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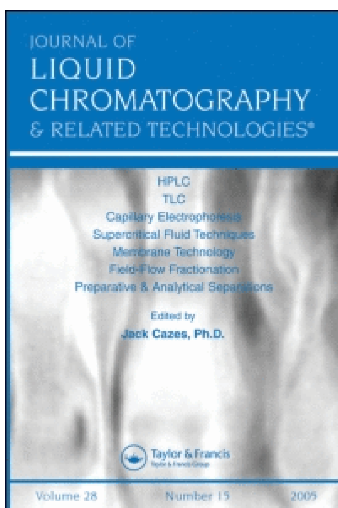

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Simultaneous Reversed-Phase Gradient-HPLC Analysis of Anthranilic Acid Derivatives in Anti-Inflammatory Drugs and Samples of Biological Interest

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SIMULTANEOUS REVERSED-PHASE GRADIENT-HPLC ANALYSIS OF ANTHRANILIC ACID DERIVATIVES IN ANTI-INFLAMMATORY DRUGS AND SAMPLES OF BIOLOGICAL INTEREST

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ABSTRACT

A simple, rapid and sensitive reversed-phase gradient High-Performance Liquid Chromatographic (HPLC) method is proposed for the determination of anthranilic acid derivatives in anti-inflammatory drugs and biological fluids.

HPLC analyses are performed with a Lichrosorb-RP 18, 10 μm, 250x4 mm I.D., column employing gradient elution for the development of the chromatogram. The gradient system is formed with the following solutions: A=0.065M ammonium acetate and B=methanol. The flow rate is 0.8 ml/min and the detection is achieved at 282 nm. The retention time is 4.63 min for flufenamic, 4.99 min for mefenamic and 5.46 min for tolfenamic acid. The absolute detection limits are 0.5 ng for flufenamic acid, 0.7 ng for mefenamic acid and 1.0 ng for tolfenamic acid. The linearity is observed up to 70 ng for the flufenamic acid, 70 ng for the mefenamic acid and 80 ng for the tolfenamic acid injected. The method involves the use of solid phase extraction for sample clean-up and subsequent separation of anthranilic acid derivatives and internal standard-caffeine-from endogenous interfering compounds on a reversed-phase column.

The proposed method is applied to the analysis of flufenamic, mefenamic and tolfenamic acid in blood serum (40 μl), urine (200 μl), tablets, capsules, suppositories, suspensions and ointment.

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INTRODUCTION

Flufenamic acid, [N-(3-trifluoromethylphenyl)] anthranilic acid, Mefenamic acid [N-(2,3-xylyl)]anthranilic acid and Tolfenamic acid [N-(2-methyl-3-chlorophenyl)]antranilic acid (fig. 1) are potent nonsteroidal analgesic and anti-inflammatory agents used in the management of rheumatoid arthritis. These compounds are used in the production of anti-inflammatory drugs which are widely prescribed.

Large doses of fenamates produce excitement, incoordination depression, and convulsions in mice, while it has been stated that the possible effects of massive overdosage in man include flaccid depression, coma, convulsions, diarrhoea, and heamotological abnormalities. With the exception of convulsions these effects have not been confirmed in practice.

Although information on therapeutic actions and side-effects of anthranilic acid derivatives are available[1-4], very little has been published on the simultaneous analysis pharmacokinetics and toxicity overdosage[5-10].

A number of methods have been published for the individual determination of some anti-inflammatory drugs in serum[11-14]. These include colorimetry, spectrofluorimetry and gas chromatography. Some of these methods are non-specific, others involve complex extraction procedures and derivatization steps. Although some methods have been presented for the analysis of these drugs in serum using high-performance liquid chromatography (HPLC)[15-16], they require at least 1 ml of serum and are only applicable to a specific drug.

During these days High-Performance Liquid Chromatography (HPLC) has been used as an acceptable technique for the simultaneous analysis of organic compounds because of the limitations of interferences, the advantages in speed, ease and sensitivity. This technique gives the analyst the opportunity to obtain quantitative results in a few minutes.

In this paper, a simple, sensitive and fast method for the simultaneous reversed-phase gradient HPLC analysis of flufenamic, mefenamic and tolfenamic acid derivatives in anti-inflammatory drugs and samples of biological interest, blood serum and urine, is presented.

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EXPERIMENTAL

Apparatus: Anthranilic acid derivatives analyses were carried out with a high performance liquid chromatograph consisting of a ternary gradient pump (Spectra Physics, Model SP 8800 California USA), a Spectra Physics Spectra Chrom 100 variable wavelength UV-VIS detector operated at 282 nm and a sensitivity setting of 0.002 absorbance units full scale (AUFS). A Rheodyne 7125 (California USA) injection valve was fitted with a 10 μl loop. The pressure was 1750 psi at a flow rate of 0.8 ml/min. The analytical column was a Lichrosorb RP-18, 10 μm ODS, 250x4 mm I.D., stainless steel from MZ Analysentechnik. Bond Elut C_{18} cartridges were obtained from Analytichem International, a division of Varian (Harbor city USA).

