Simultaneous Determination of Pentoxifylline and Three Metabolites in Biological Fluids by Liquid Chromatography

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We describe a sensitive and specific liquid-chromatographic assay for pentoxifylline and three of its metabolites in human plasma and urine. Addition of hydrochloric acid to the sample before extraction, and incorporation of acetic acid in the chromatographic eluent, allow the simultaneous determination of the four compounds plus an internal standard in one chromatographic run. Unlike gas-chromatographic procedures, this method does not involve derivatization; no similar analysis of serum or plasma samples has been described before now. The method has been applied successfully to routine analysis and to pharmacokinetic studies.

Additional Keyphrases: chromatography, reversed-phase • oxpentifylline • urine • pharmacokinetics

Pentoxifylline is a hemorheologic agent used in the therapeutics management of peripheral and cerebrovascular diseases and of defective regional microcirculation (intermittent claudication) (1). It improves peripheral circulation by increasing erythrocyte deformability (2), reducing blood viscosity (3), inhibiting platelet aggregation (2), and diminishing fibrinogen concentration (4).

Pentoxifylline undergoes extensive metabolism, resulting in a large number of metabolites (Figure 1) (5). The major metabolites are the monohydroxy-metabolite I and the carboxylic acid metabolites IV and V.

"High-performance" liquid chromatographic (HPLC) (6–12) and gas-chromatographic (GC) (13, 14) procedures have been reported for determining pentoxifylline and its hydroxy-metabolite I in plasma (6–11) and metabolites IV and V in urine (12).4 Recently, a single-run GC assay has been described for quantifying pentoxifylline and metabolites I, IV, and V in plasma (15). In 1983, Smith et al. (16) described the extraction and the HPLC separation of pentoxifylline and its metabolites I, IV, and V in microorganisms. We now present a HPLC procedure for separating and quantifying pentoxifylline and its metabolites I, IV, and V in human plasma and urine.

Materials and Methods

Instrumentation

We used a Model 8800 liquid chromatograph (DuPont Inc., Wilmington, DE 19898) equipped with an LC 95 UV/visible spectrophotometer (Perkin-Elmer Ltd., Bucks., U.K.) set at 274 nm, a Valco injector with a 50-µL loop (Valco Instruments, Houston, TX 77255), and a 15 cm x 4.6 mm (i.d.) prepacked Zorbax ODS (5 µm) reversed-phase column (also from DuPont). The column was eluted isocratically at room temperature with acetonitrile/water/acetic acid (22/77.9/0.1, by vol) at a flow rate of 0.75 mL/min. Chromatograms were recorded on a Model BD 8 recorder (Kipp en Zonen, Delft, The Netherlands) at a chart speed of 0.5 cm/min.

Reagents

Pentoxifylline [P; 1-(5'-oxohexyl)-3,7-dimethylxanthine], metabolite I [I; 1-(5'-hydroxyhexyl)-3,7-dimethylxanthine], metabolite V [V; 1-(3'-carboxypropyl)-3,7-dimethylxanthine], and the internal standard [IS; 1-(6'-oxohexyl)-3,7-dimethylxanthine] were supplied by Hoechst Pharmaceutical Research Laboratories, Milton Keynes, U.K. Metabolite IV [IV; 1-(4'-carboxybutyl)-3,7-dimethylxanthine] was a gift from Hoechst AG-Werk Albert, Wiesbaden, F.R.G. Theophylline, theobromine, caffeine, acetic acid, and the extraction solvents were from E. Merck AG, Darmstadt, F.R.G. Solvents used for chromatography were "HPLC"-grade and came from Fisher Scientific, Fair Lawn, NJ 07410.

Standard solutions. Separate stock solutions of all standards (P, I, IV, and V) and of the internal standard (IS) were prepared by dissolving weighed amounts in acetonitrile/water (22/78, by vol). The working solution was prepared by mixing 200 µL of the stock solution of P, I, and IV with 400 µL of the stock solution of V, to give final concentrations of 76.2, 49.9, 35.7, and 43.6 µmol/L, respectively. The internal standard and the other xanthines (theophylline, theobromine, and caffeine: 439, 738, and 509 µmol/L, respectively) were used directly from their respective stock solutions.

