Talanta 82 (2010) 945–951

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Rapid high-performance liquid chromatography–tandem mass spectrometry method for determination of pentoxifylline and its active metabolites M1 and M5 in human plasma and its application in bioavailability study

Laurian Vlase[a], Béla Kiss[b,*], Dana Muntean[a], Sorin E. Leucuţa[a]

[a] Department of Pharmaceutical Technology and Biopharmaceutics, Faculty of Pharmacy, University of Medicine and Pharmacy "Iuliu Hatieganu", No. 8 Victor Babeş, RO-400012, Cluj-Napoca, Romania
[b] Department of Toxicology, Faculty of Pharmacy, University of Medicine and Pharmacy "Iuliu Hatieganu", No. 6 Pasteur, RO-400349 Cluj-Napoca, Romania

ARTICLE INFO

Article history:
Received 11 February 2010
Received in revised form 21 May 2010
Accepted 30 May 2010
Available online 8 June 2010

Keywords:
Pentoxifylline
Metabolites
Liquid chromatography/mass spectrometry
Plasma
Bioequivalence

ABSTRACT

A new rapid, sensitive and selective liquid chromatography coupled with mass spectrometry method was developed and validated for the simultaneous quantification of pentoxifylline (PTX) and two major active metabolites in human plasma (M1 and M5). After a deproteinization step, chromatographic separation of the selected analytes was performed on a RP-C18 column. The detection of target compounds was in multiple reaction monitoring mode using an ion trap mass spectrometer equipped with an electrospray ion source. The method was validated and proved to be linear, accurate and precise over the range 5.08–406.14, 10.08–806.40 and 20.15–1611.60 ng/mL in case of PTX, M1 and M5, respectively. The major advantages of this method are the small sample volume, simple sample processing technique, the high sensitivity and the very good selectivity guaranteed by the MS/MS (in case of PTX) or MS/MS/MS (in case of M1 and M5) detection. The validated method has been successfully applied to a bioequivalence study.

1. Introduction

Pentoxifylline [1-(5-oxohexyl)-3,7-dimethylxanthine] is a hemorrheologic drug prescribed for the treatment of peripheral vascular disease and intermittent claudication [1–5]. This therapeutic benefit is primarily due to increased erythrocyte flexibility, reduced blood viscosity and decreased potential for platelet aggregation and thrombus formation [6,7].

Pentoxifylline (PTX) is readily absorbed from the gastrointestinal tract but undergoes extensive first-pass metabolism and its oral bioavailability is only 20–30% [8,9]. The major active metabolites in humans are 1-(5-hydroxyethyl)-3,7-dimethylxanthine (M1), 1-(4-carboxybutyl)-3,7-dimethylxanthine (M4) and 1-(3-carboxypropyl)-3,7-dimethylxanthine (M5). A single 400 mg oral dose of PTX given to human subjects produced average peak plasma concentrations of 1.6 and 2.0 mg/L, respectively for PTX and M1, whereas elimination half-life averaged 0.8 and 1.0 h. After oral administration to healthy volunteers the areas under the plasma concentration curves (AUCs) of M1 and M5 were superior to the AUC of PTX. In case of M4 the AUC was lower than for the parent compound [8–11]. Due to the rapid absorption (average time to reach the peak plasma levels, t_{max} = 1 h) and elimination of immediate-release oral forms of PTX, sustained-release tablets were developed. In case of these formulations peak plasma levels are reached after 2–3 h, while half-life is delayed to 3–4 h [8].

A major requirement for a pharmacokinetic, bioavailability or bioequivalence study is the elaboration and validation of a sensitive, selective and high-throughput quantitative analytical method.

To date, several gas chromatography methods using a nitrogen phosphorus detector (NPD) and high performance liquid chromatography (HPLC) methods with UV or MS/MS detection have been published for the quantitative analysis of PTX, with or without the simultaneous quantification of the major metabolites, in biological samples [10,12–29]. Extraction of the analytes from the biological matrices is performed either by classic liquid–liquid extraction (LLE) or solid-phase extraction (SPE). Best et al. described a HPLC–UV method for the simultaneous quantification of PTX and M1 with a previous sample treatment consisting in deproteinization and evaporation of the supernatant [30].

For human plasma samples, the GC–NPD methods presented lower limits of detection (LLOD) for PTX and its metabolites in the range of 2–10 ng/mL [12–16]. The GC–NPD method reported by Bryce and Burrows presented lower limit of quantification (LLOQ) values between 3 and 10 ng/mL [10]. The main problem of the GC–NPD methods is the need for a derivatization step, which means a prolonged, laborious sample preparation and a supplementary source of errors. Kumazawa et al. reported a GC-FID (flame ionization detector)/MS for the screening of 10 xanthine derivatives

* Corresponding author. Tel.: +40 264 450555; fax: +40 264 450555.
E-mail address: kbela@umfcluj.ro (B. Kiss).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.05.064

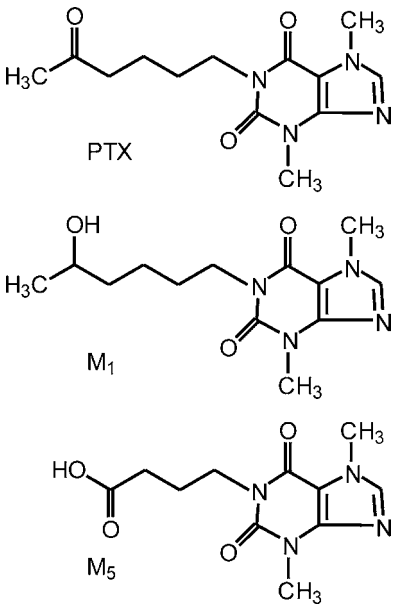

Fig. 1. Structures of pentoxifylline, and two major metabolites, M1 and M5.

in human plasma and urine after a previous SPE with LLOD values between 0.16 and 0.83 μg/mL sample [31].

