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Bioanalysis of pentoxifylline and related metabolites in plasma samples through LC-MS/MS

Daniela Iuliana Sora[a], Elena Cristea[a], Florin Albu[a], Victor David[b]* and Andrei Medvedovici[a,b]

ABSTRACT: Analytical aspects related to the assay of pentoxifylline (PTX), lisofylline (M1) and carboxypropyl dimethylxanthine (M5) metabolites are discussed through comparison of two alternative analytical methods based on liquid chromatography separation and atmospheric pressure electrospray ionization tandem mass spectrometry detection. One method is based on a 'pure' reversed-phase liquid chromatography mechanism, while the second one uses the additional polar interactions with embedded amide spacers linking octadecyl moieties to the silicagel surface (C-18 Aqua stationary phase). In both cases, elution is isocratic. Both methods are equally selective and allows separation of unknowns (four species associated to PTX, two species associated to M1) detected through specific mass transitions of the parent compounds and owning respective structural confirmation. Plasma concentration–time patterns of these compounds follow typical metabolic profiles. It has been advanced that *in-vivo* formation of conjugates of PTX and M1 is possible, such compounds being cleaved back to the parent ones within the ion source. The first method was associated with a sample preparation procedure based on plasma protein precipitation by strong organic acid addition. The second method used protein precipitation by addition of a water miscible organic solvent. Both analytical methods were fully validated and used to assess bioequivalence between a prolonged release generic formulation and the reference product, under multidose and single dose approaches. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: pentoxifylline; related metabolites; LC-(ESI)MS/MS; validation; bioequivalence

Introduction

Pentoxifylline (PTX) or 1-(5-oxohexyl)-3,7-dimethylxanthine, is a hemorrheological agent inhibiting phosphodiesterase and platelet aggregation, increasing erythrocyte and leukocyte flexibility and stimulating cytokine production (Dettelbach and Aviado, 1985; Beermann *et al*., 1985; Peterson, 1996).

PTX undergoes extensive metabolization in humans and animals. 3,7-Dimethyl-1-(5′-hydroxyhexyl)xanthine (namely lisofylline or M1), 1-(3′-carboxypropyl)-3,7-dimethylxanthyne (M5) and 1-(4′-carboxybutyl)-3,7-dimethylxanthine (M4) are the metabolites frequently cited in literature (Ward and Clissold, 1987; Bryce *et al*., 1989; Rocci *et al*., 1987), resulting from *in-vivo* reduction (M1) or oxidation (M4, M5) processes. Other four non-conjugated metabolites are known (Nicklasson *et al*., 2002). PTX is reduced to M1 in the human liver. The microsomes may oxidize back the active metabolite to the parent compound. However, erythrocytes seem to represent the main site for PTX reduction. PTX oral bioavailability is only 20–30%.

PTX and active related metabolites have been assayed in biological fluids by gas chromatography (GC) with selective nitrogen detection (NPD) through derivatization to trifluoroacetyl products (Burrows and Jolley, 1985; Burrows, 1987; Smith *et al*., 1986).

Liquid chromatography (LC) with ultraviolet spectrometry detection (UV) has been used for the same purposes (Garnier-Moiroux *et al*., 1987; Musch *et al*., 1989; Mancinelli *et al*., 1992; Srinivasu *et al*., 1999; Chmielewska *et al*., 2006). Relatively poor chromatographic resolution was reported and required sensitivity was supported through time-consuming sample preparation procedures, involving concentration steps, as liquid–liquid extraction (LLE) or solid-phase extraction (SPE). The limits of quantitation (LOQs) obtained usually by using LC-UV methods range from 12.5 to 25 ng/mL.

On our knowledge, a single approach has been reported for assaying PTX and M1 in plasma by means of liquid chromatography–atmospheric pressure electrospray ionization–tandem mass spectrometry detection [LC-(ESI)MS/MS] (Kyle *et al*., 2005). Reported LOQ value found for PTX falls in the 1 ng/mL level. However, sample preparation is still complicated, requiring protein removal via lithium precipitation, followed by LLE in chloroform, solvent evaporation at 60°C under cooled nitrogen flow and re-dissolution of the residue in water. Although the method

* Correspondence to: V. David, University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, Sos. Panduri 90, Bucharest—050663, Romania. E-mail: Vict_David@yahoo.com

[a] Labormed Pharma S.A., Splaiul Independentei 319E, Bucharest—060044, Romania

[b] University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, Sos. Panduri 90, Bucharest—050663, Romania

Abbreviation used: M1, lisofylline; M4, 1-(4′-carboxybutyl)-3,7-dimethylxanthine; M5, carboxypropyl dimethylxanthine; PTX, pentoxifylline; s, standard deviation.

was fully validated, it has not been used to assay large number of samples issued from clinical trials.

Extensive metabolic transformations and processes reversibility are responsible for the low and variable bioavailability of the drug (Pokrajac *et al*., 1997; Magnusson *et al*., 2006). The analytical techniques used to assay PTX and related metabolites may also add enhanced variability to the final quantitative results, as extensive sample manipulation steps induce experimental errors in addition to the intrinsic response variability of the detection equipments (especially when using MS or MS/MS ones). In recent years, the assessment of metabolite pharmacokinetic data has been increasingly on the rise in several types of clinical pharmacology studies including bioavailability/bioequivalence assessments of drug formulations (Srinivas, 2009).

Two methods have been developed for assaying PTX, M1 and M5 active metabolites in plasma samples. The first method uses the simple and straightforward mode of strong organic acid addition for precipitation of plasma proteins. Chromatographic separation is based on the reversed phase (RP) mechanism using isocratic elution. The second method is based on protein precipitation through water miscible organic solvent addition. The chromatographic separation is mainly produced according to the RP mechanism, with additional retention induced by polar interactions occurring between target compounds and embedded amide spacers linking octadecyl chains to silicagel. Both methods rely on (ESI)MS/MS detection. These methods were validated and used to assess bioequivalence between a prolonged release generic pharmaceutical formulation and the reference product (Trental®) containing 400 mg of PTX as active ingredient. The first method was applied following a multidose clinical study, while the second one was used for a single dose clinical approach. Specific analytical aspects related to the assay of PTX and metabolites M1 and M5 in plasma samples are discussed through comparison between the two methods. The proposed methods are equivalent, being characterized by similar quality attributes.

Experimental

Reagents

All solvents (acetonitrile and methanol) were HPLC gradient grade from Merck (Darmstadt, Germany). Water for chromatography (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument and used during experiments. Formic acid (extra pure grade), ammonia solution 25% (extra pure grade) and trichloroacetic acid (Ph. Eur., extra pure) were also from Merck. Pentoxifylline CRS, batch no.1e and 4-chloro-*N*-(2-methyl-1*H*-indol-1-yl)-3-sulfamoylbenzamide CRS (used as internal standard 2), batch no. 2a were obtained from European Pharmacopoeia (Strasburg, France). Lisofylline [1-(5-hydroxyhexyl)-3,7-dimethylxanthine] or M1 and 1-(3-carboxypropyl)-3,7-dimethylxanthine (M5) were reference substances (batches R-1004-006 and R-1004-047, respectively) from SynFine Research Inc. (Richmond Hill, Canada). 1,1′-(Propane-1,3-diyl)bis(3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione), used as internal standard 1 (IS1), was an working standard (batch 442/03) of controlled origin and purity higher than 99.8%.

