.02 (0.36)	0.02 (0.29)	0.15 (0.59)	-0.01 (-0.11)
	4	0.06 (0.75)	-0.20 (-1.97)	0.20 (0.66)	0.02 (0.15)
	5	0.03 (0.33)	-0.11 (0.88)	0.02 (0.06)	-0.01 (-0.33)
Precision: Repeatability (%) / Intermediate precision (%)	1	1.41 / 1.41	1.22 / 1.63	1.23 / 1.23	1.63/1.63
	2	1.11 / 1.42	2.22 / 2.22	1.23 / 1.23	0.79/1.10
	3	0.99 / 1.07	1.09 / 1.09	1.01 / 1.01	1.19/1.24
	4	0.73 / 0.73	1.48 / 1.54	0.68 /0.68	0.77/0.79
	5	0.53 / 0.83	2.36 / 1.62	0.74 / 1.14	1.29/1.29
Accuracy: *β*-expectation tolerance interval. (µg/mL) (Relative *β*-expectation tolerance interval (%))	1	1.94−2.07 (-3.20/3.71)	3.69−4.09 (-7.68/-2.14)	14.58-15.49 (-2.77/3.24)	3.83-4.15 (-4.14/3.84)
	2	3.89−4.23 (-2.69/5.84)	5.62−6.27 (-6.31/4.56)	19.43−20.64 (-2.83/3.19)	5.91-6.31 (-1.47/5.09)
	3	5.86−6.18 (-2.34/3.07)	7.81−8.24 (-2.38/2.96)	24.53−25.77 (-1.87/3.06)	7.74-8.24 (-3.20/2.98)
	4	7.92−8.20 (-1.03/2.53)	9.42−10.19 (-5.79/1.86)	29.70−30.70 (-1.02/2.33)	9.82-10.21 (-1.82/2.11)
	5	9.76−10.30 (-2.37/3.04)	11.29−12.92 (-5.89/7.64)	33.75−36.29 (-3.57/3.69)	11.62-12.37 (-3.18/3.14)
Uncertainty : Relative expanded uncertainty (%)	1	2.78	3.60	2.60	3.44
	2	3.12	4.68	2.60	2.42
	3	2.28	2.30	2.13	2.63
	4	1.54	3.26	1.44	1.68
	5	1.86	5.63	2.53	2.72
Linearity :	Slope	1.003	1.014	1.003	0996
	Intercept	0.017	-0.161	0.020	0.056
	R^2	0.999	0.996	0.999	0.999

4.2.4. Accuracy

The accuracy was determined by the 95% *β*-expectation tolerance interval in order to observe the closeness of agreement of every single concentration measured by the method, and the assumed true value of this concentration. It combines the uncertainties of trueness and precision and is expressed as actual values and as a percentage of the targeted concentration

(Table 4). As shown in Figure 2, all methods were found accurate all over the tested concentration levels since the lower and upper tolerance intervals were included within the 10% acceptance limits, guaranteeing that 95% of the future quantification results will be accurate in the dosing ranges evaluated. However, the four methods are able to quantify largely the corresponding analytes at the target concentration 100.0% that correspond to 8 µg/mL of quinine sulfate for tablet, 10 µg/mL of quinine bichlorhydrate for oral drop and metronidazole benzoate for oral suspension and to 30 µg/mL of amoxicillin trihydrate for capsule.

In addition, the four methods are also able to quantify largely the corresponding analytes at ±10.0% of the claimed content that can ensure the control during the release of batch production as well quantification of low or high content.

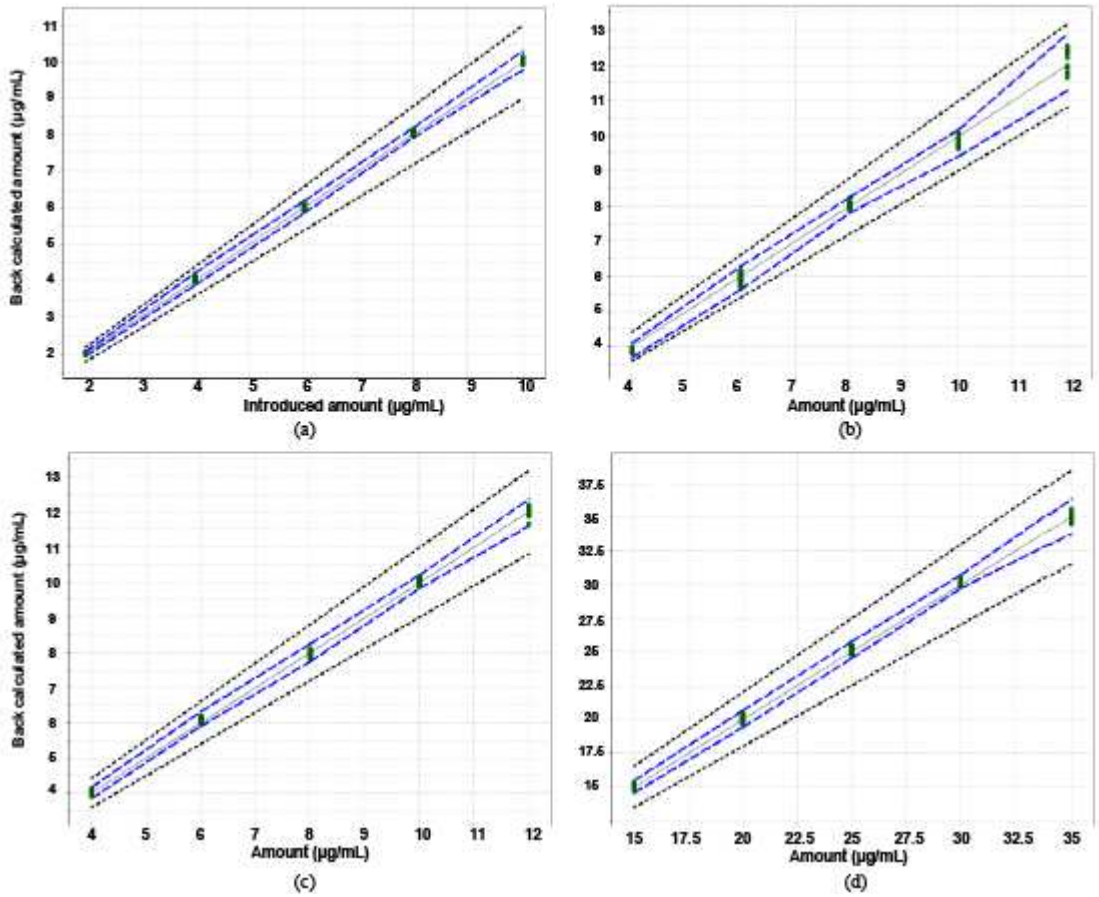


Legend: Red = bias (%), Black = acceptance limit (±10%), Blue = 95% β-expectation tolerance interval, Green = individual measures. (a) Quinine sulfate in tablet; (b) Quinine bichlorhydrate in oral drop; (c) Metronidazole benzoate in suspension; and (d) Amoxicillin trihydrate in capsule.

Figure 2. Accuracy profiles for quantitative methods validation of quinine sulfate in tablet, quinine bichlorhydrate in oral drop, metronidazole benzoate in suspension and amoxicillin trihydrate in capsule

4.2.5. Linearity

The linearity of results was also evaluated for the four tested methods. It expresses the ability of the methods to give results directly proportional to the concentrations. Indication about linearity is given by the parameters of the linearity equation, namely the coefficient of determination (R2), the slope and the y-intercept. As can be seen in Table 4, the relationship was assumed linear for the four methods as the R2 values and the slope were close to one with acceptable intercepts fairly near to zero. Figure 3 shows that, for each concentration level, the absolute 95% β-expectation tolerance limits were all within the absolute acceptance, confirming the linearity of these four UV-Visible spectrophotometric methods.



Legend: Gray = identity line, Black = acceptance limit (±10%), Blue = 95% β-expectation tolerance interval, Green = individual measures. (a) Quinine sulfate in tablet; (b) Quinine bichlorhydrate in oral drop; (c) Metronidazole benzoate in suspension; and (d) Amoxicillin trihydrate in capsule.

Figure 3. Linearity graph for quantitative methods validation of quinine sulfate in tablet, quinine bichlorhydrate in oral drop, metronidazole benzoate in suspension and amoxicillin trihydrate in capsule.

4.2.6. Limits of Quantification (LOQ)

As the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with a well-defined accuracy, the LOQ was evaluated by calculating the smallest concentration beyond which the accuracy limits or β-expectation tolerance limits go outside the acceptance limits. As the accuracy profile was included inside the acceptance limits (Figure 2), the first concentration level was considered as the lower LOQ for all molecules studied (2 µg/mL for quinine sulfate in tablet, 4 µg/mL for quinine bichlorhydrate in oral drop and for metronidazole benzoate in oral suspension and 15 µg/mL for amoxicillin trihydrate in capsule). On the other hand, the upper LOQ was evaluated as the highest quantity of the targeted substance in the sample that can be assayed under

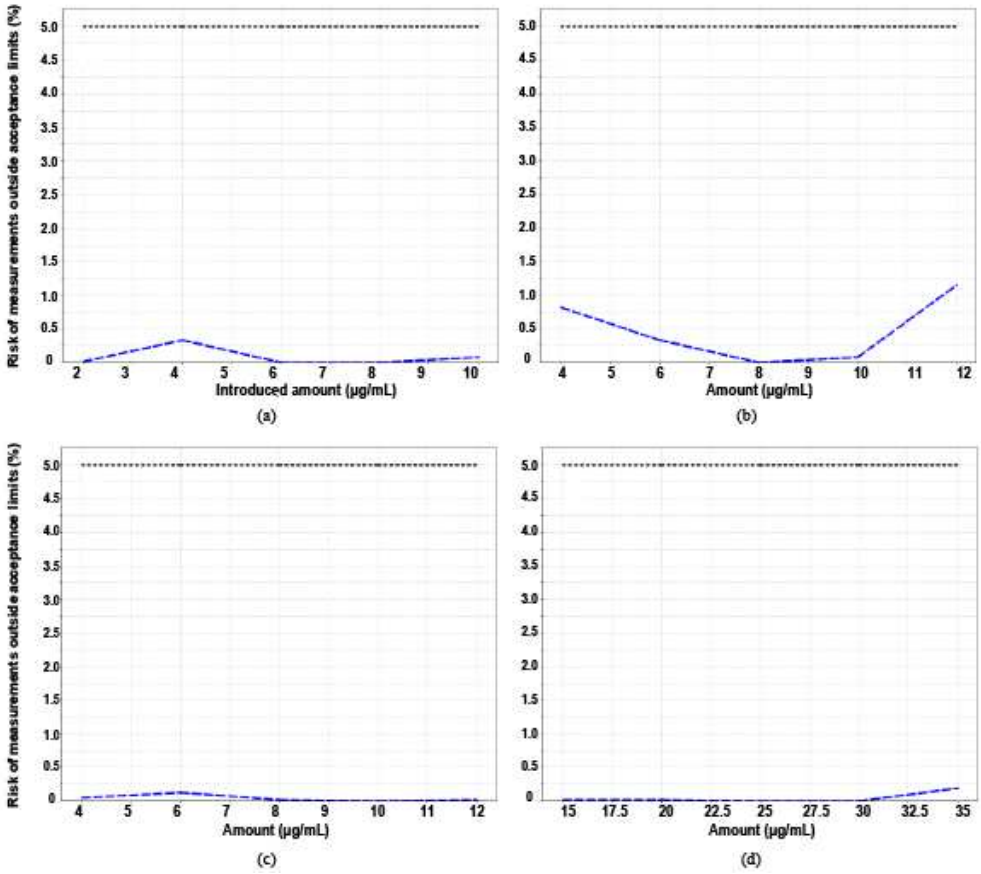
experimental conditions with a well-defined accuracy. It was estimated by calculating the highest concentration above which the accuracy limits or β-expectation tolerance limits go outside the acceptance limits. For all the four tested methods, the upper LOQ were the highest concentration levels, namely 10 µg/mL for quinine sulfate in tablet, 12 µg/mL for quinine bichlorhydrate in oral drop and for metronidazole benzoate in oral suspension and 35 µg/mL for amoxicillin trihydrate in capsule.

