Journal of Chromatography, 310 (1984) 61—69
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2187

GAS CHROMATOGRAPHIC DETERMINATION OF PENTOXIFYLLINE AND ITS MAJOR METABOLITES IN HUMAN BREAST MILK

MICHAEL T. BAUZA, ROBERT V. SMITH* and D. ELAINE KNUTSON

Drug Dynamics Institute, College of Pharmacy, University of Texas at Austin, Austin, TX 78712-1074 (U.S.A.)

and

FRANK R. WITTER

Division of Clinical Pharmacology, Department of Medicine, School of Medicine, Johns Hopkins University, Baltimore, MD 21205 (U.S.A.)

(First received December 20th, 1983; revised manuscript received April 19th, 1984)

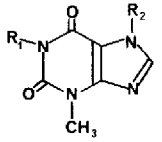
SUMMARY

A method has been developed for determination of the xanthine drug, pentoxifylline, and three of its metabolites (a secondary alcohol and two carboxylic acids) in human milk. The method requires pre-extraction with hexane to remove lipids followed by extraction with dichloromethane or dichloromethane—isopropanol (4:1). Absolute extraction recoveries were between 76—90%. Pentoxifylline and its alcohol metabolite (as the trifluoroacetate) and the carboxylic acid metabolites (as ethyl esters) were measured in separate gas chromatographic steps using a nitrogen detector. Determinations of pentoxifylline and its three metabolites were 96—99% accurate and standard deviations of 5—10% were observed for samples at or above the lower practical sensitivity limit (10 ng/ml) for the assay. Pentoxifylline and its metabolites were stable in breast milk for three weeks when stored at −15°C.

INTRODUCTION

Pentoxifylline [P; 1-(5′-oxohexyl)-3,7-dimethylxanthine] (Fig. 1) is used extensively in the treatment of cerebrovascular and peripheral vascular diseases [1—3]. The principle metabolites found in plasma after oral administration of P are a secondary alcohol metabolite (metabolite I) and two carboxylic acid metabolites (metabolites IV and V) (see Fig. 1). Since some mothers who

0378-4347/84/$03.00



P, R_1 = $CH_2CH_2CH_2CH_2COCH_3$; R_2 = CH_3

I, R_1 = $CH_2CH_2CH_2CH_2CHOHCH_3$; R_2 = CH_3

II, R_1 = $CH_2CH_2CH_2CH_2CH_2CH_2OH$; R_2 = $CH_2CH_2CH_3$

III, R_1 = $CH_2CH_2CH_2CH_2CH_2CO_2H$; R_2 = CH_3

IV, R_1 = $CH_2CH_2CH_2CH_2CO_2H$; R_2 = CH_3

V, R_1 = $CH_2CH_2CH_2CO_2H$; R_2 = CH_3

Fig. 1. Chemical structures of pentoxifylline (P), its principal metabolites (I, IV and V) and internal standards (II and III).

require therapy with P may wish to breast-feed their infants, the amounts of P and its metabolites that are excreted into breast milk must be assessed to evaluate potential drug-related risks to newborns.

Wilson [4] and Findlay [5] have recently reviewed the literature on drug excretion in breast milk. Few reports have appeared on the secretion of methylxanthine-related substances in milk. Different investigators, however, note that the milk levels of theobromine [6], theophylline [7] and caffeine [8] are similar to those found in plasma. Pentoxifylline is closely related structurally to these compounds, thus, its excretion into mother's milk may be significant.

Procedures for determining P and its metabolites have been developed for plasma [9, 10] and urine [11]; however, a suitable method has not been published for the corresponding determinations in human breast milk. We now report a gas chromatographic (GC) method which has been validated through analyses of spiked human milk samples.

MATERIALS AND METHODS

Reagents

Pentoxifylline, the internal standards II and III, and metabolites I, IV and V (Fig. 1), were supplied by Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.). These compounds were used as received and were homogeneous when submitted to thin-layer chromatography [12], high-performance liquid chromatography [12] and GC as described herein. Dichloromethane (glass-distilled), toluene (glass-distilled), and trifluoroacetic anhydride were used as obtained from MCB Manufacturing Chemists (glass-distilled i.e. Omni Solv; Cincinnati, OH, U.S.A.). Hexane (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). All other solvents and reagents were reagent grade or better. Control human breast milk was obtained from local hospitals and was generally colostrum.

