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Three optimized and validated (using accuracy profiles) LC methods for the determination of pentamidine and new analogs in rat plasma

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ABSTRACT

Three novel LC-UV methods for the determination of pentamidine (PTMD) and two of its new analogs in rat plasma are described. The chromatographic conditions (wavelength, acetonitrile percentage in the mobile phase, internal standard) were optimized to have an efficient selectivity. A pre-step of extraction was simultaneously developed for each compound. For PTMD, a solid phase extraction (SPE) with Oasis® HLB cartridges was selected, while for the analogs we used protein precipitation with acetonitrile. SPE for PTMD gave excellent results in terms of extraction yield (99.7 ± 2.8) whereas the recoveries for the analogs were not so high but were reproducible as well (64.6 ± 2.6 and 36.8 ± 1.6 for analog 1 and 2, respectively).

By means of a recent strategy based on accuracy profiles (β-expectation tolerance interval), the methods were successfully validated. β was fixed at 95% and the acceptability limits at ±15% as recommended by the FDA. The method was successfully validated for PTMD (29.6–586.54 ng/mL), analog 1 (74.23–742.3 ng/mL) and analog 2 (178.12–890.6 ng/mL). The first concentration level tested was considered as the LLOQ (lower limit of quantification) for PTMD and analog 1 whereas for analog 2, the LLOQ was not the first level tested and was raised to 178.12 ng/mL.

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1. Introduction

Pentamidine (PTMD) isethionate, an aromatic diamidine derivative, is an antiprotozoal agent used in the treatment of African trypanosomiasis (also known as sleeping sickness) and leishmaniosis [1]. This drug was released for clinical use in the eighties in therapy for *Pneumocystis Jirovicii* Pneumonia, an opportunistic infection that occurs in individuals with weakened immunity, particularly in the case of the Acquired Immune Deficiency Syndrome (AIDS) [1,2]. More recently, PTMD has been described as a potential active agent in myotonic dystrophy, a dominant genetic disorder which is the most common form of muscular dystrophy in adults [3]. Unfortunately, this therapeutic agent presents a well known toxicity. It has a variety of potential adverse reactions. Immediate reactions (hypoglycemia, nausea, and tachycardia), local reactions (pain, abscess or necrosis at the injection site) and systemic reactions (nephrotoxicity, leucopenia, abnormalities in glucose metabolism) have been described [1,2].

Several laboratories are trying to synthesize new analogs with less side effects than PTMD without reduction of efficacy [4–6]. *Piperazine-1,4-bisbenzamidines* derivatives seem to pave the way of a new trend in the research of new active drug candidates [7–9]. The development of new drugs involves pharmacokinetic studies and clinical evaluation. This implicates the need of an efficient and sensitive analytical method to quantify the new compounds and the reference drug (PTMD) in biological fluids. These bio-fluids (e.g. blood and urine) are relatively complex and a pre-step of extraction is an important and critical point.

In this paper, we propose to develop quick and sensitive analytical methods for PTMD and two of its new bisbenzamidines analogs. One of them has already shown noticeable results in antioxidant and neuroprotective activity [10]. *In vitro* and *in vivo* activity against *P. carinii*, as well as pharmacokinetic of these compounds has already been assessed and will be the topic of a forthcoming paper. Several papers from the literature deal with the determination of PTMD. Usually, this latter is analyzed by means of a high per-

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formance liquid chromatography (HPLC) apparatus coupled with ultraviolet (UV) or fluorescence detection [11–15]. Ion pairing HPLC is often cited method [11,13,15], but is known for its difficulty of use in routine due to the long time of column equilibration. Among other types of described techniques, Valnice et al. depicted electrochemical techniques using hanging mercury drop electrode [16,17], but showing a high detection limit for PTMD. Micellar electrokinetic chromatography (MEKC) or capillary electrophoresis (CE) have also been proposed but carry the same lack of sensitivity problem [18,19]. Concerning the pentamidine extraction in serum, several possibilities are retrieved in the literature: extraction by organic solvents [13], protein precipitation by addition of acetonitrile or copper sulfate [14,19] or solid phase extraction (SPE) with C-8 or C-18 cartridges and ion pairing eluant [11,19]; with variable results of success. Rabanal et al. have more recently proposed a new type of SPE extraction with Oasis® HLB (Hydrophilic Lipophilic Balance) cartridges. They obtained promising results and very high percentage of recovery although they worked on a large volume sample and at relatively high concentration [20].

Among many different analytical methods proposed for PTMD, only few are correctly validated. This might be the reason for disparity in the results shown in the bibliography. Nowadays, validation takes a more important place in the development of new analytical methods. Being part of the "lifecycle of a method", it has to take place before routine implementation [21]. The aim of the validation is to ensure that every future measurement in routine analysis will be close enough to the unknown true value of the analyte in the sample. In other terms, validation is required to "confirm the fitness for purpose of a particular analytical method" [21–24].

