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Note

High-performance liquid chromatographic analysis of pentoxifylline and 1-(5′-hydroxyhexyl)-3,7-dimethylxanthine in whole blood

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Pentoxifylline is a hemorrheologic agent currently being used in the therapeutic management of intermittent claudication. Pentoxifylline improves blood flow through the peripheral circulation by decreasing blood viscosity, inhibiting platelet aggregation, enhancing erythrocyte flexibility and diminishing fibrinogen concentration [1]. Pentoxifylline's disposition is characterized in part by erythrocyte metabolism to 1-(5′-hydroxyhexyl)-3,7-dimethylxanthine (metabolite I), which may possess pharmacologic activity. The systemic clearance of pentoxifylline greatly exceeds hepatic blood flow [2,3]; erythrocyte metabolism may contribute substantially to the total systemic clearance of pentoxifylline. In order to fully characterize the dispositional fate of pentoxifylline as well as the red blood cell distribution of this drug and its metabolite, an assay capable of measuring these substances in whole blood is needed.

Analytical methods for the quantitation of pentoxifylline and metabolite I in biological fluids have employed thin-layer chromatography and colorimetry [4], gas chromatography (GC) [5–7] and high-performance liquid chromatography (HPLC) [8–11]. While these techniques appear to be sensitive and selective, they are complicated by a number of disadvantages including: (a) a sample volume of at least 1 ml [4–10]; (b) the use of internal standards which are either not commercially available [5–9] or specified [4,10]; and (c) lengthy and cumbersome extraction procedures [10] and/or derivatizations [5–7]. Furthermore, only one of the procedures is applicable to whole blood samples [5].

The purposes of this paper are to describe a simple, sensitive and selective assay for the quantification of pentoxifylline and one of its major metabolites 1-(5′-

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hydroxyhexyl)-3,7-dimethylxanthine in whole human blood by HPLC, and to examine its applicability to laboratory animal research. The small sample size (250 μl), ease of extraction and low limits of detection (approximately 5 ng/ml) enables this assay to be employed for the analysis of clinical and research samples.

EXPERIMENTAL

Reagents

Pentoxifylline and 1-(5'-hydroxyhexyl)-3,7-dimethylxanthine (metabolite I) were supplied by Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.). The purity of these compounds was determined by differential scanning calorimetry to be 99.88 and 98.92%, respectively. The internal standard, 3-isobutyl-1-methylxanthine, was purchased from Sigma (St. Louis, MO, U.S.A.). These compounds all produced single peaks when subjected to the HPLC system described below.

The solvents employed in this assay were HPLC grade. The water used in this procedure was deionized using the Milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.). Whole blood extracts were filtered through 0.45-μm polytetrafluoroethylene (PTFE) filter pads (Acrodisc CR©) purchased from Gelman Sciences (Ann Arbor, MI, U.S.A.).

Standard preparations

Solutions of pentoxifylline and metabolite I (1.00 mg/ml) were prepared by weighing 10.00 mg of the powders in 10-ml volumetric flasks and diluting the compounds to volume with HPLC-grade water. Equal volumes of these solutions were then combined to produce a standard stock solution of 500 μg/ml. This solution was further diluted to 50.0 μg/ml and to 5.0 μg/ml for use in the preparation of high and low standards. The stability of these compounds in distilled water has been previously confirmed [8].

The internal standard, 3-isobutyl-1-methylxanthine, was diluted in methanol to a concentration of 100 μg/ml. This was further diluted to 2.0 μg/ml; 35 μl of this solution were added to each sample as an internal standard. All solutions were stored at −70°C and were not used for periods of time exceeding three months.

Sample preparation

Whole blood samples (250 μl) were collected and immediately placed into 5-ml polypropylene tubes containing 4 ml of acetonitrile. The tubes were vigorously vortexed for 30 s and were then centrifuged at 3000 *g* for 10 min. A portion of the supernatant (3.7 ml) was precisely transferred to a similar tube and stored at −70°C until analysis.

At the time of analysis, internal standard (35 μl of a 2.0 μg/ml solution) was added to each thawed sample. The samples were vortexed for 10 s and were then filtered through 0.45-μm PTFE filter pads into 14-ml conical shaped tubes, and evaporated to dryness under a stream of nitrogen at 47°C.

Hexane (2.0 ml) was added to each tube and vortexed for 15 s. This step was

followed by the addition of water (300 μl) and a 30-s vortex. The samples were then centrifuged for 15 min at 1250 *g*. A 250-μl aliquot of the aqueous phase was injected for chromatographic analysis.