Apparatus

Experiments were performed with an Agilent 1200 SL series LC/MSD (Agilent Technologies) system consisting of the following modules: degasser (G1379B), binary pump (G1312A), thermostated autosampler (G1367C and G1330B, respectively), column thermostat (G1316B), AP-ESI standard ion source (G1948B) and triple quadrupole mass spectrometric detector (G2571A). System control, data acquisition and interpretation were done with the Agilent MassHunter software version B 01.00 incorporating both qualitative and quantitative packages. The system was operationally qualified before and after the experimental study (the relative standard deviation RSD% for 500 fg of reserpine in the ion source, $n = 7$, varied from 5.5 to 11.2%).

The vortex system was model Multi Reax from Heidolph (Schwabach, Germany) and the thermostated centrifuge was model Universal 320R from Hettich (Tuttlingen, Germany).

Sample Preparation

Two sample preparation alternatives were considered. The first one was based upon acidic protein precipitation. The second sample preparation procedure dealt with protein precipitation based on organic solvent addition.

The plasma sample (0.5 mL) was combined with the internal standard (IS1) stock solution (0.05 mL, 4 µg/mL in acetonitrile). After vortexing (5 min, 2000 rpm), an aqueous solution of trichloroacetic acid (0.05 mL, 70% w/w) was added. After vortexing (10 min, 2000 rpm) ammonia solution (0.05 mL, 2.5%) was used to remove acid excess. Centrifugation was done (25°C, 10 min, 8000*g*) after another vortexing period (2 min, 2000 rpm). The supernatant was transferred to a vial and 10 µL were injected. This sample preparation procedure was applied together with the chromatographic separation achieved on the Zorbax SB-C_{18}, Rapid Resolution Cartridge and will be further addressed as Method 1.

As an alternative, the proteins from the plasma sample (0.2 mL) were precipitated by addition of a mixture of methanol–acetonitrile (2:8 v/v, 0.4 mL) also containing the IS2 (0.8 µg/mL). After vortexing for 5 min at 2000 rpm, centrifugation was applied (25°C, 5 min, 9000*g*). The supernatant was transferred to a vial and then diluted with 0.3 mL of HPLC-grade water. Homogenization through vortexing (30 s at 2000 rpm) was done prior to injection of 100 µL. This sample preparation procedure was applied together with the chromatographic separation achieved on the Zorbax SB-Aq column and will be further addressed as Method 2.

Chromatographic Methods

The chromatographic column suited to sample preparation based on acidic protein precipitation was a Zorbax SB-C_{18}, Rapid Resolution Cartridge, 30 mm length, 2.1 mm internal diameter and 3.5 µm particle size (Agilent Technologies, catalog no. 873700-902), fitted with a Phenomenex Guard Cartridge C_{18}, 4 × 2 mm (product no. AJO-4286). The column was operated at 35°C. The mobile phase was obtained by combining methanol with aqueous 0.1% formic acid solution in the volumetric proportion 1:3, under isocratic elution conditions. The flow rate was set up at 0.8 mL/min.

The column used for samples prepared through protein precipitation with organic solvent addition was a Zorbax SB-Aq, StableBond Analytical 150 mm length, 4.6 mm internal diameter and 5 µm particle size (Agilent Technologies, catalog no. 883975-914) operated at 35°C. The same Phenomenex guard cartridge was used. Elution was achieved in the isocratic mode, with a mobile phase combining methanol with aqueous 0.1% formic acid solution in the volumetric ratio of 3 to 2, at a flow rate of 0.8 mL/min. A complete chromatographic run took 7.5 min.

Both columns were qualified before and after completion of the studies, through determination of the reduced plate height corresponding to the fluoranthene peak. Minor modifications were observed after processing of more than 1100 samples per study ($\bar{h}$ varied from 3.4 to 3.8 for the first column and from 1.9 to 2.2 for the second one).

MS Parameters

The parameters controlling the AP-ESI ion source were as following: drying gas (N_2) temperature, 350°C; drying gas flow, 13 L/min; pressure of the nebulizing gas, 60 psi; capillary voltage, 4500 V. The fragmentor potential was set at 140 V. Collisional induced dissociation (CID) was

carried out at 20 V for IS1 and IS2, PTX and M5 and 15 V for M1, using N_2 as collision gas. The mass analyzers were used in the MRM mode. Transitions being monitored for each of the compounds were: for PTX, *m/z* 279 a.m.u. to *m/z* 181 a.m.u. for quantitation (quantifier transition) and to *m/z* 138 a.m.u for spectral confirmation (qualifier transition); for M1, *m/z* 263 a.m.u. to *m/z* 181 a.m.u. for quantitation (quantifier transition) and to *m/z* 193 a.m.u for spectral confirmation (qualifier transition); for M5, *m/z* 267 a.m.u. to *m/z* 221 a.m.u. for quantitation (quantifier transition) and to *m/z* 193 a.m.u for spectral confirmation (qualifier transition); for IS1, *m/z* 401 a.m.u. to *m/z* 221 a.m.u. for quantitation (quantifier transition) and to *m/z* 193 a.m.u for spectral confirmation (qualifier transition); for IS2, *m/z* 364 a.m.u. to *m/z* 130 a.m.u. for quantitation (quantifier transition) and residual *m/z* 364 a.m.u for spectral confirmation (qualifier). The isolation window for the precursor ions was set to wide (±1 a.m.u.). Chromatograms were obtained in the MRM format. Automated integration was used.

Within the ion source, IS1 and IS2 produced the molecular protonated ions (as major ones) and the sodium adducts (about 40%, relative intensity). No fragmentation was observed. PTX also ionized to the protonated molecular ion. Adducts with sodium (50% relative intensity) and potassium (10% relative intensity) were observed. No additional fragmentation arose. M1 generated intense adducts with sodium (80% relative intensity) and potassium (50% relative intensity), although the protonated molecule remained the major ion. Some fragmentation (~10% relative intensity) within the source to *m/z* 193 and 181 a.m.u. ions was also observed. M5 produced the molecular ion (major) and the sodium adduct (70% relative intensity). A fragment ion (40% relative intensity) was generated through water loss, while a minor signal (10% relative intensity) was observed at *m/z* 221 a.m.u. The behavior of the target compounds in the source did not support a sensitive assay, as long as the protonated molecular ion taken as precursor was competing with the alkaline metal adducts or fragmentation products. The collision-induced dissociation (CID) pattern for PTX is proposed in Fig. 1A. The CID of M1 and M5 produced the same ion fragments as PTX, characterized by *m/z* values of 181, 138 and 110 a.m.u. For M1, additional specific ion fragments were produced, according to schema in Fig. 1B. The active M5 metabolite underwent specific CID fragmentation processes leading to signal *m/z* = 221 a.m.u., as depicted in Fig. 1C, by stabilization through intra-molecular cyclization. IS1 produced under CID conditions the fragment *m/z* = 193 a.m.u. (specific to M1) and the same product ion as M5 (*m/z* = 221 a.m.u., see Fig. 1D). The product ion fragment characteristic to IS2 was assigned to the route described in Fig. 1E.

