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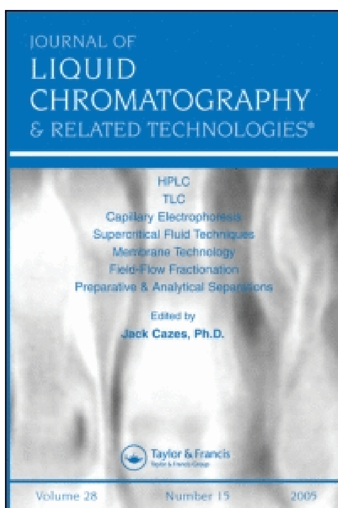

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HPLC ANALYSIS OF TIAPROFENIC ACID IN THE SAMPLES OF WHOLE BLOOD USING L-L AND S-L EXTRACTIONS

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HPLC ANALYSIS OF TIAPROFENIC ACID IN THE SAMPLES OF WHOLE BLOOD USING L-L AND S-L EXTRACTIONS

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ABSTRACTS

In order to evaluate tiaprofenic acid in the samples of whole blood, methods of extraction on solid phases (SLE) and those of extraction into an organic solvent (LLE) were worked out and the suitability of its use for HPLC analysis of tiaprofenic acid was compared. SLE was carried out on the solid phases C-18 and LLE into dichloromethane.

Prior to the commencement of extractions, stability of tiaprofenic acid was examined. After the adjustment of the sample by a selected method of extraction (SLE), tiaprofenic acid was evaluated in blood in a two-hour pharmacokinetic study.

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INTRODUCTION

Tiaprofenic acid (2-(5-benzoyl-2-thienyl)propionic acid), a nonsteroidal anti-inflammatory drug with an analgesic effect, is particularly suitable for the therapy of osteoarthrosis and rheumatism of soft tissues.[1] Of analytical methods, HPLC,[2-7] or capillary electrophoresis,[8] or radiochemical assay of labelled ^{14}C[9,10] are most widely selected to evaluate tiaprofenic acid in biological material. HPLC[11-14] has also been used to separate enantiomers of tiaprofenic acid. Most frequently, plasma,[2-5,11,13,14] serum,[6,7,12] or urine[4,5,11] samples are analysed, mainly in patients,[2-4,6,11-14] or rats[7,12] and horses.[5]

The above-mentioned HPLC method aims at the isolation technique which can most suitably adjust a rabbit whole blood sample prior to the proper HPLC analysis which will serve to determine the blood level of tiaprofenic acid in experiments on laboratory rabbits.

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EXPERIMENTAL

Reagents, Chemicals and Materials

Tiaprofenic acid and diclofenac sodium (IS) were supplied by Léèiva (Prague, Czech Republic). Methanol, methylene chloride, sodium acetate, potassium dihydrogenphosphate, disodium hydrogenphosphate, acetic acid, and H_3PO_4 were obtained from Lachema (Neratovice, Czech Republic), and perchloric acid was supplied by Merck (Darmstadt, Germany). Methanol was HPLC grade, and all other chemicals were analytical-reagent grade. Methylene chloride was distilled and water was bidistilled.

The extraction columns, Silica cart C-18 containing Separon SGX C-18, 60 μm (1 mL cartridge) were supplied by Tessek (Prague, Czech Republic).

Preparation of Buffers

Phosphate Buffer

Mixing 88.9 parts of a potassium dihydrogenphosphate solution (0.01 mol/L) and 11.1 parts of a sodium hydrogenphosphate solution (0.01 mol/L) yielded a buffer of pH 6, which was adjusted to the required pH (from pH 5 to pH 2) by adding phosphoric acid (5 % w/v).

Acetate Buffer

Mixing 95 parts of sodium acetate (0.2 mol/L) and 5 parts of acetic acid (0.2 mol/L) yielded acetate buffer of pH 6. Acidification of the buffer with acetic acid (0.2 mol/l) yielded buffers of required pH (from pH 5 to pH 2).

