A fast Ultra High Pressure Liquid chromatographic method for qualification and quantification of pharmaceutical combination preparations containing paracetamol, acetyl salicylic acid and/or antihistaminics.

E. Deconinck, P.Y. Sacré, S. Baudewyns, P. Courselle, J. De Beer*

Dept. of Pharmaco-Bromatology, Section Medicinal Products, Scientific Institute of Public Health (IPH), J. Wytmansstraat 14, 1050 Brussels, Belgium

Abstract:

A fully validated UHPLC method for the identification and quantification of pharmaceutical preparations, containing paracetamol and/or acetyl salicylic acid, combined with anti-histaminics (phenylephrine, pheniramine maleate, diphenhydramine, promethazine) and/or other additives as quinine sulphate, caffeine or codein phosphate, was developed. The proposed method uses a Waters Acquity BEH C18 column (2 x 100mm 1.7µm) with a gradient using an ammonium acetate buffer pH 4.0 as aqueous phase and methanol as organic modifier. The obtained method was fully validated based on its measurement uncertainty (accuracy profile) and robustness tests. Calibration lines for all components were linear within the studied ranges. The relative bias and the relative standard deviations for all components were respectively smaller than 1.5% and 2%, the β-expectation tolerance limits did not exceed the acceptance limits of 10% and the relative expanded uncertainties were smaller than 5% for all of the considered components.

A UHPLC method was obtained for the identification and quantification of these kind of pharmaceutical preparations, which will significantly reduce analysis times and workload for the laboratories charged with the quality control of these preparations.

Key words: pharmaceutical preparations, UHPLC, NSAID, anti-histaminics, method validation

*corresponding author: Jacques.Debeer@wiv-isp.be

Tel. +32 2 642 51 70

Fax. +32 2 642 53 27

1. Introduction

Pharmaceutical preparations containing paracetamol and/or non-steroidal anti-inflammatory drugs (NSAID) as acetyl salicylic acid or ibuprofen, are frequently used. In Belgium these preparations are often made in-house by the pharmacist. Often they are combined with anti-histaminics as diphenhydramin and pheniramin maleate and some other active components as caffeine and codein. The antihistaminics are added in formulations for the symptomatic treatment of the flue and flue-like illnesses, like a common cold. The paracetamol and/or the NSAID treats the fever and possible pains, while the anti-histaminic relieves the symptoms of nasal congestion. Caffeine and codein are added for their synergic activity with paracetamol and NSAID's. Therefore a minimal dose of 30 mg has to be present in the formulation.

Because of the extended use of this kind of preparations the authorities need to check the quality of these products. The European Pharmacopeia [1] describes only analytical methods for bulk products while the United States Pharmacopeia [2] describes the analysis of some preparations, but limiting itself to preparations containing only an NSAID or the combination with caffeine or codein. Due to the fact that these preparations are prepared in-house with or without recipe of a medical doctor, a lot of variation exists as well in composition as in the doses of the different compounds. Due to the variation in the formulations the laboratories charged with the analysis have a series of methods that were developed for the analysis of such preparations in the course of time.

In literature several methods are described for the analysis of mixtures of NSAID's and additives. Most methods describe classical HPLC methods with simple UV or DAD detection. Both normal phase [3] as reversed phase [4-13] methods can be found. Further methods can be found using LC-MS [14,15], capillary electrophoresis [16-21] and micellar electrokinetic

chromatography [17,20,22]. Other methods like fluorimetric determinations [23], sequential injection analysis [24] and thin layer chromatography [25] are also available.

The development of a generic applicable method allowing the analysis of a major part of the formulations with the same system would save resources and time.

This paper describes a validated chromatographic method capable of analysing at least ten of the frequently occurring components in the concerned formulations made by pharmacists in Belgium. In first instance the development was focused on HPLC, but soon it was seen that analysis times would be too long to be practical. Therefore it was decided to concentrate on UHPLC, allowing shorter analysis times and an important saving of organic solvents.

In a first step a method was developed to separate ten compounds, selected based on the in house database containing all pharmaceutical formulations analysed in our lab in the past. In a second part the method was validated according to the requirements of the ISO 17025 guideline [26]. The robustness of the method was tested using a full factorial design following the method proposed by Massart et al. [27] with as factors the pH, the flow and the temperature.

2. Methods and materials

2.1. Chemicals and reagents

The reference standards for Paracetamol (batch 08J09-B02-230199), Salicylic acid (batch 08H29-B01-229453) and Quinine sulphate (batch 09B12-B05-232890) were purchased from Fagron (Waregem, Belgium). Acetyl Salicylic Acid (batch 04J04GO) and Phenylephrine.HCl (batch 08C26-B04) were purchased from BUFA (Uitgeest, The Netherlands), Caffeine (batch 06D11-B01-215309) and Diphenhydramine.HCl (batch 07A22-B10-219304) from Certa (Braine-L'Alleud, Belgium), Pheniramine maleate (batch 068K1128) and Promethazine.HCl

(batch 097K1276) from Sigma-Aldrich (St. Louis, USA) and Codein Phosphate Hemihydrate (batch 06C15/V24735) from Conforma (Destelbergen, Belgium).

For the preparation of the mobile phases ammonium Acetate and ammonium solution were purchased from Merck (Darmstadt, Germany), formic acid from VWR prolabo (Fontenay-Sous-Bois, France) and MeOH and acetonitril, both HPLC-grade, from Biosolve (Valkenswaard, The Netherlands).

2.2. Instrumental conditions

Method development and validation was performed on an Acquity UPLC™ system (Waters, Milford, USA). The system consisted of a binary solvent manager, a sample manager and a photo diode array detector. The output signal was monitored and processed using the Waters Empower2 software.

The initial screening tests were performed using combinations of two stationary phases, an Acquity BEH C18 column 2.1 x 100mm 1.7µm (Waters) and a Grace Vision HT™ C18-P 2 x 100mm 1.5µm (Grace Davision Discovery Sciences, Lokeren, Belgium) two organic buffers, an ammonium formate buffer 0.025M of pH 3 and an ammonium acetate buffer 0.025 M of pH 4, and two organic modifiers acetonitril and methanol. The gradient used starts at 98% buffer and 2% organic modifier, going to a plateau of 30% buffer in 8 minutes. These conditions are held for 2 minutes before returning to the initial conditions. The gradient was linear and the flow was 0.50 ml/min. The injection volume was 2µl, the column temperature 50°C and the detection wavelength 254 nm. This wavelength was selected since all of the components showed enough sensitivity at 254 nm.

Method optimisation and validation were performed on the Acquity BEH C18 column under gradient conditions using a mobile phase composed of a 0.025 M ammonium acetate buffer of pH 4 and methanol.

2.3. Sample preparation

2.3.1. Preparation of standards

Calibration standards were prepared starting from separated stock solutions for each of the ten components. The respective stock solutions contained 5 mg/ml paracetamol, 5 mg/ml acetylsalicylic acid, 0.2 mg/ml promethazine, 0.2 mg/ml phenylephrine.HCl, 0.2 mg/ml salicylic acid, 0.3 mg/ml pheniramine maleate, 0.3 mg/ml diphenhydramin.HCl, 1.2 mg/ml codeine phosphate hemihydrate, 1.2 mg/ml caffeine and 1 mg/ml quinine hydrochloride.
Starting from these solutions standards were prepared by making dilutions of respectively 1.0, 2.5, 5.0, 7.5 and 10.0 ml in 50ml. All solutions were prepared in methanol containing 1% of formic acid to ensure the stability of acetyl salicylic acid and promethazine.HCl in solution.