4.3. Other Parameters

4.3.1. Risk Profile

In this study, the risk profile was used to investigate the risk of having future measurements falling outside of the 10% acceptance limits for each concentration level taking into account each analyte with its matrix and the selected regression model. Figure 4 shows that the risks of having future measurements outside the 10% acceptance limits were practically below 1.3% at all over the tested concentration range.

When analyzing of these products in routine, only about 98.7 times out of 100 the future measurements will be included in the 10% acceptance limits.



Legend: (a) Quinine sulfate in tablet; (b) Quinine bichlorhydrate in oral drop; (c) Metronidazole benzoate in suspension; and (d) Amoxicillin trihydrate in capsule.

Figure 4. Graph of the risk profiles with a risk level set a priori at 10%.

4.3.2. Uncertainty of Measurements

The uncertainty corresponds to the dispersion of the values that could reasonably be attributed to the measurand. The uncertainty was derived from the variance used to build the β-expectation tolerance limits at each concentration level tested, prior to intermediate precision, between and within-series variances [27].

As shown in Table 4, uncertainties of measurements of the developed methods were found acceptable since the relative expanded uncertainty were below 5.64%, confirming that all the results are within the 10% acceptance limit.

4.4. Application

The validated methods were applied for the determination of the four active ingredients (quinine sulfate, quinine bichlorhydrate, amoxicillin, and metronidazole) in several brand pharmaceutical products coded A, B, C, D, and purchased in the DRC. The samples were obtained in non legal Pharmacy located in suburban areas. As it can be noticed in Table 5, all the targeted and claimed active ingredients were quantified and only three products (code C001 and C002 suspension containing metronidazole benzoate and code D001 capsule containing amoxicillin trihydrate) were not compliant with the assay specification 90.0% - 110.0% for the analyzed compound [25].

Table 5. Assay results of fifteen pharmaceuticals marketed in DRC. Results consist in the mean percentage of claimed nominal content and the standard deviation computed on 3 independent samples. Specifications are set to 90% - 110% of the claimed nominal content (500 mg for Quinine sulfate, 3 g/15mL for quinine bichlorhydrate, 125 mg/5mL for metronidazole benzoate and 500 mg for amoxicicilline 3H2O).

Drug	Quinine sulfate Content	Quinine bichlorhydrate Content	Metronidazole benzoate Content	Amoxicillin trihydrate Content
A 001 Tablet	500 mg **97.3 ± 0.71 %**	-		-
A 002 Tablet	500 mg **102.0 ± 0.52 %**	-		-
B 003 Oral drop	-	3 g/15mL **101.0 ± 0.95**		-
B 004 Oral drop	-	3 g/15mL **99.7 ± 0.62**		-
B 005 Oral drop	-	3 g/15mL **102.9 ± 0.71**		-
B 006 Oral drop	-	3 g/15mL **95.7 ± 0.93**		-
B 007 Oral drop	-	3 g/15mL **96.9 ± 0.87**		-
B 008 Oral drop	-	3 g/15mL **104.1 ± 0.97**		-
C 001 Suspension	-	-	125 mg/5mL **87.2 ± 1.11 %**	-
C 002 Suspension	-	-	125 mg/5mL **95.7 ± 0.97 %**	-
C 003 Suspension	-	-	125 mg/5mL **88.2 ± 1.10 %**	-
D 001 Capsule	-	-	-	500mg **111.1 ± 0.91 %**
D 002 Capsule	-	-	-	500mg **97.7 ± 1.03 %**
D 003 Capsule				500mg **91.3 ± 0.57 %**
D 004 Capsule	-	-	-	500mg **96.8 ± 0.91 %**

5. Conclusion

In the framework of fighting against the phenomenon of poor quality medecines that affects mostly developing countries and ensure that the assay tests results are reliable for UV-Visible spectrophotometric methods, it was necessary to validate these methods and use them for routine analysis in the future.

In order to fight this problem, easily accessible classical analytical methods were developed. Four methods were concerned and were validated for the quantitative analysis of active substances present in pharmaceutical formulations suspected to be of poor quality.

For these UV-Visible spectrophotometric methods, the assay method of quinine sulfate in tablets was validated in the dosing range of 2 µg/mL to 10 µg/mL, the assay methods for quinine bichlorhydrate in oral drop and metronidazole benzoate in oral suspension were validated in the range of 4 µg/mL to 12 µg/mL and amoxicillin trihydrate in capsule was validated in the range of 15 µg/mL to 35 µg/mL. The strategy of the total error has been used to validate these methods that present advantages of being also simple, rapid and affordable.

Finally, the methods have been successfully applied to the determination of quinine, amoxicillin and metronidazole in fifteen pharmaceuticals marketed in DRC.

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Section IV.2.

La Spectroscopie Proche Infrarouge, une technique non destructive dans la lutte contre la contrefaçon des médicaments

Cette section se rapporte à l'article «**Near infrared spectroscopy, a non-destructive technique to fight against counterfeit medicines**» publié dans le Journal Spectra ANALYSE 298 (2014) 46-49.

Contexte

Le paracétamol sirop est un médicament très largement utilisé en RDC dans le traitement de la fièvre chez l'enfant. Comme nous l'avions déjà épinglé au point III.3, il fait partie des 15 médicaments interdits d'importation en RDC. Rappelons que le sirop de paracétamol préparé avec du diéthylène glycol, un produit toxique substitué à la place du propylène glycol avait provoqué le décès de 89 personnes en Haïti en 1995, de 30 nourrissons en Inde en 1998 et de 100 bébés au Nigéria en 2008.

Dans le souci de prévenir l'occurrence de cette situation malheureuse et tragique, de protéger la production pharmaceutique locale et d'appuyer les autorités Congolaises de la Santé Publique, nous avons voulu démontrer la capacité de la spectroscopie Proche Infrarouge à discriminer rapidement le vrai médicament des faux médicaments tant au niveau quantitatif que qualitatif de la formulation pharmaceutique sirop mais aussi d'autres formulations pharmaceutiques similaires commercialisées en monothérapie. La méthode développée a permis de discriminer avec succès le sirop de paracétamol de référence des échantillons contrefaits et sous-standards.

Résumé

La spectroscopie proche infrarouge est une méthode d'analyse en plein développement. Force est de constater qu'elle est de plus en plus utilisée dans le secteur pharmaceutique pour le contrôle de qualité des produits. En effet, la spectroscopie proche infrarouge permet l'analyse rapide, non destructive, polyvalente des échantillons et ne nécessite par d'étape de préparation de ces derniers. Compte tenu de ces avantages, elle se présente de plus en plus comme l'une des méthodes de première ligne dans la lutte contre la contrefaçon des médicaments.

Summary

Near infrared spectroscopy is a ver y promising and expanding analytical technique. It has to be noted that this technique is becoming more used in the pharmaceutical feld for the quality control of products. Indeed near infrared spectroscopy allows to perform fast, non-destructive, versatile analysis of the sample and minimization of the sample preparation. Based on those advantages, this spectroscopic method is one of the frst reliable analytical techniques for fghting against counterfeit medicines.

1. Introduction

Selon une estimation de l'Administration Américaine des Denrées Alimentaires et des Médicaments (FDA), si la part des médicaments contrefaits ou falsifiées représente plus de 10% du marché mondial des médicaments, elle s'élève jusqu'à 80% dans les pays en voie de développement [1]. Ce phénomène touche aussi bien les pays industrialisés que les pays émergents et s'accompagne de risques importants dans le domaine de la santé publique. Face à cette problématique, le développement de méthodes de détection rapide et spécifique des contrefaçons pharmaceutiques est indispensable.

Dans ce contexte, la spectroscopie proche infrarouge se présente de plus en plus comme une méthode de choix pour résoudre rapidement les problèmes d'analyse, de contrôle de la qualité des produits intervenant dans le processus de fabrication, finis ou contrefaits [2-6]. Cet engouement résulte des avantages liés à l'utilisation de cette technique spectroscopique à savoir : l'acquisition rapide, non destructive, polyvalente, peu coûteuse de spectres sans préparation de l'échantillon. Ce dernier élément est l'un des atouts majeurs de la spectroscopie proche infrarouge car il permet de réduire drastiquement la durée de l'analyse et de supprimer l'utilisation de solvants organiques employés lors de la préparation et du contrôle des échantillons [7]. Il a également permis le développement de spectrophotomètres portables autorisant l'analyse sur le terrain par les autorités locales. De par son caractère non-destructif, l'utilisation de techniques analytiques complémentaires sera possible.

La spectroscopie proche infrarouge est une technique analytique appartenant à la spectroscopie vibrationnelle et dont le principe repose sur l'absorption du rayonnement proche infrarouge par les différents constituants de l'échantillon [8]. Ce rayonnement couvre la partie du spectre électromagnétique comprise entre 12500 et 4000 Cm^{-1}. Les bandes d'absorption relativement larges dans cette région sont principalement dues aux vibrations harmoniques et de combinaisons des liaisons atomiques de type X-H où X représente les atomes de carbone (C), d'oxygène (O), d'azote (N) ou de soufre (S) et H représente l'atome d'hydrogène (H) des molécules. En effet, les liaisons d'une molécule peuvent être assimilées à des ressort vibrant à certaines longueurs d'onde suite à l'absorption d'une parte de l'énergie du rayonnement incident.

L'interprétation et l'exploitation des spectres du domaine proche infrarouge sont relativement difficiles compte tenu de la largeur des bandes d'absorption, de leur chevauchement, mais également de l'influence des caractéristiques physiques de l'échantillon. Cette superposition des informations liées aux caractéristiques chimiques et physique rend indispensable le recours à des outils chimiométrique (prétraitement des multivariée exploratoires, prédictives,…) pour extraire l'information considéré comme pertinente [9].

L'utilisation de la spectroscopie proche infrarouge dans la détection de médicaments contrefaits sera illustrée au travers de l'analyse d'une formulation pharmaceutique (sirop faiblement dosé) contenant du paracétamol.

2. Matériel et méthodes

1. La spectroscopie proche infrarouge

Les échantillons ont été analysés à l'aide d'un spectrophotomètre proche infrarouge à transformée de Fourier « MPA » (Bruker Optics, Ettlingen, Allemagne) équipé d'un détecteur semi-conducteur constitué de sulfure de plong (RT-Pbs) et d'une sonde à transmission pour les liquides avec un trajet optique de 2 mm. Les spectres ont été collectés avec le logiciel Opus 6.5 (Brukers Optics). Chaque spectre était la moyenne de 32 scans avec une résolution spectrale de 8 cm^{-1} sur une gamme allant de 12500 à 4000 cm^{-1}.

2. Echantillons

La formulation pharmaceutique analysée était un sirop constitué principalement de paracétamol (substance active) à une concentration de 2% (m/v), de glycérol, d'eau, d'éthanol, de sirop simple et d'agents conservateurs. Des échantillons sous-dosés (80% de la teneur nominale en paracétamol), sur-dosés (120% de la teneur nominale en paracétamol), placebos et dans lesquels le glycérol a été remplacé par du diéthylène glycol ont été également produits afin de tester la méthode d'identification développée.