Specimen Collection

Blood samples (8 mL) were collected in heparin-containing evacuated tubes ("Venosafe"; Terumo Europe N.V., Leuven, Belgium). The plasma was immediately separated at 4 °C to prevent in vitro erythrocyte metabolism (II), stored at −20°C, and thawed at room temperature before...
analysis. Patients received 200 to 300 mg of pentoxifylline per infusion, up to 1200 mg/day. A male human volunteer took 400 mg of the delayed-release formulation orally.

Procedures

**Extraction of plasma.** Add 20 μL of the internal standard solution to 1 mL of plasma in a 10-mL screw-cap glass tube. Add 100 μL of 3 mol/L HCl solution and extract the samples with 5 mL of an equivolume solution of dichloromethane and chloroform for 10 min on a rotary mixer (Cenco Instruments, Breda, The Netherlands) at 20 rpm. Separate the phases by centrifugation at 1000 × g for 10 min, and aspirate the aqueous (upper) layer. Transfer the organic layer to a conical glass tube and evaporate under a stream of nitrogen at room temperature. Dissolve the residue in 200 μL of the solvent for chromatography, vortex-mix, and centrifuge (1 min at 1000 × g); then inject a 20-μL aliquot into the HPLC system.

Prepare calibration samples by adding increasing volumes of the above-described working solution to 1-mL aliquots of a drug-free human plasma pool. After adding the internal standard, treat these calibration samples the same as the unknowns.

**Extraction of urine.** Extract urine (0.1–0.5 mL) similarly to plasma. In addition, purify the organic phase on a Bond Elut C18 solid-phase column (Analytichem International, Harbor City, CA 90710) that has been rinsed previously with methanol (6 mL) and water (6 mL). Then treat the purified organic phase the same as a plasma extract. After the columns are rinsed with 20 mL of methanol, they can be used a second time.

**Calculations.** Determine the concentrations of P, I, IV, and V from the respective regression equations relating peak-height ratios (for the standards vs IS) to their concentrations, as determined with the calibration samples.

**Results**

Figure 2A shows the separation of pure standards, including caffeine, theobromine, and theophylline. Figure 2B shows the trace for a plasma extract from a volunteer 4 h after an oral dose of 400-mg pentoxifylline in a sustained release tablet. Figure 3A is a chromatogram of an extract of a urine sample collected 14 h after a 400-mg oral dose of pentoxifylline. Drug-free plasma and blank urine contained no interfering compounds (Figures 2C and 3B).

For plasma and urine samples, calibration curves for pentoxifylline and for the metabolites were linear from 0 to 14.4 μmol/L, with y-intercepts not significantly different from the origin.

The extraction recovery of the compounds from plasma and urine was determined by comparing the slopes of two calibration graphs obtained by analyzing two series of samples. To one set of samples we added the IS at the beginning of the analysis; to the other, after the complete sample work-up. The results are summarized in Table 1. The within-day and day-to-day precision data are presented in Table 2.

For analysis of 1 mL of plasma or 0.5 mL of urine, the limit of detection for pentoxifylline was 53 to 71 nmol/L, i.e., at a minimum signal-to-noise ratio of 3:1.

The pharmacokinetic profile of P and its different metabolites in plasma after an oral dose of 400 mg of P in the sustained release formulation is presented in Figure 4. Over the 10 h after dosing, concentrations of P remained around 0.35 μmol/L, whereas V peaked at 3.00 μmol/L after 2 h. IV concentrations never exceeded 0.18 μmol/L; and by 2 h post-dose the concentration of I exceeded that of P itself. After oral administration of 400 mg of pentoxifylline in a sustained release preparation, urine analysis showed that 47% of the administered dose was recovered as V in the following 14 h; IV accounted for 2.3%, and I and P for <1% of the administered dose.

