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Determination of pentoxifylline and its metabolites in human plasma by high-performance liquid chromatography with solid-phase extraction

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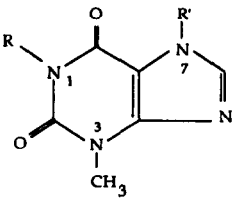
ABSTRACT

A simple and reliable method for the determination of pentoxifylline and its main metabolites in human plasma has been developed using high-performance liquid chromatography. After selective solid-phase extraction, pentoxifylline, its metabolites and an internal standard, 7-(2'-chloroethyl)theophylline, were separated on a 5-μm LiChrospher 100 RP-18 column using water–dioxan–acetonitrile (87:6.5:6.5, v/v/v) acidified with acetic acid (0.5%, v/v) as the mobile phase. The analytes were detected at 275 nm. The lowest detectable concentration for all analytes was 25 ng/ml; the recovery was 85%. The assay has been successfully applied to analysis of these compounds in human plasma after administration of an oral dose of 400 mg of pentoxifylline to healthy volunteers.

INTRODUCTION

Pentoxifylline, 1-(5-oxohexyl)-3,7-dimethylxanthine (PXF), is an active haemorheological drug widely used for the treatment of intermittent claudication and other circulatory disorders [1–4]. The drug improves perfusion in the impaired microcirculation of peripheral and cerebral vascular beds, so it has also been tried as a therapy for cerebrovascular disorders [5–7]. From biochemical investigations PXF appears to be a cyclic nucleotide phosphodiesterase inhibitor [8] and an adenosine receptor antagonist [9]. Recently, the drug was tested in human beings after injection of *Salmonella abortus equi* endotoxin with beneficial effects [10].

Pharmacokinetic and metabolic studies in man and animals show that PXF undergoes extensive



	R	R'
PXF	$CH_2(CH_2)_3COCH_3$	CH_3
M-I	$CH_2(CH_2)_3CHOHCH_3$	CH_3
M-IV	$CH_2(CH_2)_3COOH$	CH_3
M-V	$CH_2(CH_2)_2COOH$	CH_3
I.S.	CH_3	CH_2CH_2Cl

Fig. 1. Structures of pentoxifylline (PXF), its metabolites (M-V, M-IV, M-I) and the internal standard (I.S.).

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metabolism [11,12]. The major metabolites of PXF in man are 3,7-dimethyl-1-(5′-hydroxyhexyl)xanthine (M-I), formed by carbonyl reduction, and two carboxylate metabolites, 1-(3′-carboxypropyl)-3,7-dimethylxanthine (M-IV) and 1-(4′-carboxybutyl)-3,7-dimethylxanthine (M-V), formed by an oxidation process (Fig. 1).

PXF and its metabolites have been assayed in biological fluids by thin-layer chromatography [13], gas chromatography (GC) [14–16] and high-performance liquid chromatography (HPLC) [17–24].

GC procedures involve the use of a nitrogen-selective detector and trifluoroacetyl derivatization to quantify PXF and metabolites. Previously described HPLC methods involve a time-consuming extraction procedure, employ not easily available internal standard and do not allow all of the above three metabolites to be detected.

This paper describes an HPLC method for the simultaneous determination of PXF and its metabolites, M-I, M-IV and M-V, using 7-(2′-chloroethyl)theophylline as internal standard (I.S.), validated for clinical pharmacokinetic studies.

EXPERIMENTAL

Chemicals

All solvents and reagents were of analytical or HPLC grade. Solid-phase extraction (SPE) cartridges containing reversed-phase octadecylsilane-bonded silica sorbent (C_{18}, 0.1 g in a 1-ml tube) were purchased from J. T. Baker (Phillipsburg, NJ, USA). The Viseprep SPE vacuum manifold, which can accept twelve SPE cartridges simultaneously, was purchased from Supelco (Bellefonte, PA, USA). PXF metabolites (M-I, M-IV and M-V) were synthesized in Real Lab. (Villaguardia, Como, Italy); the chemical identity of these metabolites was tested by NMR. 7-(2′-Chloroethyl)theophylline used as I.S. was obtained from Aldrich (Milan, Italy). PXF was supplied by Sigma (St. Louis, MO, USA). Separate standard solutions of PXF, its metabolites and the I.S. were prepared by dissolving weighed amounts in acetonitrile–water (22:78, v/v) and were stable for at least 1 month at 4°C.

