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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF TIAPROFENIC ACID AND ITS METABOLITES IN PLASMA AND URINE BY DIRECT INJECTION*

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SUMMARY

A rapid, convenient, sensitive and selective reversed-phase high-performance liquid chromatographic method was developed to measure tiaprofenic acid, its reduced and oxidized metabolites and their conjugates in biological fluids. The method involved direct injections of plasma and urine samples into the chromatograph before and after alkaline hydrolysis of the conjugates. Concentrations as low as 0.5 μg/ml of the drug in plasma and urine were quantifiable. The method was suitable for analysis of tiaprofenic acid and its metabolites in biological fluids after administration of therapeutic doses. Several other non-steroidal anti-inflammatory drugs which were applied to the system did not interfere with the assay.

INTRODUCTION

Tiaprofenic acid (Surgam®, Roussel) is a new non-steroidal anti-inflammatory drug (NSAID) which is under clinical trial in North America. The drug is a potent inhibitor of prostaglandin synthesis [1] and, despite its short half-life ($t_{1/2}$), seems to be as effective as other NSAIDs with longer $t_{1/2}$ if given 200 mg three times a day [2]. Tiaprofenic acid (TA) and its reduced (R) and oxidized (O) metabolites (Fig. 1) have been measured in biological fluids using a thin-layer chromatographic—spectrometric method [3]. More recently, a high-performance liquid chromatographic (HPLC) method describing extraction and determination of TA in plasma of a man was reported [4]. In

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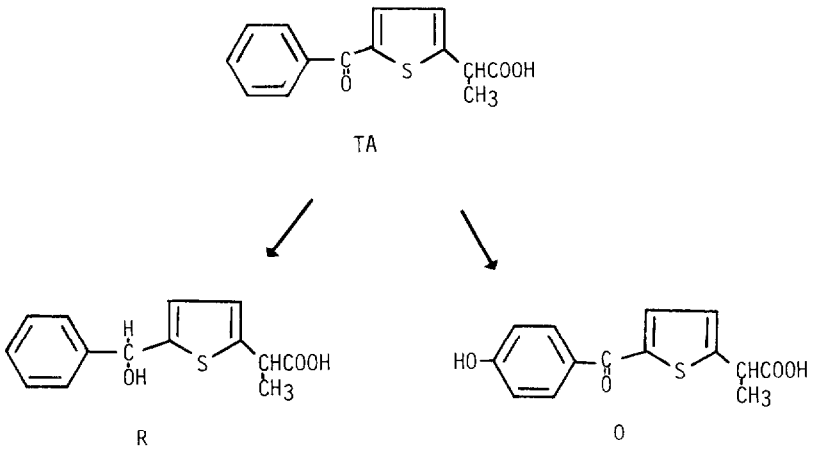


Fig. 1. Chemical structure of tiaprofenic acid (TA) and of its reduced, α-(5-benzyl alcohol-2-thienyl)propionic acid (R), and oxidized, α-[5-(4-hydroxybenzoyl)-2-thienyl]propionic acid (O).

this paper we report a convenient, selective and sensitive method for measuring TA, R and O and their conjugates in biological fluids.

EXPERIMENTAL

Apparatus

The HPLC system (Waters Sci., Mississauga, Canada) consisted of a Model 6000A pump, a Wisp autosampler, a Model 481 variable-wavelength ultraviolet (UV) detector, a Model 730 data module and a 10-cm reversed-phase column (Novapac C_{18}; 5-μm Radial-Pak cartridge, Waters Sci.). A 5-cm guard column (Whatman, Clifton, NJ, U.S.A.) packed with 10-μm C_{18} material was utilized throughout the experiment. The mobile phase, methanol—water—acetic acid (57:40:3) was pumped at a flow-rate of 1.5 ml/min and pressure of 3.5 MPa after filtering through 45-μm filters (Millipore, Mississauga, Canada).

UV spectra of TA, O and R in mobile phase showed maximum absorbances at 305, 254 and 315 nm, respectively. They all showed considerable absorbance at 254 nm while negligible absorbance was detected for R at 305 and 315 nm. Specimens were tested at 254 and 315 nm to examine the presence of the drug and its metabolites. For quantitation purposes, however, plasma samples were monitored at 315 nm and urine samples at 254 nm.