The major disadvantages of UV detection based HPLC assays are the low sensitivity (LLOD ≥ 10ng/mL) and laborious sample preparation [18–20,25–27,30]. Pokrajac et al., Wong et al., Sripalakit et al. and Teksin et al. reported HPLC–UV methods with LLOQ between 10 and 15 ng/mL, but which allowed only the quantification of the parent compound PTX, without the metabolites [21,22,28,29]. Kuroda et al. described a HPLC–UV assay with column-switching for another xanthine derivative, propentofylline, and its three metabolites in human and rat serum with direct injection of serum. The major disadvantages of this method were the very long chromatographic run time of 30 min and poor sensitivity (LLOD between 0.08 (approximately 25 ng/mL) and 0.57 nmol/mL) [32].

To our knowledge, two LC tandem mass spectrometry methods were published for quantification of PTX in human plasma with LLOQs of 1 ng/mL for PTX and M1 [23] and 2 ng/mL for PTX [24], respectively. Both methods required a previous liquid–liquid extraction, and none of them was able to quantify the M5 metabolite.

In this paper we present a simple, sensitive, high-throughput LC tandem MS method for simultaneous quantification of PTX and two major active metabolites, M1 and M5 (Fig. 1) from human plasma. Based on the higher concentration and AUC achieved by the M1 and M5 metabolites in human plasma, only these two metabolites are requested to be analyzed by the FDA (Food and Drug Administration) and EMEA (European Medicines Agency) in bioavailability and bioequivalence studies [33]. The validated method was applied successfully to quantify the selected analytes from human plasma samples in a bioequivalence study.

2. Materials and methods

2.1. Chemicals and reagents

Pentoxifylline, 1-(5-hydroxyethyl)-3,7-dimethylxanthine (M1) and 1-(3-carboxypropyl)-3,7-dimethylxanthine (M5) standards were obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Methanol (HPLC isocratic grade), 98% formic acid and 70% perchloric acid (analytical grade) were purchased from Merck (Merck KgaA, Darmstadt, Germany). Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system. Drug free human plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania.

2.2. Calibration standards and quality control samples

Primary stock solutions of PTX (967 μg/mL), M1 (960 μg/mL) and M5 (1020 μg/mL) were prepared by dissolving accurately weighed quantity of PTX, M1 and M5, respectively in methanol (weighed on an Analytical Plus balance from Mettler Toledo, USA). Working solutions of PTX (10.15 and 1.02 μg/mL), M1 (20.16 and 2.02 μg/mL) and M5 (40.29 and 4.03 μg/mL) were obtained by diluting specific volumes of stock solution with blank plasma. These working solutions were used to spike different volumes of blank human plasma, providing finally eight calibration standards containing a mixture of all three analytes with the concentrations ranged between 5.08–406.14 ng/mL (PTX), 10.08–806.40 ng/mL (M1) and 20.15–1611.60 ng/mL (M5), respectively.

Quality control (QC) samples containing all three analytes at 15.23, 152.30 and 304.61 ng/mL for PTX, 30.24, 302.40 and 604.80 ng/mL for M1 and at 60.44, 604.35 and 1208.70 ng/mL for M5 were prepared by diluting specific volumes of the analytes working standards with blank human plasma.

2.3. Chromatographic and mass spectrometry conditions

LC analysis was performed using an Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary pump, degasser, autosampler and column thermostat. Chromatographic separation was achieved on a Zorbax SB-C18 column (100 mm × 3.0 mm i.d., 3.5 μm) (Agilent Technologies, Palo Alto, CA, USA) preceded by a 0.5 μm online filter. The mobile phase consisted of a 20/80 (v/v) mixture methanol/0.1% formic acid, under isocratic conditions. Each solvent was degassed in an ultrasonic bath before use for HPLC. The mobile phase was delivered at a flow rate of 1 mL/min. The column temperature was maintained at 45 °C.

The HPLC system was coupled to an Agilent MSD VL Ion Trap detector (Bruker Daltonik, GmbH, Brehmen, Germany) equipped with an electrospray interface (ESI) operated in the positive ionization mode. Chromatographic and mass spectrometric data acquisition were performed using Chemstation software (Agilent Technologies, Palo Alto, CA, USA), version B.01.03 and LC/MSD Trap Control (Bruker Daltonik, GmbH, Brehmen, Germany), version 5.3, while data processing was performed using LC/MSD Quant Analysis (Bruker Daltonik, GmbH, Brehmen, Germany), version 1.7. Nitrogen was used as drying gas (12 L/min, 300 °C) and nebulizing gas (50 psi). The capillary voltage was set at 4800 V for M5, 3000 V for PTX and 2200 V for M1, while the capillary exit potential was set at 38 V for M5, 42 V for PTX and 54 V for M1. The trap drive was set at 26.5 V (M5), 24.2 V (PTX) and 23.4 V (M1). The scan range was 100–300 m/z. The mass spectrometer was set to monitor ion transitions as follows: $m/z = 279.0 \rightarrow 180.9$ for PTX, $m/z = 281.0 \rightarrow 263.0 \rightarrow (180.9 + 192.9)$ for M1 and $m/z = 267.0 \rightarrow 249.0 \rightarrow 221.0$ for M5.