Chromatography

The chromatographic system consisted of a Model L-6200 pump and Model AS-4000 autosampler equipped with a 20-μl loop (Merck-Hitachi, Darmstadt, Germany) and a Model SPD-6A variable-wavelength UV detector at 275 nm (Shimadzu, Kyoto, Japan). Separation was carried out on a 125 mm × 40 mm I.D. LiChrospher 100 RP-18, 5 μm particle size column (Merck, Darmstadt, Germany) at room temperature. The mobile phase consisted of water–dioxan–acetonitrile (87:6.5:6.5, v/v/v) acidified to pH 3.0 with glacial acetic acid (0.5%, v/v). The flow-rate was set at 1.0 ml/min. The output of the UV detector was fed into the chromatography data system consisting of the D-6000 interface (Merck-Hitachi), a Compaq 386 personal computer and an FX-850 printer (Epson). The raw data of each chromatogram were stored, and peak heights of analytes were calculated.

Sample preparation

A 0.5-ml volume of plasma was transferred into a disposable conical centrifuge test tube to which 0.1 ml of 0.2 *M* hydrochloric acid and 0.4 ml of phosphate buffer (0.06 *M*, pH 5.0) were added, giving a final pH of 5.3. After centrifugation (10 000 *g*, 3 min, room temperature) the supernatant was loaded on a SPE cartridge previously conditioned sequentially with 1.0 ml of acetonitrile and 1.0 ml of phosphate buffer (pH 5.0). Then, the column was rinsed twice with 1 ml of phosphate buffer (0.06 *M*, pH 5.0), dried and eluted three times with 0.2 ml of acetonitrile. The eluate was evaporated under a gentle stream of air (37°C) and reconstituted with vortex-mixing in 0.1 ml of mobile phase for HPLC analysis. A schematic and detailed SPE procedure is described in Fig. 2.

Recovery and calibration curves

For recovery and calibration curves, fresh-frozen human plasma received from a blood bank (AVIS, Rome, Italy) was used.

The percentage recovery of PXF and its metabolites from the above plasma was assessed by comparing the peak-height ratio (drug/I.S.) ob-