2.3.2. Preparation of samples

In order to validate the method following, the “total error” approach, blank spiked samples were prepared starting from stock solutions with the same concentrations as the ones used for the preparation of the standards. Stock solutions for sample preparation were prepared separately from the ones used for the standards. For the preparation of the validation samples a blank matrix consisting of lactose was used. 100 mg of the matrix was spiked with the stock solutions and brought to volume with methanol containing 1% of formic acid. The samples were brought in the ultrason bath for 10 minutes. Starting from the stock solutions three samples were prepared with different concentration levels. The concentration levels of the different components were chosen in function of the concentrations occurring in

pharmaceutical preparations previously analysed at our laboratory. Attention was paid to the fact that in all three samples the different components were present in concentrations showing the same proportions as the solutions obtained with samples from practice. Table 1 shows the concentration levels chosen for each of the analytes.

As example a real sample containing 300 mg acetyl salicylic acid, 250 mg paracetamol, 20 mg caffeine, 10 mg codeine phosphate and 20 mg of diphenhydramine was analysed. 20 capsules were emptied and homogenised. A quantity of powder corresponding to 60 mg of acetyl salicylic acid was brought in methanol containing 1% of formic acid and put on ultrason for 10 minutes. A clear solution was obtained and the solution was brought to 100 ml with methanol containing 1% of formic acid.

2.4. Experimental design

The robustness testing of the method was performed using experimental design. A three-factor three-level full factorial design was applied [27]. The experiments were randomly performed in triple and the effects of the different factors were interpreted using regression. A quadratic response surface area was constructed, represented by following general equation:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \qquad (1)$$

where b_0 represents the intercept, b_i and b_{ij} the regression coefficients and x_i the factors tested. The significance of the regression coefficients is a value for the significance of the effects of the different factors on the response. The regression coefficients of the products of two factors represent the significance of their interaction effects [27].

2.5. Method validation

The method validation was performed in accordance with the requirements of the ISO17025 guideline using the total error approach [26, 28-30].

Therefore the spiked blank samples prepared in section 2.3.2 were prepared in triple and analysed for three consecutive days. The concentrations of the spiked samples were back-calculated using the calibration lines, prepared as described in section 2.3.1., to determine the linearity between theoretical and measured concentrations, the mean relative bias, the repeatability, the intermediate precision and the β-expectation tolerance or total error intervals at the 5% level.

2.6. Statistics

The statistical analysis was performed using Statgraphics Plus 5.1 (STSC Inc.,Rockville, MD, USA) and Microsoft Excell 2003. Visualisation of the response surfaces was executed using Matlab version 7.9 R2009b (The Mathworks Inc., Matick, MA).

3. Results

3.1. Selection of the system to be optimised

The initial screening tests were performed as described in section 2.2.

Visual inspection of the obtained chromatograms led to the conclusion that the best initial separation was obtained using a mobile phase of methanol with the ammonium acetate buffer and the Acquity BEH C18 column. This method was used as starting point for further method optimisation. Figure 1 shows the corresponding chromatogram.

3.2. Optimisation of the method

From figure 1 it can be seen that phenylephrine is eluted with the void volume and that the separation between acetyl salicylic acid and caffeine is not optimal. Therefore the initial gradient was adapted by lowering the percentage of aqueous phase in the initial conditions, keeping the initial conditions for one minute and going to a plateau of 50/50 buffer/methanol in 8 minutes. These adaptations to the gradient let to a good separation (resolution > 1.5) for all ten components as well as to an improvement in peak symmetry (0.80-1.30). In principle the run time of the method could still be reduced, though we chose to keep it at 11 minutes in order to obtain a more general applicable method. Keeping a longer run time and higher resolutions improves the opportunity that a component present in a preparation that was not taken into account during the method development, can be detected and quantified with the same method.

The final gradient starts at 95% ammonium acetate buffer pH 4 and 5% methanol. The initial conditions are kept for one minute, before going to a plateau of 50% buffer and 50% methanol in nine minutes. The plateau is maintained for two minutes before returning to the initial conditions. The gradient was linear and the flow was 0.50 ml/min. Figure 2a the corresponding chromatogram.

As example figure 2b shows the chromatogram obtained for the real commercial sample described in section 2.3.2.

This method was validated following the ISO 17025 requirements in order to implement it in the routine analysis of these combined pharmaceutical preparations.

3.3. Validation

3.3.1. Selectivity

The selectivity of detection was ensured by determining the retention time of each component separately and by monitoring the UV-spectra of the different components during the different analyses.

3.3.2. Linearity of the calibration lines

For all of the ten components four calibration standards were prepared in order to evaluate the relationship between the area under the curve and the concentration. The linearity of the relationship was evaluated for each of the components in a concentration range, covering the normal range of concentrations obtained when analyzing pharmaceutical preparations.

The calibration curves were obtained using ordinary least-square linear regression and the linearity was confirmed with the R^2 values and a quality coefficient [31]. Table 2 summarizes for the ten components the concentration ranges of the calibration curves, the R^2 values and the quality coefficients. From this table it can clearly been concluded that the calibration curves for all components are linear within the chosen concentration ranges.

3.3.3. Trueness, precision, accuracy and uncertainty assessment

A statistical approach based on the “total error” profiles was applied to validate the method.

As explained in section 2.3.2 spiked blank samples were prepared at three concentration levels. Table 3 gives the exact concentrations of the 3 levels for each of the components. Every sample was prepared in triple and analysed for three consecutive days.

The concentrations of the spiked samples were back-calculated using the calibration lines, prepared as described in section 2.3.1., to determine the linearity between theoretical and measured concentrations, the mean relative bias, the repeatability, the intermediate precision and the β-expectation tolerance or total error intervals at the 5% level. All results are shown in table 4.

The relationship between the theoretical and the calculated concentrations for each of the ten components is clearly linear with R^2-values from 0.9997 to 1.000.

Trueness refers to the closeness of agreement between the average of the obtained values and the known exact concentration of the spiked samples and is a measure for the systematic errors of the method [30,32]. It is expressed in terms of relative bias. From table 4 it can be concluded that the trueness for all components is acceptable since the relative bias is always smaller than 1,5%.

The precision is a measure for the relative errors of the method and is expressed as the relative standard deviations (RSD) for repeatability and intermediate precision. From table 4 it can be seen that an acceptable precision is obtained for all components. The maximal RSD is obtained for phenylephrine and is 1.995%.

Accuracy takes into account the total error of the test results and is represented by the β-expectation tolerance intervals. The acceptance limits for the bias were set at 10 %. This is based on the fact that the general acceptance limits for the content of pharmaceutical preparations, made by a pharmacist, are from 90 to 110%. As shown in table 4 and figure 3 the relative β-expectation tolerance intervals did not exceed the acceptance limits, which means that each future measurement of unknown samples will be included in the tolerance limits for the relative bias at the 10% level.

