DETERMINATION OF PENTOXIFYLLINE AND ITS MAJOR METABOLITES IN MICROBIAL EXTRACTS BY THIN-LAYER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) methods have been developed for the determination of the xanthine drug, pentoxifylline, and three of its metabolites (a secondary alcohol and two carboxylic acids) in microbial extracts. The methods require initial extraction of acidified media with dichloromethane–2-propanol (4:1). Extracts are submitted to TLC development on silica gel G layers using three solvents and HPLC development on an C_{18} column using methanol–phosphoric acid (0.02 M, pH 5) (3:7) as mobile phase. All systems provide good separations of the drug and its metabolites. Quantitative analyses of pentoxifylline and its metabolites by HPLC were accurate and precise. The HPLC method was applied to studies of the metabolism of pentoxifylline by two microorganisms.

INTRODUCTION

Pentoxifylline [P; 1-(5′-oxohexyl)-3,7-dimethylxanthine] is widely used in the treatment of patients with cerebrovascular and peripheral vascular diseases[1–3]. Seven metabolites (I–VII) of pentoxifylline (P) have been identified in mammals including man[4]. The major metabolites are the monohydroxy metabolite I and the carboxylic acid metabolites IV and V[5]. Using the strategy of microbial models of mammalian metabolism[6,7], we have sought microorganisms that metabolize P in a manner similar to mammals. These studies required procedures for determining compounds P, I, IV and V in cultures of fungi, yeasts and bacteria.

As gas chromatography (GC)–alkali flame ionization detection method has been reported for the determination of P and I in human plasma[8]. The GC method has good sensitivity and selectivity but requires conversion of I to its trifluoroacetate to affect separation from P.

High-performance liquid chromatography (HPLC) with ultraviolet detection and a C_{18}-column with a methanol–phosphoric acid mobile phase has been used to determine the xanthine P and I in plasma[9] and carboxylic acids, IV and V in urine[10].
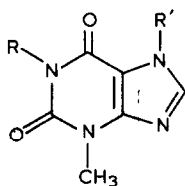
P,   R = CH$_2$–(CH$_2$)$_3$–CO–CH$_3$, R′ = CH$_3$
I,   R = CH$_2$–(CH$_2$)$_3$–CHOH–CH$_3$, R′ = CH$_3$
II,   R = CH$_2$(CH$_2$)$_3$–CHOH–CH$_2$OH, R′ = CH$_3$
III,   R = CH$_2$–(CH$_2$)$_2$–CHOH–CHOH–CH$_3$, R′ = CH$_3$
IV,   R = CH$_2$–(CH$_2$)$_3$–COOH, R′ = CH$_3$
V,   R = CH$_2$–(CH$_2$)$_2$–COOH, R′ = CH$_3$
VI,   R = CH$_2$–(CH$_2$)$_3$–CO–CH$_3$, R′ = H
VII,   R = CH$_2$–(CH$_2$)$_3$–CHOH–CH$_3$, R′ = H
VIII,   R = CH$_2$–(CH$_2$)$_2$–COOH, R′ = CH$_2$–CH$_2$–CH$_3$

Thin-layer chromatography (TLC) is well established for the detection of xanthines[11–14]. These reports were useful in developing TLC and HPLC methods for the determination of I–IV in microbial cultures. Compound VIII was used as the internal standard in the HPLC work. The developed procedures were used in a preliminary investigation of the metabolism of P by a *Nocardia corallina* and a *Rhodotorula rubra*.

EXPERIMENTAL

Materials

Compounds P, I, IV, V and VIII were supplied by Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.). These compounds were used as received and were homogeneous when submitted to the TLC and HPLC systems described below.

Water used for HPLC was deionized and double-distilled in glass. Methanol was HPLC-grade (OmniSolve; MCB Reagents, Cincinnati, OH, U.S.A.). All other reagents and chemicals were reagent-grade. Polytetrafluoroethylene filters pads (Millipore, Bedford, MA, U.S.A.) were used to filter HPLC solvents.