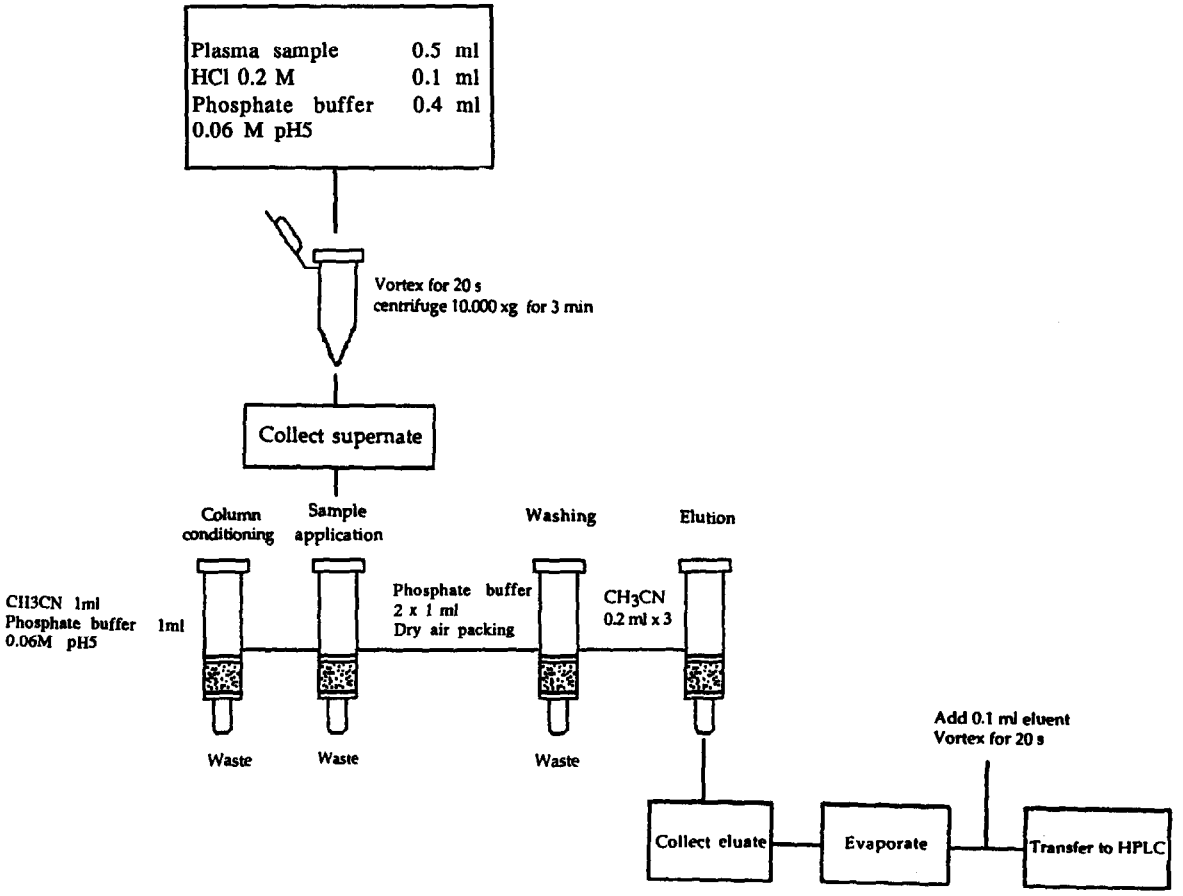

Fig. 2. Flow chart of the extraction procedure.

tained after extracting known amounts of analyte (50–1000 ng/ml range) with that obtained when identical amounts of the working standard were dispensed without extraction. Calibration curves were constructed by adding known amounts (50–1000 ng/ml) of PXF and metabolites, with the I.S. at a fixed concentration of 300 ng/ml, to the above plasma. Values of the peak-height ratio (drug/I.S.) for PXF and metabolites were plotted in calibration graphs, in order to calculate the drug concentration in the sample.

Preliminary bioavailability investigation

Two healthy male volunteers weighting 65 kg were fasted overnight before drug administration; water was available *ad libitum* throughout this period. PXF (Trental, Albert Farma, L'Aquila, Italy) in a sustained-release formulation (400 mg) was administered with a glass of water. Blood samples (10 ml) were withdrawn into heparinized tubes prior to dosing (time 0) and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 h. Blood was centrifuged immediately and the resulting plasma was stored at −20°C until the assay.

RESULTS AND DISCUSSION

Several HPLC procedures have been described for the assay of PXF and its metabolites in biological fluids. Most of these methods deal with the measurement of PXF and its hydroxy metabolite (M-1) [18,19,23,25] and fail to detect the carboxylate metabolites (M-V and M-IV). Only one paper describes the assay of PXF and M-I, M-IV