The uncertainty represents the dispersion of the values that could reasonably be attributed to the analyte. The expanded uncertainty represents an interval around the results where the unknown true value can be observed with a confidence level of 95%. The relative expanded uncertainties (%) are obtained by dividing the corresponding expanded uncertainties with the corresponding concentrations. Results are shown in table 4. Since all uncertainties are below 5% percent the method is considered to have acceptable uncertainties for all components.

3.3.4. Limits of detection and quantification

The limits of detection and quantification of the method were calculated based on the standard deviation of the analysis of a blank and the sensitivity of the method [27].

A blank was analysed 10 times and the standard deviation of the signal at the retention time of each of the components was calculated. The limit of detection was calculated as three times the standard deviation of the blank divided by the sensitivity, equal to the slope of the calibration curve. The quantification limit was calculated as ten times the standard deviation of the blank divided by the sensitivity. The detection and quantification limits for all ten components are listed in table 5.

3.3.5. Recovery

The absolute recoveries of all ten components were determined at the three concentration levels used to construct the accuracy profile. The recoveries were determined by analysing spiked blank samples and calculating their concentrations using calibration lines in analogy with what was done for the accuracy profile. Table 6 summarizes the mean recoveries obtained for all ten components for each concentration level. All recoveries are within acceptable limits, indicating that the method is suited for the analysis of these active substances in pharmaceutical preparations.

3.3.6. Robustness

Robustness is a measure for the influence of small changes in the analytical procedure/parameters on the measured response.

The test was performed by a 3-factor 3-level full factorial design, with the flow, the column temperature and the pH of the ammonium acetate buffer as factors and the resolution between caffeine and acetyl salicylic acid (critical pair) as response. The different levels were chosen

based on the errors which are common during such an analysis. Table 7 shows the experimental design performed and the corresponding resolutions obtained. All experiments were performed in random order.

The effects of the different factors were calculated and their significance at the 5% level was tested by ANOVA analysis. Table 8 shows the calculated effects of the different factors as well as their interaction effects, with their standard errors, a measure for the sampling error. Figure 4 shows the standardized Pareto chart and figure 5a-c shows the response surfaces obtained with the regression methods.

From the ANOVA analysis it could be seen that the regression is significant with an R^2 of 99.99%. From figure 4 and 5 and from the ANOVA table shown in Table 9 it could be seen that the temperature and the flow have a small significant effect on the resolution between caffeine and acetyl salicylic acid. The pH has a strong effect on the resolution between those two components. The effects of the temperature and the flow could be explained by the fact that UHPLC works with very high pressure. Little changes in temperature and flow cause an important change in the pressure, which influences retention and resolution. The strong effect of the pH on the resolution of the critical pair can be explained by the fact that pH 4.0 is close to the pK_a value of acetyl salicylic acid. Comparing the different chromatograms obtained under the different conditions revealed that the retention time of acetyl salicylic acid changes strongly in function of the pH, while the shifts in retention times of the other components are less significant.

Eventhough it was statistically proven that the pH, the temperature and the flow have significant effects on the resolution of the critical pair, this does not influence the quality of the method since the resolution of the critical pair stays always higher than 1.5 (Table 7). The method can be considered as suited for purpose.

4. Conclusions

An Ultra Fast Liquid Chromatographic method was developed and validated for the qualitative and quantitative analysis of pharmaceutical preparations containing a series of non-steroidal anti-inflammatory drugs in combination with anti-histaminics and/or caffeine and codein phosphate. The validation was performed following the ISO17025 requirements and proved that the method was suited for purpose and can be used in the routine analysis of these pharmaceutical preparations.

The method is a gradient method, using a 0.025 M ammonium acetate buffer of pH 4.0 as aqueous phase and methanol as organic phase. The gradient starts at a percentage of 95% of the buffer solution and comes to a plateau of 50% buffer at 9 minutes. The flow rate is 0.5 ml/min and the detection wavelength 254 nm.

The method was applied in the analysis of routine samples at our lab and showed a good performance. Depending on the composition of the sample the gradient could even be shortened in order to gain time and solvents. When the composition allowed it the method was also used in our laboratory to dose preparations containing ibuprofen, chlorphenamine maleate, metoclopramide, etc… , broadening the applicability of the method to preparations composed of other combination of non-steroidal anti-inflammatory drugs and anti-histaminics. When using the method for other molecules as described in this paper, one should always check the method for the APIs in the preparation first.

The fact to have a general applicable method that allows the analysis of the majority of the NSAID pharmaceutical preparations in a run time of twelve minutes represents a significant gain in time and workload for laboratories charged with the quality control of such preparations.

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Figure Captions:

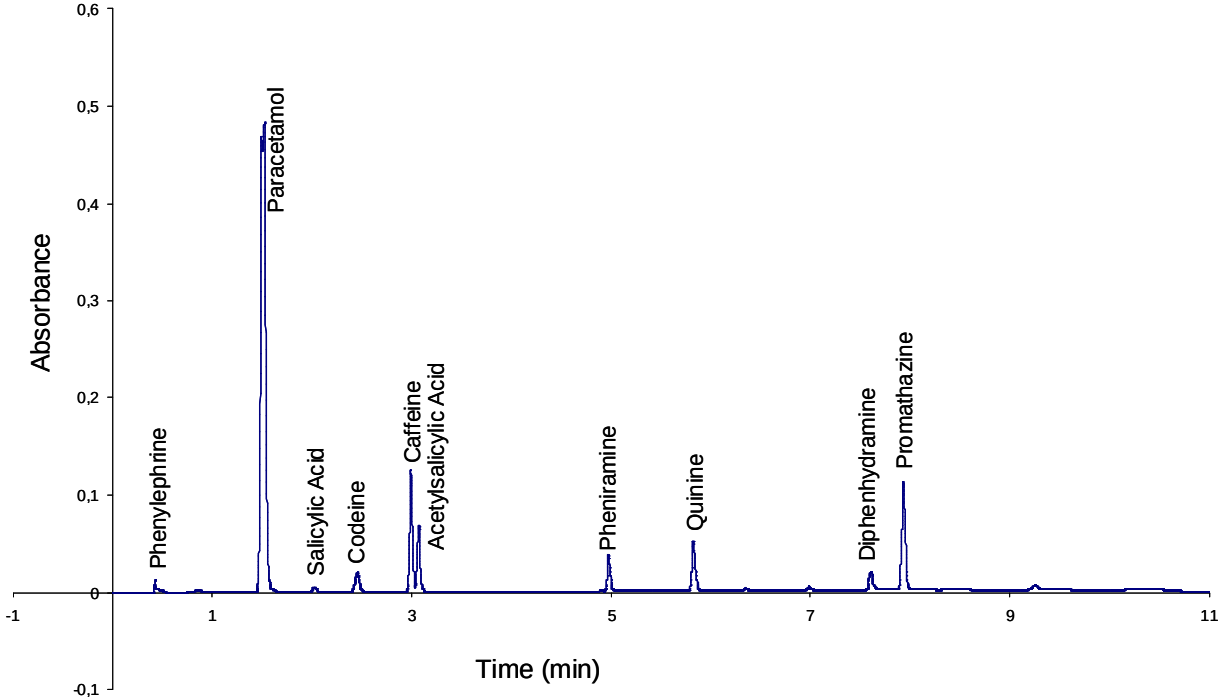
Figure 1: Chromatogram obtained with the selected method from the initial screening.

