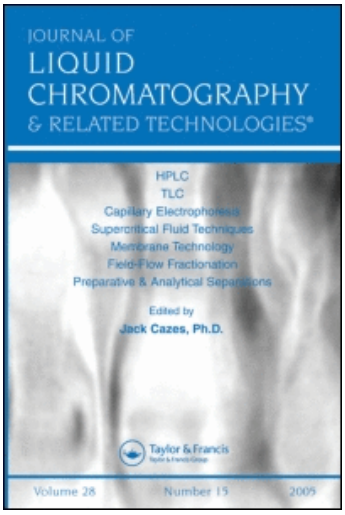

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DETERMINATION OF TOLFENAMIC ACID IN HUMAN PLASMA BY HPLC

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ABSTRACT

Tolfenamic acid is a potent prostaglandin synthetase inhibitor used clinically as non-steroidal anti-inflammatory and analgesic-antipyretic agent. A simple, sensitive, accurate, and precise reverse phase high performance liquid chromatographic method has been developed and validated for the quantitative determination of tolfenamic acid in small volumes of human plasma. The chromatographic separation of tolfenamic acid and the internal standard (phenylbutazone) was performed on a reversed phase, 5-μm C18 column (250 x 4 mm) using acetonitrile-10 mM phosphoric acid (60:40, v/v) as mobile phase with a flow rate of 1.1 ml/min and the chromatographic peaks were detected at 280 nm. Plasma was deproteinized with acetonitrile, the supernatant fraction was evaporated to dryness and the resulting residue was reconstituted in the mobile phase and injected into the HPLC system. Calibration curves were linear in the range 0.2-5.0 μg/ml with a squared correlation coefficient (r^2) of 0.999 or better and the detection limit was 50 ng/ml for 100-μl plasma samples. The method was not interfered with by other endogenous compounds or metabolites and one assay can be completed in 12 min. The within-day and between-day assay variation for three different concentrations was found to be less than 6% and the accuracy was nearly 100%.

INTRODUCTION

Tolfenamic acid, N-(2-methyl-3-chlorophenyl)anthranilic acid, is a potent non-steroidal anti-inflammatory agent with analgesic and antipyretic activities (1,2).

Like other fenamates tolfenamic acid is a prostaglandin synthetase inhibitor and its clinical efficacy has been documented in rheumatoid arthritis, dysmenorrhea, and migraine (3-5). Recently, tolfenamic acid has been introduced as a veterinary treatment and it is also used to dope horses (6).

To study the pharmacokinetics of tolfenamic acid in neonatal and pediatric patients, it is important to minimize the total volume of plasma required for the analysis. For the determination of tolfenamic acid in human plasma high performance liquid chromatographic (HPLC) methods have been previously reported (7-10). However, these methods require relatively large plasma samples, time-consuming or expensive sample preparation techniques, or use commonly used compounds as internal standards (e.g. caffeine) which may interfere with the assay, and are not directly applicable to the determination of plasma levels of tolfenamic acid in pediatric patients.

The present study was undertaken to develop a simple, sensitive, and reliable isocratic reversed phase HPLC assay for the determination of tolfenamic acid in small volumes of human plasma, with advantages over previously published methods (7-10). The method has been applied to the pharmacokinetic study of pediatric patients receiving oral administration of tolfenamic acid.

MATERIALS AND METHODS

Chemicals and reagents

HPLC-grade acetonitrile and analytical reagent grade 85% phosphoric acid were obtained from Merck (Darmstadt, Germany). Tolfenamic acid (batch 10818) was kindly supplied by ELPEN (Athens, Greece). The internal standard, phenylbutazone was purchased from Sigma (St. Louis, MO, USA).