Pentoxifylline and metabolite I

The method developed for the assay of P and metabolite I in milk is a modification of that described for plasma by Bryce and Burrows [9]. The procedure involves GC with alkali flame ionization (nitrogen-selective) detection. A P-analogue, compound II, was used as the internal standard. Metabolite I, and

compound II were converted to their trifluoroacetate derivatives to affect complete separation from P in the GC step.

Standard solutions of P and metabolite I (5 and 50 μg/ml) were prepared in toluene (glass-distilled). These solutions were stored at −15°C and were stable for several months. In screw-capped 12-ml centrifuge tubes (silylated with 2% trimethylchlorosilane (TMCS) in toluene on alternate use), 10-μl portions of a stock solution of II and sufficient volumes of stock solutions of P and metabolite I were added to yield the final concentrations shown in Table I. Following evaporation of the organic solvent at 40°C under a gentle stream of nitrogen, the residues were mixed with 1-ml portions of milk. The resulting spiked milk standards and samples were washed with single 5-ml portions of hexane to remove lipids and the aqueous phases transferred to clean tubes, taking scrupulous care to avoid transfer of interfacial material. To the washed aqueous phases were added 0.5-ml portions of 1 *M* sodium hydroxide and 5-ml portions of dichloromethane (glass-distilled) and the samples were extracted for 10 min on an inversion mixer (18 cycles per min). Following separation of the phases by centrifugation at 1875 *g* for 5 min, the milk layers were discarded and the organic phases transferred to 5-ml PTFE-capped reaction vials. Organic solvent was removed as noted above and the residues taken up in 1-ml portions of freshly prepared 5% trifluoroacetic anhydride in hexane. After mixing on a vortex shaker, the samples were heated at 60°C for 5 min to form the trifluoroacetyl derivatives of metabolite I and compound II. Following evaporation of excess derivatizing agent and solvent, the residues were taken up in 50-μl portions of glass-distilled toluene and 5-μl portions were chromatographed. GC was performed on a Varian 3700 gas chromatograph equipped with a nitrogen—phosphorus detector. The glass column (2 m × 2 mm I.D.) was packed with 3% OV-17 on Chromosorb W-HP (100—120 mesh). Flow-rates of helium carrier gas, air and hydrogen were 25 ml/min, 100 ml/min and 3.3 ml/min, respectively. Detector bias voltage was set at 4 and bead

TABLE I

STANDARD CURVE CHARACTERISTICS FOR MILK ANALYSIS OF TRENTAL AND METABOLITE I

All samples contained internal standard (II; approximately 500 ng/ml).

Concentration (ng/ml)		Normalized, integrated peak area ratios*	
Pentoxifylline	Metabolite I	Pentoxifylline	Metabolite I
10.0	10.0	0.019 (0.007)	0.022 (0.009)
20.0	20.0	0.038 (0.009)	0.044 (0.006)
51.0	51.0	0.102 (0.022)	0.113 (0.011)
102	102	0.210 (0.035)	0.228 (0.025)
255	256	0.564 (0.060)	0.587 (0.028)
511	511	1.19 (0.131)	1.19 (0.056)
1020	1020	2.41 (0.198)	2.34 (0.250)
2040	2050	4.80 (0.380)	4.88 (0.156)

*Peak area drug or metabolite divided by peak area internal standard. Mean of four determinations. Standard deviation given in parentheses.

current at 300—600, depending on bead age. Oven, injector and detector temperatures were 245°C, 300°C and 300°C, respectively. Under these conditions, chromatograms were obtained as shown in Fig. 2. Chromatograms of blank milk samples were devoid of interferences at retention times corresponding to P, metabolite I and compound II.

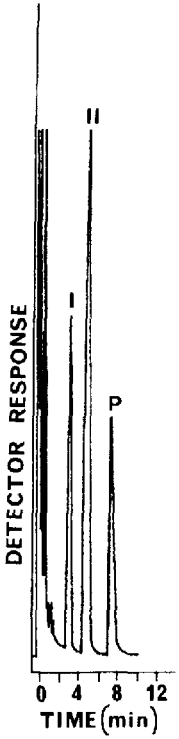


Fig. 2. Gas chromatogram of pentoxifylline (P; equivalent to 26 ng), metabolite I (I; equivalent to 26 ng as trifluoroacetate) and internal standard (II; equivalent to 52 ng as trifluoroacetate) extracted from human breast milk. Chromatographic conditions are as described in Materials and methods.