The problem in validation comes from the lack of consensus on terminology in different official documents such as the Food and Drug Administration (FDA) guide on validation of bioanalytical methods, ICHQ2R1, ISO or IUPAC [25–27]. Moreover, this official documents describe the criteria of validation to be tested, but do not suggest any experimental protocol [22]. In this context, the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) commissions started, in 2003, to elaborate validation guidelines to help scientists to apply harmonized regulatory recommendations and to validate their analytical and bio-pharmaceutical procedures [22–24]. Their suggestion is to use a novel validation strategy based on the total error (bias + standard deviation) and accuracy profiles. This holistic approach to validation establishes the expected proportion of acceptable results lying between acceptability limits (definitions given in Section 2.6.1). Currently, this new protocol of validation becomes more attractive and knows a wider spreading among the scientific community [28,29].

A procedure can be qualified as acceptable if the difference between every measurement (x) of a sample and its "true value" (μ_T) is inside the acceptance limits λ (predefined by the analyst depending to the objective of the method). The probability that the results will be in these acceptance limits should be superior or equal to a probability β. It can be translated into Eq. (1) [22–24].

$$P(|x - \mu_t| < \lambda) \geq \beta \quad (1)$$

The goal of this work is to optimize an accurate analysis of PTMD and two of its new analogs in rat plasma, by means of rapid and simple LC methods with UV detection. A pretreatment step of plasma samples was optimized for each compound. For PTMD solid phase extraction with Oasis® HLB cartridges was used while a protein precipitation with acetonitrile was performed for the analogs.

The three analytical methods developed were validated using the accuracy profiles concept and for each compound an example of pharmacokinetic determination after a subcutaneous injection is shown.

2. Experimental

2.1. Chemicals

Pentamidine {4,4′-(pentane-1,5-diylbis(oxy)bisbenzenecarboximidamide bis(2 hydroxyethanesulfonate)}, PTMD (Fig. 1a) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Analog 1 {4,4′-[1,2-ethanediyldiimino)]bisbenzenecarboximidamide dihydrochloride salt} (Fig. 1b); analog 2 {4-[(2-*N*-pheny-

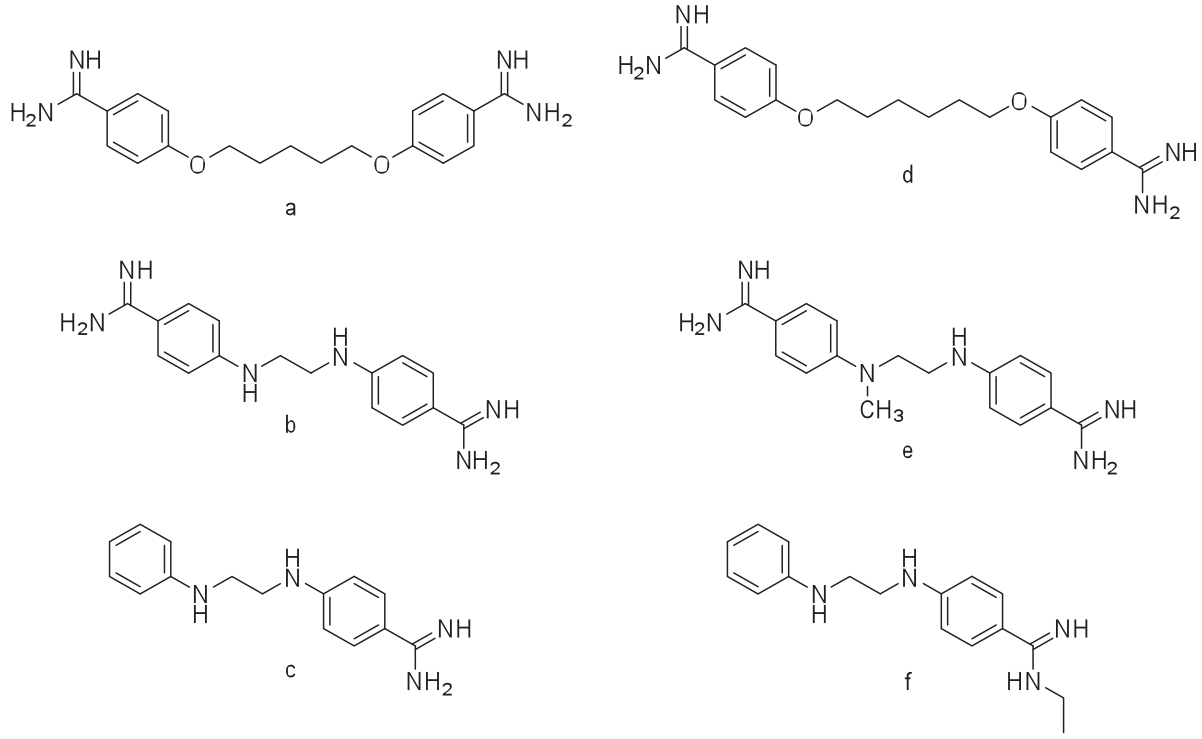

Fig. 1. Chemical structures of (a) pentamidine (PTMD), (b) analog 1 (1), (c) analog 2 (2), (d) hexamidine (HXMD), (e) analog 3 (3), and (f) analog 4 (4).

lamino)ethylamino]benzenecarboximidamide sulfate salt} (Fig. 1c); analog 3 {4,4′-((*N*-methyl-1,2-ethanediyldiimino) bisbenzenecarboximidamide) dihydrochloride salt} (Fig. 1e) and analog 4 {*N*-ethyl 4-[2-(*N*-phenylamino)ethylamino] benzenecarboximidamide hydrochloride salt} (Fig. 1f) were synthesized in the Laboratory of Organic Chemistry, Faculty of Sciences, University of Mons (Belgium). Analogs 3 and 4 were respectively used as internal standard (IS) for analogs 1 and 2.