HPLC instrumentation

The liquid chromatographic system (Varian, Palo Alto, CA, USA) consisted of a 2510 high-pressure solvent delivery pump, a 2550 variable-wavelength UV-Vis detector set at 280 nm, and a 7125 manual injector with a 20-µl fixed loop (Rheodyne, Cotati, CA, USA). The analysis was performed using a LiChrospher 100 RP-18, 5-µm particle size, analytical column (250 x 4 mm, ID) (Merck, Darmstadt, Germany) proceeded by a guard column (30 x 4.6 mm, ID) dry packed with C18 ODS (37-53 µm). The chromatograms were recorded on a 4290 integrator (Varian, Palo Alto, CA, USA), and peak heights were reported.

Chromatographic conditions

The mobile phase consisted of acetonitrile-10 mM phosphoric acid solution (60:40, v/v) and the resulting pH was approximately 2.6. The mobile phase was prepared fresh on the day of analysis and was filtered through a 0.45-µm pore size nylon filter (Alltech, Deerfield, IL, USA) and degassed by ultrasonic treatment before use. The HPLC system was allowed to equilibrate isocratically at a flow rate of 1.1 ml/min resulting in an inlet pressure of approximately 1700 psi. All chromatographic separations were carried out at ambient temperature and the effluent was monitored at 280 nm.

Preparation of standard solutions

Stock standard solutions of tolfenamic acid and the internal standard, phenylbutazone (0.1 mg/ml) were prepared in acetonitrile and stored at -20^{0}C. An aliquot of tolfenamic acid solution was diluted 1:10 with acetonitrile to produce

fresh working standard solution (0.01 mg/ml). Internal standard solution consisting of 4.0 μg/ml of phenylbutazone was prepared in acetonitrile and stored at -20°C. Calibration standards were prepared using 1.5-ml conical polypropylene tubes (Eppendorf, Hamburg, Germany) in 100 μl of drug-free plasma spiked with appropriate volumes of tolfenamic acid working solution and 250 μl of internal standard solution to prepare standards with concentrations of 0.2, 0.5, 1.0, 2.0, 3.0, and 5.0 μg/ml. Aliquots of acetonitrile were added to each tube to make the final volumes equal (400 μl).

Sample preparation

The sample preparation employed was as previously described (11). A 100 μl sample of plasma was pipetted into a 1.5 ml conical polypropylene Eppendorf tube. A 250-μl aliquot of internal standard solution and 50 μl of acetonitrile were added, mixed on a vortex mixer for 60 sec and centrifuged at 9000 g for 3 min. An aliquot of the supernatant fraction (250 μl) was transferred into a new Eppendorf tube and evaporated to dryness in a SC110A Savant SpeedVac concentrator (Farmingdale, NY, USA). The residue was then reconstituted with 50 μl of mobile phase, mixed on a vortex agitator and a 20-μl aliquot was injected into the HPLC system.

Quantitation

Tolfenamic acid concentrations in plasma samples were calculated by interpolation from the linear least-squares regression line of the standard curve plot of peak height ratio tolfenamic acid/phenylbutazone, *versus* tolfenamic acid concentration in the calibration standards. The concentrations of tolfenamic acid

standards were chosen to cover the range of tolfenamic acid concentrations in children following oral administration with this drug.

RESULTS AND DISCUSSION

The retention times for phenylbutazone (internal standard) and tolfenamic acid were 7.9 and 11.5 min respectively. The retention times were found to be reproducible and the coefficients of variation were less than 0.3%. Typical chromatograms obtained from human plasma samples are shown in Fig. 1. Fig. 1A shows the chromatogram from the analysis of drug-free human plasma. No interfering peaks due to endogenous compounds or metabolites were observed near the retention time corresponding to phenylbutazone or tolfenamic acid. Fig. 1B shows the chromatogram of a plasma sample obtained from a 22-month hospitalized feverish child who had received a 1 mg/kg oral administration of tolfenamic acid (syrup). The plasma concentration of this patient 4 h after administration was 0.6 μg/ml.