Computations were performed using a PC Vip 220 computer.

Materials: Mefenamic acid and Tolfenamic acid, anhydrous powders (Ph.Eur.) were supplied from ELPEN Athens. Flufenamic acid was obtained from SIGMA Chemical Co. (St. Louis, MO, USA).

Caffeine was from BDH Chemicals Ltd., Poole, England. These reagents were used, as provided without further purification. All standard solutions of these compounds were prepared by dissolving the appropriate amounts in methanol. Ammonium acetate, acetic acid and sodium hydroxide, pro analysi reagents were from Merck (Darmstadt Germany). HPLC-grade methanol and acetonitrile were also from Merck. All other reagents used were of analytical grade and glass-distilled water was used throughout.

Chromatographic Conditions: The gradient system was formed with the following solutions: A=0.065M ammonium acetate and B=Methanol.

The chromatogram is started with solvent B containing 25% solvent A, it is decreased from 75% to 70% by an increase from 25 to 30% of solvent A. This linear gradient is achieved from 0 to 6 min. This gradient elution solvent system was selected through a number of other mobile phases and systems on the basis of their relative polarities and low absorption at the wavelength used. The flow rate was low 0.8 ml/min, as well as the sensitivity, 0.002 AUFS.

The separations were performed at ambient temperature 22°C. The chromatographic conditions about solvent systems were selected among several ones

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examined as shown in Table 1, since they were found to be the most suitable for a good separation of the anthranilic acid derivatives.

System Suitability: The reversed phase analytical column was equilibrated with methanol and the eluting solvent system used, at a flow rate of 0.8 ml/min. After an acceptable stable baseline was achieved, the samples, the internal standard caffeine and the standards were analyzed. The resolution factors, R_t, were calculated between the four peaks[18] and found to be: 2.15 for caffeine, 5.54 for flufenamic acid, 6.57 for mefenamic acid and 6.92 for tolfenamic acid. These resolution factors signify complete separation between anthranilic acid derivatives. This separation is given in Figure 1.

The relative standard deviations of eight replicate analyses of three standards 0.5, 2 and 3 ppm were found to be at the range 2.42, to 5.54 %, 0.37 to 3.86 % and 0.27 to 2.17 % for flufenamic acid, for mefenamic acid and for tolfenamic acid respectively. These results are illustrated in Table 2.

Selectivity: The selectivity of the gradient elution HPLC method was investigated at the retention times of caffeine, flufenamic acid, mefenamic acid and tolfenamic acid. No interferences from endogenous compounds were found in chromatograms of samples extracted from blood serum. Therefore, the described method can be used in the analyses of anthranilic acid derivatives using caffeine as internal standard. The same derivatives can be determined in urine samples without internal standard, because caffeine is not separated from endogenous compounds.

Detection and Quantitation Limits: The detection limits of anthranilic acid derivatives were assessed in the presence of the internal standard, caffeine, and were considered to be the quantities producing a signal of a peak height twice the size of background noise. The minimum detectable quantities, expressed in ng injected on the column, were found to be 0.5 ng, 0.7 ng and 1.0 ng for flufenamic acid, mefenamic acid and tolfenamic acid respectively.

The quantitation limits of the above named components were assessed in the presence of the internal standard and were considered to be the quantity producing a signal five to ten times the peak height the detection limit quantity produces. These limits are 5 to 30 ng for all anthranilic acid derivatives.

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Table 1. Isocratic (Is) and Gradient (Gr) HPLC Conditions Examined in the Separation and Determination of Anthranilic Acid Derivatives.

Eluent System Isocratic (Is), Gradient (Gr)	Flow Rate (ml/min)	Retention Time (min)			
		Caffeine	Flufenamic acid	Mefenamic acid	Tolfenamic acid
(Is) A=18 B=82	1.0	1.46	1.89	2.65	2.65
(Is) A=18 B=82	1.9	1.46	1.80	2.53	2.53
(Is) C=18 B=82	1.0	2.77	2.96	3.65	3.65
(Gr) 0min A=20, B=80, D=0 7min A=30, B=60, D=10	1.2	2.31	3.19	4.42	4.42
(Gr) 0min E=20, B=80 12min E=30, B=70	1.2	2.33	3.14	4.47	4.47
(Gr) 0min E=20, B=80, D=0 7min E=30, B=60, D=10	1.2	2.29	2.55	4.15	4.16
(Gr) 0min C=25, B=75 10min C=0, B=100	0.7	4.01	4.44	5.50	5.50
(Gr) 0min C=30, B=70 7min C=0, B=100	1.2	2.45	3.70	4.10	4.60
(Gr) 0min C=30, B=70 8min C=10, B=90	1.2	2.38	3.93	3.93	4.41
(Gr) 0min C=30, B=70 8min C=70, B=30	1.2	2.48	4.01	4.01	4.52
(Gr) 0min F=40, B=60 10min F=20, B=80	1.0	3.28	8.07	8.07	8.74
(Gr) 0min C=20, B=80 10min C=10, B=90	1.0	2.34	2.63	2.96	2.96
(Gr) 0min C=30, B=70 8min C=22, B=78	0.9	2.46	3.95	4.17	4.75
(Gr) 0min C=25, B=65 8min C=35, B=65	0.8	3.73	4.66	5.05	5.54
(Gr) 0min C=25, B=75 6min C=33, B=67	0.8	3.64	4.47	4.86	5.36
(Gr) 0min C=25, B=75 8min C=30, B=70	0.8	3.62	4.55	4.90	5.38
(Gr) 0min C=25, B=75 6min C=30, B=70	0.8	3.66	4.63	4.99	5.46

