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PERFORMANCE EVALUATION OF A MIP FOR THE MISPE-LC DETERMINATION OF *P*-[^{18}F]MPPF AND A POTENTIAL METABOLITE IN HUMAN PLASMA

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ABSTRACT

Within the family of serotonin (5-HT) receptors, the 5-HT$_{1A}$ subtype is particularly interesting as it may be involved in various physiological processes or psychological disorders. The *p*-[^{18}F]MPPF, a highly selective 5-HT$_{1A}$ antagonist, is used for in vivo studies in human or animal by means of positron emission tomography (PET) [1].

In order to selectively extract *p*-[^{18}F]MPPF and its main metabolites from plasma, molecularly imprinted polymer (MIP) was prepared against these compounds by using the *p*-MPPF as template. For the control of the selectivity, non-imprinted polymer (NIP) was also synthesized without template. The MIP sorbent, packed in disposable extraction cartridges (DECs), was then evaluated as molecularly imprinted solid-phase extraction (MISPE) prior to the LC determination. The conditions of extraction were evaluated in order to obtain the highest selective retention of the *p*-[^{18}F]MPPF and its metabolites on this MIP. The MIP selectivity was exploited in the loading and washing steps by adjusting the pH of plasma samples at a suitable value and by selecting mixtures for the washing step to limit the contribution of non-specific interactions. Other important parameters involved in the conditioning and elution steps were also studied. Finally, a pre-validation was carried out with optimal extraction conditions to demonstrate the performance of this MISPE-LC method as a generic method in the context of evaluation of new MISPE for *p*-[^{18}F]MPPF and its potential for metabolites extraction from human plasma.

KEYWORDS: Molecularly imprinted polymer ; MIP ; Solid-phase extraction ; Plasma sample ; *p*-MPPF ; Radiopharmaceutical compound

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1. Introduction

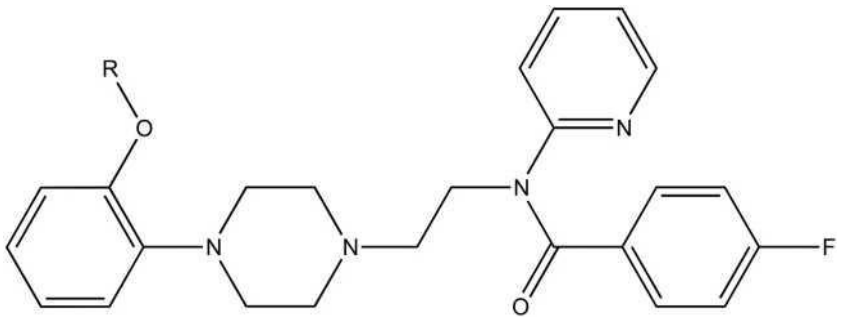
Among the 5-hydroxy-tryptamine (serotonin, 5 HT) receptors, the 5 HT_{1A} receptor is a subtype from class 1 involved in various physiological regulation processes such as sleep, and in important psychiatric disorders such as anxiety and depression. Imaging this receptor with positron emission tomography (PET) using labelled antagonist has attracted many research groups [1].

PET is a medical imaging technique developed and used for diagnostic clinical studies, basic human studies for understanding biochemical process in neurobiology and preclinical studies using animals. This technique allows the study, in living tissues, of the spatial distribution and the pharmacokinetics of tracers labelled with a positron emitter [2-5].

Today, the most usual radionuclide used in PET is fluorine-18 (half-live: 109.7 min, β^+ 97 %, Emax β^+ = 635 KeV) with a high specific activity (around 40 GBq μmol^{-1}). The total amount of substances supporting the radioactivity is very low (a few µg) [6].

In the present study, a highly selective serotonin 5-HT_{1A} antagonist 4-(2'-methoxyphenyl)-1-[2'-[N-(2"-pyridinyl)-*p*-fluorobenzamido]ethyl]-piperazine (*p*-MPPF, Fig. 1) and its radioactive form *p*-[^{18}F]MPPF were used. This compound is nowadays one of the best radiopharmaceuticals available for imaging the serotoninergic system *in vivo* with PET in humans [1]. 4-(2'-hydroxyphenyl)-1-[2'-[N-(2"-pyridinyl)-*p*-fluorobenzamido]ethyl]-piperazine (*p*-DMPPF, Fig. 1) and the radioactive form *p*-[^{18}F]DMPPF are potential metabolites of *p*-MPPF and *p*-[^{18}F]MPPF respectively.

Fig. 1. *Chemical structures of p-MPPF (R = -CH3) and its potential metabolite p-DMPPF (R= -H).*



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Positron emitters used in nuclear medicine are short-lived cyclotron-produced radionuclides (half-live from a few minutes to a few hours) and the use of radiopharmaceutical compounds in that field relies on very specific and effective methods of nuclear physic, organic synthesis and analytical chemistry.

The solid-phase extraction (SPE) is one of the most used and efficient techniques in order to purify and isolate compounds of interest. Consequently, this technique has been considered as a convenient one for the purification steps during the synthesis of PET tracers, allowing the use of the microchemistry techniques. These techniques have many advantages such as high efficiency with low quantity of sorbents at very low concentration level, fast and easy to automate, single use affordable material, easy recovery of the active compounds in small volume of solvents compatible with the formulation of an injectable solution to human [7]. As an example of its application, SPE is routinely used for GMP production of 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG), a world-wide used PET tracer for the detection of tumours, in different steps of the synthesis process [8].