Chemicals

The drug and its metabolites were gifts from Roussel (Montreal, Canada). Naproxen (Syntex, Palo Alto, CA, U.S.A.) was used as internal standard (IS). Salicylic acid (BDH Chemicals, Toronto, Canada), piroxicam (Pfizer, Kirkland, Canada), ketoprofen (Poulenc, Montreal, Canada), fenprofen (Eli Lilly, Toronto, Canada), flurbiprofen (Boots, U.K.) and etodolac (Ayerst, Montreal, Canada) were examined for their elution time using the developed HPLC conditions. All organic solvents were of analytical grade. Water was distilled and deionized.

Sample preparation

To 100—200 μl of plasma or urine were added either 50 μl of water or 50 μl of 1 *M* sodium hydroxide, 50 μl of IS solution (0.5 mg/ml in methanol) and 300 μl of acetonitrile. Samples were vortexed, centrifuged at high speed (Fisher micro-centrifuge, Model 235A) for 2 min, filtered through 2-μm disposable filter tips (Supelco, Bellefonte, PA, U.S.A.) and transferred into vials containing micro-inserts (250 μl). Depending on the concentration of samples 10—200 μl of the preparations were injected into the chromatograph. To quantify the plasma and urine contents, four sets of solutions containing different concentrations of TA (1, 2, 3, 5, 10, 20, 40, 50 and 100 μg/ml) and half of the above concentrations of R and O were prepared. To 50 μl of the above solutions were added 50 μl of blank plasma and after mixing, the preparations were treated as described for plasma and urine samples. The peak area ratios (TA/IS, O/IS and R/IS) were calculated and plotted versus corresponding concentrations. The slope, intercept and correlation coefficient of the resulting standard line were calculated using a regression equation. Samples were always tested against a set of three freshly prepared standard solutions.

Subjects, dosages and sample collections

Two patients (one male and one female) with rheumatoid arthritis (RA) and two healthy male subjects participated in the study voluntarily. Patients were amongst the participants in a clinical trial and under therapy with TA (200 mg t.i.d.). Blood (4—5 ml) and urine (total output) samples were collected from patients during the first daily dosing interval at 0, 0.25, 0.50, 0.75, 1, 2, 3, 4, 5, 6 and 8 h and 0, 1, 3, 5, 7 and 8 h, respectively. Healthy subjects took 400 mg TA 1.5 h before breakfast and their urine samples were taken hourly from 0 to 6 h and then 8, 10, 12 and 24 h post-dosing.

Blood samples were drawn into dry heparinized tubes, centrifuged and the plasma fraction was separated. The volume and pH of urine samples were recorded after collection. The specimens were either tested immediately or kept frozen until the day of analysis. Volunteers were under no other drug therapy at least one week prior to and during the experiment.

Concentrations of TA in plasma and urinary excretion rates of each component were plotted versus time. The slopes of the terminal phases of curves were calculated using a curve-fitting program (Model 41C Hewlett-Packard, Corvallis, OR, U.S.A.).

Stability tests

Solutions of TA were prepared in plasma (16 mg/l), urine (24 mg/l), methanol (22 mg/l) and 1 *M* sodium hydroxide (26 mg/l) and kept at room temperature, 4°C and −20°C. Samples were taken for twelve days to four months, freshly prepared solutions of naproxen were added and were injected into the chromatograph.