Biological Samples

Samples of whole heparinized blood were obtained from the Department of Pathological Physiology, Charles University Medical Faculty (Hradec Králové, Czech Republic). The rabbits were administered Surgam inj. siccum. The injection was given *i. v.* at a dose of 10 mg/kg body weight and blood samples were withdrawn 3, 6, 15, 30, 60, and 120 min after drug administration. All samples were frozen immediately after withdrawal.

Sample Preparations

Whole blood samples were first hemolyzed and then adjusted by solid-liquid extraction (using vacuum), or liquid-liquid extraction.

Hemolysis

A 0.25 mL volume of the blood sample was pipetted into a 5 mL glass-stoppered centrifuge test tube, 20 μL of the internal standard solution was added and the blood sample was hemolyzed by adding 0.5 mL of water, shaken for 5 minutes, exposed to the action of ultrasound for 5 minute, and allowed to stand for 5 min at ambient temperature.

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Solid-Liquid Extraction (SLE)

To the hemolyzed blood sample, 1 mL of buffer was added and after shaking (5 min) the sample was centrifuged for 10 min at 1930 g. The supernatant was transferred onto the extraction column, which was pre-activated by washing with 5 mL of methanol and then 5 mL of water. After the application of the supernatant, the column was washed with 10 mL of water. The extraction column was dried with vacuum for 1 min.

The analytes were eluted with 5 mL of methylene chloride. The eluate was evaporated to dryness with a weak nitrogen flow. The remainder was dissolved in 100 μL of the mobile phase and 10 μL was injected onto the column.

Liquid-Liquid Extraction (LLE)

To the hemolyzed blood sample 1 mL of buffer was added and after 5-min shaking, 5 mL of methylene chloride was added. After 5-min shaking and centrifugation (5 min at 1930 g), 4 mL of methylene chloride was separated and evaporated to dryness with a weak nitrogen flow. The remainder was dissolved in 100 μL of the mobile phase and 10 μL was injected onto the column.

Extraction efficacy was determined by comparing the area of the peak of tiaprofenic acid in the extract of the blood sample with the area of the peak of

the methanolic standard of tiaprofenic acid prepared in an equivalent concentration.

Preparation of Standards

The working standard solution of tiaprofenic acid was prepared in methanol by diluting the standard methanolic solution 1 mg/mL, the internal standard used was a solution of 1 mg/mL of diclofenac sodium in water.

The calibration curve was prepared by adding a sufficient amount of a standard solution of tiaprofenic acid (60, 40, 20, 10, and 6 μg/mL of blood) to a sample of whole blood with an addition of the internal standard (800 μg/mL). The samples were adjusted by the method described below.

Concentration of tiaprofenic acid was calculated from the regression equation of the calibration curve, constructed as the dependence of the ratios (y) of the areas of the peaks of tiaprofenic acid and the internal standard on the concentration (x) of tiaprofenic acid.

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Chromatography

The HPLC system consisted of a solvent delivery system SP 8700, and an integrator SP 4100 (both from Spectra-Physics, Santa Clara, CA, USA), and a detector Spectra 100 (Thermo Separation Products, Santa Clara, CA, USA).

Analytical samples were introduced onto the column using a Rheodyne injection valve (Rheodyne, Cotati, CA, USA) with a 10 μL loop. The analytical glass column contained Separon SGX C-18 (150 x 3.3 mm I.D., 5 μm) (Tessek, Prague, Czech Republic).

The mobile phase was a mixture of methanol – water (70:30, v/v) with a final apparent pH of 3.1 adjusted with a 5% (w/w) perchloric acid solution. The flow rate was set at 0.4 mL/min.

The UV absorbance of the column effluent was monitored at 313 nm (and 0.01 aufs).