In these cases, although the choices of the SPE materials are correctly adapted to the characteristics of the compound to be trapped, the mechanisms of retention are not based on a specific molecular recognition.

Molecularly imprinted polymers (MIPs) are cross-linked polymers synthesised in presence of a template molecule and are known for their capability of selective recognition of the template and its structural analogues. MIPs can be used as sorbents for SPE, to improve the the selectivity of the method for a target analyte and its structural related compounds thus rendering their analysis in real samples more reliable [9-11]. Potentially, MIPs could thus be of special interest for the radiosynthesis and evaluation of PET tracers in biological fluids. Especially, during the step of synthesis, a MIP could be useful to selectively extract a labelled compound from a mixture with the non-radioactive precursor and impurities, radioactive or not, mostly generated during the labelling step. On the other hand, MIPs could serve as selective sorbents for analytical methods used for radiopharmaceutical release activities. Finally, the capability of these selective sorbents for the extraction of a target molecule from biological fluids as plasma has a potential interest for the quantification and the biochemical validation of the tracer (determination of the percentages of metabolites and correction of the input function, for example).

Zheng et al. have developed and validated a specific, selective, and reliable LC-ESI-MS/MS analytical method to measure MEFWAY and *p*-MPPF concentrations in brain homogenates and plasma [12]. The method is descripted as simple, rapid and the LOQ (limit of quantification) is 1 ng mL^{-1}.

However the specific activity of *p*-[^{18}F]MPPF at injection time can range from around 40-150 GBq μmol^{-1} and the intravenous bolus injection in healthy human volunteers range usually from 150 to 370 MBq [1,13,14]. In these conditions, the theoretical concentration in plasma of *p*-[^{18}F]MPPF at injection time can range from 0.15 to 1.5 ng mL^{-1}.

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Considering the biodistribution of the compound and its rapid metabolism, the real concentration of *p*-[^{18}F]MPPF in plasma is even smaller[1,12-15]. For this kind of study in human volunteers other analytical methods must be used to determine *p*-[^{18}F]MPPF and its metabolite such as SPE-LC method with radioactivity detection [2,16]. Furthermore, in order to reduce analysis time of radiopharmaceuticals, especially for carbon-11 and fluorine-18, radio-high performance liquid chromatography (radio-HPLC) is the analytical method of choice [17,18].

From our knowledge, no application of MIPs against radiopharmaceutical compounds for synthesis or analysis in the PET field has been described. During the present study, the synthesis of a highly selective molecularly imprinted polymer obtained against the *p*-MPPF and *p*-DMPPF is described. This study was mainly focused on the development of a SPE-LC method with non-radioactive analytes as an analytical tool in order to evaluate for the first time the performances of new molecularly imprinted solid phase extraction (MISPE) in the context of radiopharmaceutical compounds in human plasma. For this study, the compound *p*-[^{18}F]MPPF and its potential metabolite the *p*-[^{18}F]DMPPF were selected. Therefore, the retention study in the porogen was made for the first time with a radioactive compound, the *p*-[^{18}F]MPPF, in order to proved the selectivity of the MIP, which is one of the targeted parameters selected to demonstrate the performance of the developed method. During the development phase of the SPE-LC method, non-radioactive substances were then used because it is less constraining and safer. The development of this SPE-LC method was followed by a first evaluation of its quantitative measurement capability by means of a pragmatic pre-validation step.

2. Experimental

2.1. CHEMICALS AND REAGENTS

All chemicals and solvents used were of analytical or HPLC grade. Acetonitrile (ACN), methanol (MeOH), dichloromethane (DCM), ethanol (EtOH), hydrochloric acid fuming 37 %, sodium dihydrogen phosphate dihydrate, di-sodium hydrogen phosphate heptahydrate, sodium chloride, sodium hydroxide, ammonium hydroxide 25 % (ammonia), acetic acid, formic acid, ortho-phosphoric acid (85 % w/w) were purchased from VWR International (Leuven, Belgium). Ultrapure water was generated from Milli-Q system (Merck Millipore, Billerica, MA, USA). Human plasma samples were obtained from the Blood Transfusion Centre of Liège (Liège, Belgium).

Ethylene glycol dimethacrylate (EGDMA) and 2-methacrylic acid (MAA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,2'-Azobis(2-methylpropionitrile) (AIBN) was purchased from Acros Organics (Geel, Belgium). The reagents were used without purification with some exceptions. MAA was distilled under reduced pressure. EGDMA was consecutively washed with 10 % sodium hydroxide (NaOH) in water, water and brine. AIBN was recrystallised from MeOH. The synthesis of

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radioactive *p*-[^{18}F]MPPF, for the retention study in porogen, was achieved following described procedures by Le Bars [19]. The synthesis of both non-radioactive compounds, *p*-MPPF and *p*-DMPPF, were achieved following described procedures by Le Bars [19] and Defraiteur [20], respectively. All these compounds were synthesized by the Cyclotron Research Center of the University of Liège (Liège, Belgium). Stock solutions of *p*-MPPF and *p*-DMPPF were prepared in MeOH at a concentration of 1.0 mg mL^{-1} and were stored at 4°C.