In order to maintain the ESI source clean, the column effluent was diverted to waste for the first 2 min after injection.

2.4. Sample preparation

200 μL blank plasma, calibration standards and QC samples were vortex-mixed (Vortex Genie 2, Scientific Industries) for 5 s with 100 μL 6% perchloric acid in 1.5 mL polypropylene tubes. The samples were then centrifuged at 4000 rpm for 6 min (204 Sigma centrifuge, Osterode am Harz, Germany). 150 μL of the supernatant was transferred to an autosampler vial and 6 μL were injected into the HPLC system.

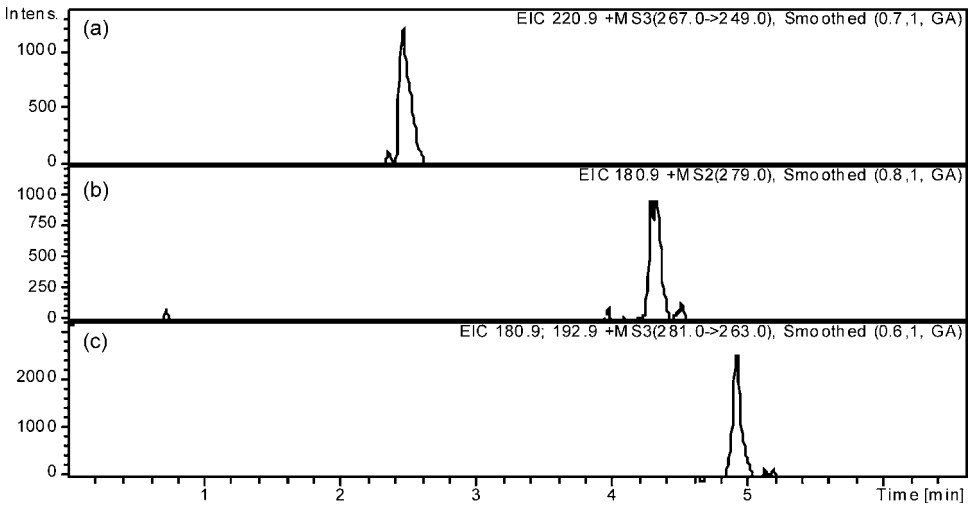

Fig. 2. Representative ion chromatogram of M5 (a), PTX (b) and M1 (c) after deproteinization of blank plasma spiked with PTX, M1 and M5 at LLOQ level (5.08 ng/mL PTX, 10.08 ng/mL M1 and 20.15 ng/mL M5).

2.5. Ion suppression testing

The ion suppression was expressed as relative difference between the analytical response for a mixture of PTX, M1 and M5 at quantification limit injected directly in mobile phase and the response of the same concentration of analytes added to preextracted blank samples [34].

2.6. Assay validation

The assay was validated in accordance to the industrial guidance for the bioanalytical method validation [35–37].

Selectivity was checked by comparing six different plasma blanks with the corresponding spiked plasma samples for interference of endogenous compounds with the analytes.

Linearity was studied by analyzing singlicate calibration standards at 8 concentration levels for each analyte. The concentration of analyte was determined automatically by the instrument data system using the external standard method. The calibration curve model was determined by the least squares analysis. The applied calibration model was $y = c + bx + ax^2$, weight $(1/y)$ quadratic response, where y = area and x = concentration. Linearity was determined by checking five calibration curves on five different working days. The calibration model was accepted if the residuals were within ±20% at the lower limit of quantification (LLOQ) and within ±15% at all other calibration levels and at least two-third of the calibration standards met this criterion, including highest and lowest calibration levels. Regarding the sensitivity of the assay, the LLOQ was set at the lowest calibration level with an accuracy and precision less than 20%.

Precision is defined as coefficient of variation (CV%) and accuracy as relative deviation expressed as percentage error of the calculated value as compared with target added concentrations (true value). Accuracy and precision were calculated for all three QC levels. Within-run accuracy and precision were determined by analysis on the same day of five different samples (plasma spiked with PTX, M1 and M5) at each QC level. The between-run accuracy and precision were determined at the same concentrations of PTX, M1 and M5 but on five different experimental days.

The relative recoveries were analyzed at each of the three QC levels and also at the LLOQ, by comparing the response of treated plasma samples with the response of untreated standards in solvent with the same concentration of PTX, M1 and M5 as the plasma QC sample.

The stability study of PTX, M1 and M5 in human plasma included the evaluation of room-temperature stability, freeze–thaw stability, long-term stability at −20 °C and post-preparative stability in the autosampler. The evaluation of stability was performed at low and high QC levels (15.23 and 304.61 ng/mL for PTX, 30.24 and 604.80 ng/mL for M1 and at 60.44 and 1208.70 ng/mL for M5). Stability was assumed when concentrations of stability test samples fell within ±15% of the nominal value.