TLC

Compounds and extracts were developed on 0.25-mm silica gel GF 254 plates (EM Labs., Darmstadt, Germany) using the following solvent systems: (A) chloroform–methanol (9:1); (B) acetone–cyclohexane (7:3); (C) ethyl acetate–light petroleum–diethyl ether (45:4:1) —multiple development twice in a water saturated chamber. Developed TLC plates were visualized by fluorescence quenching of 254-nm radiation and by spraying with Dragendorff's reagent[11] or equal parts, 10 percent aqueous ferric chloride in 20 percent tartaric acid plus 4 percent iodine in acetone[12].

HPLC

A Model 6000A pump and Model U6K injector (Waters Assoc., Milford, MA, U.S.A.) were connected to a Model 970A variable-wavelength UV–visible detector (Tracer, Austin, TX, U.S.A.) set at 275 nm and C-R1A recording integrator (Smith-Kline Beckman, Fullerton, CA, U.S.A.) set at 1 mV/min input sensitivity. A μBondapak C$_{18}$ column (300 × 3.9 mm I.D., 10 μm packing; Waters Assoc.) was
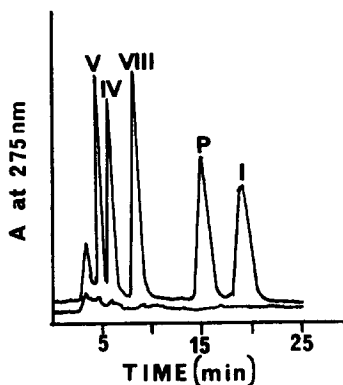
Fig. 1. Chromatograms from the HPLC of blank *N. corallina* culture extracts and extracts spiked (conc., μg/ml) with compounds: P (125), I (125), IV (75), V (75) and VIII (125). Chromatographic conditions are described in the Experimental section.

eluted at 1.0 ml/min with methanol–0.02 M phosphoric acid, pH 5.0 (3:7). Retention times (min) were: P, 15.3; I, 19.0; IV, 5.93; V, 4.73; VIII, 8.46 (Fig. 1).

HPLC assay for compounds I–IV

Separate stock solutions containing 0.5 mg/ml of compounds P, I, IV, V and VIII were prepared in methanol and stored at 24°C for up to 30 days without decomposition. A minimum of five portions (between 5 to 1000 μl = 2.5 to 500 μg) of the stock solutions of compounds P, I, IV and V were added to 125 × 16-mm glass tubes; a 50-μl portion of the stock solution of internal standard (= 25 μl of VIII) was also added to each tube. The methanol was evaporated to dryness at 24°C under nitrogen. A one-ml portion of stage 2 (ref. 15) *N. corallina* (ATTC 19071) or *R. rubra* (ATCC 20124) cultures in dextrose soybean meal medium was added to each tube. The samples were acidified with 2-ml portions of Clark's buffer (25 ml of 0.2 M potassium chloride plus 6.5 ml 0.2 M hydrochloric acid diluted to 100 ml) to pH 2.0 and extracted for 30 min with dichloromethane–2-propanol (4:1) at 18 rpm on a Lab-Tek mixer. After centrifuging for 5 min (1500 g), 1.6–3.2-ml portions of the organic phases were transferred to clean glass tubes (125 × 16 mm) and evaporated to dryness at 24°C under nitrogen. The residues were reconstituted in 0.8–2.0 ml of HPLC mobile phase and 25-μl portions were chromatographed. Peak area ratios (drug or metabolite/internal standard) were determined after each injection and plotted *versus* concentration of the drug or metabolite (2.5 to 500 μg/ml) to produce standard curves for compounds P, I, IV and V.

Accuracy and precision of assay

N. corallina stage-2 culture samples were spiked with 25, 200, and 400 μg/ml concentrations of P, 7.5, 25 and 40 μg/ml concentrations of I and IV, 25 and 40 μg/ml concentrations of V and 25 μg/ml concentrations of VIII. All spiked samples were extracted and analyzed as indicated above. Peak area ratios (drug or metabolite/internal standard) were determined for each injection. Concentrations were determined by interpolation using the standard curves generated on each day of analy-

sis. The means and standard deviations of the calculated values were determined as a measure of accuracy and precision, respectively.