3. Analyse des données spectrales

L'analyse des données spectrales a été réalisée à l'aide de la PLS Toolbox 7.0.3 (*Eigenvector research, Wenatchee*, WA, USA) pour Matlab R2013a (The Mathworks, Natick, MA, USA). Les spectres ont été analysés par analyse en composantes principales (ACP).

Cette méthode exploratoire ne nécessite pas de connaissance à priori et permet de réduire le nombre de variables (nombre d'onde) en créant des combinaisons linéaires de celle-ci. Ces combinaisons, appelées « composantes principales » (CP), sont définies de sorte à représenter le maximum de variabilité des données originales et d'être orthogonales entre-elle.

3. Résultats

La figure 1 présente les spectres proches infrarouges du principe actif (paracétamol), d'un échantillon constitué uniquement des excipients (placebo) et d'un échantillon. L'analyse de ces différents spectres a permis de sélectionner une gamme spectrale comprise entre 5400 et 9000 cm^{-1} dans laquelle le principe actif et les excipients absorbent modérément. Par ailleurs, le choix de cette dernière permettra de renforcer le caractère discriminant de l'ACP compte tenu de l'étendue des nombre d'onde couverts.

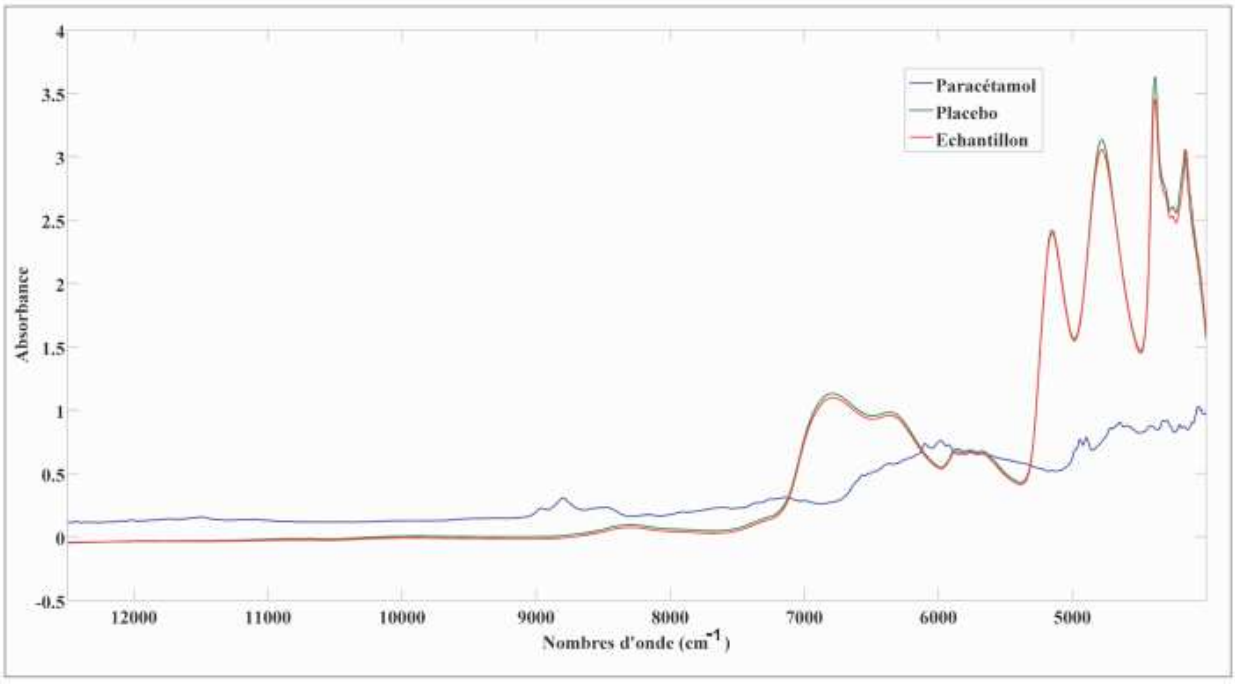

Figure 1. Spectres en proche infrarouge du paracétamol (bleu), d'un placébo (vert) et d'un échantillon de références de sirop de paracétamol (rouge).

La construction du modèle ACP a été réalisée à partir d'une collection d'échantillons de référence provenant de lots différents afin de prendre en compte la variabilité liée aux matières premières (lot, fournisseur) et au procédé de fabrication. La dispersion de ces échantillons permet de définir une ellipse, représentant la valeur T^2 de Hotelling pour un

niveau de confiance donné, en dehors de laquelle tout échantillon est considéré comme atypique.

Afin de tester le modèle ACP et de démontrer son pouvoir discriminant, plusieurs cas de figure ont été investigués : (i) échantillons de référence indépendants, (ii) des échantillons avec une concentration inadéquate en principe actif (80 ou 120% de la teneur nominale en paracétamol), (iii) des échantillons sans principe actif et (iiii) des échantillons dans lesquels le glycérol (excipient) a été remplacé par du diéthylène glycol qui est toxique.

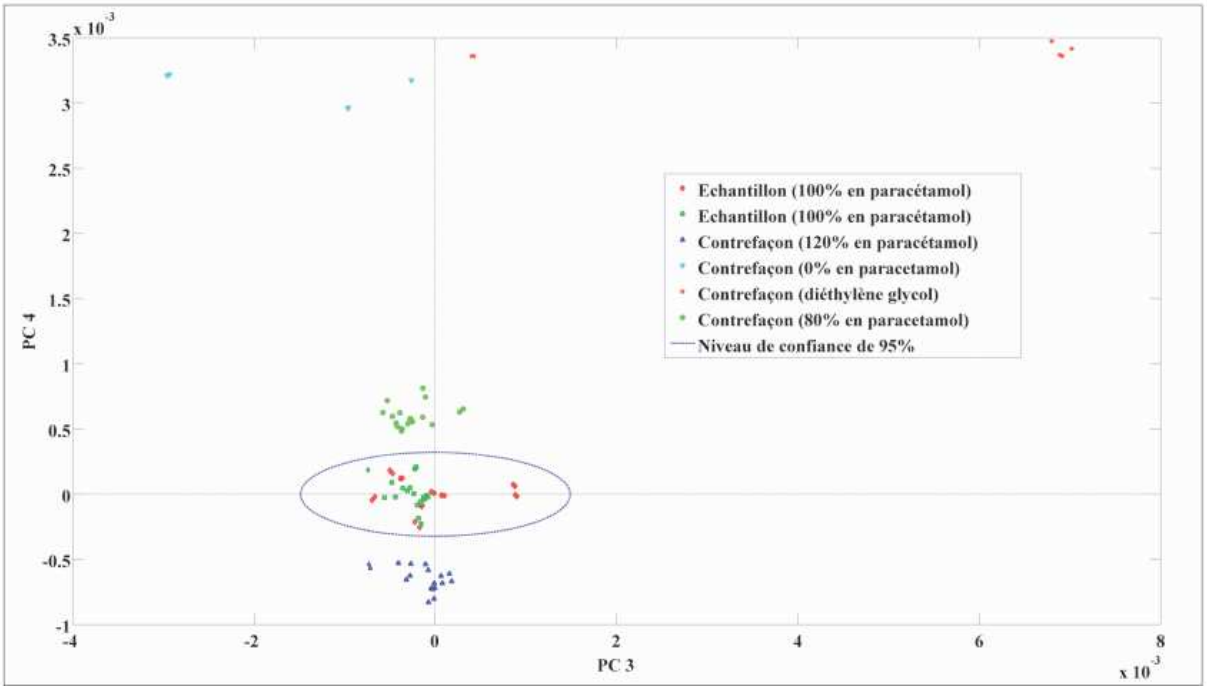

Figure 2. Graphique des scores ACP de l'analyse des spectres en proche infrarouge d'échantillons originaux et contrefaits : les losanges rouges représentent les échantillons de référence utilisés pour construire le modèle. Les carrés verts représentent les échantillons de référence indépendants utilisés pour tester le modèle. Les triangles bleus représentent les échantillons sous-dosés (80% de la teneur nominale en paracétamol). Les ronds verts représentent les échantillons sur-dosés (120% de la teneur nominale en paracétamol). Les triangles inversés cyan représentent les échantillons ne contenant pas de paracétamol. Les étoiles oranges représentent les échantillons renfermant 100% de la teneur nominale en principe actif mais dans lesquels le glycérol a été remplacé par du diéthylène glycol.

Comme le montre la figure 2, le modèle ACP développe permet de discriminer sans équivoque les échantillons de référence de ceux contrefaits. En effet, les échantillons de référence indépendants se situent bien à l'intérieure de l'ellipse pour un niveau de confiance fixé à 95% tandis que tous les autres échantillons contrefaits tombent en dehors de cette zone même si une faible différence au niveau de la teneur nominale en principe actif existe. Par

ailleurs les échantillons qui contiennent une teneur en paracétamol adéquate mais un autre excipient (diéthylène glycol) sont également reconnus comme atypiques.

4. Conclusion

L'exemple présenté dans cet article démontre le potentiel et l'intérêt de la spectrophotométrie proche infrarouge dans la détection rapide de médicaments contrefaits. En effet, l'analyse des données spectrales à l'aide d'outils chimiométriques a permis de discriminer avec succès les échantillons de référence des échantillons contrefaits tant au niveau de la teneur en principe actif que de la composition de la formulation pharmaceutique. Le développement de systèmes portables devrait renforcer sa position au niveau des méthodes de première ligne dans la lutte contre la contrefaçon des médicaments.

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Section IV.3.

Développement, validation et comparaison des méthodes Spectroscopiques Proche Infrarouge et Raman pour l'identification et le dosage de la quinine goutte orale

Cette section se réfère à l'article «**Development, validation and comparison of NIR and Raman methods for the identification and assay of poor-quality oral quinine drops**» publié dans le Journal of Pharmaceutical and Biomedical Analysis 111 (2015) 21-27.

Contexte

Comme nous l'avions déjà évoqué au point III.3, le paludisme demeure une maladie très préoccupante en RDC qui mobilise l'attention de la population mais aussi des décideurs politiques, tout comme des charlatans malheureusement. Son influence augmente avec l'apparition rapide de la résistance du parasite transmetteur (le *plasmodium falciparum*) aux différents antipaludéens. Cette résistance repose entre autre sur l'automédication parfois non justifiée mais surtout sur la mauvaise qualité des produits antipaludéens. Si la quinine n'est pas préconisée en première intention dans le traitement contre le paludisme, elle est en revanche réservée comme médicament de dernière solution et de dernier ressort en cas de paludisme sévère chez l'enfant. De ce fait, les formulations gouttes orales pédiatriques à 20% de quinine sont appropriées pour préserver la santé des enfants de 0 à 5 ans très vulnérables. Ces formulations sont fabriquées par les laboratoires pharmaceutiques locaux en RDC en réponse à l'appel du gouvernement de favoriser la production locale. Mentionnons que la quinine fait partie des 15 médicaments interdits d'importation (voir section III.1). Malheureusement à cause de la contrefaçon, les chances de promotion des producteurs locaux sont minées tout comme le traitement du paludisme.

C'est dans ce contexte que nous nous sommes attelés à mettre au point deux méthodes d'analyse de la quinine respectivement en spectroscopie Proche Infrarouge et en spectroscopie Raman. Notre souhait était d'arriver à différencier dans un temps court les vraies quinines gouttes orales des fausses.

Les résultats des analyses obtenus en appliquant les méthodes préalablement validées ont révélé des situations de non-conformités.