RESULTS AND DISCUSSION

Stability of Tiaprofenic Acid

In order to avoid complications and imprecision in the quantification of tiaprofenic acid, as well as in the elaboration of the processes of extraction, the

conditions were optimized for the preservation of tiaprofenic acid samples (both the samples of standards and the samples of whole blood or extracts from it, i.e. samples injected on the column). Tiaprofenic acid is described as a photosensitive compound.[5,10,13-15]

Its degradation products have been described by Bosca,[15] its decomposition has been monitored by Zabrzewska,[16] and the photoproduct, i.e. decarboxytiaprofenic acid, has been studied by de Vries.[10] Geisslinger[13] has reported that the substance of tiaprofenic acid was stable for at least 12 weeks when kept in the dark and at –30°C.

Methanolic solutions of tiaprofenic acid of varying pH (3,5,7, and 8.4) were kept on the one hand under common laboratory light conditions, and on the other hand, protected from light in dark glass. Under the laboratory conditions, the decomposition of tiaprofenic acid was observed in all solutions; it decomposed most slowly in a solution of pH 5 (see Figure 1).

In solutions protected from light, slow decomposition occurred only in alkaline medium (pH 8.4). Even keeping these samples in a refrigerator at 4°C did not prevent decomposition of tiaprofenic acid in a methanolic solution of pH 8.4. As reported by Delbeke,[5] tiaprofenic acid is unstable in a strongly alkaline solution even when frozen to -20°C.

Possible complications due to incorrect preservation of samples with tiaprofenic acid were eliminated by keeping the reference solutions of tiapro-

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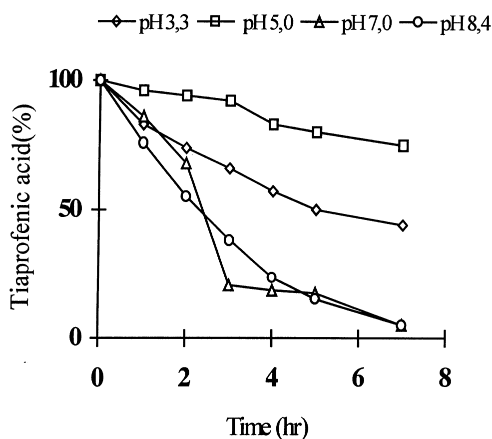

Figure 1. Stability of tiaprofenic acid in methanol solutions of other pH (samples were stored under normal laboratory-light conditions).

fenic acid in brown glass test tubes; on extraction the samples were protected from light including protection of the thickening of the organic solvent into which tiaprofenic acid was extracted.

Also, prior to the injection onto the column the sample (i.e. the remainder after extraction dissolved in the mobile phase) was protected from light in brown glass test tubes.

Isolation of Tiaprofenic Acid from Blood

Both extraction into an organic solvent and extraction on solid phases were tested to isolate tiaprofenic acid from whole blood samples.

Adjustment of plasma, serum, and urine samples prior to the analysis itself was performed either by deproteination,[2,4,7] or liquid-liquid extraction,[5,6,11,13,14] or a combination of deproteination (with trichloroacetic acid) and subsequent extraction into chloroform.[3] In liquid-liquid extraction, besides chloroform,[3,11] ether[5,12] or ether in a mixture with hexane,[13] is employed as the extraction medium. In urine[4,5] tiaprofenic acid is evaluated after alkaline hydrolysis. Solid-solid extraction is not reported in the literature.

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Methylene chloride was selected for the adjustment of whole blood samples by extraction into an organic solvent because fewer residues from blood are extracted into it than into ether or chloroform. Prior to extraction into methylene chloride, the blood sample was adjusted with phosphate buffer (pH 2 to pH 6) and/or acetate buffer of the identical pH range. Acetate buffer of pH 3 proved to be most suitable for isolation, achieving a recovery of 91.3%.

Extraction of tiaprofenic acid from whole blood samples by means of solid phases was also tested. Prior to spreading on the extraction column, the blood sample was adjusted by an addition of buffer (phosphate and/or acetate buffer of pH ranging between 2 and 6). The sample was centrifuged and the supernatant was spread on the extraction column.