Figure 2: (a) Chromatogram obtained with the optimized gradient conditions. (b) Chromatogram obtained with a real commercial sample

Figure 3: Accuracy profile of the ten components. The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits, the bold plain line are the acceptance limits (10%) and the dots represent the relative back-calculated concentrations, plotted with respect to their targeted concentration.

Figure 4: standardized Pareto chart for the resolution between caffeine and acetyl salicylic acid.

Figure 5: (a) response surface for the effect of the pH and the temperature on the resolution of caffeine and acetyl salicylic acid; (b) response surface for the effect of the flow and the temperature on the resolution of caffeine and acetyl salicylic acid.


Absorbance
Time (min)
Phenylephrine
Paracetamol
Salicylic Acid
Codeine
Caffeine
Acetylsalicylic Acid
Pheniramine
Quinine
Diphenhydramine
Promathazine
0,6
0,5
0,4
0,3
0,2
0,1
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-0,1
-1
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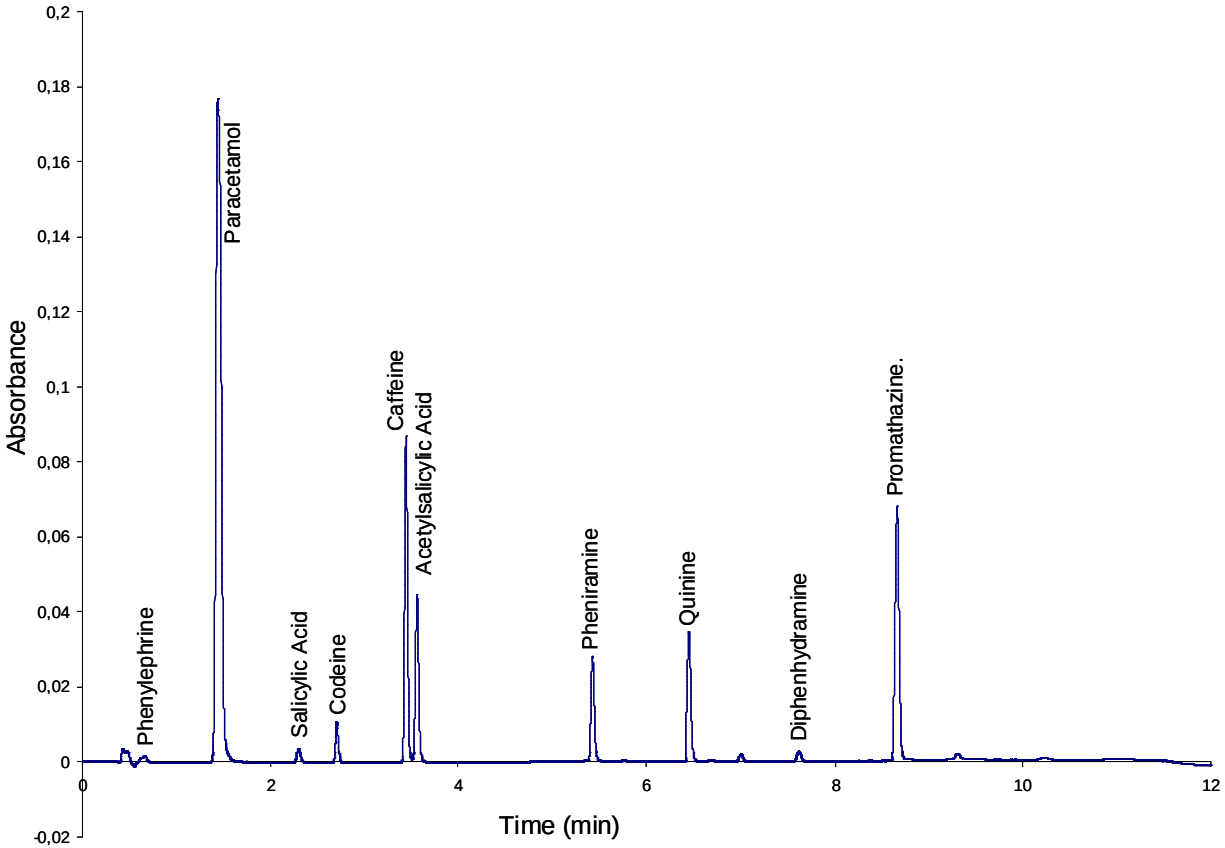
Figure 1


Absorbance
Time (min)
Phenylephrine
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Quinine
Diphenhydramine
Promathazine.

Figure 2a

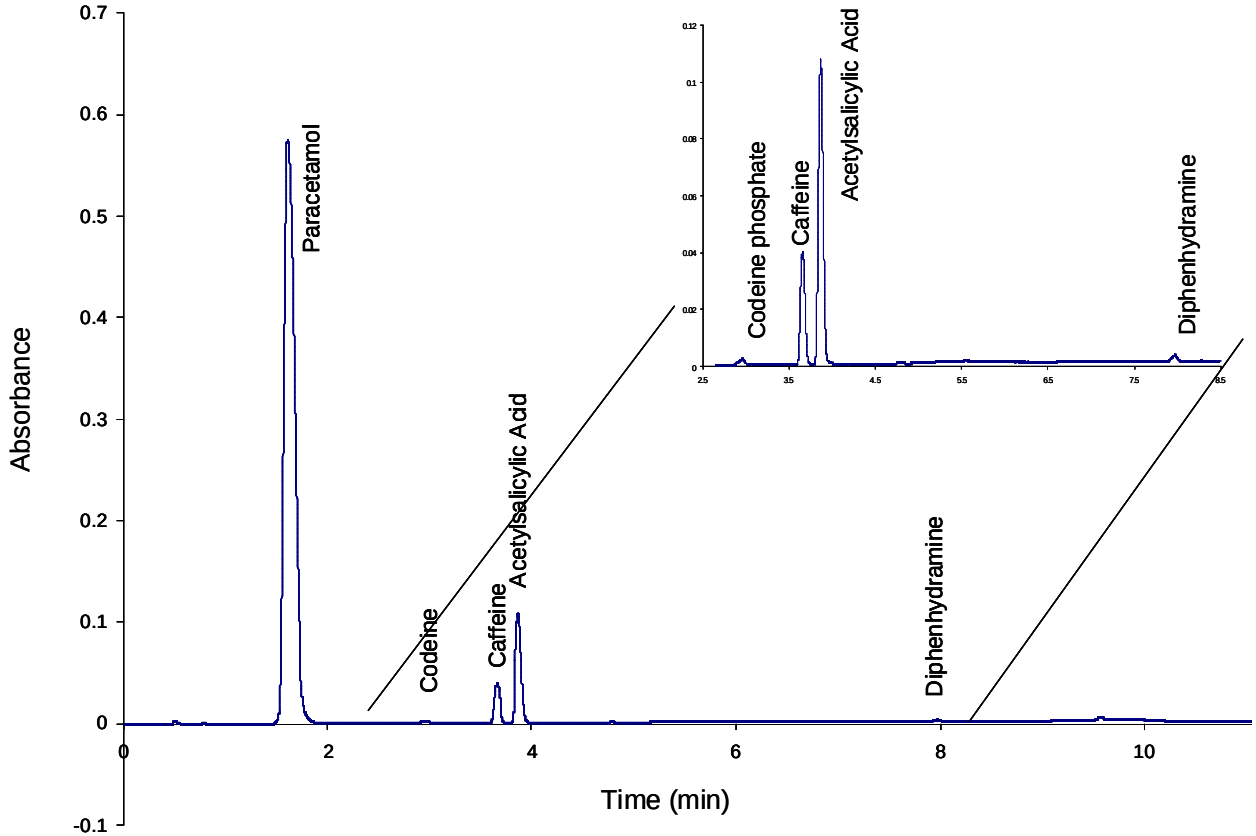
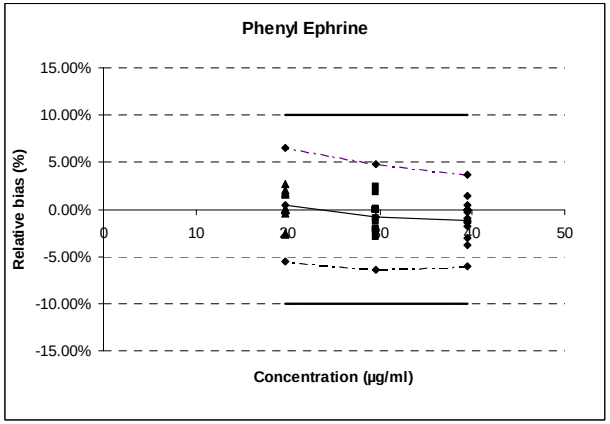