Chromatography

The HPLC system consisted of a Model 510 pump, a Model U6K injector and a Model 481 variable-wavelength UV detector set at 280 nm (Waters Assoc., Milford, MA, U.S.A.). The system was equipped with a LC-8-DB reversed-phase column (Supelco, Bellefonte, PA, U.S.A.). The column dimensions were 25 cm×4.6 mm I.D. with 5-μm particles. All chromatography was performed at ambient temperature. The mobile phase consisted of acetonitrile–water (24:76) at a flow-rate of 1.0 ml/min. Degassing of mobile phase was achieved by filtering through a 0.4-μm polycarbonate membrane filter, supplied by Nucleopore (Pleasanton, CA, U.S.A.).

Assay recovery

The percentage recovery of pentoxifylline and metabolite I was assessed at concentrations of 100 and 1000 ng/ml. The procedure consisted of extracting and injecting ten samples (250 μl) containing each compound and the internal standard. Six injections of the same amounts of these compounds in water were injected directly. The peak heights of all compounds at the two concentrations were measured. The recovery of each agent was computed using the following equation:

$$\text{recovery (\%)} = \frac{\text{peak height extract}}{\text{mean peak height of direct injections}} \times 100.$$

RESULTS AND DISCUSSION

Typical chromatograms of a blank sample of human whole blood and an extracted sample containing pentoxifylline and metabolite I (500 ng/ml) with internal standard are illustrated in Fig. 1A and B. The small peak present in the blank chromatogram, at a retention time which is similar to that of the internal standard, is a consistent finding in chromatograms of blank whole blood extracts. This peak, however, represents only 2% of the total internal standard peak height and does not appear to compromise the analysis. Retention times for metabolite I, pentoxifylline and the internal standard are 5.4, 6.4 and 7.6 min, respectively. The standard curves for pentoxifylline and metabolite I are linear over the 0–1000 ng/ml range. The assay appears to be equally sensitive for pentoxifylline and metabolite I based upon the similarities in the slopes of the standard curves for each compound.

The results of the assay recovery experiment are outlined in Table I. The assay recovery of pentoxifylline and metabolite I was independent of concentration and averaged 96.5 and 93.5%, respectively. The internal standard was extracted to a similar extent, i.e. 106%. The detection limits of the assay were approximately 5 ng/ml, based on a signal-to-noise ratio of 2.5:1.

Selected statistics which describe the variability of the assay are presented in

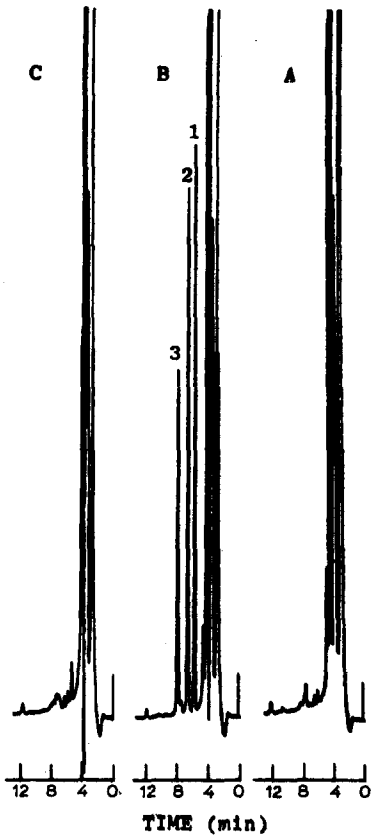

Fig. 1. Chromatograms of (A) blank extracted human whole blood; (B) extracted human whole blood spiked with pentoxifylline and metabolite I (500 ng/ml) and internal standard (35 μl of 2 μg/ml 3-isobutyl-1-methylxanthine); and (C) blank extracted rat whole blood. Peaks: 1 = metabolite I; 2 = pentoxifylline; 3 = 3-isobutyl-1-methylxanthine, the internal standard.

Table II. These values are based on the analysis of six samples of each concentration on three consecutive days. Intra- and inter-day precisions are characterized by relative standard deviations (R.S.D.) of less than 5% for both pentoxifylline and metabolite I at all concentrations. In addition, accuracy is within 2% for each compound.