2.7. Clinical application and in-study validation

The validated method was applied to the determination of PTX, M1 and M5 in plasma samples from a bioequivalence study of two dosage forms containing PTX. Plasma samples were periodically collected up to 24 h after oral dose administration of a 600 mg extended release tablet to 24 healthy volunteers.

The accuracy and precision of the validated method was monitored to ensure that it continued to perform satisfactorily during analysis of volunteer samples. To achieve this objective, a number of QC samples prepared in duplicate at three concentration levels were analyzed in each assay run and the results compared with the corresponding calibration curve. At least 67% (four out of six) of the QC samples should be within 15% of their respective nominal values; 33% of the QC samples (not all replicates at the same concentration) can be outside ±15% of the nominal value.

3. Results and discussion

3.1. Chromatographic analysis

Bioequivalence studies involve the analysis of a large number of biological samples. That is the reason why high-throughput methods are needed in order to perform this type of analysis. The method described in this paper presents a major advantage compared to most of the assays for PTX and its metabolites published earlier, as there was no need for a laborious extraction step of analytes before chromatographic analysis. The sample treatment was reduced to a minimum and included only a deproteinization step. Best et al. described also a chromatographic method for the simultaneous quantification of PTX and M1 with a previous sample treatment consisting in deproteinization, but they needed to evaporate the supernatant in order to achieve an acceptable sensitivity [30]. The absence of the extraction step reduced the need for an internal standard because in case of protein precipitation there is no partition

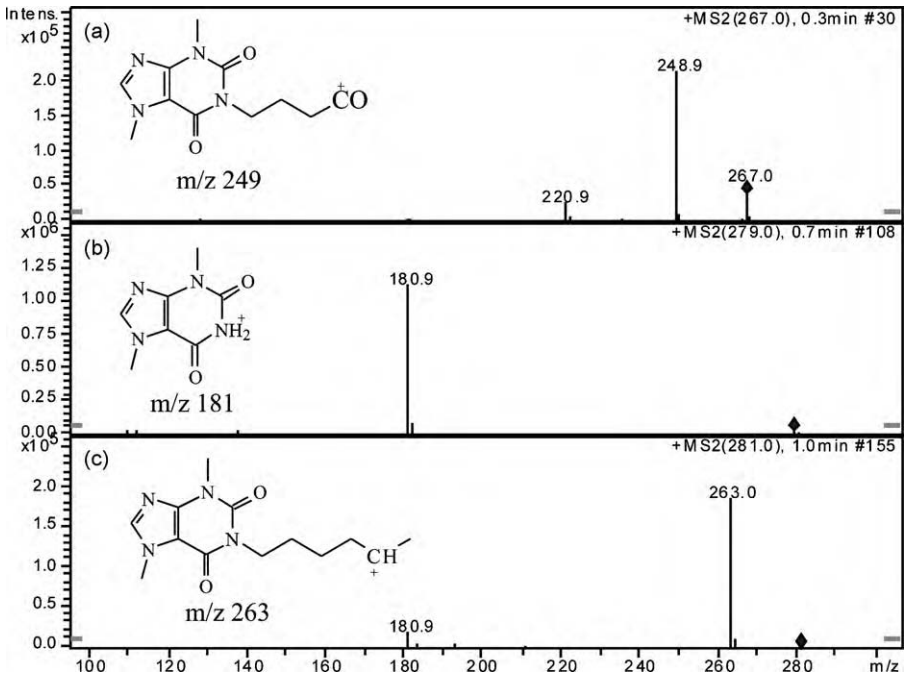

Fig. 3. Full-scan mass spectra (MS/MS) of product ions of PTX (b), M5 (a) and M1 (c).

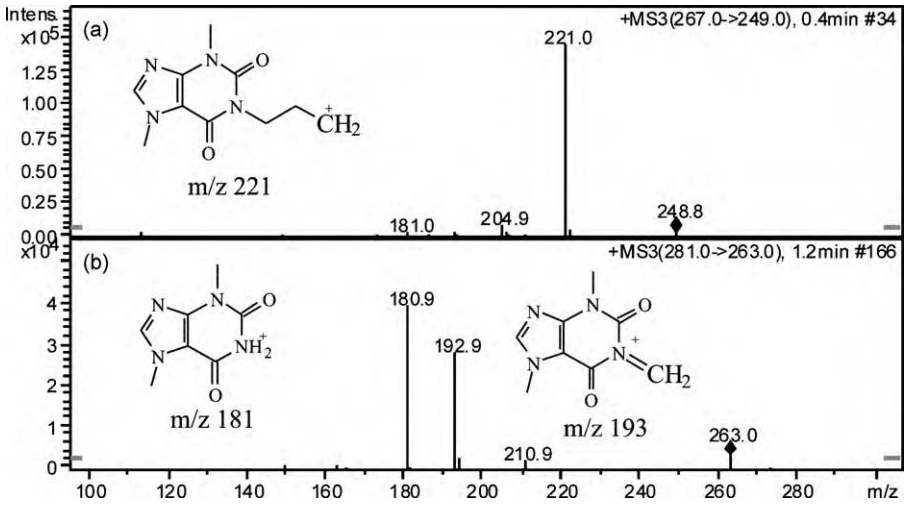

Fig. 4. Full-scan mass spectra (MS/MS/MS) of product ions of M5 (a) and M1 (b).