2.2. APPARATUS AND CHROMATOGRAPHIC CONDITIONS

Two LC systems were used. The first one consisted in a Waters 717 autosampler (Waters, Milford, MA, USA), a Croco-cil column oven (Interchim, Montluçon, France), a Varian 9012 solvent delivery pump and a Varian 9065 Polychrom diode array detector (Agilent Technologies, Santa Clara, CA, USA). The second one used for analysing plasma samples and for the pre-validation, was a Waters 2695 Separations Module with Waters 2998 Photodiode Array detector. The system was controlled by Waters Empower™2 software. Chromatographic separation was carried out on a Waters XBridge Shield RP18 analytical column (150 × 4.6 mm i.d., 3.5 µm) with a guard column (20 × 4.6 mm i.d., 3.5 µm) packed with the same material. The isocratic separation was performed at a temperature of 35° C using a mobile phase consisting of a mixture of ACN-MeOH-ammonium acetate (pH 5.0; 50 mM) (25/25/50, v/v/v). The buffer was prepared by introducing 50 mmol of acetic acid in 1.0 L water and adjusted with ammonium hydroxide (25 %) to pH 5.0. The flow rate was set to 0.7 mL min^{-1}. The sample injection volume was set at 100 µL. UV detection was operated at 210 nm and the total run time was 15 min.

During the MIP and NIP synthesis the polymerization was initiated by means of UV light emitted by a UVP SpotLite™ model SCL2-2 (Upland, CA, USA).

The empty polypropylene cartridges and frits for the MIP packaging were obtained from Biotage AB (Uppsala, Sweden).

The Sep-Pak C18 solid-phase extraction cartridges (1 g, 6 mL) were purchased from Waters.

During the validation step, plasma sample preparation was performed by means of a robotic Multiple Probe SPE 215 System from Gilson International B.V. (Den Haag, The Netherlands), controlled by Trilution™ LH software.

2.3. SYNTHESIS OF THE MIP

The polymerisation mixtures were prepared following the molar proportions 1/60/12 (template, crosslinker, and monomer). The volume of porogen was calculated to be sixty percents of the total volume of the liquid components (EGDMA, MAA and porogen). The same mixtures were used for the polymerisation of the non-imprinted polymers (NIP) without adding the template. For the MIP, *p*-MPPF were used as the template.

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In a glass tube (25 × 150 mm), the template (0.6 g, 1.38 mmol) was dissolved in a fraction of the porogen (4 mL) then mixed with EGDMA (5.47 g, 27.6 mmol) and MAA (0.48 g, 5.5 mmol) both in a mixture with porogen (4.5 mL). Finally, AIBN (54.4 mg, 0.33 mmol) was added and dissolved. The mixture was cooled at 0°C and flushed for 10 min with nitrogen in order to remove oxygen. Cooled mixture (0°C) was exposed for 22 h to an UV light at 365 nm in order to initiate the polymerization. Irradiation was conducted simultaneously for both MIP and NIP solutions. The resulting solid was manually grounded and extracted for 24 h with 250 mL of a mixture of MeOH and acetic acid (90:10, v/v) using a Soxhlet extractor. The extracted solid was left to dry for 48 h. After drying, the solid was again grounded with small volumes of MeOH and mechanically sieved with decreasing granulometries (100 µm, 63 µm, 50 µm, 36 µm, 25 µm). The 50-63 µm and the 25-36 µm fractions were kept and slurried with a mixture of MeOH and water (80:20, v/v). After 5 sedimentation steps using 20 mL of this mixture, the liquid became clear within 20 min. The resulting solid was left to dry slowly at room temperature.

The chemical and morphological characterization of this MIP was not envisaged in the first step of this project focused on the evaluation of the performance of the method. Similarly, the synthesis has also not yet repeated.

2.4. RETENTION STUDY IN POROGEN

Empty cartridges were packed with 100 mg of MIP or NIP, each in triplicate. Before use the cartridges were conditioned using the following sequence: 20 mL of ethanol, 40 mL of water, 40 mL of 1 M hydrochloric acid in water and 40 mL of water. After radio-synthesis following the procedure of Le Bars et al. [19], about 70 MBq of *p*-[^{18}F]MPPF were obtained in 2 mL of DCM and further diluted to 12 mL with the same solvent. 2mL (7-10 MBq) of this solution were passed through each MIP or NIP cartridge with a flow of 0.5 mL min^{-1} using a syringe pump. With a specific activity around 40 Gb µmol^{-1}, this amount of radioactivity is supported by about 2.10^{-4} µmol (~0.1 µg) of *p*-MPPF. The cartridges were then successively rinsed with 2 mL of DCM, 2 mL of a mixture of DCM-MeOH (99:1, v/v), 2 mL MeOH, 2 mL MeOH-acetic acid (99:1, v/v) and 2 mL MeOH-acetic acid (95:5, v/v). All liquids were recovered in the same vial for each cartridge. A volume of 10 mL of air was used to purge the cartridges after loading and after each rinsing.