The most suitable adjustment was that of using acetate buffer of pH 3 (see Figure 2) and washing the extraction column with water. Tiaprofenic acid was eluted with methylene chloride, the recovery being 90.2%.

Both methods of isolation can be employed to evaluate tiaprofenic acid (Figure 3). Its recovery is nearly identical, but on experimental evaluation of tiaprofenic acid in blood samples extraction on solid phases was employed due to better reproducibility and facility of performance.

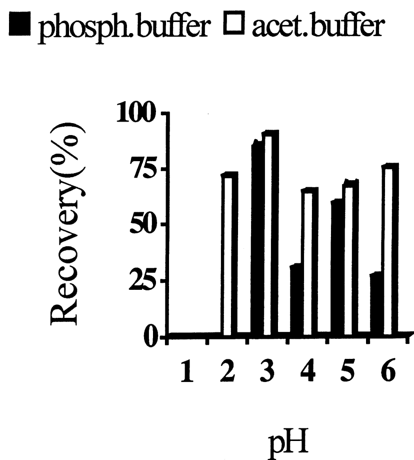

Figure 2. Recovery of tiaprofenic acid dependent on the value of pH and used buffer solutions.

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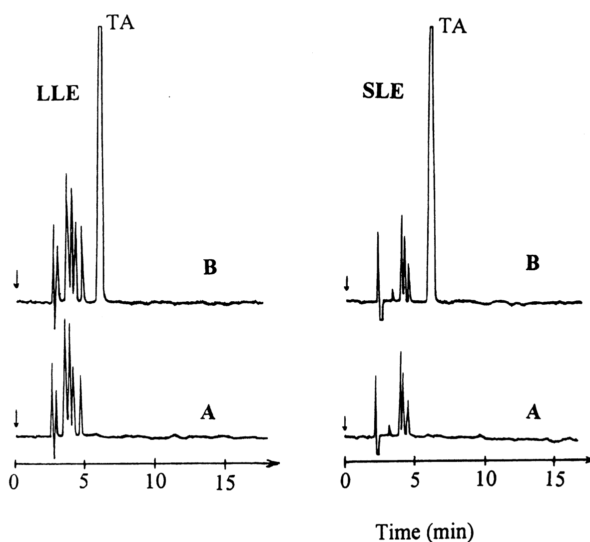

Figure 3. Typical chromatograms of blood samples adjusted with acetate buffer solutions (pH 3,0). Tiaprofenic acid (TA) was either extracted into methylene chloride (LLE) on to the solid phase (SLE). A - blank sample, B - blood sample spiked with the standard of tiaprofenic acid (4 μg/mL).

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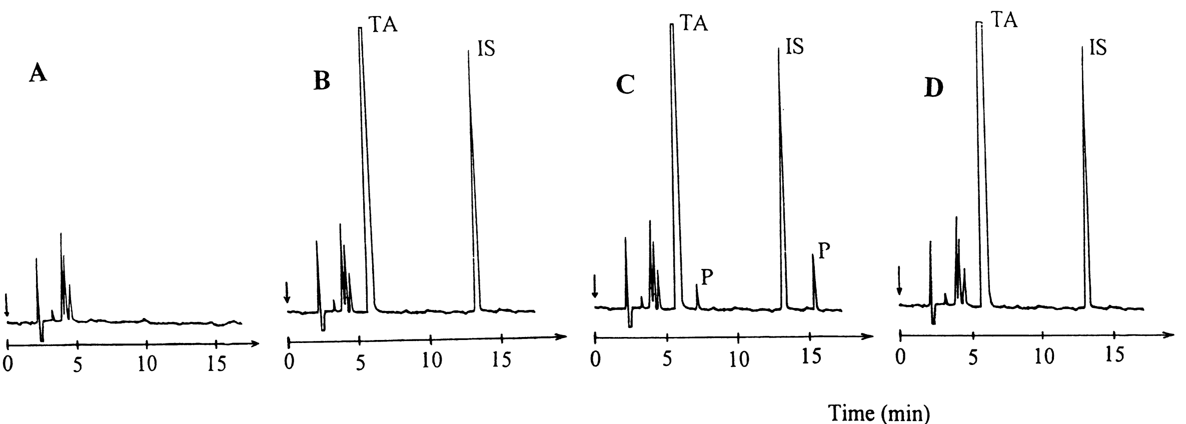