Summary

Poor quality antimalarial drugs are one of the public's major health problems in Africa. The depth of this problem may be explained in part by the lack of effective enforcement and the lack of efficient local drug analysis laboratories. To tackle part of this issue, two spectroscopic methods with the ability to detect and to quantify quinine dihydrochloride in children's oral drops formulations were developed and validated. Raman and near infrared (NIR) spectroscopy were selected for the drug analysis due to their low cost, non-destructive and rapid characteristics. Both of the methods developed were successfully validated using the total error approach in the range of 50-150% of the target concentration (20% W/V) within the 10% acceptance limits. Samples collected on the Congolese pharmaceutical market were analyzed by both techniques to detect potentially substandard drugs. After a comparison of the analytical performance of both methods, it has been decided to implement the method based on NIR spectroscopy to perform the routine analysis of quinine oral drop samples in the Quality Control Laboratory of Drugs at the University of Kinshasa (DRC).

1. Introduction

Malaria remains one of the most rampant illnesses worldwide and is one of the main causes of child mortality in developing countries [1-2]. The treatment of uncomplicated malaria is based on conventional antimalarial drugs (e.g. chloroquine, artemisinin derivatives, atovaquone, etc.). These drugs are essentially used as combinations due to the growing resistance observed with single-drug therapy [3]. However, quinine is still recommended alone in the treatment of severe and/or cerebral malaria attacks as well as for chloroquine-resistant falciparum malaria [4]. Four quinine based dosage forms are found on the pharmaceutical market in DRC: tablets (250 and 500 mg), ampuls (250 and 500 mg/2mL), syrup (100 mg /mL) and oral drops (200 mg /mL). The last three dosage forms are the most used with 0-5 year old children. In 2009, the Health Ministry of the DRC warned citizens against quinine oral drops "Quinizen 20%" that were found to have been counterfeit and substandard [5].

Poor quality (substandard, counterfeit and degraded) or substandard/spurious/falsely-labelled/falsified/counterfeit anti-malarial drugs constitute a major public health concern especially in developing countries where the pharmaceutical market is poorly regulated and controlled [6]. It has been estimated that at least a third of the drugs sold in Africa are fake. The use such drugs may lead to therapeutic failure, death and reinforce drug resistance [7, 8].

Vibrational spectroscopic techniques, such as near infrared (NIR) and Raman spectroscopies are frequently used techniques in the field of quantitative drug analysis [9-11] and in the fight against counterfeit drugs [12-15]. These techniques have the advantages of being non-destructive, fast, requiring little or no sample preparation, as well as being environmental friendly [16]. The foremost advantage for drug analysis in developing countries however is their low cost in routine analysis and the absence of consumables.

The aim of the present research was to develop NIR and Raman methods able to detect and to quantify quinine in 20% (W/V) oral drops solutions from a Congolese drug-manufacturing laboratory (manufacturer A). These methods were fully validated by the "total error" approach [17], compared by mean of a Bland and Altman analysis [18] and then tested on samples from several manufacturers.

2. Material and methods

2.1. Reagents

Ammonium formate (98.1%), hydrochloric acid (37%), and methanol (HPLC gradient grade) were purchased from Merck (Darmstadt, Germany). Benzoic acid and propylene glycol were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). The reference standard of quinine dihydrochloride (100.8%) for the HPLC analysis was purchased from Molekula Ltd (Dorset, UK). Ultrapure water was obtained from a Milli-Q Plus 185 water purification system (Millipore, Billerica, MA, USA).

2.2. NIR equipment

The oral drop samples were analyzed with a multipurpose analyzer Fourier Transform Near Infrared Spectrometer (MPA, Bruker Optics, Ettlingen, Germany) equipped with a semiconductor room temperature sulfide lead (RT-PbS) detector. A transmittance probe for liquids with a fixed optical path length of 2 mm was used to collect the NIR spectra. A background spectrum with the empty probe was acquired before each series of measurements. Between each measurement, the probe was cleaned with water. The spectra were collected with the Opus Software 6.5 (Bruker Optics). Each spectrum was the average of 32 scans and the resolution was 8 cm^{-1}, in the range of 12500-4000 cm^{-1}.

2.3. Raman equipment

Raman measurements were performed with a dispersive spectrometer RamanStation 400F (Perkin Elmer, MA, USA) equipped with a two-dimensional CCD detector (1024 × 256 pixel sensor). The laser excitation wavelength used was 785 nm with a power of 100 mW. Raman spectra were collected with a Raman reflectance probe for solids and liquids interfaced with Spectrum Software 6.3.2.0151 (Perkin Elmer). The spectral coverage was 3620-90 cm^{-1} with a spectral resolution equal to 2 cm^{-1}. Each Raman spectrum resulted from the accumulation of six spectra with a 5.0 s exposure time. NIR and Raman spectra were acquired on the same day and prior to the analysis by HPLC-UV allowing the determination of the concentration of quinine dihydrochloride corresponding to each sample.

2.4. Reference method

The HPLC experiments were performed on an Alliance 2695 HPLC system (Waters, Milford, USA) coupled to a 2996 PDA detector (Waters). Data acquisition and treatment were performed with the Empower 2® software (Waters). The analysis was performed with an XBridge™C18 (250 mm x 4.6 mm, 5µm particle size) column preceded by an XBridge™C18 (20 mm x 4.6 mm, 5 µm particle size) guard column kept at 30°C. The mobile phase consisted of a mixture (45:55, V/V) of methanol and a 10 mM ammonium formate buffer adjusted to pH 3.0 with 6N HCl. The HPLC system was operated in isocratic mode with a flow rate of 1.0 mL min^{-1} and an injected volume of 10 µL. UV detection was carried out at 235 nm.

2.5. Test samples

Six samples of quinine dihydrochloride 20% (W/V) oral drop solutions from four manufacturers (A, B, C and D) were collected at the local Congolese pharmaceutical market. The calibration samples used to build the PLS models were prepared on basis of the qualitative and quantitative compositions of manufacturer A. Samples from other manufacturers had different qualitative and quantitative compositions regarding the pharmaceutical formulation and the origin of the active ingredient. Samples from manufacturers C and D were green-coloured whereas those from manufacturers A and B were yellow-coloured. To test the ability of the developed models to detect and quantify quinine in oral drops, seven simulated substandards have been prepared with 2% and 40% (W/V) quinine dihydrochloride (corresponding to 10% and 200%, respectively, of the target value).

2.6. Sample preparation

2.6.1. Preparation of samples for reference method validation

Calibration samples for HPLC method validation were prepared from a stock solution of quinine dihydrochloride at a concentration of 1 mg mL^{-1} in ultrapure water. The stock solution was diluted to obtain solutions of 50, 100 and 150 µg mL^{-1}. The calibration standard solution was composed of three series of three replicates per concentration level (27 samples in total). Validation samples for HPLC method validation were prepared from a stock solution composed of 20% (W/V) quinine dihydrochloride, dissolved in an excipient solution composed of propylene glycol and benzoic acid in ultrapure water. The stock solution was

diluted to obtain solutions of 50, 75, 100, 125 and 150 µg mL^{-1}. The validation standard solution was composed of three series of three replicates per concentration level (45 samples in total).

2.6.2. Preparation of solutions for HPLC analysis of samples

Two independent standard solutions were prepared by dissolving quinine dihydrochloride in ultrapure water to achieve a final concentration of 100 µg mL^{-1}. Calibration, validation and test samples were diluted in ultrapure water to obtain a final concentration of 100 µg mL^{-1}.

2.6.3. Preparation of calibration and validation samples for NIR and Raman

The target (100%) sample composition is 20% (W/V) quinine dihydrochloride dissolved in an excipient solution composed of propylene glycol and benzoic acid in ultrapure water. Calibration and validation standards were prepared by dissolving the appropriate amount of quinine dihydrochloride in the excipients solution to achieve concentrations of 50, 75, 100, 125 and 150% of the target amount. Three series of both calibration (C1, C2, C3) and validation (V1, V2, V3) samples were prepared with three replicates for each concentration level. C1, C2, V2 and V3 series were prepared using quinine dihydrochloride from Pharmakina (Bukavu, DRC). While C3 and V1 series were prepared using quinine dihydrochloride from A.V. Pharma (Kinshasa, DRC).

2.7. Multivariate data analysis

Partial least squares (PLS) regression models were built with NIR and Raman data using HPLC assay values as reference. Several PLS models were built using different pre-processing methods. Best models were selected based on their Root Mean Square Error of Prediction (RMSEP) computed as follows:

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n} \left(y_i - \hat{y}_i\right)^2}{n}} \quad (1)$$

where y_i is the reference value determined by HPLC, $\hat{y}_i$ is the predicted value given by the PLS model and n is the total number of samples. All data were mean centred and the number of latent variables of each PLS models was selected based on the RMSECV versus latent

variables plot. Cross validation consisted of random subsets with ten data splits and ten iterations. First and second derivatives were computed using the Savitsky-Golay algorithm [19] with a polynomial order of 2 and 15 smoothing points. Asymmetric least squares have been used for baseline correction of Raman spectra. A value λ of 10^5 and a value *p* of 10^{-3} were used. PLS models were built using PLS_Toolbox 7.0.3 (Wenatchee, WA, USA) running on Matlab® R2013a (The Mathworks, Natick, MA, USA).

The validation of a multivariate calibration model is often performed by checking at the R^2 and RMSEP values. However, as described in De Bleye et al. [20], these performance parameters are not sufficient to ensure that the developed method will provide reliable results over the complete dosing range. Therefore, both NIR and Raman predictive models were validated through the "total error" approach. All validation calculations were performed with e-noval® version 3.0 (Arlenda S.A., Liège, Belgium).

3. Results and discussion

3.1. Validation of the reference method

The method was successfully validated using the "total error" approach in the range of 50 µg mL^{-1} to 150 µg mL^{-1} with acceptance limits set at 10% according to the USP for quinine sulphate tablet assay [21]. Trueness, precision (repeatability and intermediate precision), accuracy and linearity of the method were found to be acceptable (see also Table 1).

Table 1 ICH Q2 (R1) validation criteria of the reference HPLC, NIR and Raman methods.

	concentration level	HPLC	NIR	Raman
Trueness				
	50	3.98	-0.19	2.44
	75	2.09	0.05	-0.95
Relative bias (%)	100	-0.18	1.63	-0.31
	125	0.76	0.37	0.65
	150	2.13	-0.98	-1.35
Intra-assay precision				
	50	0.78	1.12	1.40
	75	0.32	0.63	0.53
Repeatability (RSD%)	100	0.44	1.24	1.17
	125	0.21	0.29	0.53
	150	0.37	0.79	1.23
Between-assay precision				
	50	0.78	1.36	1.40
	75	0.62	0.76	0.97
Intermediate precision (RSD%)	100	0.62	1.24	1.41
	125	0.63	1.33	1.02
	150	0.62	0.82	1.23
Accuracy				
	50	[2.08 ; 5.88]	[-3.90 ; 3.51]	[-1.00 ; 5.87]
	75	[-0.16 ; 4.35]	[-2.02 ; 2.12]	[-4.38 ; 2.49]
Relative β-expectation tolerance limits (%)	100	[-2.07 ; 1.71]	[-1.41 ; 4.66]	[-4.42 ; 3.80]
	125	[-1.97 ; 3.48]	[-5.84 ; 6.57]	[-3.16 ; 4.46]
	150	[0.00 ; 4.27]	[-3.01 ; 1.06]	[-4.36 ; 1.67]
Uncertainty				
	50	1.64	2.95	2.96
	75	1.39	1.65	2.18
Relative expanded uncertainty (%)	100	1.38	2.62	3.09
	125	1.44	3.06	2.32
	150	1.40	1.74	2.60

3.2. Quantitative NIR study

Quantifying an API in an aqueous matrix may be a difficult task with NIR spectroscopy. Indeed, the matrix absorbance spectrum shows that the multiple absorption maxima characteristic of water and detector saturation occurs between 5250 and 5050 cm^{-1}. Therefore, the spectral range was selected between 8937 and 7278, 6318 and 5396 and 4733-4428 cm^{-1} to build PLS models (see Fig. 1a). By doing so, perturbations due to matrix absorptions were avoided while keeping the information dealing with the API. Table 2 shows the different pre-treatments tested as well as the figures of merit for the corresponding models. As one can see, both models gave similar values of RMSEP that were inferior to 2%. However, the different models have varying complexity with a number of latent variables (LV) ranging from 2 to 4. The simplest model was obtained by applying a standard normal variate (SNV) normalization computed as follows:

$$x_{ij,SNV} = \frac{x_{ij} - \bar{x}_i}{s_i} \qquad (2)$$

where $x_{ij,SNV}$ is the transformed portion of the original element x_{ij}, $\bar{x}_i$ is the mean value of the spectrum I and s_i is the standard deviation of the spectrum I. This choice was driven by the fact that it is one of the simplest models (only 3 LVs) which limits the risk of over fitting. This model should, therefore, be more robust for any future analysis of unknown samples. As can be determined from the accuracy profile (Fig. 2b), the β-expectation tolerance intervals of each concentration level are inside the limits of acceptation set at 10%. This indicates that 95% of future measurements will lie within these limits. The largest β-expectation tolerance intervals have relative values of -5.84% and 6.57% (see Table 1).