General fermentation procedure

Cultures of *N. corallina* (ATTC 19070) and *R. rubra* (ATCC 20129) were maintained on refrigerated (2°C) slants of Sabouraud-maltose agar (Difco Labs., Detroit, MI, U.S.A.), and transferred every 6 months. The medium used in these studies consisted of the following: dextrose, 20 g; soybean meal (20 mesh; Capital Feeds, Austin, TX, U.S.A.), 5 g; sodium chloride, 5 g; potassium phosphate (dibasic), 5 g; yeast extract (Difco), 5 g; distilled water, 1000 ml; pH adjusted to 7.0 with 6 M hydrochloric acid. The medium was sterilized in individual flasks at 121°C for 15 min. Incubations were conducted in 125-ml Bellco DeLong culture flasks containing 25 ml of the medium, using NBS model G-25R environmental shaker (New Brunswick Scientific, Edison, NJ, U.S.A.) at 250 rpm and 27°C. For each culture examined, first-stage flasks were initiated by suspending surface growth on agar slants in 25 ml of sterile medium and transferring the suspension to a 125-ml flask under aseptic conditions. After incubation for 72 h, a 2-ml portion was used to inoculate a second-stage flask of the same composition, and the incubation was allowed to continue for 24 h. For analytical development, cultures were harvested and frozen. Twenty-four hour, two-stage cultures were used in the metabolism studies. An 8- to 12.5-mg portion of P in 50 μl dimethylformamide was added aseptically to each flask (final concentration in the culture = 500 μlg/ml). One-ml samples were harvested at 30 min, 1, 2, 3, and 6 days and frozen. Samples were then thawed and analyzed by HPLC as described above. The identity of metabolites formed was confirmed by TLC in the three solvent systems noted above.

RESULTS AND DISCUSSION

Literature references[12,13] to the TLC of xanthine compounds (*e.g.* caffeine, theophylline) served as a starting point for the development of TLC systems for compounds P, I, IV and V. Three systems were devised using silica gel layers and the R_F values for compounds P, I, IV and V in systems A–C are indicated in Table I. Systems A and B are primarily based on adsorption chromatography and provide good separation of all four compounds. System C functions best in a tank saturated

TABLE I

TLC OF PENTOXIFYLLINE AND ITS METABOLITES

Values are $R_F \times 100$.

Compound	Solvent systems		
	A	B	C*
P	70	41	40
I	41	34	29
IV	23	22	24
V	16	15	17

* After development twice in a water saturated chamber.

with water vapor and following multiple development. Each development is slow (50 min), yet it provided the best separation of pentoxifylline and its metabolites due to the consolidation of spots. Compounds P, I, IV and V are readily detected in the low μg range after TLC by UV-quenching or spraying with Dragendorff's reagent or ferric chloride and iodine in aqueous acetone.

A satisfactory separation of compounds P, I, IV, V and VIII was also achieved by HPLC using a μBondapak C_{18} column and a mobile phase consisting of methanol–0.02 M phosphoric acid, pH 5.0 (3:7) (see Fig. 1). A similar stationary- mobile phase combination has been used for the analysis of compounds P and I in plasma[9] and IV and V in urine[10]. The developed HPLC system provides good reso- lution of all compounds within 20 minutes.

Halogenated solvents such as dichloromethane have been used in the extrac- tion of xanthines from complex mixtures[16]. Bryce and Burrows[8] used this solvent to extract pentoxifylline (P) and its alcohol metabolite (I) from plasma. We confirmed the efficiency of dichloromethane in extracting compounds P and I from biological material but poorer recoveries were found with the carboxylic acid metabolites IV and V. Addition of 20 percent 2-propanol to dichloromethane improved recoveries and provided the following percent absolute recoveries from microbial cultures [stan- dard deviation; concentration ($\mu g/ml$); n]: P, 102 (2.7; 125; $n = 5$); I, 90.4 (4.6; 125; $n = 5$); IV, 86.4 (7.1; 75; $n = 4$); V, 76.7 (13.3; 125; $n = 3$); VIII, 89.3 (4.4; 125; $n = 10$).

