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Note

Determination of pentoxifylline and a major metabolite, 3,7-dimethyl-1-(5'-hydroxyhexyl)xanthine, by high-performance liquid chromatography

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Pentoxifylline is a new hemorrheologic agent used in the treatment of intermittent claudication and other vascular disorders [1—4]. The currently available high-performance liquid chromatographic (HPLC) techniques for pentoxifylline quantitation in biologic fluids are complicated by the use of microbial extracts [5], lengthy and confusing extraction procedures [6], and internal standards not routinely available [7]. Furthermore, all of these techniques use at least 1 ml of plasma.

The purpose of the present paper is to describe an assay which has been used to determine the concentrations of pentoxifylline and one of its major metabolites in rat plasma by HPLC. This procedure is simple, sensitive, requiring small volumes of plasma, and is capable of quantitating pentoxifylline and its metabolite in concentrations ranging from 25 to 1000 ng/ml.

EXPERIMENTAL

Reagents and standards

Pure samples of pentoxifylline and its metabolite, 3,7-dimethyl-1-(5'-hydroxyhexyl)xanthine (metabolite I), were supplied by Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.). The internal standard, acetophenetidin (phenacetin), was supplied by Sigma (St. Louis, MO, U.S.A.).

The water used in this procedure was deionized using the Milli-Q® Reagent

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Water system (Millipore, Bedford, MA, U.S.A.). Acetonitrile was HPLC-grade. Extracts were filtered using polytetrafluoroethylene filter pads (Millipore).

A stock solution (500 μg/ml) was prepared by dissolving 10 mg each of pentoxifylline and metabolite I in 20 ml of 24% acetonitrile in water. Plasma standards were then prepared by diluting appropriate volumes of the stock solution in drug-free plasma to give final concentrations of pentoxifylline and metabolite I of 25, 75, 100, 250, 500 and 1000 ng/ml. The internal standard solution (phenacetin, 20 μg/ml) was prepared in 24% acetonitrile in water. All solutions were stored at − 70°C.

Chromatography

The high-performance liquid chromatograph consisted of a Model 510 pump, a Model U6K injector and a Model 440 fixed-wavelength absorbance detector using a 280-nm filter.

The chromatograph was equipped with a Waters Assoc. (Milford, MA, U.S.A.) reversed-phase μBondapak® C_{18} column (5 μm particle size) operating at ambient temperature. The mobile phase consisted of 24% acetonitrile in water (sparged with helium), at a flow-rate of 1.0 ml/min.

Sample preparation

Following collection, whole-blood samples were immediately centrifuged to avoid in vitro red blood cell metabolism. The plasma was then harvested and stored at −70°C until analysis.

To 250 μl of plasma contained in a 10-ml polypropylene tube, 20 μl of internal standard solution were added. The combination was vortexed for 15 s. Acetonitrile (2.5 ml) was then added, the mixture vortexed for 15 s, and then centrifuged (1980 *g*) for 10 min. The supernatant was passed through a PTFE-filter pad into a borosilicate glass tube. The filtered organic phase was evaporated at 70°C under a constant nitrogen stream. The residue was reconstituted with 125 μl water, vortexed, and 100 μl of this solution were injected into the chromatograph.

Assay recovery

The assay recovery of pentoxifylline and metabolite I was assessed at concentrations of 50 and 750 ng/ml. Five samples (250 μl) containing both compounds were extracted and injected. Five injections of the same amounts of these compounds in mobile phase were injected directly. The peak heights of the compounds in both sets of samples were measured. The recovery of each agent was computed using the following equation:

$$\text{Percentage recovery} = \frac{\text{peak height extract}}{\text{mean peak height of direct injections}} \times 100$$

RESULTS AND DISCUSSION

A representative chromatogram of a blank extracted plasma sample and an extracted plasma sample containing 500 ng/ml pentoxifylline, 500 ng/ml metabolite I and internal standard is illustrated in Fig. 1a and b. Blank plasma

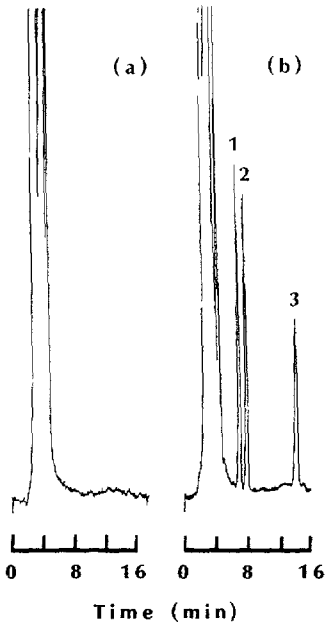

Fig. 1. Chromatograms of (a) blank plasma extract; (b) plasma extract spiked with 500 ng of metabolite I, 500 ng of pentoxifylline and 350 ng of phenacetin, the internal standard. Peaks: 1 = metabolite I; 2 = pentoxifylline; 3 = phenacetin, the internal standard.

TABLE I

EXTRACTION RECOVERIES OF PENTOXIFYLLINE AND METABOLITE I AT TWO CONCENTRATIONS AND SENSITIVITY LIMITS OF THE ASSAY

Drug	Recovery (mean ± S.D., n = 5) (%)		Mean recovery (%)	Sensitivity limit* (ng/ml)
	50 ng/ml	750 ng/ml		
Pentoxifylline	99 ± 3.2	104 ± 2 7	101.5	25
Metabolite I	98 ± 4 1	102 ± 2.6	100.0	25

*Based on a signal-to-noise ratio of 4:1.

samples demonstrated no interfering peaks. Retention times for metabolite I, pentoxifylline and the internal standard are 6.7, 8.0 and 14.7 min, respectively.