The selectivity of the assay was examined by injecting a number of related or commonly administered substances into the chromatograph to see if any compounds produced interfering peaks. The compounds injected and their respective retention times are illustrated in Table III. It is evident from the retention time values that interference would not be expected from any of the compounds tested. Pentobarbital and 5-*sec.*-butyl-5-ethyl-2-thiobarbituric acid are substances used as anesthetics in laboratory animals. In our experience, these compounds are extracted from blood by our method and produce large broad peaks on the chromatogram. As a result, barbiturates should not be used as anesthetics when employing this assay in animal studies.

TABLE I

EXTRACTION RECOVERIES OF PENTOXIFYLLINE AND METABOLITE I FROM HUMAN WHOLE BLOOD AT TWO CONCENTRATIONS AND DETECTION LIMITS OF THE ASSAY

Drug	Recovery (mean ± S.D., $n=12$) (%)		Mean recovery★ (%)	Detection limit★★ (ng/ml)
	100 ng/ml	1000 ng/ml		
Pentoxifylline	94 ± 2.3	99 ± 2.7	96.5	5.4
Metabolite I	95 ± 2.1	92 ± 4.6	93.5	5.1
3-Isobutyl-1-methylxanthine	106 ± 2.5★★★		106	

★The mean recovery from rat whole blood was 72, 74 and 81% for pentoxifylline, metabolite I and 3-isobutyl-1-methylxanthine, respectively. These recoveries were concentration-independent when tested at 100 and 1000 ng/ml.

★★Based on a signal-to-noise ratio of 2.5:1.

★★★Based on direct injection of 35 μl of a 2 μg/ml internal standard solution.

The ability to employ this assay in animal species is of importance in our laboratories. Fig. 1C illustrates a chromatogram resulting from the analysis of a blank blood sample from a rat. While a number of unknown substances elute off of the column at times similar to the compounds of interest, they do not appreciably hamper accurate quantitation of pentoxifylline and metabolite I. Table II contains the accuracy and precision data for the quantitation of pentoxifylline

TABLE II

ASSAY VARIABILITY FOR PENTOXIFYLLINE AND METABOLITE I IN HUMAN AND RAT WHOLE BLOOD AT LOW AND HIGH CONCENTRATIONS

Species	Nominal concentration (ng/ml)	Drug	Mean ★ concentration found (ng/ml)	Accuracy (%)	Precision			
					Intra-day		Inter-day	
					S.D. (ng/ml)	R.S.D. (%)	S.D. (ng/ml)	R.S.D. (%)
Human★★	50	Pentoxifylline	50.9	+1.8	0.42	0.82	2.43	4.78
		Metabolite I	49.5	−1.0	0.65	1.32	2.28	4.61
	750	Pentoxifylline	737	−1.7	8.5	1.16	12.7	1.72
		Metabolite I	745	−0.67	7.8	1.05	2.08	0.28
Rat★★★	100	Pentoxifylline	106	+6.0	8.2	7.7		
		Metabolite I	98.7	−1.3	8.7	8.8		
	1000	Pentoxifylline	1022	+2.2	12	1.2		
		Metabolite I	1054	+5.4	32	3.0		

★The means for the human data were derived by pooling all measurements, i.e. $n=18$. The means for the rat data were derived from a single run with $n=6$ and $n=5$ for 100 ng/ml and 1000 ng/ml concentrations, respectively.

★★All variability statistics for human whole blood samples are based on six measurements daily for three consecutive days.

★★★Assessment of the ability of the assay to accurately quantitate concentrations of pentoxifylline and metabolite I in rat whole blood from a standard curve prepared in human whole blood.

TABLE III

RETENTION TIMES OF SELECTED COMPOUNDS

Compound	Retention time (min)
Theophylline-7-acetic acid	1.4
Uric acid	2.0
1-Methyluric acid	2.4
3-Methyluric acid	2.4
Xanthine	2.6
1,3-Dimethyluric acid	2.6
8-Chlorotheophylline	2.6
Hypoxanthine	2.8
1-Methylxanthine	3.0
3-Methylxanthine	3.0
7-Methylxanthine	3.0
3,7-Dimethylxanthine (theobromine)	3.0
Dyphylline	3.0
7-(β-Hydroxyethyl)theophylline	3.2
1,3-Dimethylxanthine (theophylline)	3.4
7-(Piperidinomethyl)theophylline	3.4
7-(β-Hydroxypropyl)theophylline	3.6
1,3,7-Trimethylxanthine (caffeine)	3.8
Pentobarbital	9.0
Antipyrine	10.8
Phenacetin	12.8
8-Phenyltheophylline	16.0
5-*sec.*-Butyl-5-ethyl-2-thiobarbituric acid	32.0
1-Hexyl-3,7-dimethylxanthine (pentifylline)	77.0