Table 2 Figure of merits of the different tested PLS models.

NIR	RMSEC (%)	RMSECV (%)	RMSEP (%)	LV
MC	0.86	0.96	1.59	4
1D-MC	0.81	0.95	1.69	3
2D-MC	1.00	1.09	1.46	2
SNV-MC	**0.90**	**1.03**	**1.42**	**3**
Raman	**RMSEC (%)**	**RMSECV (%)**	**RMSEP (%)**	**LV**
MC	1.04	1.27	1.81	3
AsLS-MC	**1.11**	**1.75**	**1.88**	**2**
2D-MC	0.45	1.41	2.01	4
SNV-MC	3.27	4.69	7.27	4

RMSE: root mean square error; C: calibration; CV: cross-validation; P: prediction; LV: number of latent variables considered; MC: mean center; 1D: SavitskyGolay's first derivative; 2D: SavitskyGolay's second derivative; SNV: standard normal variate; AsLS: asymmetric least squares.

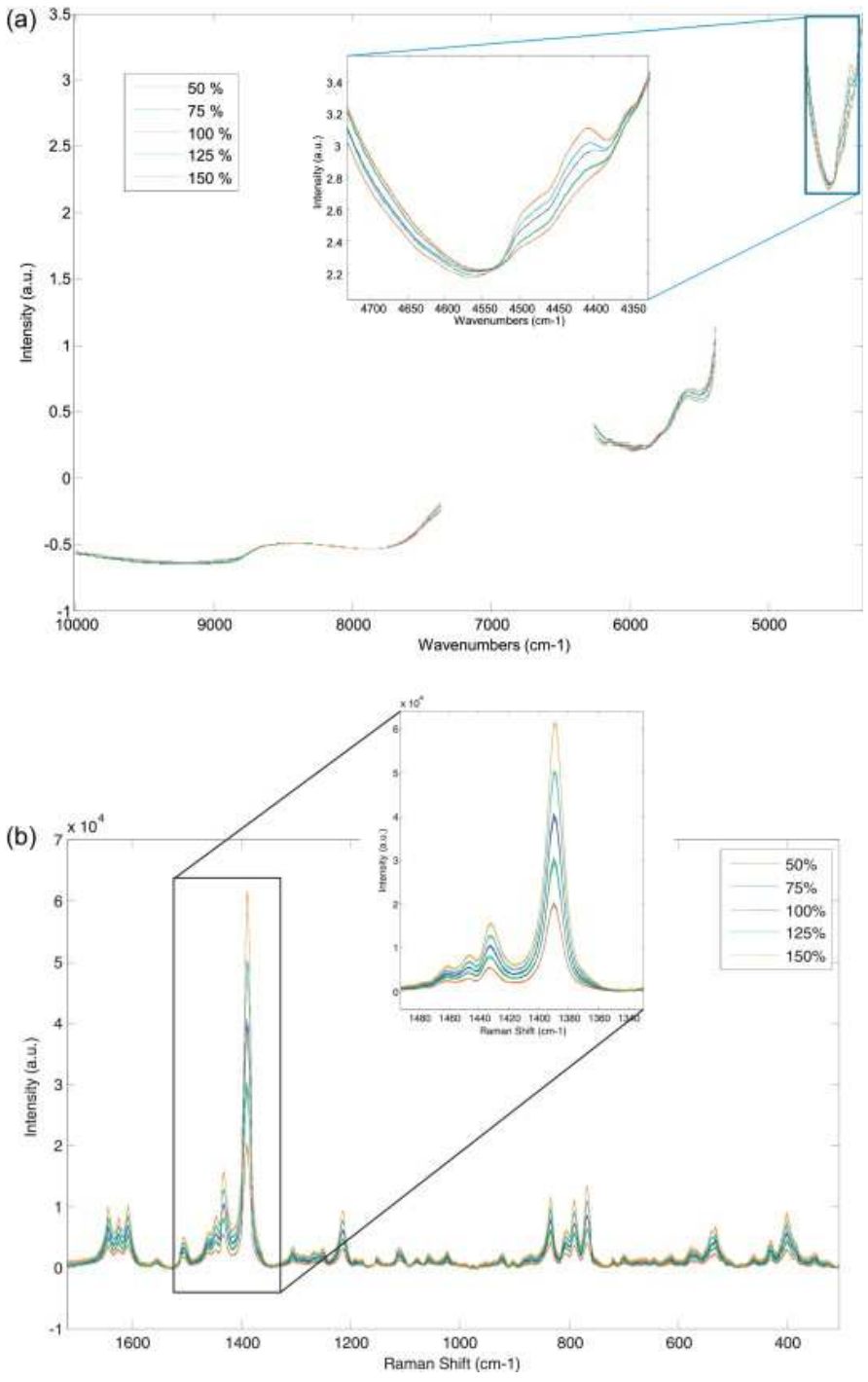

Fig.1. (a) Selected spectral ranges of SNV pre-processed calibration NIR spectra. (b) Selected spectral ranges of asymmetric least squares baseline corrected calibration Raman spectra.

3.3. Quantitative Raman study

Compared to NIR spectroscopy, the main advantage of Raman spectroscopy in quantifying an API in an aqueous matrix is due to the weak Raman scattering effect of water.

Raman spectroscopy, however is often limited to pharmaceutical applications due to the fluorescence of samples. The spectral range was selected to be 1720-306 cm^{-1} to build PLS models. The selected model was the one obtained by applying baseline correction by asymmetric least squares (AsLS) with a RMSEP of 1.88% and two latent variables (see Fig. 1b). As can be seen in Fig. 2a, the selected Raman quantitative model was also validated with the acceptance limits fixed at 10%.

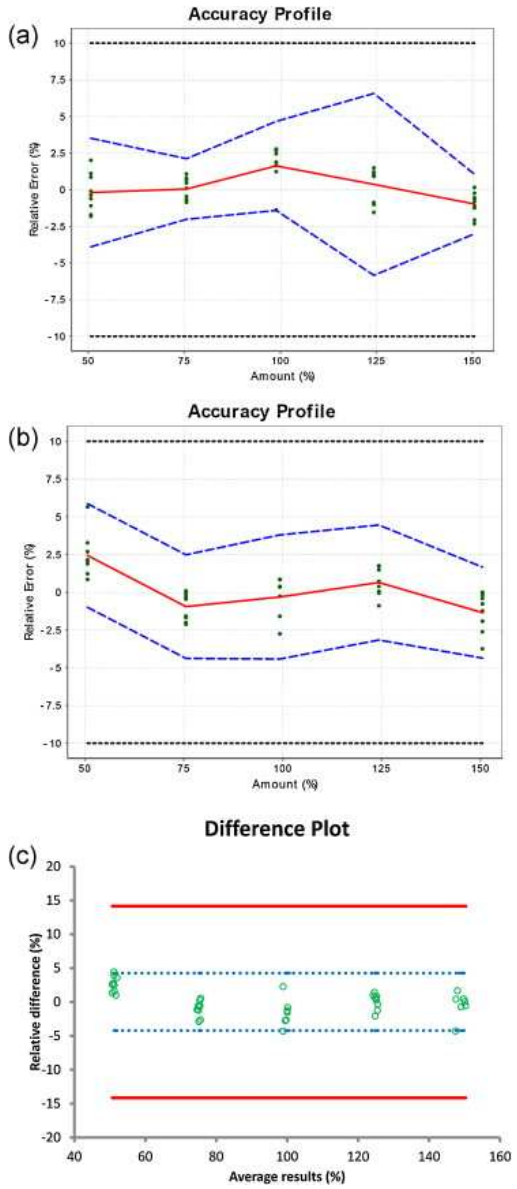

Fig.2. (a) Accuracy profile of the Raman quantitative PLS model. The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits (β=95%) and the bold plain lines are the acceptance limits set at 10 %. The dots represent the relative back-calculated concentrations of the validation samples, plotted with regards to their target concentration. (b) Accuracy profiles of the NIR quantitative PLS. The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits (β=95%) and the bold plain lines are the acceptance limits set at 10 %. The dots represent the relative back-calculated concentrations of the validation samples, plotted with regards to their target concentration. (c) Bland and Altman plot of the relative differences (%) of the results obtained by the NIR quantitative model and the Raman quantitative model against the average content of quinine (%) for the five concentration levels results of the two methods. Dashed blue lines: 95% agreement limits of the relative differences; Continuous red lines: maximum acceptable relative difference between the two methods set at ±14.14% based on the maximum acceptable error of 10% for each method; Dots: relative differences.

3.4. Comparison of methods

The analytical performances of both NIR and Raman models were compared using a Bland and Altman analysis [18] (see results in Fig. 2c). The plot represents the relative difference between the Raman and NIR methods against the average relative content of quinine at each concentration level. As shown in this figure, 95% of the agreement limits with values of [- 4.20%; 4.26%] are inside the acceptance limits [-14.14 %; 14.14 %] that were set based on the maximum acceptable error of 10% for each method. These results guarantee that each future difference between the result obtained using the Raman method and the one obtained with the NIR method has a probability of 0.95 of falling within the acceptance limits. Thus both methods agree sufficiently to quantify quinine in the oral drops allowing the analyst to select the method according to the advantages and drawbacks of each method.

3.5. Analysis of test samples

The two methods developed were applied to six samples of quinine dihydrochloride 20 % (W/V) oral drops solutions as mentioned in Section 2.5 and on seven reconstituted substandard solutions. Samples from manufacturer A were considered as being genuine samples since they had the same qualitative and quantitative composition as the calibration samples used to build the PLS models. Neither NIR spectroscopy nor Raman spectroscopy could qualitatively discriminate between samples of manufacturers A, B, C and D. This is not surprising since these samples are constituted mainly of quinine dihydrochloride 20% (W/V) and water 79% (W/V), whereas the excipients are less than 1% (W/V). The spectral variations due to differences in qualitative composition were below the detection limits of both techniques thus did not allow a distinction between the manufacturers. Test samples were then analyzed with the two developed PLS models. As shown in Table 3, the two methods generally predicted each sample correctly. In addition, the samples from each of the manufacturers are predicted correctly, thus demonstrating that the developed NIR and Raman methods could be applied to the detection of placebo or sub-dosed samples.