Having been in use for more than 10 months, the method has already proved useful for routine determinations. It is absolutely necessary to separate the plasma from the erythrocytes as soon as possible, to prevent any further metabolism of P to I in whole blood. In one experiment we added a known amount of P to different aliquots of whole blood. After incubation at 37 °C for different periods (up to 9 h) we analyzed the separate plasma samples. Over this 9-h period, the concentration of P decreased from 1.44 to 0.72 μmol/L at the same time, the concentration of I was already 0.64 μmol/L.

**Discussion**

Earlier HPLC procedures—except one for the analysis of microorganisms (16)—have concentrated on the measurement of P and its hydroxy metabolite I (6–11) and ignored the acidic metabolites (IV and V), one of which (V) is the major species in plasma. Only one research group described
Table 1. Analytical Recovery of Pentoxifylline and Its Metabolites from Plasma and Urine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plasma</th>
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<th></th>
<th>Urine</th>
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<tbody>
<tr>
<td></td>
<td>Range,</td>
<td>Recovery,</td>
<td>CV,</td>
<td>Range,</td>
<td>Recovery,</td>
</tr>
<tr>
<td></td>
<td>μmol/L</td>
<td>%</td>
<td></td>
<td>μmol/L</td>
<td>%</td>
</tr>
<tr>
<td>V</td>
<td>0-6.8</td>
<td>67.0 (2.9)*</td>
<td>4.3</td>
<td>0-13.9</td>
<td>64.0 (3.7)*</td>
</tr>
<tr>
<td>IV</td>
<td>0-5.7</td>
<td>88.2 (2.8)</td>
<td>3.2</td>
<td>0-11.4</td>
<td>86.3 (3.3)</td>
</tr>
<tr>
<td>I</td>
<td>0-7.8</td>
<td>93.2 (1.9)</td>
<td>2.0</td>
<td>0-15.7</td>
<td>92.1 (2.8)</td>
</tr>
<tr>
<td>P</td>
<td>0-12.2</td>
<td>98.4 (2.7)</td>
<td>2.7</td>
<td>0-24.1</td>
<td>95.5 (3.5)</td>
</tr>
</tbody>
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*Mean (and SD) values, n = 5 each.

Table 2. Within-Day and Day-to-Day Reproducibility

<table>
<thead>
<tr>
<th>Compound</th>
<th>Within-day (n = 6)*</th>
<th>Day-to-day (n = 11)*</th>
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<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>CV,</td>
</tr>
<tr>
<td></td>
<td>μmol/L</td>
<td>%</td>
</tr>
<tr>
<td>V</td>
<td>638 (24.9)</td>
<td>3.9</td>
</tr>
<tr>
<td>IV</td>
<td>642 (12.2)</td>
<td>1.9</td>
</tr>
<tr>
<td>I</td>
<td>965 (18.3)</td>
<td>1.9</td>
</tr>
<tr>
<td>P</td>
<td>1509 (34.7)</td>
<td>2.3</td>
</tr>
</tbody>
</table>

*Drug-free plasma, supplemented with various concentrations of each metabolite.

This HPLC system allows a simple, sensitive, and reproducible determination of pentoxifylline and three of its metabolites in biological matrices from human origin in one run. Furthermore, the method has proven useful for routine determinations over a long period, as well as for pharmacokinetic studies. The results of our pharmacokinetic studies agree with those obtained by GC procedures (13, 15) and demonstrate once more the reliability of the HPLC procedure described.