RESULTS AND DISCUSSION

Fig. 2 depicts chromatograms of blank plasma and that of a patient 8 h after administration of 200 mg TA. The drug and IS appeared 7.8 and 10.9 min after

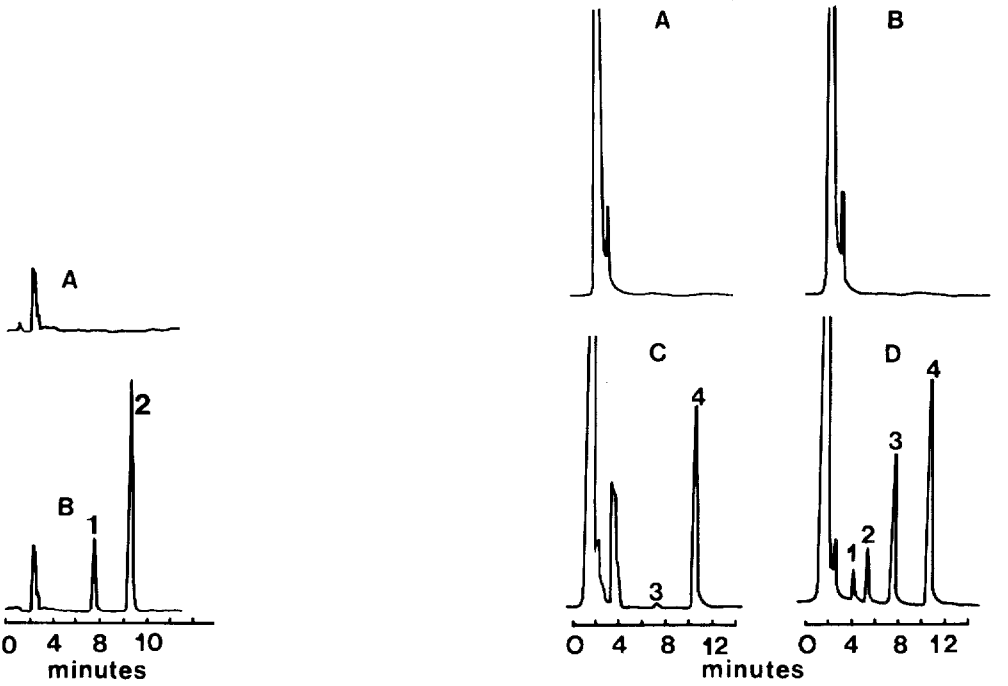


Fig. 2. Chromatograms of blank plasma (A) and plasma of a patient (B) 8 h after a 200-mg dose of tiaprofenic acid (1). Peak 2 is internal standard.

Fig. 3. Chromatograms of blank urine (A and B) and urine of a subject (C and D) 8 h after a 400-mg dose of tiaprofenic acid (3) before (A and C) and after (B and D) alkaline hydrolysis. Peaks 1, 2 and 4 represent reduced and oxidized metabolites and internal standard, respectively.

injection into the instrument, respectively, and no interfering peaks were observed. Although no interfering peak appeared at 305 nm (TA maximum absorbance) a more steady baseline was observed when samples were monitored at 315 nm. None of the metabolites were found in plasma. In urine samples, however, different pictures were observed (Fig. 3). No significant amount of intact TA, R and O were found in urine samples analyzed immediately after collection. Instead, a cluster of few peaks appeared 3 to 4 min after injection (Fig. 3). These peaks seem to be conjugates of TA, R and O. Pottier et al. [3] have reported conjugates of TA in urine. However, due, perhaps, to problems inherent in their assay procedure, they did not separate the intact drug and metabolites from their conjugates. Our direct injection assay method provided a clear picture of TA metabolism in man. Similar to probenecid, naproxen and ketoprofen [5], TA seems to be excreted mainly as ester conjugates. These conjugates are so unstable that their hydrolysis to intact drug takes place even in the bladder and results in conflicting interpretation of data [5]. Conversion of conjugates to TA, R and O was observed even during storage of urine samples at 4° C and after freezing and thawing.

After addition of alkali to the urine of patients, TA, R and O were instantly and completely hydrolyzed to intact compounds (Fig. 3). Moreover, the addition of alkali was accompanied by appearance of a transient red color

which was clearly noticeable when TA concentrations greater than 5 mg/l were present in urine. The color intensity appeared to be concentration-dependent. The nature of the color producing reaction was not studied. Retention times for R and O were 3.8 and 5.6 min, respectively. No interfering peak was eluted from blank urine (Fig. 3).