Methodology and Pharmacokinetic Parameters

The first method was use to assess bioequivalence between the Test and Reference (Trental®, Aventis Pharma Deutschland GmbH, Germany) products presented as prolonged-release film-coated tablets containing 400 mg of PTX. The approach was a multiple-dose, block-randomized, two-period, two-sequence, crossover clinical study. Fourteen repeated oral doses of PTX 400 mg prolonged-release tablets were administrated twice a day. Pre-dose blood samples were collected before the first administration and the 12th, 13th and 14th one (namely 0, 132, 144 and 156 h). The following collection blood samples program was applied after the last dose: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 10.0 and 12.0 h. The washout period between clinical phases was 21 days. The study was applied to 27 subjects, males or females from the Caucasian race, aged between 18 and 45 years, having a body mass index between 19 and 27.5. The plasma concentration profiles of PTX, M1 and M5 were used to determine the following pharmacokinetic parameters: AUC_{SS} (area under the concentration vs time curve at steady state), c^{SS}_{max} (maximum plasma concentration at steady state) and c^{SS}_{min} (minimum plasma concentration at steady state). The test and the reference products were compared through ANOVA (effects: period, subject nested in sequence, treatment and sequence) after log transformation of data for primary pharmacokinetic parameters, with determination of the 90% confidence intervals for the intra-individual ratios (test/reference). The range needed to conclude bioequivalence was 0.8–1.25 around the geometric means ratios test–reference of log-transformed data for AUC_{SS}, c_{max} and c_{min} of PTX, M1 and M5, respectively.

The second method was used to achieve a two-way, cross-over, randomized, single-dose bioequivalence study between the same test and reference products. Blood samples were withdrawn pre-dose (0) and 0.167, 0.33, 0.5, 0.75, 1.0, 1.33, 1.67, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 24.0 and 36.0 h after administration on a single dose, under fasting conditions. The washout period was 15 days. The study was made on 46 subjects, males or females from the Caucasian race, aged between 18 and 45 years, having a body mass index between 19 and 27.5. The pharmacokinetic parameters AUC_{last} (area under the concentration/time plot until the last sampling point), AUC_{tot} (area under the concentration/time plot extrapolated to infinity) and c_{max} (maximum plasma concentration) were assessed for PTX, M1 and M5 through ANOVA treatment, under the same conditions as described above.

All volunteers signed an informed written consent before initiation of the screening procedure. The protocols of the studies were formally accepted by the evaluation department of the Romanian National Drug Agency and received the approval of the Institutional Ethics Committee. Pharmacokinetic parameters were determined by means of the Kinetica™ software (version 4.4.1.) from Thermo Electron Corporation, USA.

Results and Discussion

Method Development

The reversed-phase mechanism has the ability to achieve selective separation of PTX, M1, M5 and IS. The observed elution order (see Fig. 2A–C) complied with octanol–water partition constant values ($\log K_{ow}$), calculated by the fragment methodology (0.39 for M5, 0.56 for PTX, and 1.07 for M1). The elution order for IS1 and IS2 did not match to their $\log K_{ow}$ (0.45 and 2.63, respectively) values; a possible explanation may be given based on their increased solubility in methanol. When the major interaction between analytes and the stationary phase was mainly based on the hydrophobic character, the mobile phase had an increased content of aqueous component. When an amide moiety was embedded in the stationary phase (as a spacer between octadecyl chains and the silicagel surface), additional polar interactions appeared between target compounds and the stationary phase. Consequently, elution from the C_{18} Aqua column requires a mobile phase containing an increased amount of organic modifier (60% methanol), adequately balancing retention. The separation on C_{18} stationary phase is thus adequate for a sample preparation schema based on protein precipitation through addition of a strong organic acid (sample dilution is minimal and injection of a small sample volume is possible). On the C_{18} Aqua column, a large volume injection may be used without suspecting appearance of focusing phenomena, even in a sample solvent having a higher content of organic component. Protein precipitation through addition of water-miscible organic solvents is thus allowed. Sample dilution effects are compensated by an increased injection volume.

When monitoring concentration profiles of PTX and M1 in plasma samples obtained during completion of the bioequivalence study, for the specific mass transitions of the target compounds, additional peaks were observed (two partially co-eluted on the PTX transitions and two on the M1 transitions, as in Fig. 2A). It is well known that PTX is extensively metabolized with the formation of seven compounds, M4 [1-3′(-carboxypropyl)-3,7-dimethylxanthine] being more often cited together with M1 and M5. However, such compounds should generate molecular protonated ions with different *m/z* values compared with PTX, and consequently should be detected through different mass

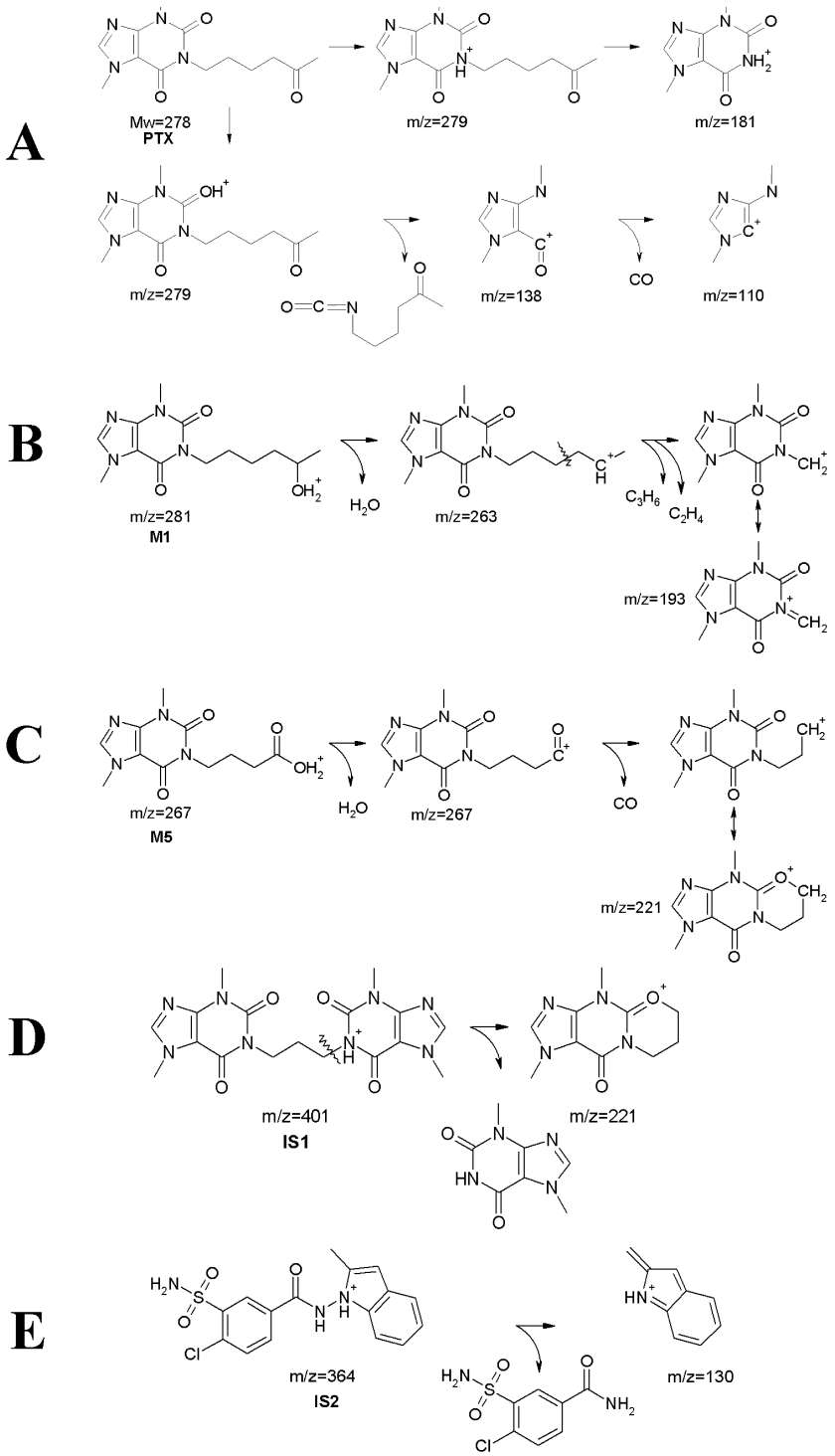

Figure 1. Suggested CID patterns for pentoxifylline (PTX), metabolites M1, M5, IS1 and IS2.