Metabolites IV and V

The assay method for P-carboxylic acid metabolites, IV and V, was a modification of the procedure described by Bryce [10] for the determination of these compounds in blood plasma. Standard stock solutions (100 μg/ml) of metabolites IV and V and internal standard III were prepared in the minimum amount of 0.1 *M* sodium hydroxide required for dissolution, the remainder of the required volume being water. These stock solutions were diluted with water to yield working stock solutions of 5 μg/ml (metabolites IV and V) and 2.5 μg/ml (III). Standard curve samples were prepared using serial dilutions of the working stock solutions. The following were added to 12-ml screw-capped centrifuge tubes (silylated with 2% TMCS on alternate use): 1-ml portions of milk, 0.1-ml portions of internal standard stock solution, and in the case of standard curve samples, sufficient amounts of working stock solutions of metabolites IV and V to give the final concentrations listed in Table II. Samples were washed with 5-ml portions of hexane to remove lipids and the aqueous

TABLE II

STANDARD CURVE CHARACTERISTICS FOR MILK ANALYSIS OF METABOLITES IV AND V

All samples contained internal standard (III; approximately 250 ng/ml).

Concentration (ng/ml)		Normalized, integrated peak area ratios*	
Metabolite IV	Metabolite V	Metabolite IV	Metabolite I
10.0	10.0	0.046 (0.017)	0.039 (0.008)
25.1	25.0	0 093 (0.012)	0.099 (0.017)
50.3	50.1	0.209 (0.013)	0.232 (0.062)
100	100	0.542 (0.040)	0.532 (0.042)
251	250	1.06 (0.087)	1.07 (0.092)
503	501	2.18 (0.137)	2.22 (0.146)
1000	1000	4.50 (0.434)	4.45 (0.427)
2010	2000	9.56 (1.52)	9.28 (0.996)

*Peak area metabolite divided by peak area internal standard. Mean of five determinations. Standard deviation given in parentheses.

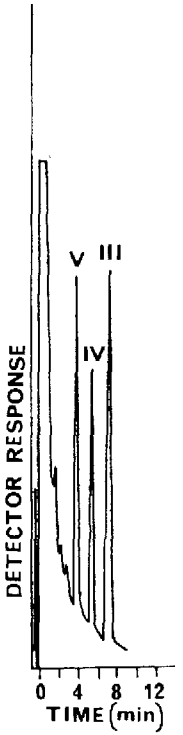


Fig. 3. Gas chromatogram of metabolite IV (IV; equivalent to 10 ng as ethyl ester), metabolite V (V; equivalent to 10 ng as ethyl ester) and internal standard (III; equivalent to 25 ng as ethyl ester) extracted from human breast milk. Chromatographic conditions are as described in Materials and methods.

phases transferred to clean tubes, taking scrupulous care to avoid transfer of interfacial material. In order to remove interfering P and metabolite I, hexane-pre-extracted milk samples were treated with 0.5-ml portions of 1 *M* sodium

hydroxide and extracted by shaking for 10 min with single 5-ml portions of dichloromethane on an inversion mixer (18 cycles per min). Following centrifugation at 1875 *g* for 5 min to separate phases, the aqueous phases were transferred to clean tubes and acidified with 1-ml portions of 1 *M* hydrochloric acid. The carboxylic acid metabolites were then extracted into 5 ml dichloromethane—isopropanol (4:1) using the technique outlined above. The organic phases were transferred to 5-ml PTFE-capped reaction vials, evaporated as above, and the metabolites were esterified with 0.1-ml portions of acidified ethanol (1 *M* hydrochloric acid in ethanol prepared by bubbling dry hydrogen chloride through ethanol and adjusting the hydrochloric acid molarity by addition of ethanol) at 60°C for 30 min. At the end of the reaction period, excess derivatizing reagent was removed by evaporation as above and the residues taken up in 50-μl portions of butyl acetate, of which 5-μl portions were injected into the chromatograph.

GC was performed as described for P and metabolite I (above). Under these conditions, chromatograms were obtained as shown in Fig. 3. Chromatograms of blank milk samples were devoid of interfering peaks at retention times corresponding to metabolites IV and V and the internal standard III.