No chromatographic interferences were observed for compounds P, I, and VII in dichloromethane-2-propanol extracts of dextrose soybean meal medium or cul- tures of *N. corallina* and *R. rubra*. Small potentially interfering peaks appeared in the region of compounds IV and V but the area contributions could be subtracted after HPLC analysis of blank extracts. Results of replicate analyses of pentoxifylline and its metabolites in stage 2 *N. corallina* cultures are given in Table II. Standard curves were prepared daily and showed consistently high correlation coefficients ($r = 0.995$). The determined concentrations are relevant to substrate and metabolite levels in microbial transformation work[6,7]. The percent recoveries and relative standard de- viations (R.S.D.) (Table II) obtained indicate that the accuracy and precision of the HPLC method are good.

Applications of the described HPLC system to study the microbial metabolism of P are depicted in Fig. 2 for *N. corallina* and in Fig. 3 for *R. rubra*. Both cultures produce the reduction product, metabolite I, as the sole metabolite, and reduction was nearly quantitative with the *R. rubra*. Other cultures under investigation appear

TABLE II

ACCURACY AND PRECISION OF HPLC ASSAY FOR PENTOXIFYLLINE AND ITS METAB- OLITES IN MICROBIAL CULTURES

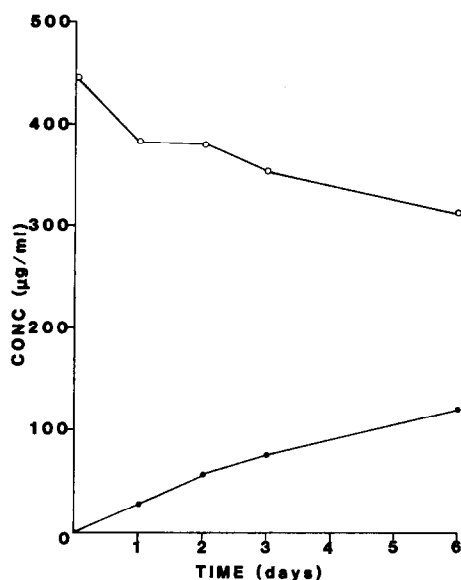| Compound	Conc. prepared ($\mu g/ml$)	Mean recovery (%)	R.S.D. (%)	n
P	25.4–406	105	5.9	7
I	7.50–39.8	101	5.5	7
IV	7.50–39.8	95.9	6.6	7
V	25.7–41.1	98.9	5.5	6

Fig. 2. Time course of reduction of pentoxifylline (P, ○) to its alcohol metabolite (I, ●) by *N. corallina* (ATCC 19070).
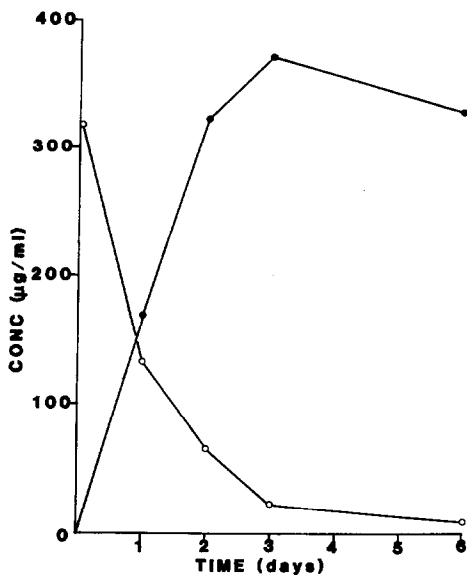
Fig. 3. Time course of reduction of pentoxifylline (P, ○) to its alcohol metabolite (I, ●) by *R. rubra* (ATCC 20129).

to produce the carboxylic acids IV and V. Extensive studies of the microbial metabolism of P are underway and will be published elsewhere.

In summary, TLC and HPLC methods have been devised for the detection and quantitative analysis of pentoxifylline and three of its metabolites in microbial extracts. The methods are selective and permit detection of $\mu g/ml$ concentrations of the xanthine compounds. Quantitative analyses of pentoxifylline and its metabolites by HPLC were accurate and precise. The HPLC method has been applied to studies of the metabolism of pentoxifylline by two microorganisms.

ACKNOWLEDGEMENT

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