Absorbance
Time (min)
Paracetamol
Codeine
Caffeine
Acetylsalicylic Acid
Diphenhydramine
Codeine phosphate
Caffeine
Acetylsalicylic Acid
Diphenhydramine

Figure 2b

1


Phenyl Ephrine
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
Relative bias (%)
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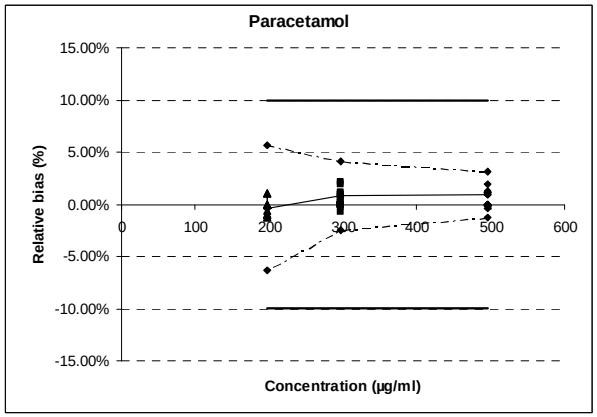
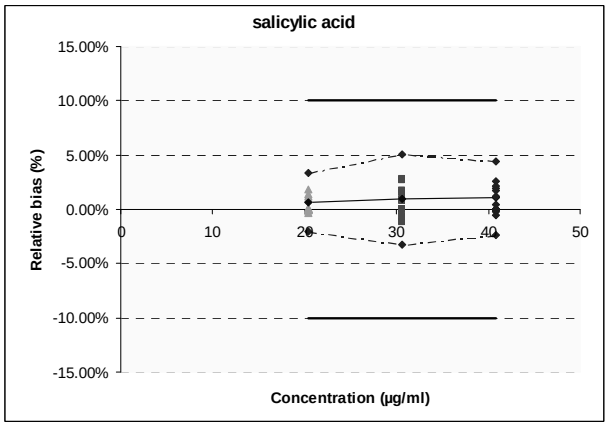
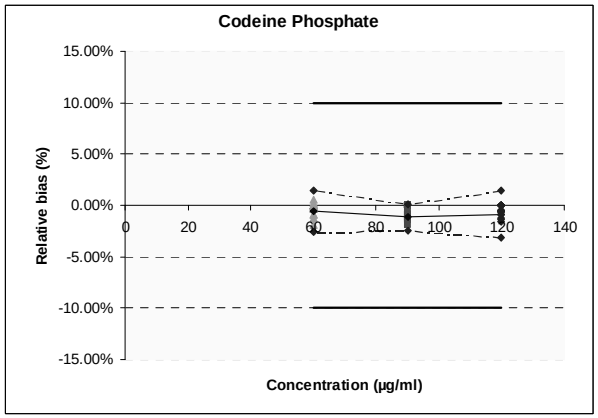
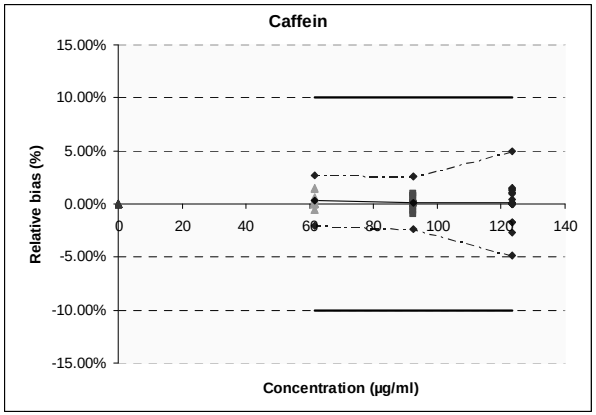
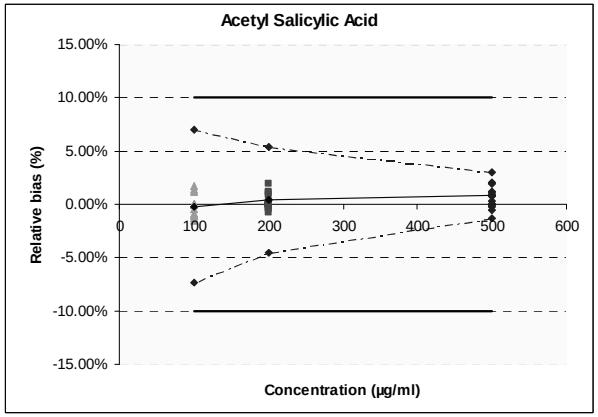
10
20
30
40
50
Concentration (µg/ml)

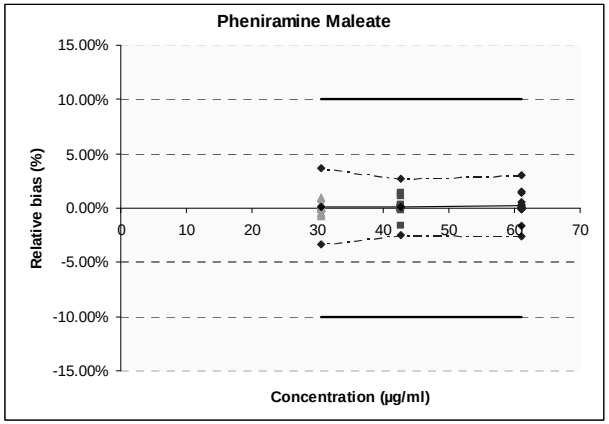

Paracetamol
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
Relative bias (%)
0
100
200
300
400
500
600
Concentration (µg/ml)