An excellent correlation was observed between the peak area ratios and concentrations of the components. For urine the best-fit lines through the points were described by $Y = 0.070X - 0.005$, $Y = 0.054X - 0.002$ and $Y = 0.036X - 0.001$ for TA, R and O, respectively. For TA in plasma $Y = 0.084X + 0.040$ was observed. Correlation coefficients of greater than 0.998 were observed for all lines. Concentrations as low as 0.5 μg/ml of TA, R and O in plasma and urine were quantifiable. The coefficients of variation (C.V.) varied from 2% to 5% within the examined range.

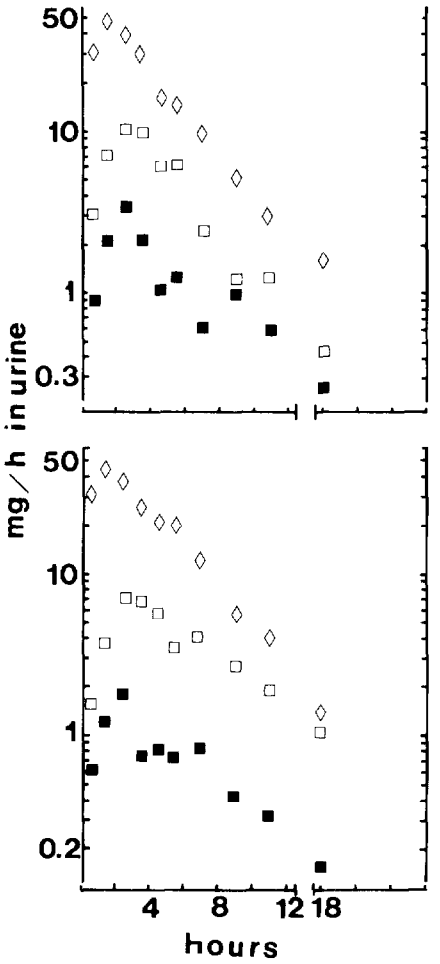


Fig. 4. Urinary excretion rates of tiaprofenic acid (◇) and of its reduced (□) and oxidized (■) metabolites after alkaline hydrolysis in healthy subjects who took 400-mg doses of the drug.

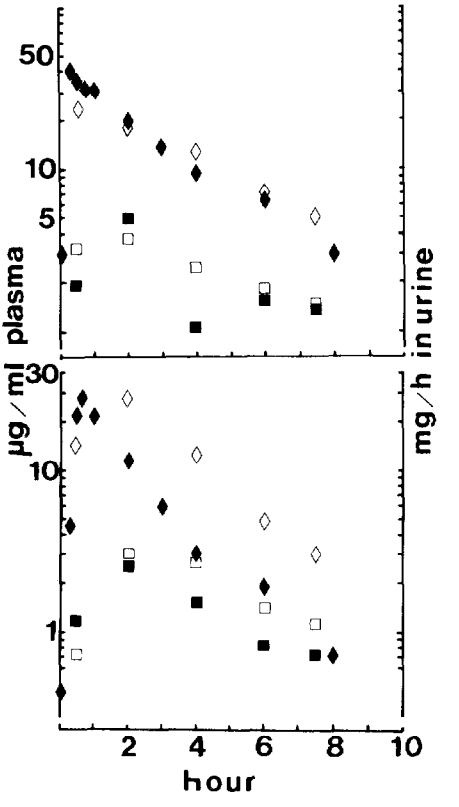


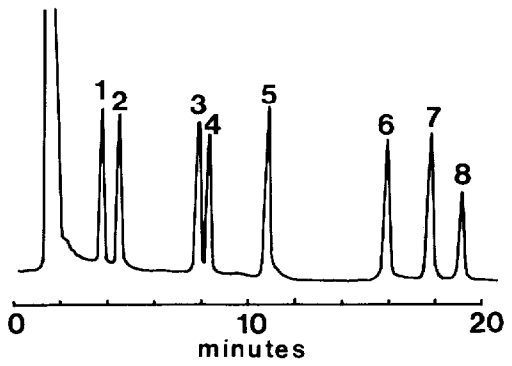
Fig. 5. Plasma tiaprofenic acid (♦) and urinary excretion rates of the conjugates of the acid (◇) and of its reduced (□) and oxidized (■) metabolites in patients under therapy with 200 mg t.i.d.