Figure 4. Typical chromatograms of tiaprofenic acid in samples of whole blood. A - blank sample, B - control sample spiked with the standard of tiaprofenic acid TA (4µg/mL) and IS (80 µg/mL), C - control sample after 2 hours under normal laboratory-light conditions(decomposition products P), D - 30-min sample from a rabbit given a dose of 50 mg/kg of tiaprofenic acid.

HPLC Analysis

Under the selected chromatographic conditions, the peaks of tiaprofenic acid and the internal standard were well separated from the peaks of the residues of endogeneous substances (Figure 4). Also, in the selection of the internal standard, a possibility of verification of the stability of tiaprofenic acid was taken into consideration which meant that the peak of the internal standard was not allowed to interfere with the peaks of potential decomposition products, so that possible decomposition products could be detected in the HPLC chromatogram (see Figure 4 C). These requirements for the internal standard were best fulfilled by diclofenac.

Concentration of tiaprofenic acid in whole blood, adjusted by extraction on solid phases, was determined from the calibration curve $y = 0.06863\ x - 0.3093$ ($r = 0.9867$). Precision and reproducibility in series and time was verified by analysing four samples in whole blood. Reproducibility in time was verified during a three-week period of preservation in brown glass.

The found values of concentrations are shown in Table 1; the values of the relative standard deviation (R.S.D.) do not exceed 5%. The limit of quantification of tiaprofenic acid, determined experimentally, was 0.2 μg/mL in blood and the detection limit was 0.06 μg/mL of blood.

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Table 1

Precision and Accuracy of the Assay for Tiaprofenic Acid in Blood

Added (μg/mL)	Mean ± SD (%)
Within-day (n = 6)	
10.0	98.33 ± 3.84
24.0	99.46 ± 2.63
48.0	99.46 ± 2.17
60.0	99.50 ± 1.68
Day-to-day (n = 10)	
10.0	96.22 ± 4.24
24.0	98.71 ± 3.46
48.0	99.01 ± 2.35
60.0	98.69 ± 1.88

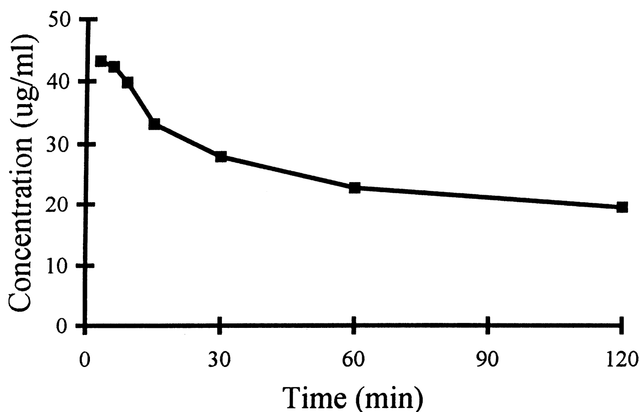

Figure 5. Concentration of tiaprofenic acid in rabbit blood following intravenous administration.

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Pharmacokinetic Study

The elaborated method was used in the pharmacokinetic examination of tiaprofenic acid in blood. Blood samples were frozen immediately after withdrawal and protected from light. The found levels of tiaprofenic acid in blood are shown in Figure 5.

CONCLUSION

The present HPLC method was developed in co-operation with the Department of Pathological Physiology of the Charles University Medical Faculty in Hradec Králové. In laboratory rabbits which were administered tiaprofenic acid whose level in blood was examined, various physiological or pathophysiological parameters influenced by the drug will be investigated.

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