and metabolite I in rat whole blood from a standard curve prepared in human whole blood. The values are based on the analysis of rat whole blood samples with pentoxifylline and metabolite I added to produce concentrations of 100 ng/ml ($n=6$) and 1000 ng/ml ($n=5$). Evident from the results is the ability of our assay to predict whole blood concentrations within 6% of the nominal concentrations in a consistent manner. In addition, the extraction efficiencies from rat blood are fairly reproducible with R.S.D. values of 6.1, 6.2 and 2.4% for pentoxifylline, metabolite I and 3-isobutyl-1-methylxanthine, respectively.

The application of this assay in the analysis of samples obtained from an incubation (37°C) of pentoxifylline at two different concentrations (250 and 500 ng/ml) in rat whole blood is presented in Fig. 2. The graph illustrates the concomitant metabolic generation of metabolite I and decline in pentoxifylline concentrations with time. Evident from the plot is the ability of the assay to accurately predict initial concentrations [528 versus 500 and 246 versus 250 ng/ml (percentage accuracy 5.6% and −0.8%, respectively)] of pentoxifylline, as well as a 1:1 stoichiometric production of metabolite I from pentoxifylline.

To our knowledge, only one other assay exists in the literature which will quantitate pentoxifylline and metabolite I in whole blood [5]. The method is a GC assay which is not as facile and widely applicable as the method described herein

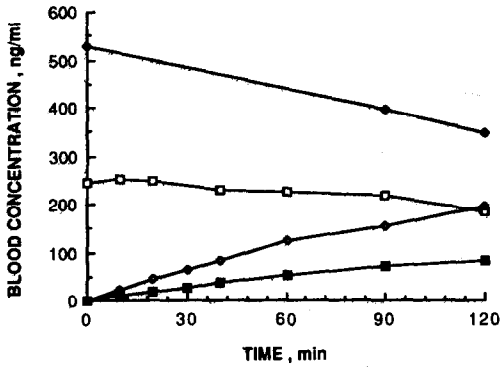

Fig. 2. Blood concentration of pentoxifylline (◆,□) and metabolite I (◇,■) in whole blood incubations with initial pentoxifylline concentrations of 500 and 250 ng/ml, respectively.

since it (a) requires a sample volume of 2 ml, (b) uses an internal standard which is not commercially available and (c) requires sample derivatization prior to analysis.

In summary, the assay described provides a simple, sensitive and selective means of analyzing human and rat whole blood for pentoxifylline and metabolite I. It employs an inexpensive, commercially available internal standard, 3-isobutyl-1-methylxanthine, and can be employed in the presence of congeners such as caffeine and theophylline without a threat of interference from the parent compounds nor any of their metabolites. The small sample size (250 μl) required and its applicability to rat whole blood make this assay ideal for both clinical and animal samples.

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REFERENCES

1 D.E. Baker and R.K. Campbell, Drug Intell. Clin. Pharm., 19 (1985) 345.
2 R.M.J. Ings, F. Nudemberg, J.L. Burrows and T.A. Bryce, Eur. J. Clin. Pharmacol., 23 (1982) 539.
3 B. Beermann, R. Ings, J. Mansby, J. Chamberlain and A. McDonald, Clin. Pharmacol. Ther., 37 (1985) 25.
4 K. Fujimoto, S. Yoshida, Y. Moriyama and T. Sakaguchi, Chem. Pharm. Bull., 6 (1976) 1137.
5 T.A. Bryce and J.L. Burrows, J. Chromatogr., 181 (1980) 355.
6 M.T. Bauza, R.V. Smith, D.E. Knutson and F.R. Witter, J. Chromatogr., 310 (1984) 61.
7 J.L. Burrows and K.W. Jolley, J. Chromatogr., 344 (1985) 187.
8 D.A. Chivers, D.J. Birkett and J.O. Miners, J. Chromatogr., 225 (1981) 261.
9 R.V. Smith, S.-K. Yang, P.J. Davis and M.T. Bauza, J. Chromatogr., 281 (1983) 281.
10 W. Rieck and D. Platt, J. Chromatogr., 305 (1984) 419.
11 D.R. Luke and M.L. Rocci, Jr., J. Chromatogr., 374 (1986) 191.