transitions (although accepting that the resulting product ions are less characteristic, as observed from Fig. 1). On the C_{18}-Aqua column additional separation was observed within peaks appearing under the transition of PTX. When using a longer C_{18} column (Zorbax Eclipse XDB-C_{18}, 100 mm length, 2.1 mm internal diameter and 3.5 µm particle size from Agilent Technologies, catalog no. 961753-902), under the same operating temperature, flow rate and same components of the mobile phase but under gradient elution conditions (from 25 to 50% methanol in 5 min), separation between compounds observable under PTX mass transition was enhanced (Fig. 2C). Four compounds could be observed. All secondary products marked in Fig. 2B and C under mass transitions of PTX and M1 exhibited structural confirmation factors (ratios between qualifier and quantifier transitions × 100) within the normal variation interval of the corresponding parameter belonging to the parent compound whose specific MRM

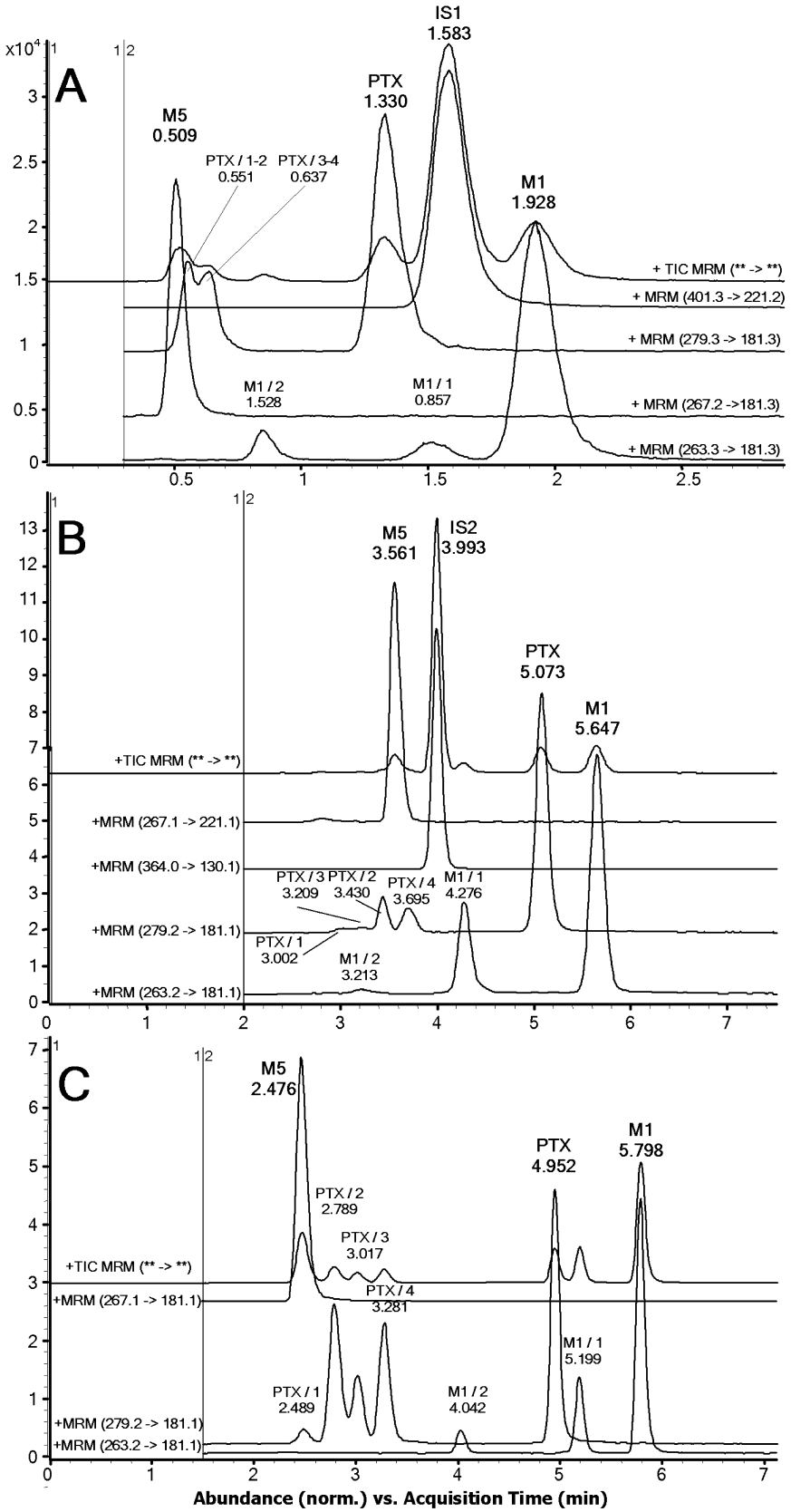

Figure 2. Chromatographic separation of pentoxifylline and related metabolites M1 and M5 from plasma samples: (A) conditions according to method 1, sample from a subject participating to the clinical trial (concentrations are 40 ng/mL for PTX, 106 ng/mL for M1 and 228 ng/mL for M5); (B) conditions according to method 2, sample from a subject participating to the clinical trial (concentrations are 75 ng/mL for PTX, 81 ng/mL for M1 and 922 ng/mL for M5); (C) chromatographic conditions described in text, sample from a subject participating to the clinical trial (concentrations are 56 ng/mL for PTX, 160 ng/mL for M1 and 631 ng/mL for M5). Compounds noted PTX/1–4 and M1/1–2 were attributed to conjugated forms of PTX and M1 respectively.

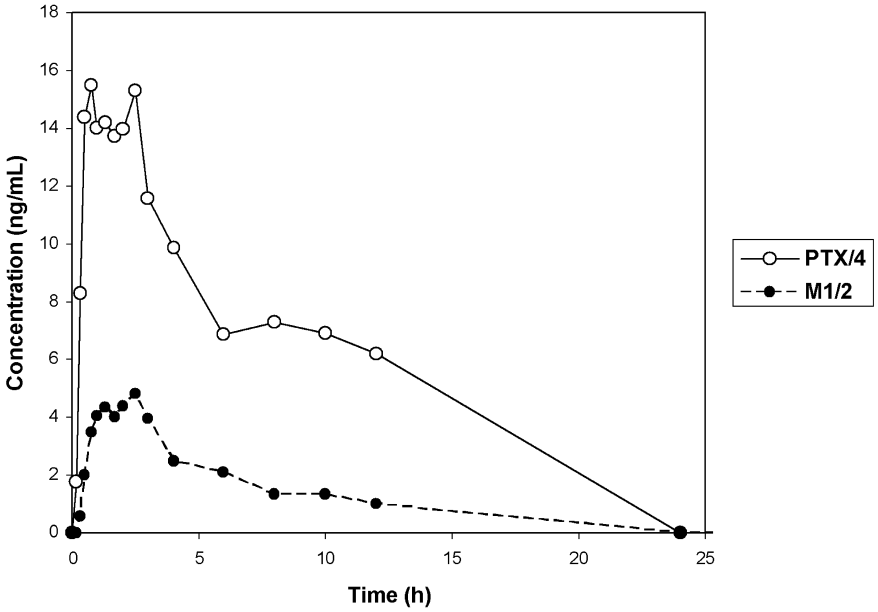

Figure 3. Plasma concentration–time profiles for possible conjugates PTX/4 and M1/2 in samples collected for one subject and one phase during the single dose clinical trial. Applied conditions according to method 2 (concentration values resulted from interpolation in PTX and M1 calibrations associated with the analytical sequence).