Elemental analyses were performed for all synthesized compounds at the Centre Wallon de Recherches Agronomiques (Libramont-Chevigny, Belgium) or at the Laboratoire de Microanalyse Organique of the Institut des Sciences Appliqués de Rouen (France) and confirmed the purity of the synthesized compounds.

Hexamidine (4,4′-(hexamethylenedioxy)dibenzamidine bis(2-hydroxyéthanesulfonate), HXMD, Fig. 1d), was used as IS for PTMD, from a commercial solution named Hexomedine® (Melisana, Switzerland). Blank chromatograms of the hexamidine solution were performed in order to verify the absence of interferences due to excipients.

Potassium dihydrogen phosphate, phosphoric acid, sodium hydroxide, methanol (HPLC grade) and acetonitrile (HPLC grade) came from ChemLab (Zedelgem, Belgium). Dimethylsulfoxide (DMSO) was from Baker (Mallinckrodt Baker, Deventer, Netherlands). Prior to use, mobile phase was degassed for 15 min by sonication (Branson 310 ultrasonic bath (Danbury, USA)). Ultrapure water (18.2 MΩ cm) was obtained with a Reference A+ Milli Q water purification system (Millipore, Brussels, Belgium).

2.2. Apparatus and chromatographic conditions

For all experiments a Lachrom D-7000 HPLC system was used, piloted by the program D-7000 HPLC System Manager (Merck-Hitachi, Darmstadt, Germany). The analyses were carried out on a Waters Atlantis reversed phase C18 column (100 × 4.6 mm, 3 μm particle size) (Milford, MA, USA) at room temperature. The mobile phase was composed of phosphate buffer 0.025 M (pH adjusted to 3.2 with phosphoric acid on a Metrohm 827 pH meter (Antwerp, Belgium)) and adequate percentage of acetonitrile depending on the compound to separate. The solvent flow-rate was maintained at 1 mL/min. The UV detector wavelength was set at 270 nm for the PTMD and at 320 nm for the two analogs. The injection volume was 20 μL.

SPE was carried out using Oasis® HLB (1 mL, WAT 094225, Waters) cartridges by means of a manifold apparatus (Waters). The dryness was achieved by use of a VLM evaporator (Bielefeld, Germany) and nitrogen of 99.99% purity (Air Liquide, Belgium).

2.3. Stock and standard solutions

Stock solutions of PTMD, analogs 1 and 2 as well as IS (compounds 3 and 4) were prepared by dissolving suitable amounts of each pure substance to have a final concentration of 1 mg/mL. For compounds 1, 2, 3 and 4 a small quantity of dimethylsulfoxide (DMSO) was added to enhance the solubility of the products (the maximum percentage of DMSO in stock solutions was 7% (v/v)). Stock solutions were aliquoted and stored at −20 °C. HXMD was purchased in a 1 mg/mL commercial solution form.

Standard solutions (between 1 and 10 μg/mL) were prepared daily by diluting the stock solutions in ultrapure water. All the dilutions were performed in plastic centrifugation tubes in order to avoid adsorption of PTMD (or its related compounds) onto the silanols groups of the glass [11].

2.4. Rat plasma sampling

For the development and validation procedures, plasma samples were prepared from blood from healthy rats by centrifugation at 6000 × *g* during 10 min. The supernatant (plasma) was transferred and aliquoted into plastic centrifugation tubes and frozen at −20 °C until analysis.

2.5. Sample pre-treatment

2.5.1. SPE pre-treatment

The SPE pre-treatment was carried out using Oasis® HLB cartridges (1 mL), conditioned with 3 × 1 mL of methanol and equilibrated with 1 mL of ultrapure water. The sample, constituted by 100 μL of rat plasma spiked with PTMD and its corresponding IS (standard solution of HXMD) and 900 μL of ultrapure water (in order to obtain enough volume to perform SPE) was loaded onto the conditioned cartridge. 20 μL of concentrated phosphoric acid was also added to the sample to prevent loss of linked-drug due to its large interaction with proteins. After a first wash with 1 mL of methanol 5% (v/v), the analytes were eluted with 1 mL of pure methanol. The eluate was evaporated to dryness under a gentle nitrogen stream and reconstituted in 100 μL of mobile phase.