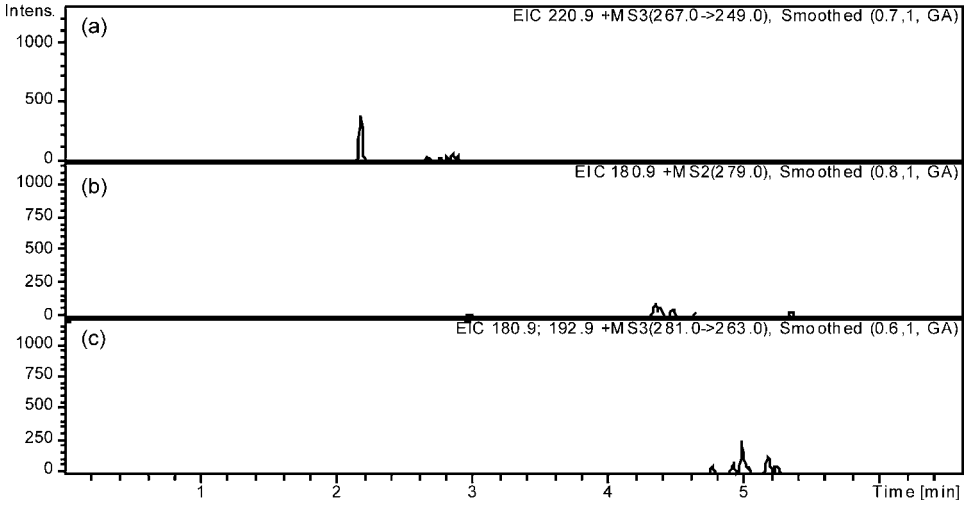

Fig. 5. Representative extracted ion chromatograms of blank plasma obtained in MRM mode, by monitoring the selected ion transitions for M5 (a), PTX (b) and M1 (c).

Table 1
Within-run precision and accuracy for PTX, M1 and M5.

Analyte	$c_{nominal}$ (ng/mL)	$c_{measured}$ (mean ± SD)	Precision (CV%)	Inaccuracy %	Recovery % (mean ± SD)
PTX	5.08(LLOQ)	5.2 ± 0.4	8.6	1.6	99 ± 10
	15.23	15 ± 1	9.1	−4.4	100 ± 10
	152.30	154 ± 4	2.8	0.9	100 ± 3
	304.61	301 ± 11	3.8	−1.3	101 ± 4
M1	10.08(LLOQ)	11 ± 1	12.2	8.1	102 ± 14
	30.24	29 ± 1	5.0	−2.6	96 ± 5
	302.40	311 ± 10	3.3	2.7	98 ± 4
	604.80	606 ± 18	3.0	0.2	96 ± 3
M5	20.15(LLOQ)	20 ± 3	12.3	0.8	93 ± 12
	60.44	62 ± 2	4.0	1.8	99 ± 4
	604.35	597 ± 35	5.9	−1.3	91 ± 6
	1208.70	1183 ± 33	2.8	−2.1	96 ± 3

$c_{nominal}$: nominal concentration, $c_{measured}$: measured concentration, SD: standard deviation; CV: coefficient of variation.

process of analytes between liquid–liquid or liquid–solid phases, making the recovery near 100% and very reproducible.

Another important aspect concerning the bioequivalence studies is the sample volume. It is recommended that this volume to be reduced as much as possible, which allows, if necessary, to collect samples more frequently from the volunteers. The method described here presents an advantage from this point of view also, since the plasma volume needed to perform the analysis is 0.2 mL.

Using the HPLC conditions described in the *Chromatographic and mass spectrometry conditions* section, PTX, M1 and M5 were well resolved, with retention times of 4.40, 5.00 and 2.50 min, respectively (Fig. 2).

In order to avoid any interferences from other compounds (exogenous or endogenous) which may co-elute with the selected compounds MS/MS (for PTX) and MS/MS/MS (for M1 and M5) detection was performed in MRM (multiple reaction monitoring) mode. Ionization of analytes was carried out using an ESI (electrospray ionization) ion source, operated in the positive ion mode.

Regarding the mass spectrometric detection, the first step consisted in recording the MS spectra of the target compounds. From full-scan mass spectra the protonated molecular ions $[M+H]^+$, with *m*/*z* 279.0 for PTX, *m*/*z* 281.0 for M1 and *m*/*z* 267.0 for M5 were selected as precursor ions and fragmented in MS/MS mode.

In case of PTX the major fragment (with highest signal intensity) was at *m*/*z* 180.9 (Fig. 3). According to Kyle et al. this transition from *m*/*z* 279.0 to 180.9 is consistent with the cleavage of the dimethylxanthine ring and oxohexyl moiety of PTX [23].