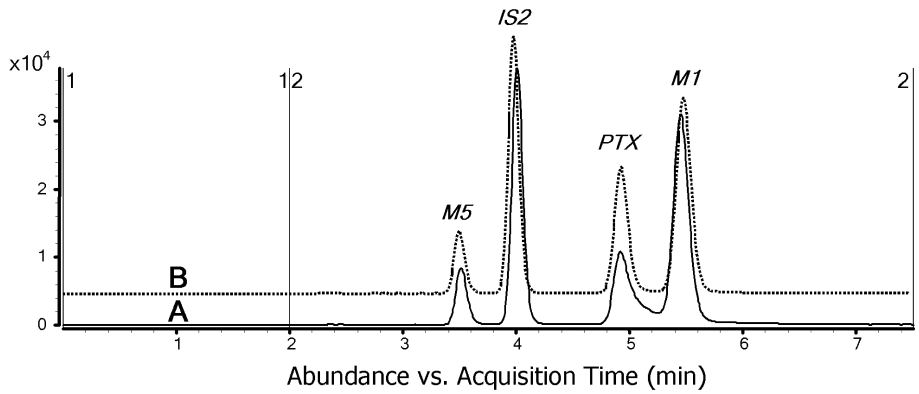

Figure 4. Separation of PTX and related metabolites in a spiked plasma sample, analyzed according method 2: (A) sample prepared and kept refrigerated (2–8°C) for 96 h; (B) freshly prepared sample (same blank plasma sample and same stock solutions).

were observed. The concentration profiles of these compounds in samples from volunteers participating to the clinical trial followed the known metabolic pattern, as observable in Fig. 3.

Retention of the metabolites is lower compared with the parent compound for all cases, reflecting increased polar characteristics. A possible explanation of the observed phenomena considers the *in-vivo* formation of conjugates of PTX and M1. In the ion source, conjugates are cleaved back to the parent compound, which undergoes formation of the protonated ion, then following the specified mass transitions. Conjugation confirms the earlier retention as the polar molecular characteristics are enhanced. One can conclude that chromatographic separations dedicated to bioanalysis should be rigorously tested for selectivity with samples obtained *in-vivo*, as metabolization patterns lead to compounds that produce interferences even in selective detectors such as MS/MS, at least for PTX and M1.

PTX seems to produce *in-vitro* interactions even with residual plasma matrix components. As can be observed in Fig. 4A, a spiked plasma sample prepared through protein precipitation with organic solvent, kept refrigerated for 96 h, produces a tailing effect on the PTX peak. The preparation of a new sample (from the same stock solutions and blank plasma) produces unaltered peak shape in the chromatogram (Fig. 4B). The observed behavior may be explained through association of PTX with a residual plasma matrix component (i.e. ion pairing), which alters the partition process. Declusterization arises in the ion source, producing free PTX, consequently detected through its specific mass transitions.

Method Validation

Validation of the presented methods was achieved according to regulations in force (US Department of Health and Human Services *et al.*, 2001; European Medicines Agency, 2008). The selectivity was tested for both methods by comparing the residual peak areas appearing at the characteristic retention of the

target compounds in chromatograms of six different blank plasma samples with the mean peak areas ($n = 6$) from chromatograms of spiked plasma samples at the lower level of quantitation (LLOQ). Residual peak areas corresponding to less than 20% from values at LLOQ were considered to belong to the noise. Results are given in Table 1.

Both methods exhibited good selectivity. Sample preparation through protein precipitation in acidic conditions produced less residual response. All samples from the clinical studies taken predose administration were producing chromatograms with residual peak areas situated below 20% from the mean values at LLOQ (samples at LLOQ concentration level from calibrations were considered for calculation of the mean).

Table 1. Selectivity of the methods used for quantitation of PTX and active metabolites M1 and M5 in plasma samples

Analyte	Percentage of the residual peak areas in blank samples compared with LLOQ			
	Method 1		Method 2	
	Min.	Max.	Min.	Max.
PTX	2.1	7.9	15.0	18.6
M1	1.2	4.7	2.3	2.7
M5	2.4	7.7	10.0	15.4

Data resulting from the studies of detector response functions generated through application of the considered methods are given in Table 2. For the first method, the linearization of the response function was achieved through representation of the log (decimal) of the peak area ratio (analyte/IS) against the log (decimal) of the concentration (Georgita *et al*., 2008; Srinivas, 2008). For the second method, the weighted $1/x^2$ linear regression was directly applied. Both mathematical approaches provide identical LLOQ values and linear correlation domains. LLOQs were also verified with respect to the S/N ratio criteria (~5) and the procedure confirmed the calculated values. In all cases, the regression parameters (slope and intercept) found during method validation were included in the variation intervals (mean ± s) determined on completion of calibrations associated with the analytical sequences. The log–log linearization procedure seemed to perform better than the weighted regression, although the variability of the IS peak area over the whole study was two times higher when applying the first method compared with the second one.

Results obtained from the precision evaluation procedure are presented in Table 3. One can observe that the inter-day variability of the response of the MS-MS equipment mainly affects results at the lower concentration levels. In all cases, RSD% values were within the accepted limits. Sample preparation based on protein precipitation through addition of acetonitrile produced a slightly increased variability compared with the method based

Table 2. Data resulting from linearity studies carried out on the two methods (including data from calibrations associated with the analytical working sequences)

Analyte/parameter	Method 1	Method 2
PTX, M1, M5/concentration levels	11	8
PTX, M1, M5/no. of samples per concentration level (linearity procedure/calibration)	6/1	6/1
PTX, M1, M5/linearization of the response function	log–log	Linear weight $1/x^2$
PTX, M1, M5/no. of calibrations	27	46
PTX, M1, M5/RSD% of the IS peak area over linearity	5.3	10.5
PTX/linearity concentration domain (ng/mL)	1–500	1–500
PTX/slope (B)	1.013	0.00071
PTX/intercept (A)	−2.6	2.4×10^{-4}
PTX/correlation coefficient	0.9996	0.9961
PTX/LLOQ {[(5 × S_A − A)/B] and S/N ratio ≈ 5} ng/mL	1	1
PTX/structural confirmation factor	±	30.2 (± 10%)
PTX/mean B ± s_B (from calibrations)	1.009 ± 0.018	0.0010 ± 0.003
PTX/mean A ± s_A (from calibrations)	−2.5 ± 0.1	$(0.04 \pm 3.4) \times 10^{-4}$
M1/linearity concentration domain (ng/mL)	1–2000	1–2000
M1/slope (B)	1.013	6.1×10^{-4}
M1/intercept (A)	−2.8	-0.8×10^{-4}
M1/correlation coefficient	0.9993	0.9956
M1/LLOQ {[(5 × S_A − A)/B] and S/N ratio ≈ 5} ng/mL	5	5
M1/structural confirmation factor	—	94.5 (± 10%)
M1/mean B ± s_B (from calibrations)	0.999 ± 0.016	$(6 \pm 2) \times 10^{-4}$
M1/mean A ± s_A (from calibrations)	−2.7 ± 0.2	$(-3 \pm 6) \times 10^{-4}$
M5/linearity concentration domain (ng/mL)	1–2000	1–2000
M5/slope (B)	0.988	4.1×10^{-5}
M5/intercept (A)	−3.8	0.5×10^{-4}
M5/correlation coefficient	0.9993	0.9961
M5/LLOQ {[(5 × S_A − A)/B] and S/N ratio ≈ 5} ng/mL	5	5
M5/structural confirmation factor	—	79.9 (± 10%)
M5/mean B ± s_B (from calibrations)	0.970 ± 0.03	$(7 \pm 4) \times 10^{-5}$
M5/mean A ± s_A (from calibrations)	−3.7 ± 0.2	$(-1 \pm 2) \times 10^{-4}$