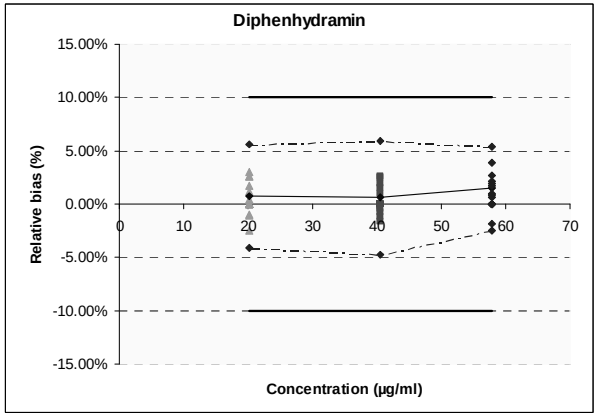

salicylic acid
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
Relative bias (%)
0
10
20
30
40
50
Concentration (µg/ml)

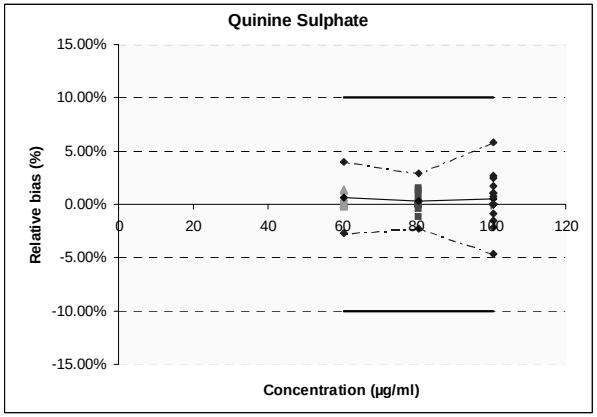

Codeine Phosphate
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
Relative bias (%)
0
20
40
60
80
100
120
140
Concentration (µg/ml)

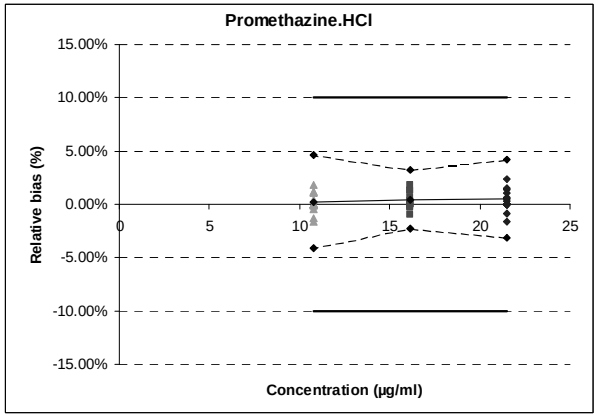

Caffein
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
Relative bias (%)
0
20
40
60
80
100
120
140
Concentration (µg/ml)


Acetyl Salicylic Acid
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
Relative bias (%)
0
100
200
300
400
500
600
Concentration (µg/ml)


Pheniramine Maleate
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
Relative bias (%)
0
10
20
30
40
50
60
70
Concentration (µg/ml)


Diphenhydramin
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
Relative bias (%)
0
10
20
30
40
50
60
70
Concentration (µg/ml)


Quinine Sulphate
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
Relative bias (%)
0
20
40
60
80
100
120
Concentration (µg/ml)


Promethazine.HCl
15.00%
10.00%
5.00%
0.00%
-5.00%
-10.00%
-15.00%
Relative bias (%)
0
5
10
15
20
25
Concentration (µg/ml)

2

3 Figure 3

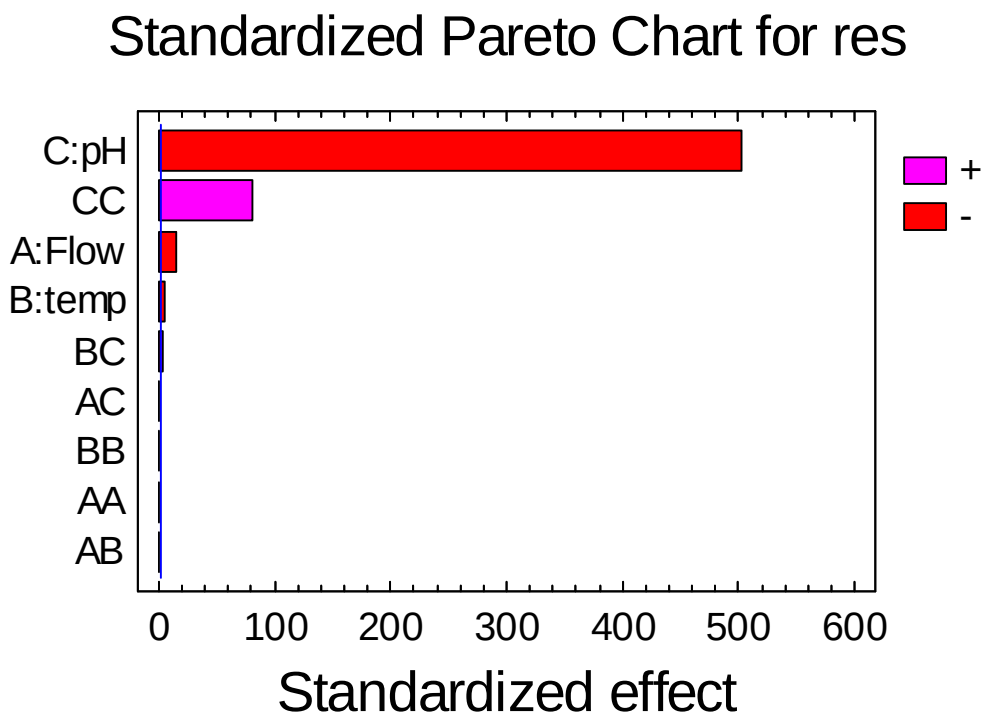

Standardized Pareto Chart for res
C:pH
CC
A:Flow
B:temp
BC
AC
BB
AA
AB
+
-
0
100
200
300
400
500
600
Standardized effect