2.5. RETENTION STUDY IN AQUEOUS MEDIA

For the study in aqueous media, different mixtures of phosphate buffers 50 mM ranging from pH 5-8 and ACN (50:50, v / v) were tested during the second conditioning step and loading step. The phosphate buffers at 50 mM were prepared while mixing stock solutions of sodium dihydrogen phosphate and di-sodium hydrogen phosphate. The pH value was adjusted with NaOH at 100 mM. Moreover, a constant sodium concentration level at 140 mM was maintained by addition of NaCl in each tested buffer [21].

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Stock solution of *p*-MPPF in MeOH was diluted in a mixture of phosphate buffer and ACN in order to obtain a sample at a final concentration of 50 ng mL^{-1}. Different liquids were tested for the conditioning step and the washing step in order to obtain the highest selective retention. MeOH and ACN were tested for the first conditioning step. Mixture of ACN-water (50:50, v/v) and ACN were tested for the first and the second washing steps respectively. Finally, in order to complete this extraction procedure with the highest recovery various mixtures were also tested for the elution step such as DCM-MeOH-acetic acid (90:9:1, v / v / v), MeOH-ammonia (95:5, v / v), MeOH-water-ammonia (80:15:5, v/v/v) and ACN-MeOH-ammonium acetate (pH 5.0; 50 mM) (25:25:50, v/v/v).

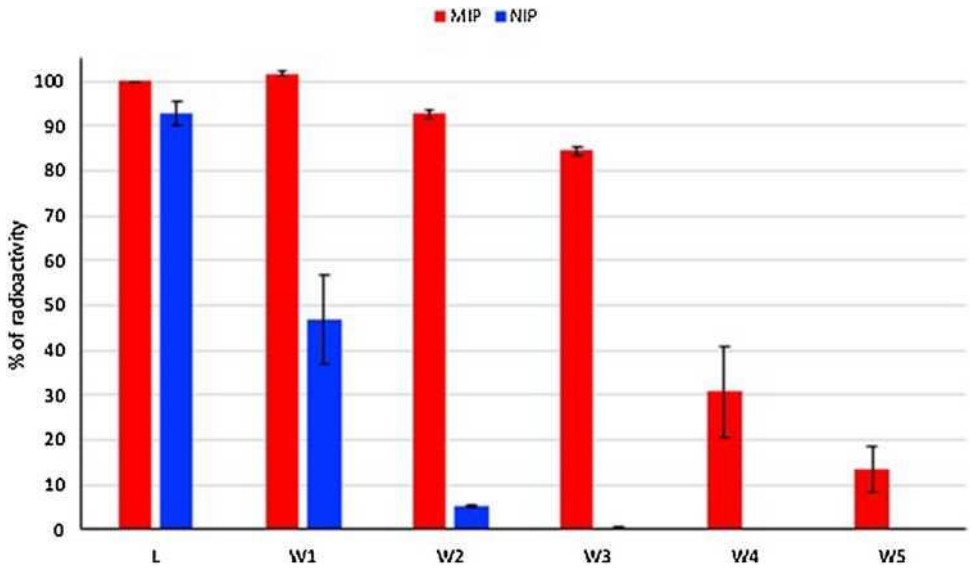
The percolated volume of all liquids was 1 mL excepted for the first washing step where the percolated volume was 750 µL.

Each fraction from the loading step, the washing steps and the elution steps except the elution with mobile phase was evaporated to dryness under nitrogen stream and reconstituted in 500 µL of mobile phase.

2.6. EXTRACTION OF *P*-MPPF AND *P*-DMPPF FROM PLASMA SAMPLE

Considering the extraction of *p*-MPPF and *p*-DMPPF from plasma, we have reduced the quantity of polymer packed in each cartridge to 25 mg.

Fig. 2. *Means and standard deviations (n = 3) for the radioactivity measured once on 3 independent cartridges expressed as the percentage of the total radioactivity assayed after each purge (sum of the decay corrected activities on the cartridge and in the solution). L: Loading of p-[^{18}F]MPPF in DCM; W1: washing with DCM; W2: washing with DCM-MeOH (99:1; v / v); W3: washing with MeOH; W4: washing with MeOH-Acetic acid (99:1; v / v); W5: washing MeOH-Acetic acid (95:5; v / v).*



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Consequently, in order to evaluate the selectivity of the method, simultaneous extractions on cartridges packed with MIP and with NIP, at a concentration of 150 ng mL^{-1}, were conducted. Plasma proteins precipitation was performed on 1 mL of plasma sample mixed with 1 mL of an ortho-phosphoric acid solution at 0.17 % (v / v) in ACN [22,23]. After a brief stirring with a vortex mixer, the samples were left standing in the dark for 15 min prior to centrifugation at 4200 x g (5000 rpm) for 15 min. 1 mL of the supernatant was collected for sample handling by means of the robotic SPE 215 System. The supernatant was then loaded onto extraction cartridges. The cartridges were previously conditioned twice with 1 mL of ACN. 750 µL of a mixture of ACN-water (50:50, v / v) and 1 mL of ACN were then applied onto the cartridges in order to perform the washing step. The elution step was then performed using 500 µL of a mixture of MeOH-ammonia (95:5, v / v). All these operations were performed automatically. Each fraction was evaporated to dryness under nitrogen stream and reconstituted in 500 µL of mobile phase. The resulting extracts were transferred manually to the LC system.

2.7. PRE-VALIDATION AND COMPUTATION

Previously to a formal validation [24-26], a simplified and pragmatic pre-validation was conducted in this study with the total error approach allowing to increase the confidence of the predictive aspect provided by this pre-validation [27,28].