A principal component analysis (PCA) was performed on the different test samples and the prepared substandard samples. This model was built using the 100% target concentration calibration and validation samples. The main goal was to check whether it was possible to quickly discriminate substandard samples without building and validating a PLS

model. As can be seen in Fig. 3, the scores of PC2 (for NIR spectroscopy) and PC1 (for Raman spectroscopy) allowed for the discrimination of genuine and substandard samples. Moreover, the third sample of manufacturer A is slightly outside the 95% T^2 Hotelling's confidence level for the PCA based on Raman spectroscopy. This is in accordance with the quantitative results obtained (89 % of the target value predicted by PLS). These results indicate that it is possible to use PCA as a discriminating method to detect substandard samples.

Table 3 Results of quantification of 6 samples with NIR and Raman PLS models. Results are presented as predicted content (%) of the active ingredient and relative expanded uncertainty (Ux). Results obtained with HPLC consist in the mean percentage of claimed nominal content and the standard deviation computed on 3 independent samples.

	NIR	Raman	HPLC	Relative Error (%)	
Drug	**(%) ± Ux**	**(%) ± Ux**	**n=3, % ± SD**	**NIR/HPLC**	**Raman/HPLC**
A1	98.3 ± 2.6	96.2 ± 3.1	96.7 ± 0.1	1.68	-0.47
A2	98.6 ± 2.6	101.4 ± 3.1	100.7 ± 0.1	-2.12	0.68
A3	90.8 ± 2.6	89.0 ± 3.1	91.1 ± 0.1	-0.33	-2.33
B	97.8 ± 2.6	92.9 ± 3.1	95.7 ± 0.1	2.22	-2.92
C	106.6 ± 2.6	100.6 ± 3.1	102.6 ± 0.1	3.86	-1.99
D	99.5 ± 2.6	98.8 ± 3.1	99.5 ± 0.1	0.54	0.68

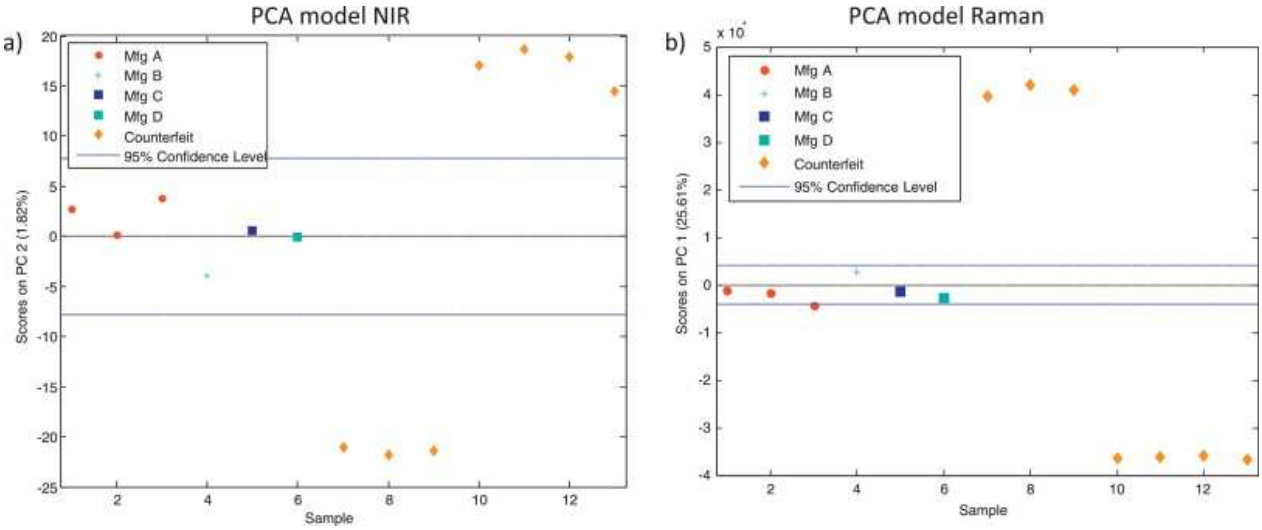

Fig.3. (a) PC2 scores of eight samples of oral quinine drop and seven substandard quinine drops based on their NIR spectra. (b) PC1 scores of eight samples of oral quinine drop and seven substandard quinine drops based on their Raman spectra.

4. Conclusion

The main objective of this study was to develop and validate efficient, rapid and cost-effective analytical methods for the analysis of quinine dihydrochloride 20% (W/V) presented as an oral drop formulation manufactured and marketed in the DRC.

To meet these requirements, NIR and Raman spectroscopic methods were successfully developed and validated using the total error approach with acceptance limits fixed at 10% in the range of 50-150% of the target concentration. A comparison of the two methods showed that they provided comparable results. Six samples collected in the Congolese pharmaceutical market were analyzed by both techniques. All samples were conform since their quinine content was within in ± 10% of the theoretical value.

The NIR spectroscopy qualitative model developed will soon implemented for routine analysis in the Quality Control Laboratory of Drugs at the University of Kinshasa (D.R. Congo) to replace the existing HPLC method. This study and its implementation are part of the fight against the traffic of poor quality medicines that endanger the public health and socio-economic aspects of developing countries.

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Chapitre V

Conclusions et perspectives

V.1. Conclusion

La problématique des médicaments de qualité inférieure dans les pays en voie de développement nous a amené à réfléchir sur la qualité des médicaments commercialisés en RDC mais surtout à poser des actes contributifs au niveau du volet analytique. Ainsi l'objectif principal de notre travail a été de développer des méthodes fiables pour le contrôle de qualité des médicaments en vue d'appuyer le Ministère Congolais de la Santé Publique dans la lutte contre la prolifération des médicaments de qualité inférieure.

Trois classes pharmacologiques ont été choisies en raison de leur large utilisation. Il s'agit des anti-inflammatoires non stéroïdiens (AINS), des antibiotiques et des antipaludéens.

Dans un premier volet, nous nous sommes focalisé au chapitre III sur la technique séparative de la chromatographie liquide couplée au détecteur Ultraviolet en vue de développer des méthodes génériques. Rappelons l'avantage que présente cette technique de faire un criblage permettant de détecter simultanément plusieurs molécules actives d'une même classe pharmacologique mais aussi d'analyser les produits de dégradation dans un médicament.

Il est intéressant de signaler que les 4 phases du cycle de vie d'une méthode analytique telles que nous les avons présentées dans l'introduction au chapitre I ont été suivies lors de l'utilisation de la technique de chromatographie liquide (CL) pour analyser les 3 classes pharmacologiques citées ci-haut. D'abord en 1^{e} phase de ce cycle nous avons pu sélectionner les facteurs analytiques qui ont montré une influence sur la réponse analytique à savoir la séparation entre les paires de pics critiques. Ainsi, la température de thermostatisation de la colonne, le pH de la partie aqueuse de la phase mobile et le temps de gradient ont été sélectionnés comme facteurs pour optimiser la séparation des AINS et des antibiotiques en mode gradient tandis que le débit de la phase mobile, le pourcentage de modificateur organique et à nouveau la température de thermostatisation de la colonne ont été sélectionnés dans le cas des antipaludéens en mode isocratique. Ensuite en 2^{e} phase du cycle, nous avons développé 7 méthodes analytiques suivant la stratégie basée sur la planification expérimentale et l'espace de conception « *Design Space* » pour optimiser simultanément les facteurs analytiques sélectionnés lors de la phase précédente. Une seule colonne chromatographique à

servi lors de la planification expérimentale, il s'agit d'une XBridge C18, 250 × 4,6 mm, (5 µm dp) précédée d'une colonne de garde XBridge C18, 20 × 4,6 mm. Cette colonne présente l'avantage de supporter des valeurs de pH allant de 1 à 12 et permet également de remédier au phénomène de tailing des pics observés surtout lorsqu'on travaille avec les composés à caractère basique par exemple l'halofantrine, la luméfantrine etc, qui ont une affinité importante pour les groupements silanols libres présents à la surface de la silice greffée. Etant donné que nous nous sommes retrouvé en présence d'un nombre important de composés à séparer (54 molécules pour les trois classes pharmacologiques), nous avons opté pour une colonne de 250 mm de longueur afin d'offrir un maximun de chance de la séparation des pics. Par ailleurs, seul le méthanol a été utilisé comme modificateur organique dans notre travail, compte tenu de son coût abordable comparé à l'acétonitrile. Mentionnons en passant que les méthodes optimisées ont été transférées géométriquement en utilisant des colonnes de courtes dimensions notamment Acquity BEH C18, 50 × 2,1 mm, (1,7 µm dp) en UPLC pour séparer les AINS et les antibiotiques et XBridge C18, 100 × 4,6 mm (3,5 µm dp) en HPLC pour séparer les antipaludéens. Le transfert géométrique a permis de réduire le temps d'analyse et la consommation de solvant organique, avantages à explorer dans un contexte de réduction du coût des analyses. Les longueurs d'onde de détection ont été fixées à 220 nm pour les AINS et les antibiotiques et à 230 nm pour les antipaludéens. La stratégie de l'erreur totale a été utilisée pour valider les méthodes développées en phase 3 du cycle de vie avant leur application en routine en phase 4.

Nous nous sommes tout d'abord intéressés dans la section III.1 au développement de méthodes d'analyse des AINS. Le plan composite centré a été appliqué pour optimiser le criblage de 27 molécules actives dont 18 AINS, 5 agents conservateurs et 4 produits associés. Le domaine expérimental des facteurs sélectionnés était : pH (1,85 à 7,00), température (20 à 35°C) et le temps de gradient nécessaire pour passer linéairement de 15% à 95 % en méthanol (20 à 60 minutes). Grâce à cette planification, nous avons pu développer trois méthodes génériques dont les conditions optimales sont les suivantes :

- Tampon formiate d'ammonium 20 mM ajusté à pH 3,05 – temps de gradient de 49,30 minutes – température de 34,5°C
- Tampon formiate d'ammonium 20 mM ajusté à pH 4,05 – temps de gradient de 53,14 minutes – température de 23,0°C

- Tampon hydrogénocarbonante d'ammonium 20 mM ajusté à pH 7,00 – temps de gradient de 60,00 minutes – température de 21,7°C.

Nous avons ensuite considéré la situation des associations des AINS commercialisées en RDC sous formes solides et liquides. Dans ce contexte, nous sommes parvenus à développer une seule méthode pour analyser 8 associations des AINS présentées sous formes solides dont la condition optimale est la suivante: tampon formiate d'ammonium 20 mM ajusté à pH 3,00 – temps de gradient de 20,00 minutes – température de 27,0°C. Nous sommes également parvenus à développer une autre méthode pour analyser 5 associations des formes liquides dont la condition optimale suivante: tampon hydrogénocarbonante d'ammonium 20 mM ajusté à pH 6,14 – temps de gradient de 35,00 minutes – température de 29,4°C.

Ensuite au cours de la section III.2, nous avons élucidé le développement de méthodes pour l'analyse des antibiotiques. A cet effet le plan D-optimal a été mis en œuvre pour optimiser le criblage de 19 molécules actives dont 16 antibiotiques et 3 inhibiteurs de bêta-lactamase. Le domaine expérimental des facteurs testés était : pH (2,70 à 7,00), température (23,0 à 30,0°C) et le temps de gradient nécessaire pour passer linéairement de 8% à 95 % en méthanol (20,00 à 60,00 minutes). Suite à cette planification, nous avons abouti à une seule méthode générique dont la condition optimale est la suivante : tampon acétate d'ammonium 10 mM ajusté à 6,00 – temps de gradient 40,00 minutes – température de 25,0°C. Cette même condition optimale a été trouvé également susceptible d'analyser 7 associations à base des antibiotiques commercialisés en RDC.