Figure 4

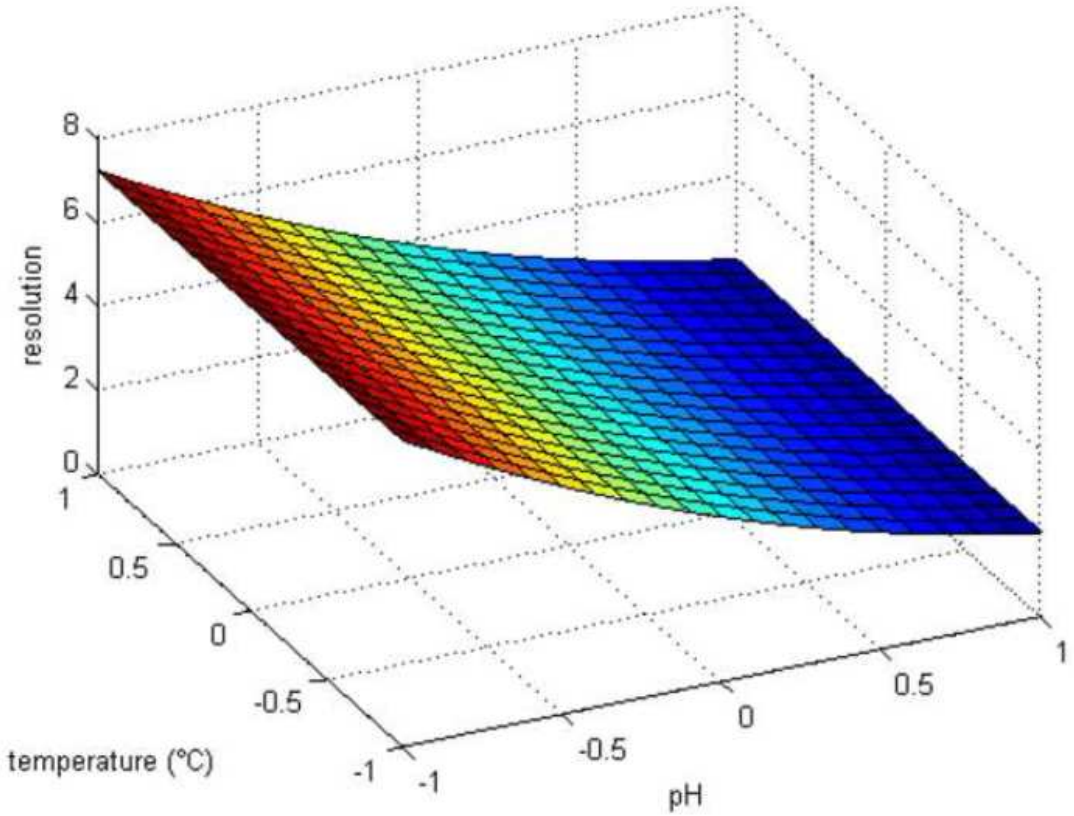


Figure 5a

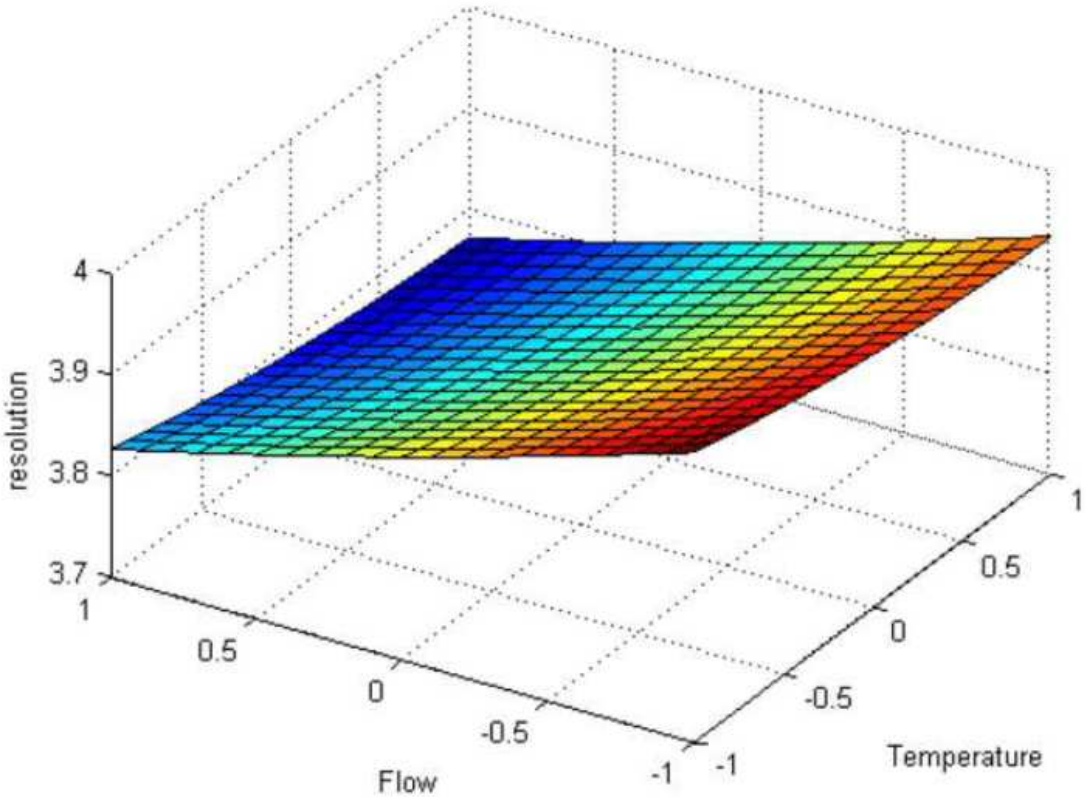


Figure 5b

21 Table 1: concentration levels for the samples used for method validation

Concentration level	Phenylephrine.HCl (mg/ml)	Paracetamol (mg/ml)	Salicylic acid (mg/ml)	Codeine phosphate hemihydrate (mg/ml)	Caffeine (mg/ml)	Acetyl salicylic acid (mg/ml)	Pheniramine maleate (mg/ml)	Quinine sulphate (mg/ml)	Diphenhydramine. HCl (mg/ml)	Promethazine. HCL
Level 1	0.04	0.5	0.04	0.12	0.12	0.5	0.06	0.1	0.06	0.02
Level 2	0.03	0.3	0.03	0.09	0.09	0.2	0.042	0.08	0.042	0.015
Level 3	0.02	0.2	0.02	0.06	0.06	0.1	0.03	0.06	0.03	0.01

22

23 Table 2: summary of the quality of the calibration curves for the different components

Component	Concentration range	R^2 value	Quality Coefficient
Phenylephrine.HCl	0.008 - 0.8 mg/ml	0.9999	0.521 %
Paracetamol	0.1 – 1.0 mg/ml	0.9997	0.120 %
Salicylic acid	0.008 - 0.8 mg/ml	0.9998	1.118 %
Codeine Phosphate	0.024 – 0.24 mg/ml	0.9998	1.650 %
Caffein	0.024 – 0.24 mg/ml	0.9998	1.546%
Acetyl Salicylic acid	0.1 – 1.0 mg/ml	0.9997	0.170 %
Pheniramine Maleate	0.012 – 0.12 mg/ml	1.0000	0.710 %
Quinine sulphate	0.02 – 0.20 mg/ml	0.9999	1.630 %
Diphenhydramine.HCl	0.012 – 0.12 mg/ml	1.0000	0.984 %
Promethazine.HCl	0.004 – 0.04 mg/ml	0.9997	0.185 %