2.5.2. Acetonitrile precipitation pre-treatment

The sample, constituted by 100 μL of rat plasma successively spiked with analog 1 or 2, the adequate IS (standard solution), 20 μL of sodium hydroxide 1 M and 400 μL of acetonitrile, was vortexed and centrifuged during 3.5 min at 7200 × *g*. 450 μL of the supernatant was evaporated to dryness under a gentle nitrogen stream and reconstituted in 100 μL of mobile phase.

2.6. Method validation

2.6.1. Terminology

In order to avoid any confusion, it seems important to us to explicit the further used terms.

Trueness (ISO 5725 definition [22–24,26]) expresses the "closeness of agreement between the mean value obtained from a series of measurements and the value which is accepted either as a conventional true value or an accepted reference value". The measure of trueness, which is related to systematic error, is generally expressed in terms of recovery and of absolute or relative bias.

Precision (ICH Q2R definition [22–25]) refers to the "closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions". It measures the random error linked to an analytical procedure (expressed as standard deviation (s), variance (s^2), relative standard deviation (RSD) or coefficient of variation (cv)).

Accuracy (ICH Q2R definition [22–25]) is the "closeness agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found." It refers to a total error measurement (sum of the trueness and precision).

Calibration standards (CS) [22–24]: Samples of known concentrations which are prepared according to the protocol that will be applied in routine and which are used to draw the calibration curve.

Validation standards (VS) [22–24]: Samples in the matrix which served to validate the analytical procedure (they represent the future samples that the analytical procedure will have to quantify).

Response function [22–24]: relationship between the signal (Y) and the quantity (concentration) X.

2.6.2. Validation protocol

Calibration standards (CS) were prepared in the matrix at 5 levels of concentrations. Right amounts of standard solutions of the

Table 1
Optimized chromatographic conditions.

Compound	Wavelength (nm)	% ACN	Retention time compound (min)	Internal standard (IS)	Retention time IS (min)
Pentamidine	270	17	9	Hexamidine	21.1
Analog 1	320	8	9.5	Analog 3	21
Analog 2	320	21	9.4	Analog 4	14.6

studied analyte and its corresponding IS were spiked to 100 μL of rat plasma and the sample was treated in accordance with the adequate previously explained sample pre-treatment protocol (Sections 2.5.1 and 2.5.2). The concentration range for PTMD was from 29.33 to 586.54 ng (free drug)/mL plasma. For analog 1, the range reached from 74.23 to 742.3 ng (free drug)/mL plasma. For analog 2 the range reached from 89.06 to 890.6 ng (free drug)/mL plasma. The correct IS (Hexamidine, analogs 3 and 4) was added in an adequate quantity to obtain a final concentration about 200 ng (free drug)/mL plasma. Each CS was analyzed in triplicate on three consecutive days. For each day the CS/IS peak area ratios were plotted as a function of the corresponding analyte concentrations.

Validation standards (VS) were prepared in the matrix at 4 levels of concentrations for each compound, in the same concentration range than for CS. Right amounts of standard solutions of the considered analyte and its corresponding IS were spiked to 100 μl of rat plasma and the sample was treated in accordance with the adequate sample pre-treatment protocol set in Sections 2.5.1 and 2.5.2. Each VS was analyzed four times on three consecutive days.

2.6.3. Building accuracy profile

According to the SFSTP validation guidelines [22–24], the experimental results are analyzed as summarized below:

- Select the acceptance limits taking into account the intended use of the method.
- Identify, using CSs, the better response function and draw the calibration curve.
- Back-calculate the concentrations of the VSs using the equation of the calibration curve.
- For each concentration level *k* of VSs, compute the average concentration calculated.
- For each level *k*, compute the validation criteria (i.e. relative bias, repeatability, and intermediate precision).
- Calculate β-expectation tolerance interval for each level of VS. It defines an interval where the expected proportion of future results will fall in β.
- Draw the accuracy profile by connecting the lower and upper limits of tolerance for each concentration. The result is a graphical decision-making tool which integrates all the elements essential