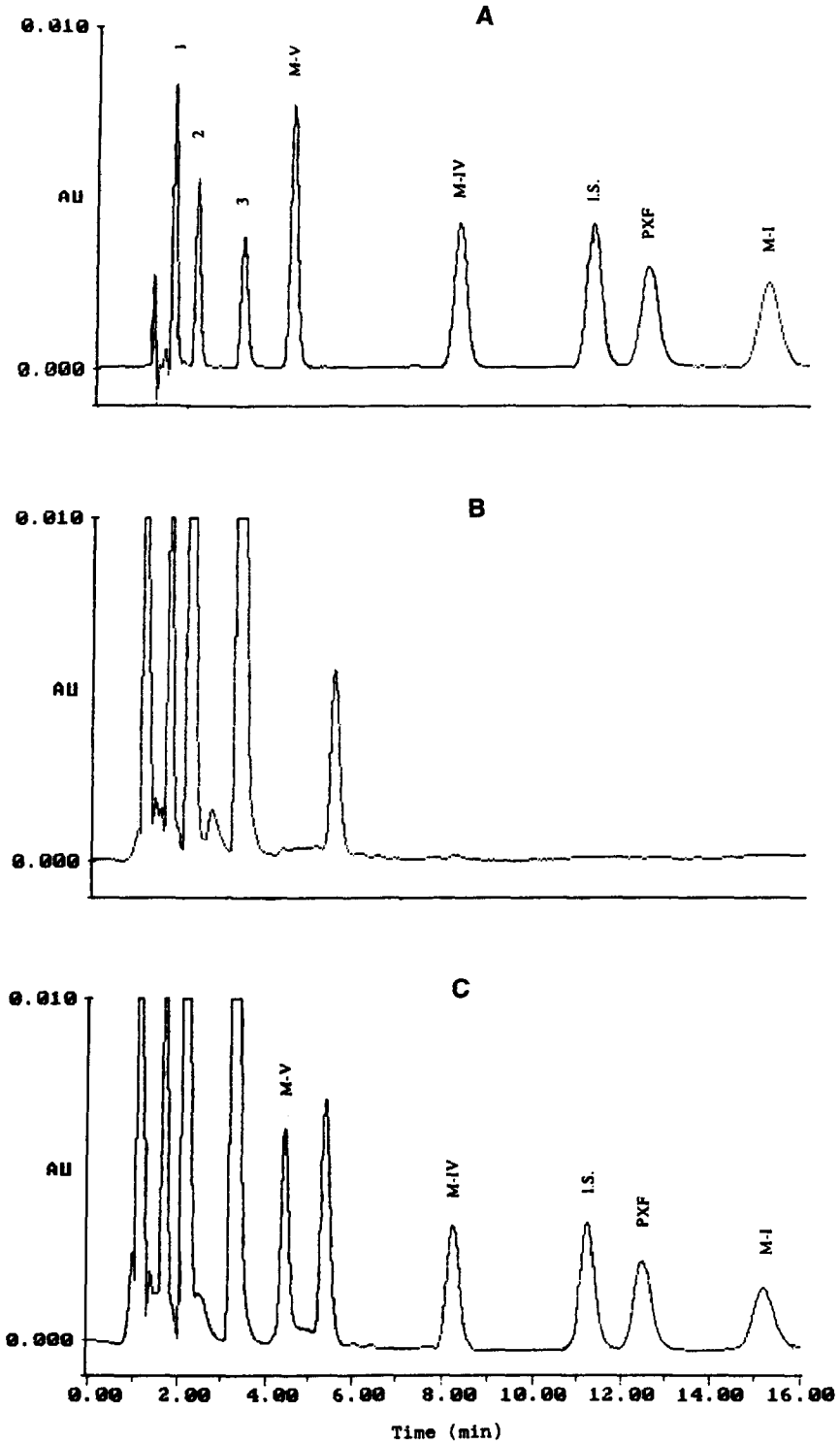

Fig. 3. Typical HPLC chromatograms of (A) analytes as authentic standards including theobromine (1), theophylline (2) and caffeine (3), (B) drug-free plasma after SPE and (C) analytes (250 ng/ml) and I.S. (300 ng/ml) added to drug-free plasma and processed according to SPE.

and M-V metabolites in human plasma [24]. This procedure however, requires a liquid–liquid extraction and an internal standard which is not readily available. In this paper an HPLC method for the measurement of PXF and its main metabolites is described using as a readily available internal standard, 7-(2'-chloroethyl)theophylline. The method proved to afford good selectivity in that no peak interfering with the analytes was detected. In addition, some structurally related substances such as caffeine, theophylline and theobromine do not interfere. Fig. 3 depicts typical chromatograms from (A) standard solution including caffeine, theophylline and theobromine, (B) drug-free plasma after SPE and (C) plasma spiked with analytes examined (250 ng/ml) and I.S. (300 ng/ml) after SPE. M-V was eluted between two endogenous peaks, with a good resolution. The SPE procedure described in this paper allows PXF, M-V, M-IV and M-I to be quantified in a single run. The recovery of all the analytes tested was 93.2, 85.6, 85.6, 74.9 and 90.2% for PXF, M-V, M-IV, M-I and I.S., respectively (Table I). When cartridges were conditioned with

TABLE I

EXTRACTION RECOVERY OF PENTOXIFYLLINE (PXF), ITS METABOLITES, M-V, M-IV, M-I, AND INTERNAL STANDARD (I.S.) FROM HUMAN PLASMA

Each value is the mean ± S.D. of five to six determinations.

Compound	Concentration (ng/ml)	Recovery (%)	Coefficient of variation (%)
PXF	50	102.0 ± 6.7	6.6
	100	87.8 ± 3.2	3.6
	250	91.2 ± 3.1	3.4
	500	91.0 ± 3.8	4.2
	1000	94.2 ± 2.5	2.6
Mean ± S.D.		93.2 ± 5.4	
C.V. (%)		5.8	
M-V	50	85.4 ± 5.7	6.7
	100	77.2 ± 5.1	6.6
	250	88.3 ± 5.3	6.0
	500	85.0 ± 3.5	4.1
	1000	92.0 ± 4.0	4.3
Mean ± S.D.		85.6 ± 5.5	
C.V. (%)		6.4	
M-IV	50	92.6 ± 7.3	7.9
	100	73.0 ± 4.2	5.7
	250	80.3 ± 4.3	5.3
	500	86.2 ± 4.6	5.3
	1000	95.8 ± 2.2	2.3
Mean ± S.D.		85.6 ± 9.2	
C.V. (%)		10.8	
M-I	50	77.2 ± 2.7	3.5
	100	80.0 ± 5.5	6.9
	250	76.5 ± 3.4	4.4
	500	73.7 ± 6.2	8.4
	1000	67.3 ± 9.2	13.7
Mean ± S.D.		74.9 ± 4.8	
C.V. (%)		6.4	
I.S.	300	90.2 ± 2.3	2.5

TABLE II

ACCURACY FOR PXF AND ITS METABOLITES

Values are mean ± S.D., $n = 5$.