24 Table 3: The different concentration levels

Concentration level	Phenylephrine.HCl (mg/ml)	Paracetamol (mg/ml)	Salicylic acid (mg/ml)	Codeine phosphate hemihydrate (mg/ml)	Caffeine (mg/ml)	Acetyl salicylic acid (mg/ml)	Pheniramine maleate (mg/ml)	Quinine sulphate (mg/ml)	Diphenhydramine. HCl (mg/ml)	Promethazine. HCL (mg/ml)
Level 1	0.0394	0.495	0.0408	0.120	0.123	0.500	0.0610	0.101	0.0578	0.0215
Level 2	0.0296	0.297	0.0306	0.090	0.0926	0.200	0.0427	0.0805	0.0405	0.0161
Level 3	0.0197	0.198	0.0204	0.060	0.0617	0.100	0.0305	0.0604	0.0202	0.0108

Table 4: Trueness, precision, accuracy and uncertainty

	Level	Phenylephrine.HCl	Paracetamol	salicylic acid	codein phosphate	caffein	acetyl salicylic acid	pheniramine maleate	quinine sulphate	diphenhydramine.HCl	Promethazine.HCl
Trueness											
	1	-1,19	0,89	1,03	-0,86	0,06	0,85	0,2	0,59	1,48	0,53
Relative bias (%)	2	-0,85	0,85	0,96	-1,17	0,1	0,39	0,13	0,3	0,60	0,46
	3	0,93	-0,33	0,67	-0,55	0,36	-0,18	0,13	0,61	0,77	0,26
Intra-assay precision											
	1	1,64	0,72	1,13	0,18	1,65	0,87	0,92	1,72	1,57	1,22
Repeatability (RSD %)	2	1,88	0,68	1,37	0,37	0,49	0,47	0,86	0,86	1,24	0,91
	3	2,00	0,21	0,63	0,73	0,69	0,29	0,26	0,29	1,93	1,04
Between-assay precision											
	1	1,64	0,72	1,13	0,47	1,65	0,87	0,92	1,72	1,57	1,22
Intermediate precision (RSD %)	2	1,88	0,92	1,37	0,47	0,70	1,01	0,86	0,86	1,74	0,91
	3	2,00	1,22	0,77	0,78	0,78	1,45	0,72	0,68	1,93	1,24
Accuracy											
	1	[-6,05;3,68]	[-1,30;3,08]	[-2,41;4,46]	[-3,14;1,41]	[-4,89;5,01]	[-1.33;3.03]	[-2,59;2,98]	[-4,62;5,79]	[-2,49;5,45]	[-3,15;4,21]
β-expectation tolerance limits (%)	2	[-6.41;4.78]	[-2,43;4,13]	[-3,19;5,11]	[-2,48;0,13]	[-2,39;2,58]	[-4.56;5.34]	[-2,47;2,72]	[-2,30;2,89]	[-4,76;5,96]	[-2,27;3,19]
	3	[-5.54;6.48]	[-6.36;5.70]	[-2,04;3,38]	[-2,58;1,49]	[-2,01;2,74]	[-7.37;7.01]	[-3,40;3,66]	[-2,73;3,95]	[-4,07;5,96]	[-4,11;4,37]
Uncertainty											
	1	3,50	2,54	2,48	1,06	3,57	1,84	2,01	3,74	3,36	2,65
Relative expanded uncertainty (%)	2	4,03	2,68	2,99	1,01	1,56	2.30	1,87	1,87	3,86	1,97
	3	4,33	2,80	1,70	1,66	1,71	3.34	1,64	1,55	4,10	2,74

27 Table 5: detection and quantification limits.

28

	LOD (µg/ml)	LOQ (µg/ml)
Phenylephrine.HCl	0.34	1.14
Paracetamol	0.016	0.054
Salicylic Acid	0.41	1.37
Codein Phosphate	0.032	0.11
Caffein	0.095	0.32
Acetyl Salicylic Acid	0.093	0.31
Pheniramin Maleate	0.020	0.068
Quinine sulphate	0.017	0.055
Diphenhydramin.HCl	0.22	0.73
Promethazine.HCl	0.084	0.28

29

30 Table 6: Summary of the recoveries obtained for the ten components

Concentration level	Phenylephrine.HCl	paracetamol	Salicylic acid	Codeine phosphate hemihydrate	Caffeine	Acetyl salicylic acid	Pheniramine maleaat	Quinine sulphate	Diphenhydramine. HCl	Promethazine. HCL
Level 1	97.87 %	100.24 %	99.18 %	100.05 %	98.30 %	100.43 %	100.58 %	100.58 %	101.25 %	101.09 %
Level 2	99.60 %	99.64 %	98.87 %	100.10 %	98.34 %	99.24 %	100.78 %	100.44 %	101.08 %	100.86 %
Level 3	100.94 %	97.93 %	97.58 %	100.72 %	99.45 %	97.24 %	101.20 %	100.91 %	101.38 %	100.65 %

Table 7: 3-factor 3-level full factorial design for robustness testing

Nr. experiment	Flow (ml/min)	Temperature (°C)	pH	Resolution for the critical pair
1	0.49	49	3.9	7.38
2	0.50	49	3.9	7.26
3	0.51	49	3.9	7.19
4	0.49	50	3.9	7.27
5	0.50	50	3.9	7.24
6	0.51	50	3.9	7.14
7	0.49	51	3.9	7.25
8	0.50	51	3.9	7.20
9	0.51	51	3.9	7.10
10	0.49	49	4.0	4.00
11	0.50	49	4.0	3.92
12	0.51	49	4.0	3.81
13	0.49	50	4.0	3.95
14	0.50	50	4.0	3.86
15	0.51	50	4.0	3.78
16	0.49	51	4.0	3.96
17	0.50	51	4.0	3.83
18	0.51	51	4.0	3.75
19	0.49	49	4.1	2.04
20	0.50	49	4.1	1.96
21	0.51	49	4.1	1.95
22	0.49	50	4.1	2.06
23	0.50	50	4.1	1.96
24	0.51	50	4.1	1.89
25	0.49	51	4.1	2.05
26	0.50	51	4.1	1.97
27	0.51	51	4.1	1.91

Table 8: Calculated effects for the different factors of the robustness test

Factor	**Effect (**± standard error*)
Intercept	3.863 ± 0.01
Flow (A)	-0.161 ± 0.01
Temperature (B)	-0.054 ± 0.01
pH (C)	-5.248 ± 0.01
AA	0.012 ± 0.02
AB	-0.002 ± 0.01
AC	0.015 ± 0.01
BB	0.020 ± 0.02
BC	0.042 ± 0.01
CC	1.454 ± 0.02

* standard errors are based on the total error with 17 degrees of freedom.

Table 9: Analysis of variance for the resolution of the critical pair

Factor	Sum of square	Degrees of freedom	Mean square	F-ratio	P-values
Flow (A)	0.11	1	0.12	239.34	< 0.00001
Temperature (B)	0.013	1	0.013	27.21	0.0001
pH (C)	123.96	1	123.96	252839.53	< 0.00001
AA	< 0.001	1	< 0.001	0.49	0.4955
AB	< 0.001	1	< 0.001	0.03	0.8640
AC	< 0.001	1	< 0.001	1.38	0.2569
BB	< 0.001	1	< 0.001	1.27	0.2755
BC	< 0.001	1	< 0.001	10.62	0.0046
CC	3.17	1	3.17	6465.5	< 0.00001
Total error	0.008	17	< 0.001		