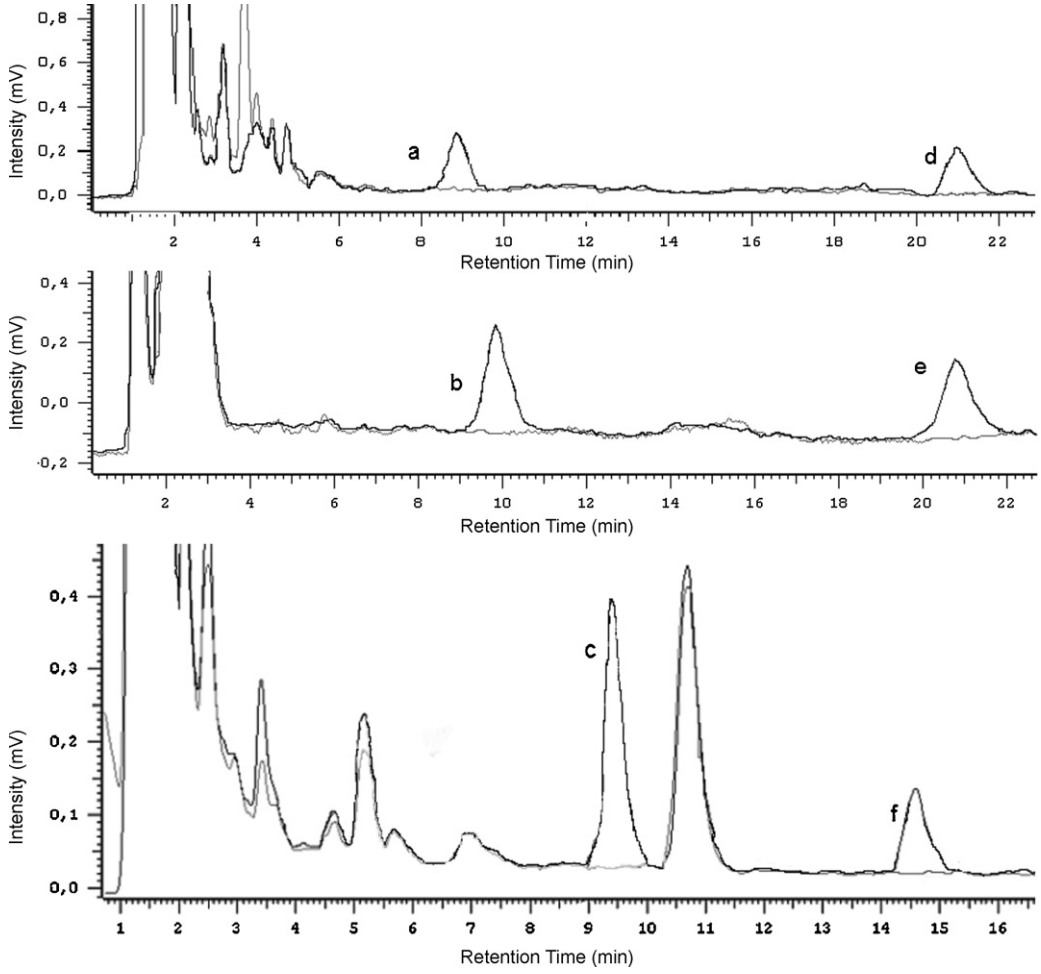

Fig. 2. Selectivity: recorded chromatograms of spiked plasmas (superposition of spiked and blank plasma) with (a) pentamidine: 29.33 ng/mL; (b) analog 1: 74.23 ng/mL; (c) analog 2: 178.12 ng/mL; (d) hexamidine: 116.8 ng/mL; (e) analog 3: 220.7 ng/mL; and (f) analog 4: 181.9 ng/mL.

for the validation, i.e. the bias, the precision, the accuracy, the risk and the quantification limits.

2.7. Practical application

In a frame of an ongoing study, the three methods were successfully applied for the determination of the pharmacokinetics of the studied products. Example is given of the behavior of each drug in the blood of one rat. Blood was drawn on the tail vein before (0 h) and after (0.5, 1, 2, 4 and 24 h) a single subcutaneous injection (20 mg/kg) on an immunodepressed *nude Lou* rat (Rat from *Laboratory of "Biology and Diversity of Eukaryotic Emerging Pathogens (BDEEP), Center for Infection and Immunity"*—Lille). The samplings were treated according to Sections 2.4 and 2.5.

3. Results and discussion

3.1. Method development

3.1.1. Chromatographic conditions – selectivity

Based on data from the literature, the mobile phase was selected as a mixture of acetonitrile (ACN)/phosphate buffer (PB) [14]. The percentage of acetonitrile increases the retention time as well as the final separation efficiency. An optimization of the ratio ACN/PB was made for each compound to obtain the best peaks shape (data not shown). The selected chromatographic conditions give the best resolution, avoiding interferences for each compound with acceptable run times (<23 min) (see Table 1). The selectivity of the methods was demonstrated after the analysis of plasma spiked with the compound of interest and blank samples from six different sources of rat plasma (Fig. 2).

3.1.2. Choice of internal standard (IS)

Adequate internal standard was added to the samples in the same amount for each method in order to limit run-to-run variation in extraction efficiency and chromatographic response. A good internal standard has to be quite similar in the chemical behavior and analytical response to the target analyte. Previous works described different IS for PTMD such as hexamidine [11,13], melphalan [12] or sulfadiazine [14]; hexamidine was selected regarding the pre-cited definition and its easy availability in a ready to use commercial solution.

For the new analogs, we met the defined conditions among structurally similar synthesized compounds from the same laboratory. The different IS for each compound are listed in Table 1.

Table 2
Extraction efficiency for PTMD, HXMD and analogs 1–4.