Following MS/MS analysis the selected metabolites of PTX generated each a main fragment consistent with the loss of water $[M-H_2O+H]^+$, with *m*/*z* 263.0 for M1 and 248.9 for M5, respectively (Fig. 3). Since the transitions due to the loss of water are not specific, a high background noise level is usually observed in chromatograms, lowering the analyte signal to noise ratio and decreasing the method specificity and sensitivity [38]. In an attempt to improve the sensitivity for M1 and M5, further fragmentation of their MS/MS product ions $[M-H_2O+H]^+$ was performed by a MS^3 (MS/MS/MS) analysis, when new ions were observed at *m*/*z* 180.9 and 192.9 for M1 and *m*/*z* 221.0 for M5, respectively (Fig. 4). For both M1 and M5, the mass transitions in MS^3 mode are more specific than in MS/MS mode and were used for quantitative analysis.

The final selected ion transitions for mass spectrometric detection and quantification of the selected three analytes after the chromatographic separation were 279.0 → 180.9 for PTX, 281.0 → 263.0 → (180.9 + 192.9) for M1 and 267.0 → 249.0 → 221.0 for M5.

3.2. Method validation

The selectivity study showed that there are no significant interferences or ion suppression effects from endogenous compounds at the retention time of the analytes (Fig. 5).

The calibration curves showed good linearity over the studied concentration range (5.08–406.14 ng/mL PTX, 10.08–806.40 ng/mL M1 and 20.15–1611.60 ng/mL M5), with correlation coefficients (*r*) 0.9993 ± 0.000606, 0.9988 ± 0.001018 and 0.9980 ± 0.001687 (mean ± SD, *n* = 5) for PTX, M1 and M5, respectively.

Having the advantage of simple and rapid sample preparation and short chromatographic run time, the method showed similar or even superior sensitivity to methods described in other scientific papers, based on LC or GC analysis and a more laborious sample preparation. The LLOQs were set at the level of the lowest calibrators for all three analytes (5.08, 10.08 and 20.15 ng/mL for PTX, M1 and M5, respectively), with accuracy and precision less than 20% (Tables 1 and 2).

Table 2
Between-run precision and accuracy for PTX, M1 and M5.

Analyte	$c_{nominal}$ (ng/mL)	$c_{measured}$ (mean ± SD)	Precision (CV%)	Inaccuracy %	Recovery % (mean ± SD)
PTX	5.08(LLOQ)	5.1 ± 0.6	11.9	1.2	103 ± 15
	15.23	15 ± 1	6.5	−1.9	106 ± 17
	152.30	160 ± 5	2.9	4.8	102 ± 7
	304.61	304 ± 8	2.6	0.0	99 ± 1
M1	10.08(LLOQ)	11 ± 1	6.8	7.1	106 ± 2
	30.24	30 ± 1	2.6	−1.8	101 ± 8
	302.40	316 ± 14	4.3	4.6	100 ± 4
	604.80	611 ± 13	2.1	1.0	99 ± 2
M5	20.15(LLOQ)	21 ± 2	11.2	2.3	89 ± 12
	60.44	60 ± 3	4.8	−0.2	100 ± 10
	604.35	622 ± 35	5.7	2.9	98 ± 12
	1208.70	1219 ± 53	4.4	0.8	99 ± 6

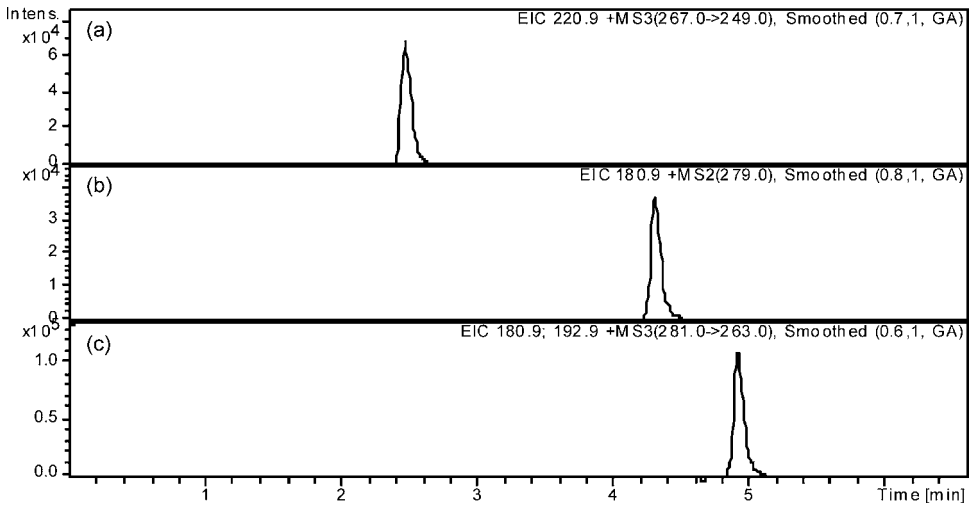

Fig. 6. Ion chromatogram of M5 (a), PTX (b) and M1 (c) after deproteinization of a real human plasma sample collected at 2.5 h after the administration of a 600 mg extended release tablet of PTX.