Table 3. Precision characterizing the two bioanalytical methods

Analyte/ procedure	Method 1				Method 2			
	Concentration (ng/mL)	RSD (%)	Lower recovery (%)	Highest recovery (%)	Concentration (ng/mL)	RSD (%)	Lower recovery (%)	Highest recovery (%)
PTX	3	3.3	102.3	108.9	3	3.4	95.6	106.7
repeatability	25	1.9	97.9	103.9	15	2.6	93.4	100.3
($n = 10$)	400	0.7	111.3	114.0	75	1.2	108.8	112.8
	—	—	—	—	225	1.4	110.2	114.3
PTX	3	4.7	93.6	105.1	3	7.8	88.6	107.1
intermediate	25	2.4	92.7	99.1	15	1.0	90.2	92.8
precision	400	1.0	106.0	108.6	75	1.2	107.6	111.3
($n = 6$)	—	—	—	—	225	1.3	110.2	114.0
M1	15	2.6	100.8	109.1	15	1.9	95.8	100.8
repeatability	100	1.3	104.7	108.5	75	1.3	103.2	106.5
($n = 10$)	1600	0.8	96.2	98.4	200	1.4	106.2	111.2
	—	—	—	—	450	1.7	104.6	110.4
M1	15	3.5	97.9	106.5	15	4.6	95.4	106.6
intermediate	100	2.6	99.4	106.2	75	1.5	103.0	107.8
precision	1600	1.0	94.8	97.3	200	1.5	106.5	111.2
($n = 6$)	—	—	—	—	450	1.2	105.7	108.2
M5	15	5.5	91.8	108.7	15	7.7	92.5	104.2
repeatability	100	2.1	102.2	108.9	30	6.3	88.3	106.4
($n = 10$)	1600	0.7	110.5	112.6	500	1.5	100.1	104.9
	—	—	—	—	1200	2.8	102.5	112.0
M5	15	3.6	88.7	97.3	15	9.1	91.5	113.1
intermediate	100	1.6	85.9	89.7	30	4.1	88.1	98.4
precision	1600	1.1	91.8	94.0	500	2.3	94.9	101.4
($n = 6$)	—	—	—	—	1200	2.8	100.1	107.0

Table 4. Recoveries of PTX and related metabolites

Analyte/procedure	Method 1			Method 2		
	Concentration (ng/mL)	Mean recovery (%)	RSD (%)	Concentration (ng/mL)	Mean recovery (%)	RSD (%)
PTX[a]	3; 25; 400	61.3	11.4	3; 15; 225	87.8	9.9
M1[a]	15; 100; 1600	53.1	9.4	15; 75; 450	65.1	14.9
M5[a]	15; 100; 1600	39.7	13.1	15; 30; 1200	41.4	9.8
IS[a]	400 (IS1)	48.3	10.4	1600 (IS2)	51.1	9.2
PTX[b]	3; 25; 400	77.7	9.5	3; 15; 225	101.6	6.2
M1[b]	15; 100; 1600	76.5	6.6	15; 75; 450	102.0	2.2
M5[b]	15; 100; 1600	79.8	8.6	15; 30; 1200	102.8	8.0
IS2[b]	400 (IS1)	76.2	6.3	1600 (IS2)	96.8	2.1

[a] Recovery is calculated between spiked plasma samples and spikes in water.
[b] Recovery is calculated between spiked plasma samples and spikes in bulk protein precipitated blank plasma.

on acid addition. Samples used to evaluate precision were also checked for accuracy, by interpolation in the calibration associated with the analysis sequence. Biases expressed as percentage from the known concentration values were within the ± 15% interval.

Recoveries of PTX and related metabolites from plasma samples were evaluated compared with spikes in water (sample preparation procedure was applied) and in bulk protein-precipitated blank plasma samples (post spikes), according to data in Table 4. Recoveries were relatively low when comparison

was made with processed water samples. Isolation yields were found to be higher when comparison was made against post-spiked plasma samples. Method 2 produced quantitative yields compared with post spikes but only about 80% of target compounds was recovered on the use of acidic protein precipitation. This indicates that analytes are adsorbed on the precipitated proteins and their removal is facilitated by the addition of the organic solvent. Results also supported the fact that residual plasma matrix accumulation within the ion source had no major suppression or enhancing effects on ionization yields. Recoveries were determined for six replicates at each of the concentration levels mentioned in Table 4.

PTX and metabolites M1 and M5 were stable in plasma matrix for five consecutive freeze and thaw cycles, during 3 months at −40 or −20°C, and at room temperature during 24 h. The samples processed through acid precipitation of plasma proteins, without consequent neutralization, were stable for less than 6 h at room temperature. The compensation of the strong acid character by ammonia addition led to a stability of the analytes in the prepared samples for a 24 h period. As the pH of the supernatant remained acidic, one can observe that, after 48 h, detector response fell outside the allowed variation interval. The samples processed according to method 2 were found stable at room temperature for 24 h. After more than 48 h, the association with residual plasma matrix led to a deterioration of the PTX peak shape within the chromatogram (as already pointed out previously). IS1 and IS2 stock solution was found to be stable for 7 and 40 days, respectively.

As the variability of the peak area values integrated for the internal standards (IS1 and IS2) during the two bioequivalence studies was consistent (RSD% of 40% for IS1/method 1 and 17% for IS2/method 2, *n* being 1026 and 1656, respectively), it seemed necessary to demonstrate that the choice of the length of the analytical sequences was adequate to compensate for the observed phenomena. Figure 5 illustrates the results obtained for the quality control samples (QC) at the low concentration levels (1.5 × LLOQ, representing the 'worst case') for PTX and related metabolites M1 and M5. Mean values and normal variation intervals (mean ± 2 s) are shown together with the allowed variation limits. It seems obvious that analytical sequences containing one calibration, one QC set and samples from one volunteer may adequately compensate for the response variation of the IS.

Method robustness was focused on the way that the operational parameters of the ion source as well as the acid content of the mobile phase may influence the response given by the detection system. Consequently the temperature of the drying gas was varied within a 295–305°C interval, the dry gas flow from 8 to 12 L/min, the nebulizer gas pressure from 55 to 60 psi, the capillary voltage from 4000 to 5000 V and the percentage of the formic acid in the aqueous component of the mobile phase from 0.05 to 0.15%. Spiked plasma samples at the medium QC concentration level were tested ($n = 5$) for each of the sample preparation methods. RSD% values were thus calculated for peak areas. None of the considered parameters placed the RSD% associated with peak area values outside of the accepted limits (15%) for PTX, IS1, IS2 and metabolites M1 and M5, except for the dry gas flow. For this parameter, the variation of the IS2 peak area was placed outside the accepted limit (RSD = 21.2%). Results returned to within the allowed limits when the variation interval for this operational parameter was limited to 9.5–10.5 L/min.