Analyte	Concentration (ng/mL)	Analyte recovery (%), $n = 3$
Pentamidine	29.33	99.8 ± 4.2
	293.27	96.8 ± 2.8
	586.54	102.5 ± 3.1
	Mean	99.7 ± 2.8
Analog 1	74.23	62.7 ± 2.3
	371.15	67.6 ± 2.7
	742.3	63.6 ± 4.3
	Mean	64.6 ± 2.6
Analog 2	80.06	38.3 ± 6
	222.6	35.2 ± 4.5
	890.6	37 ± 5
	Mean	36.8 ± 1.6
Hexamidine	116.8	98.0 ± 2.1
Analog 3	220.7	55.5 ± 6
Analog 4	181.9	47.8 ± 5.1

3.1.3. Extraction step

Based on positive results for PTMD using SPE HLB cartridges [20], the same protocol was performed for our applications rather than other SPE cartridges or extraction procedures mentioned in bibliography but giving less satisfying results [11–14]. No optimization of the basic protocol (Waters®) was made for the PTMD HLB extraction, except for the conditioning step. The repetition of this step seems to improve the efficiency of the SPE cartridge. SPE extraction was similarly performed for the two analogs and their IS. Due to the lack of reproducibility (data not shown), several conditions of extraction were tested: different washing solution compositions (methanol percentage 5, 10, 15% (v/v)), different eluting times as well as various volumes of the different solutions (conditioning, washing, and eluting). These modifications of the Waters® protocol did not improve the extraction yield reproducibility and therefore the procedure described at Section 2.5.2 was applied.

3.2. Recovery

Standard solutions at 3 different concentrations of PTMD, analogs 1 or 2 were added to 100 μL plasma in order to obtain final concentration for PTMD of 29.33, 293.27 and 586.54 ng/mL; 74.23, 371.15 and 742.3 ng/mL for analyte 1 and 89.05, 222.6 and 890.6 ng/mL for analyte 2.

For the ISs, only the used concentration level was tested. Extraction and runs were performed in triplicate as described before

Table 3
Results of the validation of LC-UV method for the quantification of PTMD and analogs in rat plasma.

	PTMD		Analog 1		Analog 2	
Regression model	Linear		Linear		Linear	
Trueness ($k = 4$; $n = 4$), relative bias (%)						
1	29.33 ng/mL	4.14	74.23 ng/mL	−0.36	89.06 ng/mL	11.63
2	73.32 ng/mL	2.23	148.46 ng/mL	−0.07	178.12 ng/mL	−2.18
3	293.27 /mL	2.45	371.15 ng/mL	0.02	445.3 ng/mL	−1.44
4	586.54 /mL	2.73	742.30 ng/mL	1.07	890.6 ng/mL	−1.74
Precision ($k = 4$; $n = 4$), repeatability/intermediate precision (RSD %)						
1	29.33 ng/mL	1.9/1.6	74.23 ng/mL	1.3/3.9	89.06 ng/mL	4.5/5.8
2	73.32 ng/mL	2.7/2.4	148.46 ng/mL	1.6/2.3	178.12 ng/mL	1.7/2.8
3	293.27 ng/mL	0.6/1	371.15 ng/mL	1.2/1.9	445.3 ng/mL	2.1/2.2
4	586.54 ng/mL	0.8/2.1	742.30 ng/mL	1.3/1.2	890.6 ng/mL	0.9/2.0
Accuracy ($k = 4$; $n = 4$) β-expectation (95%) lower and upper tolerance limits of the relative error (%)						
1	29.33 ng/mL	[0.6, 7.7]	74.23 ng/mL	[−10.2, 9.5]	89.06 ng/mL	[−2.4, 25.6]
2	73.32 ng/mL	[−3.0, 7.5]	148.46 ng/mL	[−5.6, 5.4]	178.12 ng/mL	[−9.0, 4.6]
3	293.27 ng/mL	[0.05, 4, 8]	371.15 ng/mL	[−4.6, 4.6]	445.3 ng/mL	[−6.6, 3.7]
4	586.54 ng/mL	[−2.5, 8.0]	742.30 ng/mL	[−1.6, 3.8]	890.6 ng/mL	[−6.6, 3.1]

k = number of SV, n = number of repetitions.