References


Patterns of Lactate Dehydrogenase Isoenzymes 1 and 2 in Serum of Patients Performing an Exercise Test

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Values for total lactate dehydrogenase (LD, EC 1.1.1.27) activity and LD isoenzymes were determined in serum from 56 patients and 40 healthy subjects before and 24, 48, and 72 h after they performed an exercise test. The mean (for all four times) total LD activity concentration and proportion of LD-2 were within the normal range for all 96 subjects. Mean LD-1 values for serum, although within the normal range in all subjects, were significantly higher in patients with positive exercise test results than in subjects with negative results: 75 (SD 12) U/L in 35 patients with ST depression >2 mm; 63 (SD 14) U/L in 16 patients with ST depression of 1–2 mm; 43 (SD 11) U/L in subjects with negative test results, by 48 h after the test. The LD 1:2 ratio was also markedly higher in the group of patients with positive test exercise results, especially in those with ST depression >2 mm (1.02, SD 0.06), compared with those subjects with negative results (0.60, SD 0.04). A similar trend was also found 24 and 72 h after the exercise test. We conclude that exercise-myocardial ischemia may lead to an increased LD 1:2 ratio in serum, and demonstrate a correlation between the degree of ischemia and the LD 1:2 ratio. Determination of the LD 1:2 ratio, even in the presence of normal total LD activity, may assist in the clinical evaluation of patients performing an exercise test.

Additional Keyphrases: myocardial ischemia • variation, source of • LD 1:2 ratio

Lactate dehydrogenase (LD, EC 1.1.1.27) isoenzymes in serum are sensitive and specific indicators of acute myocardial infarction (1–3). In about 80% of patients with acute myocardial infarction the LD 1:2 ratio is >1.0 (1–5). In myocardial ischemia, however, the activities of cardiac enzymes are seldom increased, there being only a few reports (6, 7) of an increased creatine kinase (CK, EC 2.7.3.2) MB fraction (CK-MB) in some patients with acute myocardial ischemia as long as 24 h after onset of chest pain. A possible pathological basis for this finding may be myocardial damage such as myocytolysis and coagulation, as have been described in postmortem examination of patients with acute myocardial ischemia (8–12).

The aim of our study was to determine the patterns of LD isoenzymes 1 and 2 in sera from patients performing an exercise test and to discover whether there is a correlation between the LD 1:2 ratio and the degree of exercise-induced myocardial ischemia.

Materials and Methods

Subjects. The study population consisted of two groups:

Group A. Fifty-six male patients with proven coronary artery disease (history of acute myocardial infarction, or significant angiographically proven coronary artery obstruction, or both). Patients with congestive heart failure were excluded from the study. None of the 56 patients had clinical or laboratory findings suggesting hemolysis or renal injury. Their mean age was 60 (range 45–63) y.

Group B. Forty healthy men, not suffering from ischemic heart disease, mean age 65 (range 40–60) y.

Exercise test. The two groups of subjects performed a submaximal exercise test according to the modified Bruce protocol (13). Results of the test were classified as positive, when there was horizontal or downsloping depression or elevation of the ST segment by at least 1 mm and a duration of 0.08 s in any of the leads. This positive test result was subdivided into two groups: (a) ST depression 1–2 mm, (b) ST depression >2 mm. Test results were negative when the ST segment was depressed by <1 mm in the presence of a submaximal heart rate. Results were considered indeterminate when there was an absence of ischemic changes in patients who were unable to reach a submaximal heart rate.

LD assays. Total serum LD activity and proportions of LD isoenzymes were determined in each subject before and 24, 48, and 72 h after the exercise test. Total LD was measured by the method of Wacker et al. (14) at 37°C, in a GEMSAEC centrifugal analyzer (Electro-Nucleonics, Fairfield, NJ). The normal reference interval in our laboratory is 100–225 U/L and the CVs for normal and abnormal concentrations of LD in serum are 3.2% and 4.2%, respectively. The proportions of the LD isoenzymes were determined by electrophoresis on cellulose acetate plates, with the Helena kit (cat. no. 5451) and instrumentation (Helena Laboratories, Beaumont, TX). The normal reference interval for LD-1 in our laboratory is 30–90 U/L; for LD-2, 35–100 U/L. The reference interval for the LD 1:2 ratio, based on data obtained in our laboratory for 150 healthy subjects and based on values in the literature (4), obtained by the same methods, for 200 subjects is 0.45–0.74.