Pharmacokinetic parameters obtained for PTX and related metabolites M1 and M5 during multi- and the single-dose clinical trials are given in Table 5. The inter-subject variability of data is increased for both reference and tested products, as already indicated in the literature. The bioequivalence of the two products could be established based on the experimental data.

Conclusions

Two methods were developed to assay PTX and related metabolites M1 and M5 in plasma samples. These methods were used to assess bioequivalence of a generic prolonged release formulation against the reference product, considering both multi- and single-dose clinical trials.

The first method achieved separation of the target compounds on a C_{18} end-capped stationary phase. Elution is isocratic and fast, but the mobile phase has an increased amount of aqueous component (75%). Sample preparation procedure associated with the chromatographic method is based upon plasma protein precipitation with a strong organic acid. Following protein precipitation, the strong acidic character of the supernatant is controlled through ammonia addition. Sample dilution is minimal, and low injection volume is required.

The second method achieved separation of the target compounds on a C_{18} Aqua stationary phase (polar embedded linker of C_{18} to silicagel). Because of additional polar interaction between the spacer and the target compounds, an increased content of organic modifier was used within the mobile phase (60%). The sample preparation procedure was based on plasma protein precipitation through addition of a water-miscible organic solvent.

ESI-MS/MS detection was used in both cases, using specific mass transitions for each of the target compounds. It was observed that both methods were able to separate four additional compounds detected under PTX transitions and two compounds under the M1 transitions. These compounds follow the concentration–time profile more often associated with metabolites. A possible explanation was advanced, relating to conjugation of PTX and M1, followed by their breakdown to the parent compounds within the ion source. Obviously, bioanalytical applications requiring the assay of PTX needed verification of chromatographic selectivity with samples obtained *in vivo*, to overcome the lack of the selectivity of the MS/MS detector for conjugates reforming parent compounds in the ionization source. PTX may interact on storage with residual plasma matrix components, leading to alteration of the peak symmetry. The process is observable through monitoring of the specific PTX transitions as declustering arises within the ion source. These analytical aspects are prone to produce additional troubles in the assay of an active ingredient already known for its highly variable pharmacokinetic characteristics. Difficulty in finding consistent clinical effects of PTX may also be explained by the drug action at least in part through formation of metabolites, the rate and extent of which may significantly vary between individuals.

Both methods were validated according to international regulations. Identical LLOQ values were obtained (1 ng/mL for PTX and 5 ng/mL for M1 and M5). The response functions needed linearization over the investigated concentration intervals, an operation achieved through logarithmation or through $1/x^2$ weighed linear regression. Precision and accuracy fell within the specified limits (± 15%).

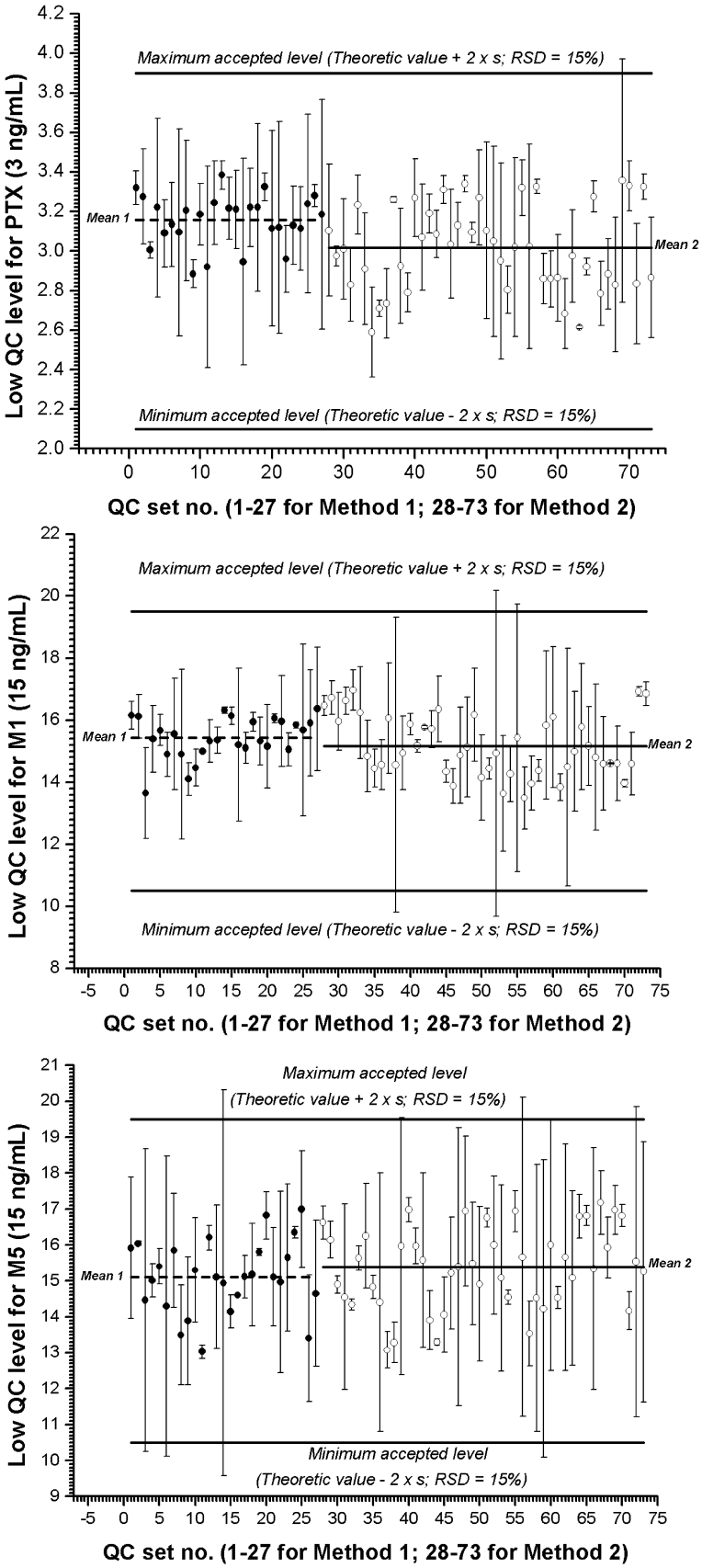

Figure 5. Normal variation intervals resulting from analysis of the low concentration level QC sets processed on completion of both multi- and single-dose clinical trials.