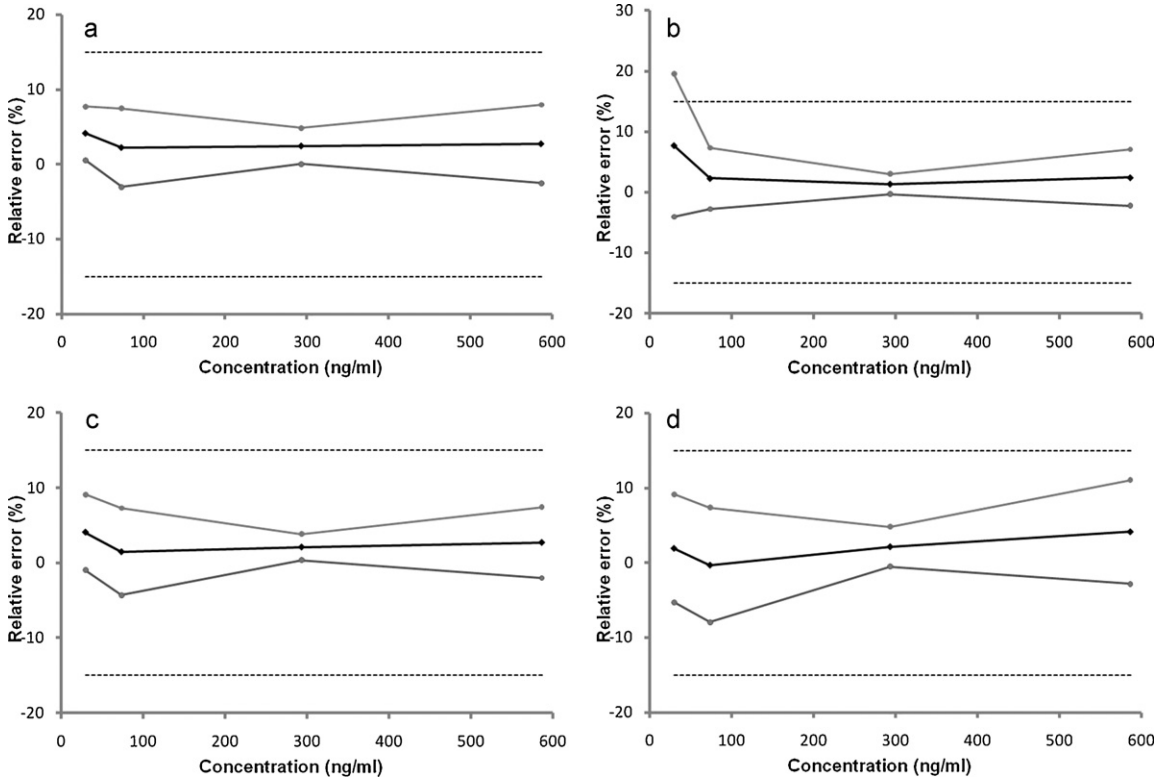

Fig. 3. Accuracy profiles for PTMD using (a) a linear model, (b) a quadratic model, (c) a weighted 1/X linear regression model and (d) a weighted $1/X^2$ linear regression model; β = 95%, λ (acceptance limits) = ± 15%.

for all the compounds. Comparison was made between the peak areas of spiked samples with those obtained with standard solutions in mobile phase at the same theoretical concentrations, and the percentage extraction yield calculated (Table 2). A very high and reproducible extraction yield for PTMD was obtained while the recoveries for the analogs were as well reproducible but not as high as for the reference drug.

3.3. Validation

The protocol described above was applied to validate our methods. The method is declared as valid if the β-expectation tolerance interval at each concentration is fully included in the acceptance limits. The probability β was settled at 95%, which means that, on average, 95% of the future results will fall in the computed tolerance intervals.

The acceptance limits were settled to ±15% according to regulatory requirements for bioanalysis [30].

We fitted four different response functions for each method. From every response function tested (linear, quadratic, weighted linear $1/X$ and $1/X^2$), the concentrations of the validation standards were back-calculated. With this data, we computed trueness, precision and accuracy for each method and each response function (Table 3 tabulates data of the finally selected response function). Trueness was expressed in terms of relative bias (%). Precision was evaluated at two levels: intermediate precision and repeatability. The β-expectation tolerance limits calculated at 95% were determined and four accuracy profiles were plotted for each method. Fig. 3 shows the different accuracy profiles obtained for PTMD. This graphical tool allowed to control, in an easy way, if the method is validated on the tested concentration range. Three of the tested response functions for PTMD (linear, weighted $1/X$ and weighted $1/X^2$) have their tolerance intervals comprised within the acceptability limits of ±15%. The best accuracy profiles are obtained with the linear and weighted $1/X$ linear regressions. Although quite similar, we can just point out, for the weighted $1/X$ model linear, that the imprecision at the first tested level concentration is a slight more important than for the linear model. Taking account of this element and the ease of use of the linear model, this latter was adopted for PTMD. The model chosen shows a limited positive bias with a relatively limited dispersion.

In the case of the two analogs, similar analysis was performed (data not shown) and pointed out as the best regression model the linear one (see Fig. 4).

Considering Fig. 4a for analog 1, all the tolerance intervals are comprised within the acceptability limits and allow: (i) to consider our analytical method as validated, (ii) to fix the lower limit of quantification (LLOQ) at the first tested concentration level. Compared to PTMD (Fig. 3a), the results for analog 1 show a wider dispersion, especially at low concentrations, but the method does not present a bias.

For analog 2, the accuracy profile obtained by linear regression (Fig. 4b), exhibits a tolerance interval at the first tested level not include in the limits. The other tested models do not give satisfaction and enlarge the dispersion around the relative bias. In this case, LLOQ was set at 178.12 ng/mL (second SV). The method is fruitfully validated on the range 178.12–890.6 ng/mL. This higher LLOQ could be likely attributed to the low rate of efficiency of the extraction for this compound. Furthermore, more variability in the extraction results of analog 2 than for PTMD and analog 1 was observed. A further improvement of the extraction step should enlarge the concentration range.