Compound	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	Error (%)
PXF	100	96.3 ± 9.1	9.4	−3.7
M-V	100	103.1 ± 7.1	6.8	3.1
M-IV	100	93.8 ± 5.4	5.7	−6.2
M-I	100	103.9 ± 9.6	9.3	3.9

acetonitrile (1 ml) recovery of carboxylated metabolites was negligible.

The accuracy of the method was also determined (Table II). Mean errors, at 100 ng/ml, ranged from -6.2% to 3.9% for all analytes tested. Similar results were observed at lower concentrations.

Good linear relationships between peak-height ratio and drug concentration in the range 50–1000 ng/ml were found for all the analytes. The parameters of linear regression are listed in Table III. The coefficient of variation (C.V.) of slope data varied from 3.6% to 9.8%, indicating good inter-day reproducibility (Table III). Analysis of variance (ANOVA) was used to confirm the linearity of calibration curves.

The lowest detectable concentration, determined by decreasing the concentration of all the analytes until reaching a signal-to-noise ratio higher than 3, was 25 ng/ml, with C.V. of 9.67, 4.00, 9.79 and 10.0% for M-V, M-IV, PXF and M-I, respectively.

The described method was successfully applied to the analysis of PXF and metabolites in plasma samples from two healthy male volunteers after an oral dose of PXF (400 mg) (Fig. 4). Peak plasma concentrations of PXF and metabolites ranged from 0.5 to 2 h after dosing; thereafter PXF and M-IV remained at about 50–100 ng/ml until the last time interval tested (12 h). M-IV was not detectable in subject B. In both volunteers M-V and M-I appeared rapidly (0.5 h) and largely exceeded PXF concentrations at all the sampling intervals examined. Data from this study are in agreement with those previously reported by other authors [15,24,25].

TABLE III

LINEARITY OF PENTOXIFYLLINE (PXF) AND ITS METABOLITES ASSAY IN HUMAN PLASMA OBTAINED WITH THE LINEAR REGRESSION METHOD

Values are mean ± S.D.; $n = 4$. Values in parentheses are coefficients of variation (%).

Compound	Concentration range (ng/ml)	Slope	Intercept	Correlation coefficient
PXF	50–1000	0.0055 ± 0.0002 (3.6)	−0.0133 ± 0.0312	0.9987 ± 0.0005
M-V	50–1000	0.0132 ± 0.0008 (6.1)	−0.0657 ± 0.1168	0.9975 ± 0.0024
M-IV	50–1000	0.0076 ± 0.0005 (6.6)	0.0070 ± 0.0580	0.9987 ± 0.0005
M-I	50–1000	0.0027 ± 0.0004 (9.8)	0.0007 ± 0.0390	0.9967 ± 0.0021

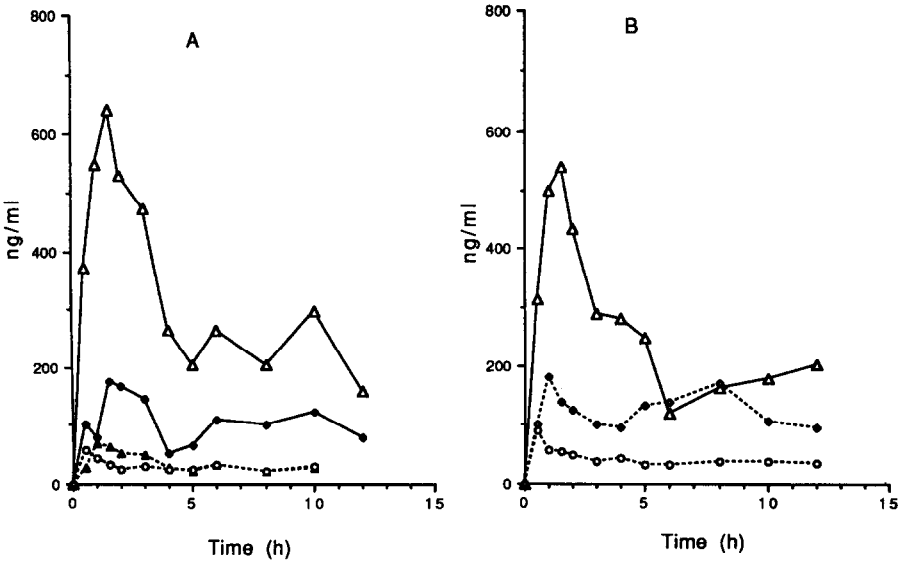

Fig. 4. Plasma concentration–time profiles of pentoxifylline (○) and its metabolites M-I (◇), M-IV (▲) and M-V (△) in two healthy volunteers (A and B) after a 400-mg oral dose of PXF.

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