Table 5. Pharmacokinetic parameters for PTX and related metabolites M1 and M5 resulting following the application of bioanalytical methods 1 and 2 on samples obtained on completion of bioequivalence studies (multi- and single-dose approaches)

Analyte	Pharmacokinetic parameter	Measuring unit	Test product		Reference product		90% Confidence interval (%)
			Mean	s	Mean	s	
			Method 1/multidose study				
PTX	c^{SS}_{max}	ng/mL	199.1	112.9	216.0	120.5	85.73–100.72
PTX	c^{SS}_{min}	ng/mL	17.0	11.4	18.7	14.2	86.58–104.84
PTX	AUC_{SS}	ng/mL*h	811.2	463.3	848.9	443.1	85.50–103.01
M1	c^{SS}_{max}	ng/mL	377.7	171.8	365.9	184.2	95.76–119.50
M1	c^{SS}_{min}	ng/mL	57.4	24.8	66.5	37.2	82.06–108.02
M1	AUC_{SS}	ng/mL*h	2094.8	902.2	2150.4	1020.9	89.33–109.78
M5	c^{SS}_{max}	ng/mL	885.5	253.4	1053.5	454.8	90.92–94.59
M5	c^{SS}_{min}	ng/mL	163.5	75.5	190.2	90.1	79.96–102.16
M5	AUC_{SS}	ng/mL*h	5083.5	1484.5	5759.9	1537.2	82.68–92.56
			Method 2/single-dose study				
PTX	c_{max}	ng/mL	155.9	74.4	148.6	97.3	103.11–123.29
PTX	AUC_{last}	ng/mL*h	762.2	407.7	767.1	473.2	95.72–110.94
PTX	AUC_{tot}	ng/mL*h	766.7	407.6	771.4	474.0	95.74–110.83
PTX	t_{max}	h	1.21	0.90	2.04	2.64	—
PTX	t_{half}	h	4.98	0.84	4.92	0.81	—
M1	c_{max}	ng/mL	279.1	148.9	256.3	146.3	104.29–121.85
M1	AUC_{last}	ng/mL*h	1895.7	877.9	2000.7	1176.6	91.7–106.52
M1	AUC_{tot}	ng/mL*h	1927.1	872.7	2027.0	1118.8	92.04–106.59
M1	t_{max}	h	2.71	0.79	3.27	2.66	—
M1	t_{half}	h	6.41	1.74	5.96		—
M5	c_{max}	ng/mL	662.7	195.1	571.5	173.4	111.5–121.69
M5	AUC_{last}	ng/mL*h	4574.3	1133.2	4409.8	1211.7	99.52–109.23
M5	AUC_{tot}	ng/mL*h	4627.8	1132.1	4458.0	1210.5	99.61–109.23
M5	t_{max}	h	1.99	0.69	2.47	1.87	—
M5	t_{half}	h	5.52	0.75	5.43	0.54	—

References

Beermann B, Ings R, Mansby J, Chamberlain J and McDonald A. Kinetics of intravenous and oral pentoxifylline in healthy subjects. *Clinical Pharmacology and Therapeutics* 1985; **37**: 25–28.

Bryce TA, Chamberlain J, Hillbeck D and MacDonald CM. Metabolism and pharmacokinetics of 14C-pentoxifylline in healthy volunteers. *Arzneimittel-Forschung* 1989; **39**: 512–517.

Burrows JL. Determination of pentoxifylline and three metabolites in plasma by capillary gas chromatography using nitrogen-selective detection. *Journal of Chromatography B: Biomedical Sciences and Applications* 1987; **423**: 139–146.

Burrows JL and Jolley KW. Determination of oxpentifylline and four metabolites in plasma and urine by automated capillary gas chromatography using nitrogen-selective detection. *Journal of Chromatography B: Biomedical Sciences and Applications* 1985; **344**: 187–198.

Chmielewska A, Konieczna L, Plenis A and Lamparczyk H. Quantitative determination of pentoxifylline in human plasma. *Acta Chromatographica* 2006; **16**: 70–79.

Dettelbach HR and Aviado DM. Clinical pharmacology of pentoxifylline with special reference to its hemorrheologic effect for the treatment of intermittent claudication. *Journal of Clinical Pharmacology* 1985; **25**: 8–26.

European Medicines Agency. *Guideline of the Investigation of Bioequivalence* (draft), CPMP/EWP/QWP/1401/98 rev. 1, 2008.

Garnier-Moiroux A, Poirier JM, Cheymol G and Jaillon P. High-performance liquid chromatographic determination of pentoxifylline and its hydroxy metabolite in human plasma. *Journal of Chromatography* 1987; **416**: 183–188.

Georgita C, Albu F, David V and Medvedovici A. Non-linear calibrations on the assay of diltiazem and two of its metabolites from plasma samples by means of liquid chromatography and ESI/MS^2 detection: application to a bioequivalence study. *Biomedical Chromatography* 2008; **22**: 289–297.

Kyle PB, Adcock KG, Kramer RE and Baker RC. Use of liquid chromatography–tandem mass spectrometry for the analysis of pentoxifylline and lisofylline in plasma. *Biomedical Chromatography* 2005; **19**: 231–236.

Magnusson M, Bergstrand IC, Bjorkman S, Heijl A, Roth B and Hoglund P. A placebo-controlled study of retinal blood flow changes by pentoxifylline and metabolites in humans. *British Journal of Clinical Pharmacology* 2006; **61**: 138–147.

Mancinelli A, Pace S, Marzo A, Arrigoni Martelli E and Passetti G. Determination of pentoxifylline and its metabolites in human plasma by high-performance liquid chromatography with solid-phase extraction. *Journal of Chromatography B: Biomedical Sciences and Applications* 1992; **575**: 101–107.

Musch G, Hamoir T and Massart DL. Determination of pentoxifylline and its 5-hydroxy metabolite in human plasma by solid-phase extraction and high-performance liquid chromatography with ultraviolet detection. *Journal of Chromatography B: Biomedical Sciences and Applications* 1989; **495**: 215–226.

Nicklasson M, Bjorkman S, Roth B, Jonsson M and Hoglund P. Stereoselective metabolism of pentoxifylline in vitro and in vivo in humans. *Chirality* 2002; **14**: 643–652.

Peterson TC. Inhibition of fibroproliferation by pentoxifylline—activity of metabolite-1 and lack of role of adenosine receptors. *Biochemical Pharmacology* 1996; **52**: 597–602.

Pokrajac M, Miljkovic B, Simic D, Brzakovic B and Galetin A. Pharmacokinetics and bioavailability of pentoxifylline in healthy volunteers—a comparative study of three oral formulations. *European Journal of Pharmaceutics and Biopharmaceutics* 1997; **43**: 193–196.

Rocci ML, Luke DR and Saccar CL. Pharmacokinetics of pentoxifylline during concomitant theophylline administration to rats. *Pharmaceutical Research* 1987; **4**: 433–435.

Smith RV, Waller ES, Doluisio JT, Bauza MT, Puri SK, Ho I and Lassman HB. Pharmacokinetics of orally administered pentoxifylline in humans. *Journal of Pharmaceutical Sciences* 1986; **75**: 47–52.
Srinivas NR. Applicability of nonlinear calibration regression for quantitative determination of parent and metabolite(s) in bioequivalence assessment. *Biomedical Chromatography* 2008; **22**: 1315–1317.
Srinivas NR. Considerations for metabolite pharmacokinetic data in bioavailability.bioequivalence assessments. Overview of the recent trends. *Arzniemittel-Forschung/Drug Research* 2009; **59**: 155–165.
Srinivasu P, Rambhau D, Rao BR and Rao YM. Pharmacokinetics of pentoxifylline after oral administration of a sustained release tablet at two different times of the day. *Arzneimittel-Forschung* 1999; **48**: 750–753.
US Department of Health and Human Services, FDA, CDER and CVM. 2001. *Guidance for Industry. Bioanalytical Method Validation*, 2001.
Ward A and Clissold SP. Pentoxifylline. A review of its pharmacodynamic and pharmacokinetic properties and its therapeutic efficacy. *Drugs* 1987; **34**: 50–97.