3.4. Application to a preliminary pharmacokinetic study

Fig. 5 illustrates the drug plasma concentration curve obtained for each compound after a single subcutaneous administration (20 mg/kg). The absorption and distribution for the three drugs

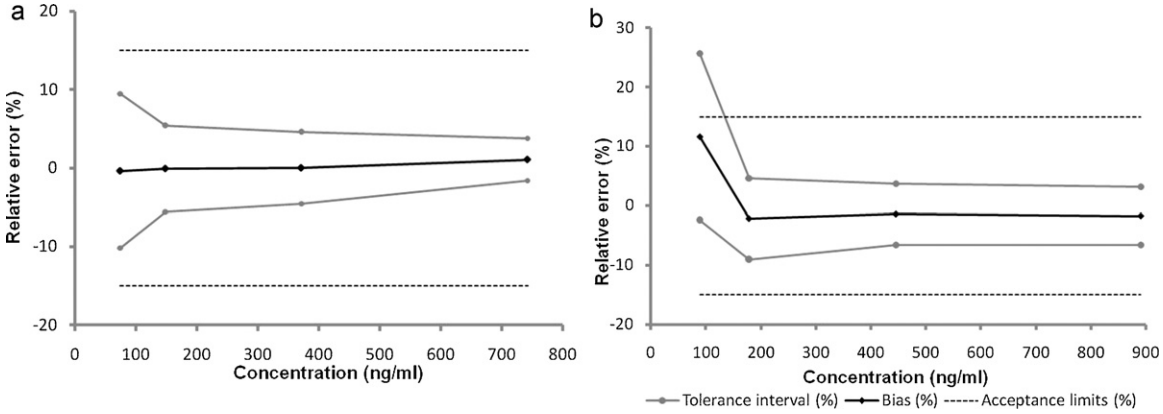

Fig. 4. Accuracy profiles for (a) analog 1 and (b) analog 2; $\beta = 95\%$, $\lambda = \pm 15\%$.

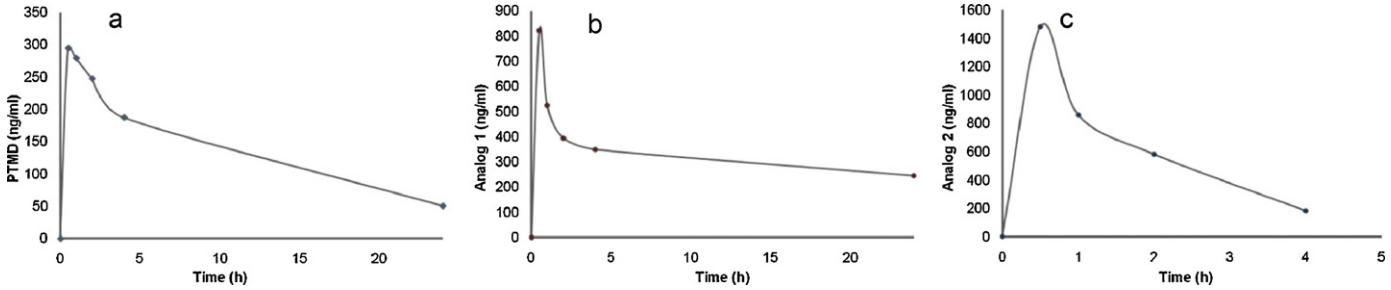

Fig. 5. Pharmacokinetic profile of rat plasma concentration of PTMD (a), analog 1 (b) and analog 2 (c) after single subcutaneous administration of the corresponding drug (20 mg/kg).

seem extremely rapid, reaching a peak after only 30 min. The peak concentrations are 294.2 ng/mL, 821.6 ng/mL and 1482.3 ng/mL for PTMD, analog 1 and analog 2, respectively. For analog 2, the elimination phase seems to be a rapid phenomenon as well, as no drug was detected on the last sampling (24 h). However, these preliminary results have to be completed with further studies to allow the determination of pharmacokinetics parameters.

4. Conclusion

The three developed SPE/LC-UV methods allow a rapid and sensitive determination of PTMD and two of its new promising benzamidine analogs in rat plasma. The three whole protocols contain a SPE or acetonitrile precipitation extraction and give rise to high resolute chromatograms, in the absence of interferences, in reasonable times of run.

A recent validation strategy based on the accuracy profiles was applied to demonstrate the ability of the new methods to quantify PTMD, analog 1 and analog 2 in rat plasma (acceptability limits and β were fixed at 15% and 95% respectively). The validation for PTMD and analog 1 were fulfilled on all the concentration range tested (29.33–586.54 ng (free drug)/mL plasma and 74.23–742.3 ng (free drug)/mL plasma for PTMD and analog 1 respectively). For analog 2, the bias and precision for the first level tested was very high but the method was considered as validated on the concentration range (178.12–890.6 ng (free drug)/mL plasma).

Compared to the published literature, the developed SPE method in this paper presents, an good extraction capacity, efficient on very low volume samples at a low concentration range. Moreover, the LLOQ obtained by our validated method is very low compared to the previously available data for PTMD.

Our practical application demonstrates that our optimized protocols are able to match the requirements for further *in vivo* evaluation and clinical monitoring.

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