The e.noval software v3.0 (Arlenda S.A., Liège, Belgium) was used to compute the pre-validation results as well as to obtain all the accuracy profiles.

3. Results and discussion

3.1. EVALUATION OF RETENTION IN POROGEN

As a proof of concept for the potential use of MIP for the selective extraction of a radiopharmaceutical compound, the retention in porogen was evaluated by means of [^{18}F] labelled compound. The retention study in porogen was therefore made with the radioactive *p*-[^{18}F]MPPF. DCM used as porogen has a non-protic character and a low polarity. These properties promote the creation of specific interactions between template and methacrylic chains/residues such as hydrogen bonds during the polymerisation.

A solution of *p*-[^{18}F]MPPF in porogen was percolated through cartridges of MIP and cartridges of NIP. The cartridges were then percolated with different washing solutions containing increasing amount of methanol to improve their elution strength. After each purge, the cartridges and the percolated solutions were assayed for radioactivity using an ionisation chamber.

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The values of activity were corrected for decay. Fig. 2 illustrates the measured results after correction for each step.

As shown in Fig. 2, following loading of *p*-[^{18}F]MPPF in DCM (L) and first washing step with DCM (W1), no decrease of radioactivity was measured on the MIP cartridges (< 0.05 MBq). Same experiments were conducted on the NIP cartridges showing that 7-8 % were not trapped during the loading step (L) and that about 45 % were lost during the first washing step with the porogen (W1). When adding 1 % of MeOH in DCM as washing solution, less than 10 % were lost from the MIP cartridges but about additional 35 % were lost from the NIP cartridges (W2). The different of comportment shows that, at these low amount levels of loaded analyte, the retention on the MIP in the presence of the porogen processes by a mechanism of interaction with specific sites that are not present in the NIP. The use of DCM promotes the creation of specific interactions such as hydrogen bonds. This evaluation of the retention in the porogen demonstrates this specific interaction between the *p*-[^{18}F]MPPF and the specific sites of the MIP. Addition of MeOH in the DCM washing solution leads to disruption of hydrogen bonds between MIP and *p*-MPPF due to the protic character and the polarity of MeOH resulting to the elution of the analyte. The third washing step envisaged was made with MeOH and showed that less than 10 % were lost from the MIP while *p*-[^{18}F]MPPF was totally removed from the NIP (W3).

Addition of acetic acid to MeOH washing solution W4 and W5, respectively 1 % and 5 %, results on a loss of about 70 %. Acid acetic disrupts hydrogen bonds between MIP and *p*-[^{18}F]MPPF and ionises the analyte leading to its elution.

3.2. EVALUATION OF RETENTION IN AQUEOUS MEDIA

The study of the retention mechanism in aqueous media is a very important preliminary step to investigate the retention in biofluids and more particularly in plasma sample for this MIP. The conditions of extraction were evaluated in order to obtain the highest selective retention of the *p*-[^{18}F]MPPF and its metabolites on the MIP. ACN was selected for this step due to its non-protic character and its low polarity. These properties seem to promote the creation of interactions between the target and the MIP during the loading step.

Different mixtures of phosphate buffers 50 mM ranging from pH 5-8 and ACN (50:50, v / v) were tested during the loading step. The MIP selectivity was also exploited in the loading and washing steps by adjusting the pH of aqueous samples at a suitable value and by selecting liquids for these steps that promote the transformation of non-specific interactions into more specific interactions. The most appropriate pH for the retention of the analyte to be applied during the loading step was investigated in order to promote the selectivity of the MIP.

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Table 1. *Final extractions conditions.*

Step	Liquid	Volume (mL)	Air push Volume (mL)
Conditioning	ACN	2.0	1.0
*Loading (L)	Phosphate buffer pH 5.5 - ACN (50:50, v / v)	1.0	1.0
*Washing	(W1) ACN - Water (50:50, v / v)	0.75	1.0
	(W2) ACN	1.0	1.5
*Elution (E)	MeOH - Ammonia (95:5, v / v)	1.0	2.0

Fig. 3*. Chromatograms of (a) a standard at 50 ng mL^{-1} of p-MPPF and p-DMPPF, (b) a spiked plasma at 50 ng mL^{-1} of both compounds loaded onto a MIP, (c) a spiked plasma at 50 ng mL^{-1} of both compounds loaded onto a NIP and (d) a blank plasma loaded onto a MIP. Peak identification: (1) p-MPPF, (2) p-DMPPF, (3) endogenous compound.*