RESULTS AND DISCUSSION

The determination of drugs and their metabolites in human breast milk presents unique challenges. Human breast milk contains large amounts of endogenous lipids [4] that can compromise both sample extraction and chromatographic separation steps. To circumvent losses in extraction efficiency of pentoxifylline and its metabolites, we pre-extracted all milk samples with hexane and scrupulously avoided contamination of organic extracts with interfacial matter from separated phases. The detector used in the GC separation step was a nitrogen-selective detector. This also favored selectivity since more than 95% of milk lipids are di- and triglycerides, sterol and sterol esters, and free fatty acids [13], all compounds that are transparent in the detection system.

TABLE III

ABSOLUTE RECOVERIES OF PENTOXIFYLLINE AND ITS METABOLITES FROM HUMAN BREAST MILK

Compound	Absolute recovery* (%)	*n*
Pentoxifylline	89.7 (4.75)	11
Metabolite I	86.5 (4.30)	10
Internal standard II	81.8 (2.87)	11
Metabolite IV	92.1 (16.3)	11
Metabolite V	76.4 (5.57)	12
Internal standard III	85.8 (7.34)	11

*Mean (standard deviation in parentheses) of *n* determinations, expressed as percent. Concentration of internal standard II was 517 ng/ml; concentration of internal standard III was 254 ng/ml. Concentrations of pentoxifylline and its metabolites were sampled over the concentration range 10—2000 ng/ml.

TABLE IV

EFFECT OF SODIUM HYDROXIDE ON AQUEOUS PENTOXIFYLLINE SOLUTIONS AS A FUNCTION OF TIME

From a 10-ml aqueous solution of pentoxifylline (approximately 1000 ng/ml), replicate 1-ml aliquots were removed, extracted and chromatographed. To the remaining 8 ml of solution, a 4-ml portion of 1 *M* sodium hydroxide was added and the solution thoroughly mixed. At the time intervals indicated in the table, replicate 1-ml samples were withdrawn, extracted and chromatographed according to the procedure outlined in Materials and methods. Values for non-zero time samples have been corrected for dilution by base.

Time (min)	Pentoxifylline remaining at 25°C (%)	*n*	Pentoxifylline remaining at 4°C (%)	*n*
0	(100)	3	(100)	6
5	95.2	3	94.8	4
10	92.8	3	95.1	5
20	101	3	97.5	5
30	94.6	3	90.9	4

TABLE V

SUMMARY OF REGRESSION DATA

Values of correlation coefficient (r), slope and ordinate for lower and upper concentration ranges. Standard deviation given in parentheses. Values for concentration ranges and n as in Tables I and II. See text for details.

	r	Slope × 10^3	Y-Intercept × 10^3
Pentoxifylline			
Lower	0.9989 (0.0009)	2.08 (0.323)	−3.16
Upper	0.9997 (0.0002)	2.36 (0.173)	−26.4
Metabolite I			
Lower	0.9994 (0.0004)	2.25 (0.196)	−1.28
Upper	0.9990 (0.0016)	2.40 (0.085)	−12.0
Metabolite IV			
Lower	0.9916 (0.0025)	5.63 (0.572)	−38.7
Upper	0.9984 (0.0015)	4.76 (0.793)	−113
Metabolite V			
Lower	0.9923 (0.0044)	5.55 (0.542)	−31.5
Upper	0.9989 (0.0012)	4.63 (0.520)	−59.6

After pre-extraction with hexane, separate milk samples are extracted with dichloromethane for P and metabolite I, or dichloromethane–isopropanol (4:1) for metabolites IV and V. The assay of metabolites IV and V requires a dichloromethane pre-extraction of P and I which interfere in the GC step. No interferences from metabolites IV and V are observed in the determination of P and I because P and I are extracted at an alkaline pH which prevents contamination by the carboxylic acid metabolites. Table III contains absolute recovery values of P, its metabolites and internal standards. Extraction efficiencies for all of these compounds are acceptable [14] when the prescribed extraction schemes are used.

The possibility existed that pentoxifylline undergoes an aldol condensation

when exposed to the strong alkali of the extraction step. This could result in losses of detectable parent drug. We, therefore, tested the stability of P in sodium hydroxide at both 4°C and room temperature. The results of these experiments are shown in Table IV. As indicated, P appears to be stable for at least 20 min at both temperatures. Thus, the extraction methods reported herein can be performed without significant loss of drug.