Enfin, au cours de la section III.3, nous nous sommes focalisés sur le développement de méthodes d'analyse des antipaludéens. Nous avons utilisé le plan factoriel complet pour optimiser le criblage de 8 antipaludéens commercialisés en association. Le domaine expérimental des facteurs testés était : débit de la phase mobile (0,3 à 0,7 mL/minute), température (25,0 à 35,0°C) et pourcentage de méthanol utilisé comme modificateur organique (80,0 à 90,0 %). Le pH de la phase mobile était fixé à 2,80 (10 mM de tampon formiate d'ammonium). Cette planification nous a permis d'affiner une seule méthode générique dont la condition optimale est la suivante : débit de la phase mobile 0,5 mL/minute – 80,0% de méthanol – température de 25,0°C. En considerant la recommandation thérapeutique antipaludéenne de l'OMS, nous avons pu développer une autre méthode pour

analyser 2 associations pharmaceutiques dont la condition optimale est la suivante : débit de la phase mobile 0,6 mL/minute – 82,5% de méthanol – température de 25,0°C.

Dans le deuxième volet détaillé au chapitre IV, nous avons épinglé le développement des méthodes en utilisant les techniques non-séparatives. Celles-ci sont connues pour être moins favorables à la quantification des produits de dégradation dans un produit fini ou pour la polythérapie mais par contre elles présentent les avantages d'être rapides, simples et faciles à mettre en œuvre. Dans ce contexte, nous nous sommes d'abord focalisé sur la spectrophotométrie UV-visible à la section IV.1 vis-à-vis d'un antipaludéen et de deux antibiotiques. Nous avons développé une méthode de dosage pour la quinine sulfate dans les comprimés de 500 mg, une méthode de dosage pour la quinine bichlorhydrate dans les gouttes orales à 20 %, une méthode de dosage du benzoate de métronidazole dans la suspension orale à 125mg/5mL et une méthode de dosage d'amoxicilline trihydrate dans les capsules à 500mg.

Ensuite, nous avons démontré à la section IV.2 *via* les tests effectués sur le paracétamol sirop à 2%, le potentiel et l'intérêt de la Spectrophotométrie Proche Infrarouge (SPIR) dans la détection rapide des médicaments contrefaits et sous-standards. Rappelons que c'est une technique non destructive par rapport à la spectrophotométrie Ultraviolet et la chromatographie. La méthode développée pour l'analyse de paracétamol sirop a permis de distinguer le vrai sirop de paracétamol et le paracétamol sirop de qualité inférieure. Au cours de la section IV.3, deux méthodes ont pu être développées en Spectroscopie Infrarouge et Raman pour analyser la quinine goutte orale à 20% utilisée comme traitement de choix dans le cas du paludisme sévère chez l'enfant. Elles ont permis de distinguer rapidement les vraies et les fausses quinines gouttes sans traitement de l'échantillon ni consommation de solvant organique.

Dans la phase finale de routine de leur cycle de vie, nous avons pu appliquer avec succès toutes les méthodes développées et validées dans le cadre de notre travail. Au total 32 échantillons comprenant des AINS, des antibiotiques et des antipaludéens commercialisés à Kinshasa ont pu être analysés. Les résultats globaux et présentés dans le tableau 1 ont révélé des non-conformités par rapport aux spécifications Européennes (EMA : European Medicines Agency) et Américaines (USP : United State Pharmacopoeia). Cette situation en rapport avec

le choix des spécifications comme signalé au point I.5 mérite d'être définie et clarifiée par les autorités du Ministère Congolais de la Santé Publique pour éviter toute ambiguité favorisant la circulation des médicaments sous-standards.

Tableau 1. Les Résultats globaux de dosage des échantillons commercialisés à Kinshasa

Normes	**95,0 – 105,0% (EMA)**		**90,0 – 110,0% et 90,0 – 120,0%* (USP)**	
Décision	Non-conforme	Conforme	Non-conforme	Conforme
AINS	5/5	0/5	3/5	2/5
Antibiotiques	6/10	4/10	3/10	7/10
Antipaludéens	4/17	13/17	1/17	16/17
Total	15/32 (46,9%)	17/32 (53,1%)	7/32 (21,9%)	25/32 (78,1%)

* Spécifications pour les antibiotiques.

Ces résultats globaux confirment le phénomène de la circulation des médicaments de qualité inférieure en RDC en tant que problème réel de Santé Publique.

Au terme de notre travail, nous livrons notre impression d'une grande satisfaction puisque d'une part, nous avons développé, validé et testé en routine en Belgique toutes ces méthodes analytiques, et d'autre part ces méthodes sont en voie d'être transférées en RDC particulièrement à l'Université de Kinshasa pour appuyer le Ministère de la Santé Publique dans le contrôle local des médicaments du « package médical » en RDC. Ainsi pour faire face efficacement au fléau des médicaments sous-standards en RDC, nous proposons une stratégie analytique simple, rapide et moins couteuse schématisée dans le logigramme suivant :

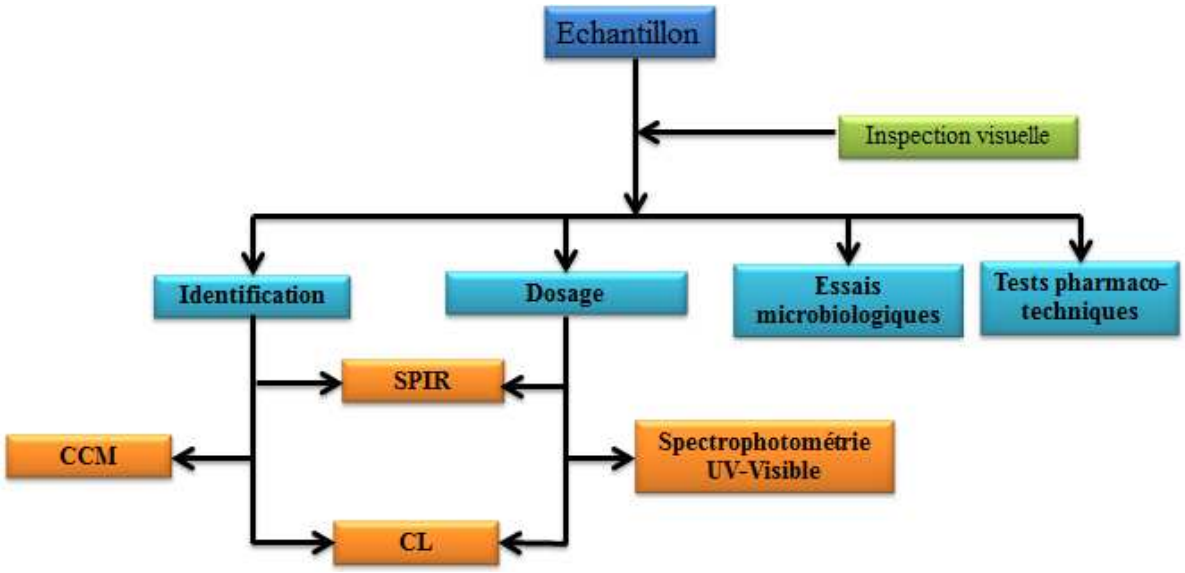

Fig. 1. Logigramme de la stratégie analytique à proposer aux laboratoires de contrôle qualité via le Ministère de la Santé Publique en RDC

Quelque soit l'échantillon pour être analysé au laboratoire de contrôle qualité à l'Université de Kinshasa, l'inspection visuelle des emballages primaire et secondaire, de l'étiquettage et du médicament constituera le premier test à réaliser. Ensuite, selon la demande du client les tests physico-chimiques (uniformité de masse, détermination du pH, identification et dosage etc.), les tests microbiologiques (essai de sterilité) et les tests pharmacotechniques (désintégration, friabilité, dissolution, etc.) seront envisagés.

Concernant l'identification et le dosage du principe actif par les tests physico-chimiques, nous proposons quatre orientations selon le cas qui sera présenté :

- 1er cas : lorsque nous disposons d'informations sur l'échantillon et que le modèle de quantification en Spectroscopie Proche Infra-Rouge (SPIR) est disponible, le contrôle se fera d'abord par cette technique qui ne nécessite pas de consommables. Si l'identification est positive alors suivra le dosage par la même technique pour libérer le produit. Si par contre l'identification en SPIR est négative nous proposerons en accord avec le client l'utilisation de la technique de chromatographie: la Chromatographie sur Couche Mince (CCM) pour l'identification ou bien la chromatographie liquide (CL) pour l'identification et le dosage si cette dernière technique a permis l'identification du composé recherché. Cette technique sera

préférée également dans le cas de polythérapie. Par contre la spectrophotométrie Ultraviolet-Visible sera préférée pour l'analyse des produits en monothérapie.

- 2ème cas : lorsque nous disposons d'informations sur l'échantillon mais pas le modèle de quantification en SPIR, l'idenfication se fera d'abord par comparaison spectrale en SPIR. Si le résultat d'identification est positif, alors le dosage sera réalisé soit par CL soit par spectrophotométrie Ultraviolet-visible. Par la suite une méthode de quantification en SPIR sera dévéloppée pour des analyses ultérieures. Si par contre l'identification en SPIR est négative, le deuxième volet du 1er cas sera exploité c'est-à-dire l'utilisation de la technique de chromatographie en accord avec le client.
- 3ème cas : lorsque nous disposons d'informations sur l'échantillon et le modèle de quantification en SPIR est disponible ou non, en cas de recherche d'impuretés de dégradation dans le produit fini, nous proposerons l'utilisation de la CL.
- 4ème cas : lorsque nous ne disposons pas d'informations sur l'échantillon, une recherche sur la nature du produit sera envisagée. La SPIR sera d'abord utilisée pour comparer le spectre du produit inconnu à la banque de données spectrales. Ensuite le spectrophotomètre Ultraviolet-Visible sera utilisé dans la même optique. Quelques tests physico-chimiques comme le poids moyen et le pH serviront d'orientation pour renforcer la recherche sur la nature du produit. Si le doute est levé, nous proposerons l'utilisation de la chromatographie (CCM ou CL) pour l'identification et le dosage. Par contre si l'identification au moyen de techniques ci-présentées est négative, nous proposerons une consultation des partenaires Nord pour des recherches plus poussées en ayant recourt à la chromatographie liquide couplée au spectromètre de masse et à la Résonance Magnétique Nucléaire.

Dans tous les cas, les méthodes appliquées pour analyser les produits finis en utilisant toutes ces techniques analytiques seront soient de références soit validées.

V.2. Perspectives

Notre travail s'étant essentiellement focalisé sur le développement et la validation des méthodes analytiques avec des applications, nous pensons qu'il serait intéressant de le poursuivre au travers des pistes suivantes :

- Transférer les méthodes qui ont été développées et validées en Belgique pour l'application à grande échelle en RDC.
- Contrôler la qualité des médicaments commercialisés dans les zones péri-urbaines des villes de la RDC pour s'assurer de leur qualité.

Pour ces deux perspectives, nous comptons travailler en étroite collaboration avec la Division 5 de la Direction de la Pharmacie, Médicaments et Plantes Médicinale du Ministère de la Santé Publique.

- Evaluer les impuretés résiduelles contenues dans les médicaments commercialisés en RDC pour protéger la population. Le plomb par exemple, responsable du saturnisme et provenant des matériaux ainsi que de l'eau pour la fabrication des médicaments peut être analysé par spectrophotométrie d'absorption atomique.
- Considérer la situation des médicaments traditionnels améliorés fabriqués en RDC et dont on ne dispose pas encore de méthodes analytiques fiables suite à leur composition complexe. Il y a moyen d'analyser ces médicaments traditionnels améliorés au moyen de la chromatographie liquide en utilisant un marqueur externe.