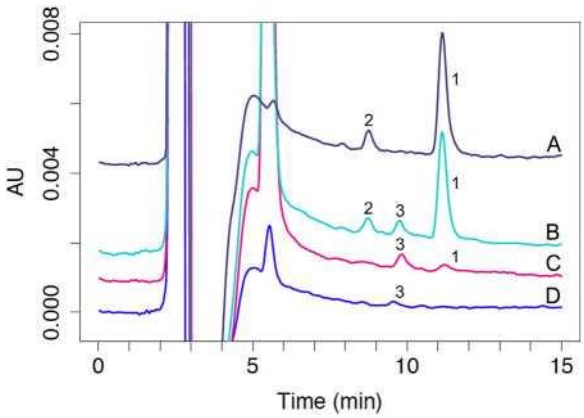
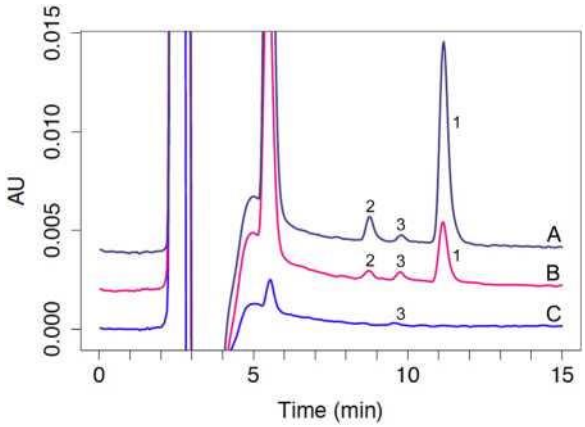


Fig. 4. *Chromatograms of (a) a spiked plasma at 150 ng mL^{-1}, (b) a spiked plasma at 50 ng mL^{-1} of p-MPPF and p-DMPPF, (c) a blank plasma. Peak identification: (1) p-MPPF, (2) p-DMPPF, (3) endogenous compound.*



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Different mixtures of ACN and phosphate buffers 50 mM ranging from pH 5-8 were tested. The goal of these tests is to modify the ionisations of the analyte and the methacrylic chains in order to promote the development of interactions such as electrostatic interactions [21]. A constant sodium concentration level was kept at 140 mM in each phosphate buffer. The addition of sodium was made to obtain a similar level than that in human plasma allowing the evaluation of the potential effect of this cation on the interactions between the MIP and the analytes. Use of ACN in the loading solution is necessary to mimic the plasma sample as ACN is considered for the plasma protein's precipitation. The washing step was made with a mixture of water-ACN (50:50, v / v) and with ACN in orderto conserve the specific interactions. The elution was carried out with a mixture of MeOH-ammonia (95:5, v / v). This protic solvent combined with a base disrupts hydrogen bonds between MIP and *p*-MPPF promoting the elution.

Finally, in order to complete this extraction procedure with the highest recovery various mixtures were also tested for the elution step such as DCM-MeOH-acetic acid (90:9:1, v / v / v), MeOH-ammonia (95:5, v / v), MeOH-water-ammonia (80:15:5, v / v / v) and ACN-MeOH-ammonium acetate (pH 5.0; 50 mM) (25:25:50, v / v / v). The final extraction conditions were shown in Table 1.

By using the SPE conditions presented in Table 1, recoveries of the MIP and the NIP were 99 % and 0 %, respectively. The difference in analyte recovery between the MIP and the NIP demonstrates the high selectivity of the developed procedure.

3.3. EXTRACTION OF *P*-MPPF AND *P*-DMPPF FROM PLASMA SAMPLE

The selected conditions in pure aqueous media were extrapolated to the extraction of *p*-MPPF and *p*-DMPPF from human plasma. For this, the extraction procedure was the same as the one described in Table 1. The plasma sample spiked with analyte was diluted with an ortho-phosphoric acid solution at 0.17 % (v / v) in ACN. This treatment of plasma sample is necessary in order to precipitate the proteins and to adjust the apparent pH of samples. The apparent pH must be the same as measured for ACN-phosphate buffer (pH 5.5; 50 mM) (50/50; v / v) mixture used during the evaluation of retention mechanism in aqueous media.

***Table 2**. Extraction rate obtained for three different concentrations of p-MPPF and p-DMPPF ranging from 50 to 150 ng mL^{-1} considering the optimal extraction conditions.*

Concentration (ng mL^{-1})	Numberof replicates (n)	*p*-MPPF Extraction rate ± SD (%)	*p*-DMPPF Extractionrate ± SD (%)
50	4	93.7 ± 3.1	71.6 ± 4.3
100	4	105.7 ± 5.6	65.9 ± 2.6
150	4	100.8 ± 0.9	112.1 ± 5.2

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Fig. 5. *Pre-validation study - Accuracy profiles for the quantification of (a) p-MPPF in plasma using linear regression model and (b) p-DMPPF in plasma using linear regression model through 0 fitted with the highest level only. Continuous line: Relative bias; Dotted lines: ±30 % acceptance limits; Dashed lines: 95 % β-expectation tolerance limits; Dots: relative back-calculated concentrations of the validation standards.*