In Fig. 4, plasma TA concentration—time curves are shown. Our observations agree with those of Ward et al. [4] who reported an HPLC method for determination of TA in plasma of a healthy subject. Their sample preparation method involved extraction with chloroform in acidic medium, evaporation and reconstitution in mobile phase. The method presented here does not require extraction, and, therefore, decreases the preparation time and increases precision. Although our sample contained TA concentrations > 0.5 μg/ml, levels as low as 0.25 μg/ml were also quantifiable with acceptable reproducibility (C.V. $< 9\%$).

After hydrolysis of conjugates of TA, R and O, their urinary excretion rates were plotted versus time as shown in Fig. 4 and 5. The slopes of excretion rate plots were parallel with those of TA concentration—time curves. A total of 76.16—79.50% of the dose, consisting of 59.32—60.60% TA, 14.30—14.35% R and 2.54—4.56% O conjugates, was recovered from urine of healthy subjects. Since patients were at the steady-state drug concentration, the cumulative amount of the drug excreted in one dosing time-interval was considered as total recovery from a given dose. They excreted 67.30—72.24% of the given dose as conjugates of TA (53.46—54.34%), R (8.04—10.65%) and O (5.81—6.57%).

Plasma TA concentration—time curves appeared as multiexponential curves. The half-lives of the terminal phase varied from 2.00 to 2.65 h and values from plasma data were similar to those calculated from urinary data.

Under our HPLC conditions, salicylic acid, piroxicam, ketoprofen, naproxen, fenprofen, flurbiprofen and etodolac had elution times of 3.7, 4.5, 8.3, 10.9, 16.0, 17.8 and 19.2 min, respectively (Fig. 6). Although peaks representing salicylic acid and piroxicam were clearly detectable in the presence of TA metabolites, their peak distances were not long enough to permit quantitation of the components in urine. In plasma, however, since none of the TA metabolites are present, determination of TA in the presence of all the above NSAIDs is possible.



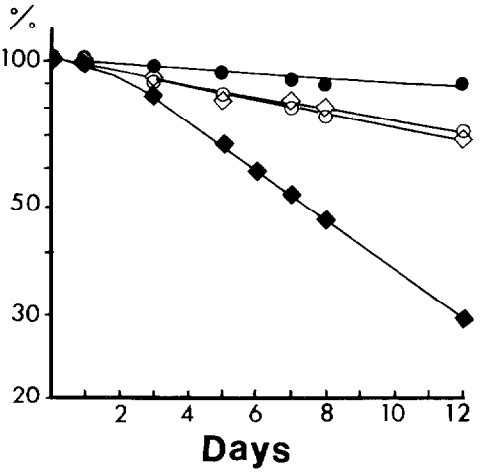


Fig. 6. Blank plasma spiked with salicylic acid (1), piroxicam (2), tiaprofenic acid (3), ketoprofen (4), naproxen (5), fenprofen (6), flurbiprofen (7) and etodolac (8).

Fig. 7. Percent tiaprofenic acid remaining in methanol (•), plasma (◇), urine (○) and 1 *M* sodium hydroxide (♦) at room temperature as a function of time.

No changes in retention times of components and separation properties of the analytical column were observed during the experiment indicating a relatively long useful column life.

TA seems to be stable for at least four months if stored frozen (−20°C) regardless of the nature of the solvent. At 4°C, the drug was stable in methanol, plasma and urine for at least two weeks but not in 1 *M* sodium hydroxide. In the latter solvent, 50% of the added TA was decomposed in twelve days.

Fig. 7 depicts stability of TA in methanol, plasma, urine and 1 *M* sodium hydroxide at room temperature. The drug seems to have limited stability at room temperature in all solvents. The decomposition products of TA appeared as four sharp peaks 6.9, 8.3, 11.3 and 28.2 min after injection.

The method presented here is sensitive and specific, and requires less preparation time than that reported by Ward et al. [4]. It also provides a clear picture of the urinary excretion of TA and its metabolites. The method can be used to measure plasma TA concentrations in presence of other NSAIDs.

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