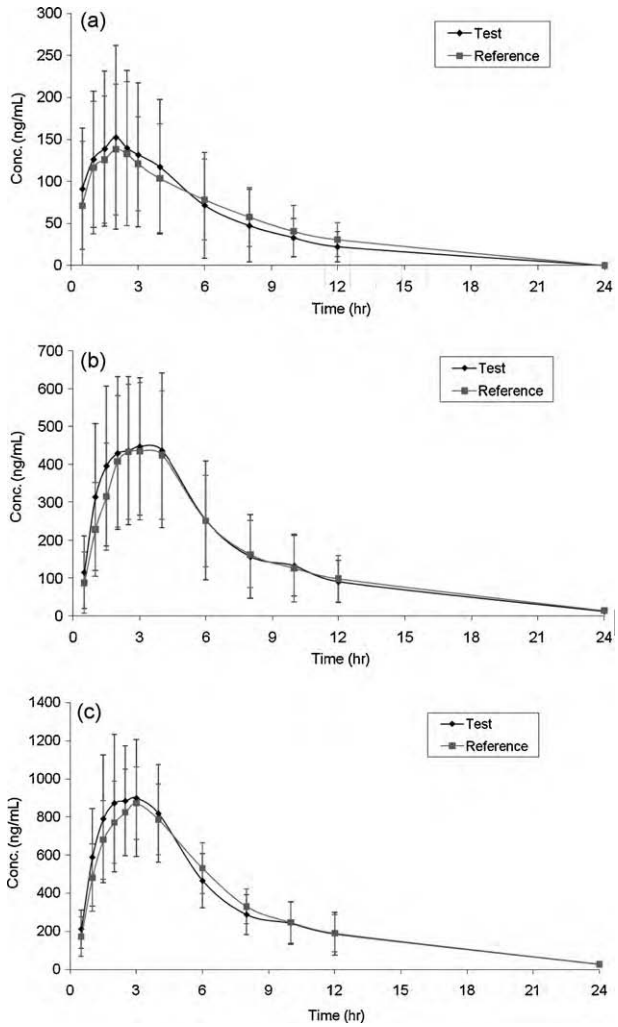

Fig. 7. Mean plasma levels ($n = 24$) of PTX (a), M1 (b) and M5 (c) after single dose administration of 600 mg PTX as slow release formulation to healthy volunteers. Test: Generic formulation product, Reference: Trental® (Sanofi-Aventis, France).

No significant ion suppression was observed for any of the three analytes. The calculated ion suppression was 5.5% for PTX, 5.9 for M1 and 8.3% for M5.

Since the method involved only a simple protein precipitation and centrifugation, without any extraction process, the mean recoveries of all target compounds were very good (superior to 88.9%) (Tables 1 and 2).

The within- and between-run precision and accuracy data are summarized in Tables 1 and 2. According to these results the assay is accurate and precise in the studied concentration range.

The results of stability study showed that no significant degradation of target compounds occurred under the tested conditions. In case of storage at room temperature the analytes proved to be stable in plasma samples for at least 4 h, the mean change in analyte content being of −3.4% and 1.3% in case of PTX, −1.9% and −0.2% in case of M1 and 2.0% and 3.0% in case of M5, at the two concentration levels tested. The post-preparative stability study showed the stability of PTX, M1 and M5 after sample preparation for at least 48 h at room temperature (inaccuracy < 15%). The mean changes in PTX, M1 and M5 concentration after three freeze–thaw cycles indicated no stability problems under these conditions. Furthermore the selected analytes were found to be stable in human plasma at least 8 months when stored at −20 °C.

3.3. Clinical application in healthy subjects

The validated method was verified during analysis of clinical samples from a bioequivalence study of two medicines containing 600 mg PTX. The method continued to perform in terms of accuracy, in each analytical run, not more than two out of six QC samples being outside of ±15% nominal value, but not all two at the same concentration.

Fig. 6 shows a representative ion chromatogram corresponding to plasma sample collected from a volunteer at 2.5 h after administration of a single oral dose of 600 mg PTX. The corresponding concentrations of PTX, M1 and M5 were of 175.69, 490.99 and 1061.14 ng/mL, respectively. The mean plasma levels of PTX, M1 and M5 obtained from the bioequivalence study are shown in Fig. 7.

4. Conclusions

This article describes a high-throughput analytical method for the simultaneous quantification of PTX, M1 and M5 in human plasma. The major advantages of this method are small sample

volume, simple and rapid sample processing technique (deproteinization), high sensitivity and a very good selectivity guaranteed by the MS/MS (in case of PTX) or MS/MS/MS (in case of M1 and M5) detection. The method was fully validated and successfully applied to a bioequivalence study of PTX in healthy volunteers.