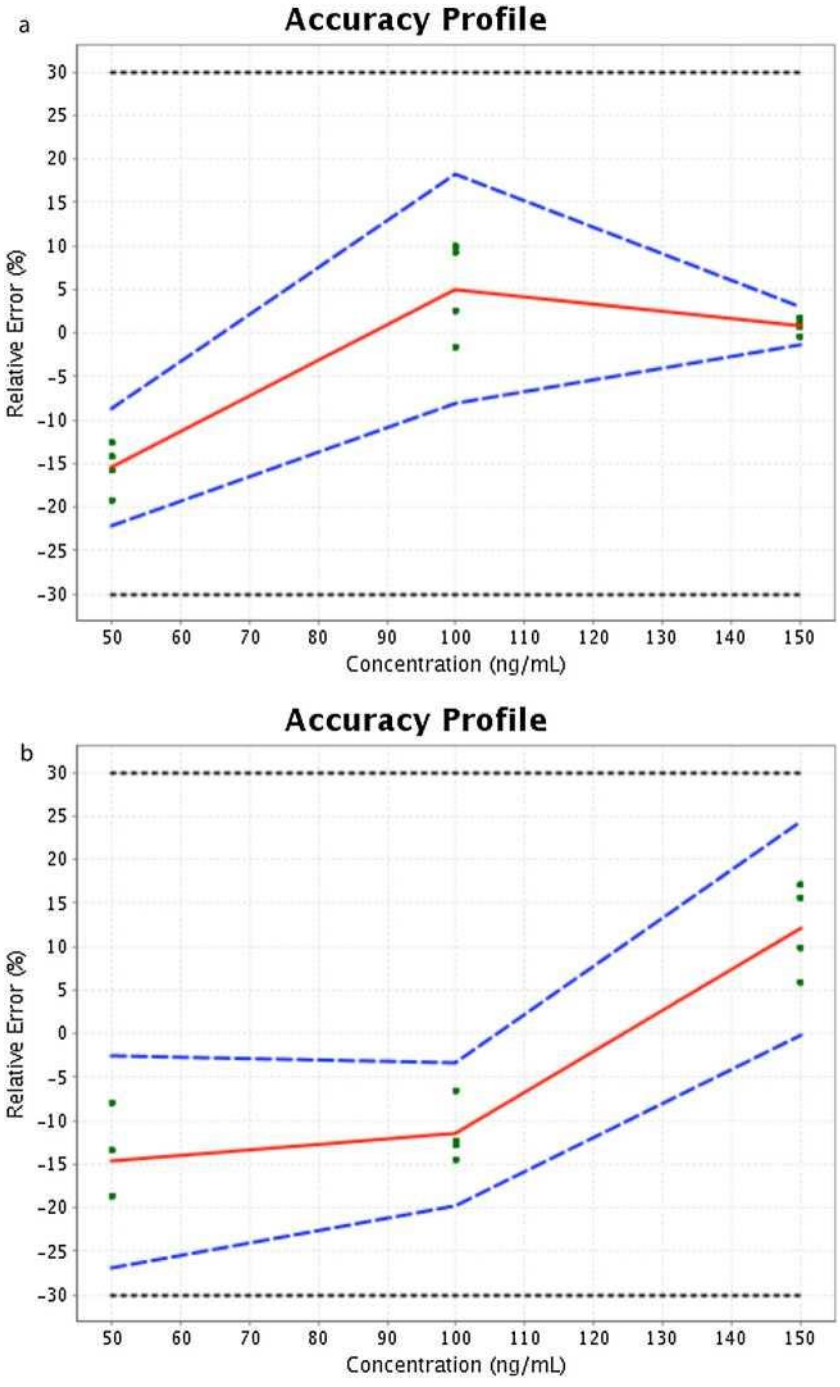

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Fig. 3 shows that the separation between *p*-MPPF and *p*-DMPPF is efficient. A peak of an endogenous compound can be observed between the peaks of the two analytes but this endogenous compound does not interfere with the quantification. These chromatograms suggest that the performances of this new MISPE is acceptable for the extraction of *p*-MPPF and *p*-DMPPF from human plasma.

Finally, a pre-validation of the methods was carried out in order to confirm the efficiency of this extraction procedure with plasma sample.

3.4. PRE-VALIDATION

The first step of the evaluation of the quantitative performances of a bioanalytical method is the pre-validation. This essential step of the analytical method lifecycle allows to ensure that the analytical procedure is fit for its intended purpose [24-26]. According to the objectives a simplified and pragmatic approach was used in this study.

This study was mainly focused on three parameters: recovery, selectivity and response function. The accuracy was also estimated to obtain some indications about the quality of the process and the performance of this analytical method. The target analytes are the *p*-MPPF and one of its metabolites, the *p*-DMPPF. The quantitative objective of this new MISPE-LC method is simultaneous determination of *p*-[^{18}F]MPPF and its principal metabolites in human plasma by using a radioactivity detector at a lower concentrations than 1 ng mL^{-1}. As a consequence, during this pre-validation step, the lower limit of quantitation (LLOQ), the limit of detection (LOD) and the linearity of the results were not relevant parameters according to the main goal of this project.

A total error approach using the accuracy profile as a decision tool was conducted [27,28]. The accuracy profile is a predictive tool that can be used to investigate the capability of this MISPE-LC protocol to provide future results within defined acceptance limits.

In summary, this accuracy profile predicts the quantitative performances of the analytical method using tolerance intervals with a probability β (e.g. 0.95 or 95 %) to be within *a priori* set acceptance limits [29].