The results of the recoveries are outlined in Table I. The assay recovery for pentoxifylline and metabolite I was independent of concentration and averaged 101.5 and 100.0%, respectively.

Typical calibration curves for pentoxifylline and metabolite I are presented in Fig. 2. Each curve was linear throughout the range of standard concentrations (25—1000 ng/ml). The inter- and intra-day coefficients of variation for two quality control samples (50 and 750 ng/ml of both pentoxifylline and metabolite I) are presented in Table II. All coefficients of variation were less than 10%.

In order to determine if commonly administered drugs interfered with the assay, plasma samples were prepared containing either theophylline, theo-

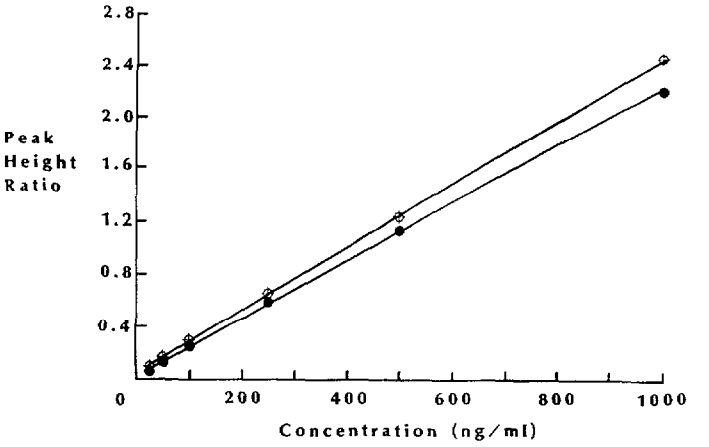

Fig. 2. A typical calibration curve for pentoxifylline (•) and metabolite I (⊕).

TABLE II

INTRA- AND INTER-DAY COEFFICIENTS OF VARIATION FOR PENTOXIFYLLINE AND METABOLITE I AT LOW (50 ng/ml) AND HIGH (750 ng/ml) CONCENTRATIONS

All variability statistics are based on six measurements daily for three consecutive days.

Drug	Intra-day coefficient of variation (%)		Inter-day coefficient of variation (%)	
	50 ng/ml	750 ng/ml	50 ng/ml	750 ng/ml
Pentoxifylline	7.33	3.17	6.99	4.25
Metabolite I	7.60	6.87	6.71	7.25

TABLE III

RETENTION TIMES OF SELECTED COMPOUNDS

Compound	Retention time (min)	Compound	Retention time (min)
Aminopyrine	36.2	Nifedipine	15.0
Aspirin	3.6	Penicillamine	3.0
Benzylpenicillin	4.0	Phenoxymethylpenicillin	5.1
β-Hydroxypropyltheophylline	3.9	Phthalic acid	2.6
β-Hydroxyethyltheophylline	3.2	Probenecid	6.3
Caffeine	2.6	Salicylic acid	2.7
Carbidopa	4.0	Sodium nafcillin	2.0
Methicillin	1.7	Theobromine	3.3
Methyldopa	3.0	Theophylline	2.2

bromine, caffeine, acetaminophen, acetylsalicylic acid, acetanilide, cimetidine, penicillin or propranolol in pharmacologic concentrations and analyzed by the present method. No interferences with the peaks of interest were detected. The retention times of the various compounds tested are listed in Table III.

A rat model was employed to demonstrate the applicability of the assay. A single intravenous dose (1 mg/kg) of pentoxifylline was administered with serial

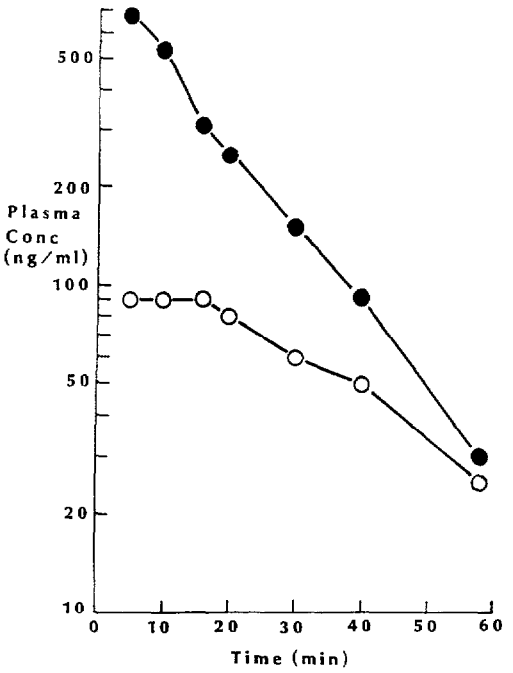

Fig. 3. Profiles of pentoxifylline (•) and metabolite I (○) plasma concentrations versus time after an intravenous bolus dose of 1 mg/kg in the rat.

blood sampling every 5 min for the first 20 min and then every 10 min for a total time of 60 min. For the purposes of this study, the volume of plasma used in the extraction procedure was 100 μl. Pentoxifylline and metabolite I plasma concentrations were determined and are graphically illustrated in Fig. 3.

In conclusion, an assay to detect pentoxifylline and metabolite I has been outlined with sufficient sensitivity to detect low concentrations of both compounds using small volumes of plasma. The assay is both sensitive and specific and circumvents the cumbersome extraction processes and relatively large plasma volume requirements of other assays currently in use.

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