Standard curve samples for P and metabolites I, IV and V were prepared as described in Materials and methods. Tables I and II contain standard curve behavior and Table V summarizes regression analysis data for these compounds determined in milk. Excellent correlations were obtained for the regressions of peak area ratios versus concentrations. Day-to-day variations in detector response due to detector bead aging, however, necessitated daily preparation of standard curves.

Our experience with analyses of P and its metabolites in plasma indicated a statistically significant difference (95% confidence level) between slopes of the lower range of standard curve concentrations (typically 10—100 ng/ml) and those for the upper range of standard curve concentrations (typically 100—2000 ng/ml). Thus, concentrations of P and its metabolites have been calculated from two separate regression line analyses, the first bracketing the lower concentration range, and the second bracketing the upper concentration range. The lower sensitivity limits for P and its metabolites were approximately 2 to 6 ng/ml at a signal-to-noise ratio of 5; however, the practical lower limit of detection was routinely 10 ng/ml for these compounds.

TABLE VI

ACCURACY AND PRECISION OF GC ASSAYS FOR PENTOXIFYLLINE AND ITS METABOLITES IN HUMAN BREAST MILK

Assay	Concentration range (ng/ml)	Accuracy*	*n*
Pentoxifylline	26.0—1760	96.1 (8.73)	19
Metabolite I	26.0—1750	98.9 (4.69)	19
Metabolite IV	15.1—1610	96.9 (10.2)	10
Metabolite V	15.0—1600	97.4 (8.07)	10

*Amount found/amount added × 100%; standard deviation given in parentheses.

Table VI contains percent accuracy and standard deviations for analyses of spiked milk samples containing P and its metabolites, I, IV and V. It can be concluded from these data (reflecting within-day variations) that the accuracy and precision of the GC assays are good.

In summary, the sensitivity, selectivity, accuracy and precision of the methods described herein are good, and compare favorably with the same characteristics of determinations of pentoxifylline and metabolite I in plasma described by Bryce and Burrows [9] and Chivers et al. [15]. An extended study of the stability of P and its metabolites in milk samples stored at −15°C was initiated. Preliminary data indicate that little or no loss of these compounds occurred over a period of three weeks.

ACKNOWLEDGEMENT

This work was supported by Hoechst-Roussel Pharmaceuticals, Somerville, NJ, U.S.A.

REFERENCES

1 H.G. Mansfield, Deut. Med. J., 23 (1972) 1.
2 T. Lehrach and R. Muller, Arzneim.-Forsch., 21 (1972) 1171.
3 H. Denck, Arzneim.-Forsch., 23 (1973) 571.
4 J.T. Wilson, Drug Metab. Rev., 14 (1983) 619.
5 J.W.A. Findlay, Drug Metab. Rev., 14 (1983) 653.
6 B.H. Resman, H.P. Blumenthal and W.J. Jusko, Ped. Pharmacol. Ther., 91 (1977) 477.
7 A.M. Yurchak and W.J. Jusko, Pediatrics, 57 (1976) 518.
8 R.M. Welch and J.W.A. Findlay, Drug Metab. Rev., 12 (1981) 261.
9 T.A. Bryce and J.L. Burrows, J. Chromatogr., 181 (1980) 355.
10 T.A. Bryce, Evaluation of the Quantitative Analytical Method for the Pentoxifylline Metabolites IV and V in Plasma, Hoechst UK Ltd., Milton Keynes, August 20, 1982, Document No. TB1D090882.
11 K.W. Jolley, The Analysis of Two Major Carboxylic Acid Metabolites of Oxpentifylline in Urine by High Pressure Liquid Chromatography, Hoechst UK Ltd., Milton Keynes, November 26, 1981, Document No. KJ1D061081.
12 R.V. Smith, S-K. Yang, P.J. Davis and M.T. Bauza, J. Chromatogr., 281 (1983) 281.
13 B.L. Nichols and V.N. Nichols, Compr. Ther., 4 (1979) 63.
14 R.V. Smith and J.T. Stewart, Textbook of Biopharmaceutic Analysis — A Description of Methods for the Determination of Drugs in Biologic Fluids, Lea and Febiger, Philadelphia, PA, 1981, pp. 95—114.
15 D.A. Chivers, D.J. Birkett and J.O. Miners, J. Chromatogr., 225 (1981) 261.