A least-squares linear regression analysis was used to calculate the equation relating the peak height ratio tolfenamic acid/internal standard (Y) *versus* tolfenamic acid concentration (μg/ml) in spiked plasma samples (X). The calibration curves were linear (r^2>0.999) in the range 0.2-5.0 μg/ml tolfenamic acid concentration and the intercepts did not differ significantly from the origin. A typical equation calculated from a calibration curve with six points was: Y = 0.005 + 0.200X with a squared correlation coefficient (r^2) of 0.9997. The lower limit of detection for tolfenamic acid was 50 ng/ml. At this concentration the signal to noise ratio was greater than 5:1.

Table 1 summarizes the within-day and between-day precision and accuracy data of the method at three different concentrations in spiked plasma samples, 0.5,

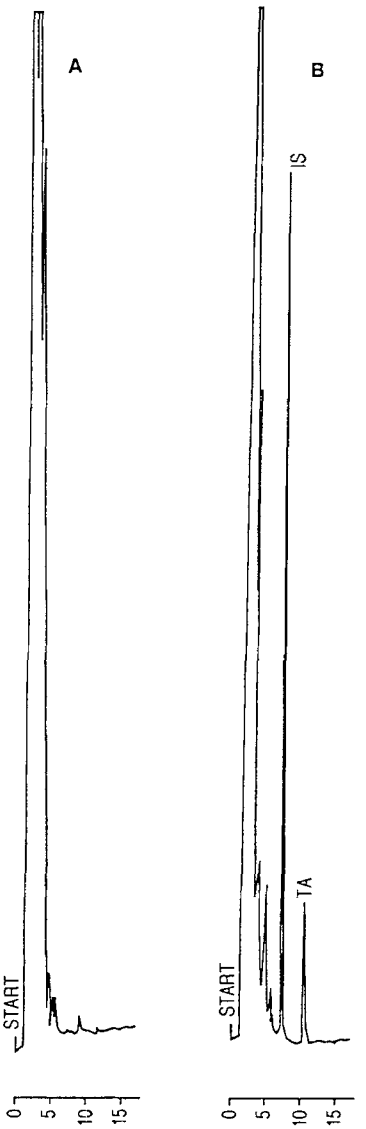

FIGURE 1. HPLC of plasma samples: (A) drug-free plasma; (B) sample obtained from a child 4 h after a single 1 mg/ml oral dose of tolfenamic acid containing 0.6 μg/ml of the drug. Peaks: IS = phenylbutazone (internal standard); TA = tolfenamic acid.

TABLE 1

Within-day and Betwen-day Precision and Accuracy Data of the Determination of Tolfenamic Acid in Human Plasma.

Concentration added (μg/ml)	Concentration found (μg/ml)	Precision CV (%) (n=6)	Accuracy Concentration found/added (%)
Within-day			
0.5	0.48	4.7	96
2.0	1.94	3.9	97
5.0	5.05	4.6	101
Between-day			
0.5	0.47	5.9	94
2.0	1.96	5.1	98
5.0	5.15	4.9	103

2.0, and 5.0 μg/ml for tolfenamic acid. The within-day precision of the method has been evaluated by calculating the coefficient of variation for six determinations at each concentration and was found to be less than 5%. The between-day precision of the method was evaluated by determining the coefficient of variation for six samples at each concentration analyzed on three different days and was found to be less than 6%. The accuracy of the method has been quantified as the percent of the estimated concentration divided by the nominal concentration and was found to be nearly 100%. These data indicate that the method developed provides good accuracy and precision.

In conclusion, the assay utilized a simple, rapid and inexpensive sample preparation procedure required a minimal volume of human plasma (100 μl) from which proteins were removed by acetonitrile precipitation. The HPLC method developed for the quantitation of tolfenamic acid in human plasma is easy, simple,

sensitive, precise, and accurate and seems well suited to characterize tolfenamic acid pharmacokinetics in pediatric patients.

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