Chapitre VI

Résumé de la thèse

Chapitre VI. Résumé de la thèse

Le présent travail de thèse s'inscrit dans le cadre d'une collaboration entre l'université de Liège et l'université de Kinshasa dans le domaine des Sciences Pharmaceutiques. Il vise la mise au point des méthodes fiables pour le contrôle de qualité des médicaments commercialisés dans le but d'appuyer le Ministère Congolais de la Santé Publique dans la lutte contre la prolifération des médicaments de qualité inférieure.

Dans un premier temps, trois classes pharmacologiques des médicaments largement utilisés et susceptible d'être contrefaits en RDC ont été sélectionnées à savoir les anti-inflammatoires non stéroïdiens, les antibiotiques et les antipaludéens.

Par la suite, pour analyser les molécules ciblées dans ces trois classes pharmacologiques deux techniques différentes ont été choisies à savoir la technique séparative de chromatographie liquide et les techniques non séparatives (spectroscopie UV-Visible, spectroscopie NIR et spectroscopie Raman) afin de mettre au point des méthodes analytiques.

Ainsi au travers trois sections se rapportant à la technique séparative de la chromatographique liquide, huit méthodes analytiques génériques ont été développées afin de tracer simultanément plusieurs molécules dans le but de les détecter, de mettre en évidence des falsifications ou des contrefaçons, et dans un second temps de quantifier ces molécules. La stratégie de la planification expérimentale et l'espace de conception a été appliquée à cet effet.

D'autre part, au travers trois sections se rapportant aux techniques non séparatives, sept méthodes analytiques ont été mises au point : quatre méthodes en spectroscopie Ultraviolet-Visible pour analyser respectivement la quinine bichlorhydrate goutte orale à 20%, la quinine sulfate comprimé à 500mg, l'amoxicilline trihydrate capsule à 500mg et le métronidazole benzoate suspension orale à 125mg/5mL, une méthode en spectroscopie Proche Infrarouge pour analyser le paracétamol sirop à 2% et deux méthodes en spectroscopie vibrationnelle Proche Infrarouge et Raman pour analyser la quinine bichlorhydrate goutte orale à 20%.

Toutes ces méthodes développées ont été ensuite validées selon l'approche de l'erreur totale utilisant le profil d'exactitude comme outil de décision. Ces différents profils obtenus ont permis de visualiser les performances analytiques de différentes méthodes concernées et de garantir qu'au moins 95% des futurs résultats seraient compris à l'intérieur des limites d'acceptation fixées.

Ensuite, les méthodes développées et validées ont servi à connaitre la teneur des principes actifs dans les médicaments commercialisés à Kinshasa. Les résultats des analyses obtenus ont confirmés que le trafic de la commercialisation de médicaments de qualité inférieure est un problème réel de la santé publique en RDC qu'il faut combattre.

Ainsi, les méthodes analytiques développées et validées dans le présent travail constituent des outils analytiques pertinents pour lutter contre le phénomène de la circulation de médicaments de qualité inférieure en RDC.

Thesis summary

The present thesis work is part of a collaboration between the University of Liège and the University of Kinshasa in the field of Pharmaceutical Sciences. It aims to develop reliable methods for quality control of marketed drugs in order to support the Congolese Ministry of Health in the fight against the proliferation of poor quality medicines.

Initially, three pharmacological classes of medicines widely used and likely to be counterfeit in the DRC were selected namely non-steroidal anti -inflammatories, antibiotics and antimalarials.

Subsequently, to analyze target molecules in these three pharmacological classes two different techniques were chosen namely the separative techniques of liquid chromatography and non-separative techniques (UV- Visible spectroscopy, NIR spectroscopy and Raman spectroscopy) in order to develop analytical methods.

Subsequently, we developed analytical methods using the separative techniques of liquid chromatography and non-separation techniques (UV-visible spectroscopy, NIR spectroscopy and Raman spectroscopy) for to analyze the molecules selects in these three pharmacological classes.

Thus, through three sections related to the separative techniques of liquid chromatography, eight generic analytical methods have been developed to simultaneously trace several molecules to detect them, highlight falsifications or counterfeits, and in a second time to quantify these molecules. The strategy of the experimental design and of the design space has been applied for this purpose.

On the other hand, through three sections related to non-separative techniques, seven analytical methods have been developed: four methods in ultraviolet visible spectroscopy to analyze quinine dihydrochloride oral drop at 20%, quinine sulfate tablet at 500mg, capsule of amoxicillin trihydrate at 500mg and metronidazole benzoate oral suspension at 125mg/5mL, respectively, one method in NIR spectroscopy to analyze paracetamol at 2% in syrup and two

methods in vibrational spectroscopy Near Infrared and Raman to analyze quinine dihydrochloride oral drop at 20%.

All these methods have been developed then validated by the total error approach using the accuracy profile as a decision tool. These different obtained profiles allowed visualizing the analytical performances of different concerned methods and ensure that at least 95% of future results would be included within the acceptance limits.

Then, the developed and validated methods were used to know the content of active ingredients in medicines marketed in Kinshasa. The analysis results obtained have confirmed that trafficking of marketing poor quality medicines is a real problem of public health in the DRC that must be fought.

Thus, the analytical methods developed and validated in this work are relevant analytical tools to fight against the phenomenon of the circulation of poor quality medicines in the DRC.

Chapitre VII

Productions scientifiques

1. Publications

1. Roland Djang'eing'a Marini, **J.K. Mbinze**, María de Lourdes Aja montes, Benjamin Bebrus, Pierre Lebrun, Jérôme Mantanus, Eric Ziemons, Claude Rohrbasser, Serge Rudaz, Philippe Hubert, **Analytical tools to figth against counterfeit medicines**, Chimica Oggi/Chemistry today 28 (2010) 10-14.

2. B. Debrus, P. Lebrun, **J. K. Mbinze**, F. Lecomte, A. Ceccato, G. Caliaro, J. Mavar Tayey Mbay, R.D. Marini , B. Boulanger, E. Rozet, Ph. Hubert, **Innovative HPLC method development for the screening of 19 antimalarial drugs based on a generic approach, using design of experiments and design space**, Journal of Chromatography A 1218 (2011) 5205-5215.

3. **J.K. Mbinze**, P. Lebrun, B. Debrus, A. Dispas, J. Mavar Tayey Mbay, T. Schofield, B. Boulanger, E. Rozet, Ph. Hubert, R.D. Marini, **Application of an innovative design space optimization strategy to the development of liquid chromatographic methods to combat potentially counterfeit nonsteroidal anti-inflammatory drugs**, Journal of Chromatography A 1263 (2012) 113-124.

4. **J.K. Mbinze**, A. Dispas, P. Lebrun, J. Mavar Tayey Mbay, V. Habyalimana, N. Kalenda, E. Rozet, Ph. Hubert, R.D. Marini, **Application of an innovative design space optimization strategy to the development of LC methods for the simultaneous screening of antibiotics to combat poor quality medicines**, Journal of Pharmaceutical and Biomedical Analysis 85 (2013) 83-92.

5. B. Debrus, P. Lebrun, E. Rozet, T. Schofield, **J.K. Mbinze**, R.D. Marini, S. Rudaz, B. Boulanger, and Ph. Hubert, **A New Method for Quality by Design Robust Optimization in Liquid Chromatography**, LC-GC Europe 26 (2013) 370- 377.

6. **J.K. Mbinze**, Patrick B. Memvanga, Eric Rozet, Philippe Hubert, Véronique Préat, Roland D. Marini, **Development of a liquid chromatographic method for the simultaneous quantification of curcumin, β-arteether, tetrahydrocurcumin and**

dihydroartemisinin. Application to lipid-based formulations, Journal of Pharmaceutical and Biomedical Analysis 88 (2014) 447-456.

7. V. Habyalimana, N. Kalenda Tshilombo, A. Dispas, **J.K. Mbinze**, J.L. Kadima Ntokamunda, P. Lebrun, Ph. Hubert, R.D. Marini, **Méthodes chromatographiques génériques de criblage pour lutter contre les médicaments de qualité inférieure**, Spectra ANALYSE 298 (2014) 30-36.

8. **J.K. Mbinze**, N. Kalenda Tshilombo, P.-F. Chavez, C. De Bleye, P.-Y. Sacre, J. Mavar Tayey Mbay, Ph. Hubert, R.D. Marini, E. Ziemons, **La spectroscopie proche infrarouge, une technique non destructive dans la lutte contre la contrefaçon des médicaments**, Spectra ANALYSE 298 (2014) 46-49.

9. **JK Mbinze**, A. Yemoa, P. Lebrun, P.-Y. Sacré, V. Habyalimana, N. Kalenda, A. Bigot, E. Atindehou, Ph. Hubert, R.D. Marini, **Fighting Poor Quality Medicines: Development, Transfer and Validation of Generic HPLC Methods for Analyzing two WHO Recommended Antimalarial Tablets**, American Journal of Analytical Chemistry 6 (2015) 127-144.

10. **J.K. Mbinze**, J. Nsangu Mpasi, E. Maghe, S. Kobo, R. Mwanda, G. Mulumba, J. Bolavie Bolande, T.M. Bayebila, M. Borive Amani, Ph. Hubert, R.D. Marini, **Application of Total Error Strategy in Vali-dation of Affordable and Accessible UV-Visible Spectrophotometric methods for Quality Control of Poor Medicines**, American Journal of Analytical Chemistry 6 (2015) 106-117.

11. **J.K. Mbinze**, P.-Y. Sacré, A. Yemoa, J. Mavar Tayey Mbay, V. Habyalimana, N. Kalenda, Ph. Hubert, R.D. Marini, E. Ziemons, **Development, validation and comparison of NIR and Raman methods for the identification and assay of poor-quality oral quinine drops**, Journal of Pharmaceutical and Biomedical Analysis 111 (2015) 21-27.

2. Posters

1. **J. K. Mbinze**, R.D. Marini, B. Debrus, P. Lebrun, F. Lecomte, B. Boulanger, Ph. Hubert, **Développement d'une méthode pour la séparation de 19 antipaludéens par HPLC au moyen de la planification expérimentale et du Design Space**, 7ème congrès francophone de l'AfSep sur les sciences séparatives et les couplages (SEP'09) 2009, Marseille, France.

2. I. Nistor, F. Lecomte, **J. K. Mbinze**, B. Debrus, P. Lebrun, R.D. Marini, R. Oprean, **Ph.Hubert, transfer of a conventional lc method for the screening of counterfeit antimalarial medicines to UHPLC**, International Symposium on Drug Analysis 2010, Anvers, Belgique.

3. R.D. Marini, **J. K. Mbinze**, M.L.A. Montes, B. Debrus, P. Lebrun, J. Mantanus, E. Ziemons, C. Rohrbasser, S. Rudaz, Ph. Hubert, **Contribution to fight against counterfeit medicines applying several analytical tools**, International Symposium on Drug Analysis 2010, Anvers, Belgique.

4. **J.K. Mbinze**, P. Lebrun, B. Debrus, A. Dispas, C. Hubert, E. Rozet, Ph. Hubert, R.D. Marini, **Application of an innovative design space optimization strategy to the development of lc methods to combat potentially counterfeit NSAIDs**, HPLC 2012, Californie, USA. Award among the HPLC 2012 posters.

3. Communication orale

1. **J.K. Mbinze**, P. Lebrun, B. Debrus, A. Dispas, R.D. Marini, Ph. Hubert, **The development of lc generic analytical methods to fight counterfeit Nsaids using design space optimisation strategy**, 16ème Forum de la Société Belge des Sciences Pharmaceutiques 2012, Blankenberge, Belgique.