References

[1] R. Muller, J. Med. 12 (1981) 209–235.
[2] B. Accetto, Am. Heart J. 103 (1982) 864–869.
[3] A.G. Dettori, M. Pini, A. Moratti, M. Paolicelli, P. Basevi, R. Quintavalla, C. Manotti, C. Di Lecce, Angiology 40 (1989) 237–248.
[4] J.P. De La Cruz, M.M. Romero, P. Sanchez, F. Sanchez De La Cuesta, Gen. Pharmacol. 24 (1993) 605–609.
[5] D. Moher, B. Pham, M. Ausejo, A. Saenz, S. Hood, G.G. Barber, Drugs 59 (2000) 1057–1070.
[6] B. Angelkort, N. Maurin, K. Boateng, Curr. Med. Res. Opin. 6 (1979) 255–258.
[7] A. Ward, S.P. Clissold, Drugs 34 (1987) 50–97.
[8] B. Beermann, R. Ings, J. Mansby, J. Chamberlain, A. McDonald, Clin. Pharmacol. Ther. 37 (1985) 25–28.
[9] R.V. Smith, E.S. Waller, J.T. Doluisio, M.T. Bauza, S.K. Puri, I. Ho, H.B. Lassman, J. Pharm. Sci. 75 (1986) 47–52.
[10] T.A. Bryce, J.L. Burrows, J. Chromatogr. 181 (1980) 355–361.
[11] M. Nicklasson, S. Björkman, B. Roth, M. Jönsson, P. Höglund, Chirality 14 (2002) 643–652.
[12] M.T. Bauza, R.V. Smith, D.E. Knutson, F.R. Witte, J. Chromatogr. B: Biomed. Sci. Appl. 310 (1984) 61–69.
[13] J.L. Burrows, K.W. Jolley, J. Chromatogr. B: Biomed. Sci. Appl. 344 (1985) 187–198.
[14] J.L. Burrows, J. Chromatogr. B: Biomed. Sci. Appl. 423 (1987) 139–146.
[15] D.A. Engelhart, E.S. Lavins, S.S. Seligman, C.A. Sutheimer, J. Anal. Toxicol. 21 (1997) 576–579.
[16] V. Marko, K. Bauerova, Biomed. Chromatogr. 5 (2005) 256–261.
[17] W. Rieck, D. Platt, J. Chromatogr. B: Biomed. Sci. Appl. 305 (1984) 419–427.
[18] G. Musch, T. Hamoir, D.L. Massart, J. Chromatogr. B: Biomed. Sci. Appl. 495 (1989) 215–226.
[19] W.E. Lambert, M.A. Yousouf, B.M. Van Liedekerke, J.E. De Roose, A.P. De Leenheer, Clin. Chem. 35 (1989) 298–301.
[20] A. Mancinelli, S. Pace, A. Marzo, E. Arrigoni Martelli, G. Passetti, J. Chromatogr. B: Biomed. Sci. Appl. 575 (1992) 101–107.
[21] M. Pokrajac, B. Miljkovic, D. Simic, B. Brzakovic, A. Galetin, Eur. J. Pharm. Biopharm. 43 (1997) 193–196.
[22] J.W. Wong, K.H. Yuen, K.K. Peh, J. Chromatogr. B 716 (1998) 387–391.
[23] P.B. Kyle, K.G. Adcock, R.E. Kramer, R.C. Baker, Biomed. Chromatogr. 19 (2005) 231–236.
[24] N.V.S. Ramakrishna, K.N. Vishwottam, S. Manoj, M. Koteshwara, M. Santosh, Y. Anjaneyulu, Chromatographia 63 (2006) 135–141.
[25] A. Chmielewska, L. Konieczna, A. Plenis, H. Lamparczyk, Acta Chromatogr. 16 (2006) 70–79.
[26] J.M. Raoul, M.R. Peterson, T.C. Peterson, Biochem. Pharmacol. 74 (2007) 639–646.
[27] A.K. Starodubtsev, I.V. Zolkina, S.N. Kondratenko, G.A. Belyakova, Pharm. Chem. J. 42 (2008) 1–3.
[28] P. Sripalakit, A. Saraphanchotiwitthaya, J. AOAC Int. 92 (2009) 837–845.
[29] Z.S. Teksin, I. Agabeyoglu, K. Yamac, J. Bioequiv. Availab. 1 (2009) 115–120.
[30] B.M. Best, J.C. Burns, J. DeVincenzo, S.J. Phelps, J.L. Blumer, J.T. Wilson, E.V. Capparelli, J.D. Connor, Curr. Ther. Res. 64 (2003) 96–115.
[31] T. Kumazawa, K. Sato, S. Hiroshi, A. Ishii, O. Suzuki, Forensic Sci. Int. 68 (1994) 53–67.
[32] N. Kuroda, Y. Hamachi, N. Aoki, M. Wada, M. Tanigawa, K. Nakashima, Biomed. Chromatogr. 13 (1999) 340–343.
[33] In vivo bioequivalence guidances, Drug protocols, Pentoxifylline Extended-Release Tablets—In Vivo Bioequivalence and In Vitro Dissolution Testing [online] [cited 2010 May 21]. Available from: URL: http://www.drugfuture.com/Pharmacopoeia/USP32/pub/data/v32270/usp32nf27s0_c1090.html.
[34] T.M. Annesley, Clin. Chem. 49 (2003) 1041–1044.
[35] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Guidance for Industry-Bioanalytical Method Validation, 2001, May, http://www.fda.gov/cder/guidance/4252fnl.pdf.
[36] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Guidance for Industry. Bioavailability and Bioequivalence Studies for Orally Administered Drug Products-General Considerations, March 2003, Rockville, USA, http://www.fda.gov/cder/guidance/5356fnl.pdf.
[37] The European Agency for the Evaluation of Medicinal Products, Note for Guidance on the Investigation of Bioavailability and Bioequivalence, CPMP/EWP/QWP/1401/98, July 2001, London, UK, http://www.emea.europa.eu/pdfs/human/qwp/140198enfin.pdf.
[38] M.M. Kushnir, A.L. Rockwood, G.J. Nelson, B. Yue, F.M. Urry, Clin. Biochem. 38 (2005) 319–327.