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Table 3. *Results of the pre-validation of the MISPE-LC-UV method.*

Response function (p = 1; n = 4)	*p*-MPPF	*p*-DMPPF
	Linear Regression (m = 3)	Linear Regression Through 0 fitted with the level 3 only (m = 3)
X	1458	125.2
Intercept	-93.4	-
r^2	0.99	ND
Trueness (p = 1; n = 4)	Relative bias (%)	Relative bias (%)
50.0 ng mL^{-1}	6.6	-14.6
100.0 ng mL^{-1}	6.6	-12.5
150.0 ng mL^{-1}	0.4	12.1
Precision (p = 1; n = 4)	Repeatability (RSD%)	Repeatability (RSD%)
50.0 ng mL^{-1}	2.5	4.8
100.0 ng mL^{-1}	4.9	3.2
150.0 ng mL^{-1}	0.8	4.8
Accuracy (p = 1; n = 4)	Relative β-Expectationtolerance limit (%)	Relative β-Expectationtolerance limit (%)
50.0 ng mL^{-1}	[-13.0; -0.2]	[-26.8; -2.5]
100.0 ng mL^{-1}	[-6.0; 19.1]	[-19.7; -3.3]
150.0 ng mL^{-1}	[-1.7; 2.5]	[-0.1; 24.4]

p : number of days (series) of analysis; n: number of repetitions per day of analysis; m: number of analytes concentration levels; ND: not determined.

3.4.1. EXTRACTION PROCESS EFFICIENCY

The recoveries of *p*-MPPF and *p*-DMPPF were determined at three different concentrations ranging from 50 to 150 ng mL^{-1} [25,26,30]. The mean recoveries are shown in Table 2. Those recoveries were calculated by comparing peak areas of analytes from freshly prepared plasma samples treated according to the described procedure with peaks areas found after the direct injection on the analytical column of standard solutions at the same concentration levels as required by regulatory guidance [25,26,30]. The recoveries demonstrate the extraction efficiency of the process [25,26,30].

3.4.2. SELECTIVITY

Calibration and validation standards of *p*-MPPF *and p*-DMPPF were prepared by spiking blank human plasma. The calibration standards are used to set up the calibration model while the validation standards are used to estimate precision, trueness and accuracy of the method. The calibration and validation standards were prepared at three concentration levels, ranging from 50 to 150 ng mL^{-1}.

Selectivity of the analytical method was assessed by analysing six independent sources of plasma [25,26]. No endogenous source of interference was observed at the retention time of *p*-MPPF and *p*-DMPPF.

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Typical chromatograms obtained with a blank plasma chromatogram, a plasma sample spiked with 50 ng mL^{-1} and a plasma sample spiked with 150 ng mL^{-1} of both compounds are presented in Fig. 4.

3.4.3. RESPONSE FUNCTIONS AND QUANTITATIVE PERFORMANCES

The response function of a LC method is an important criterion that must be considered in the pre-validation of a method since it corresponds to the assessment of the relationship between the chromatographic response and the concentration of the analyte [27]. The approach based on the β-expectation tolerance intervals for total error measurement was used to determine the most appropriate response function.

The optimal model is the one which allows an accurate quantification of analytes over the widest concentration range together with the smallest bias and variability at each concentration level investigated.

From each response function tested, the concentrations of the validation standards were back-calculated in order to determine the upper and lower β-expectation tolerance limits at β = 95 %. The acceptance limits were set at ± 30 %. Response functions that complied with the defined criteria as well as the simplest, are the linear regression model and the linear regression through 0 fitted with the highest concentration level only for *p*-MPPF and *p*-DMPPF, respectively. Fig. 5 illustrates the accuracy profiles obtained for both compounds. Pre-validation results obtained considering these response functions are presented in Table 3 for both compounds.

Considering the selected response functions, trueness [27,29] expressed in terms of relative bias (%) was assessed from the prevalidation standards at 3 concentrations levels, ranging from 50 to 150 ng mL^{-1} (Table 3). According to the regulatory requirements [29,30], trueness was found to be acceptable for both compounds. The relative bias values range from -14.63 % to 12.13 % for the *p*-DMPPF and do not exceed 7 % even at the low concentration level for the *p*-MPPF.

The precision of this bioanalytical method was then evaluated for the both compounds by computing the relative standard deviations (RSD, %) of repeatability at each concentration level of the validation standards [25-28]. The RSD values presented in Table 3 are acceptable for all concentration levels [25,26]. These RSD values did not exceed 5 % irrespectively of the concentration level for both compounds.

As shown in Table 3 and in Fig. 5, the upper and lower relative β-expectation tolerance limits (%) did not exceed 26.8 % for each concentration level for both compounds demonstrating the adequacy of the developed method for its intended purpose.

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4. Conclusions

The final aim of this project is the simultaneous determination of *p*-[^{18}F]MPPF and its principal metabolites, such as *p*-[^{18}F]DMPPF, in plasma by means of a radioactivity detector during studies with PET scan in human volunteers.

For this purpose, a MIP was synthetized in order to develop a highly specific SPE-LC method for the analysis of plasma sample. The preliminary evaluation of the selective recovery of the radiolabelled *p*-[^{18}F]MPPF shows the interaction of this analyte in porogen with specific sites in the MIP. Therefore, a SPE-LC method was developed based on these specific interactions. The analytical performances of this method were estimated by means of a total error approach using the accuracy profile according to the main goal of this project.

The results show that this SPE-LC method is valuable analytical tool in order to evaluate the performances of a new MISPE for the extraction of *p*-[^{18}F]MPPF and its potential metabolite the *p*-[^{18}F]DMPPF from human plasma. A further study could now be envisaged to characterize the MIP and to investigate the variability between different batches of this MIP in order to complete the development of a new SPE-LC method coupled with a radioactivity detector.

DECLARATION OF COMPETING INTEREST

Not applicable

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