A=Acetate buffer[17] pH=4.6
B=Methanol
C=Ammonium acetate 0.065 M
Sensitivity (AUFS)=0.002
D=Acetonitrile
E=Alkaline Solution of pH value 7-8, with NaOH 1M.
F=0.7% Ammonia-Ammonium chloride buffer

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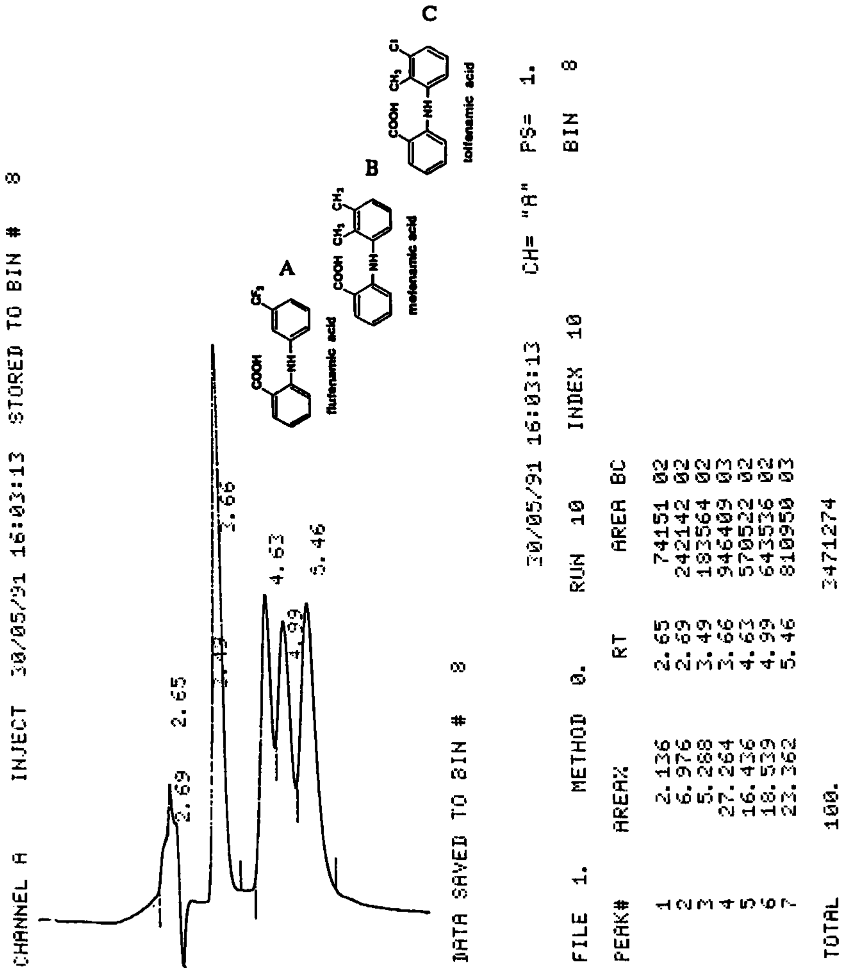

Figure 1. High-Performance Liquid Chromatogram of Anthranilic Acid Derivatives using Caffeine as Internal Standard. Peaks: (3.66) = Caffeine [1 ppm], (4.63) = Flufenamic acid [1 ppm], (4.99) = Mefenamic acid [1 ppm] and (5.46) = Tolfenamic acid [1 ppm]. Chromatographic conditions as decribed in text.
A = Flufenamic acid, B = Mefenamic acid, C = Tolfenamic acid.

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Table 2. Peak Area Ratios of Anthanilic Acid Derivatives to Internal Standard Caffeine over the concentration ranges 0.5-3 ppm in methanolic solutions, equivalent quantities injected 5-30 ng.

Concentration of Anthranilic Acid Derivatives (ppm)	Equivalent Quantity Injected (ng)	Peak Area Ratios of Anthranilic Acid Derivatives to Internal Standard Caffeine										
Flufenamic Acid		1	2	3	4	5	6	7	8	Mean value	SD	RSD (%)
0.5	5	0.2365	0.2258	0.2289	0.2192	0.2256	0.2311	0.2395	0.2288	0.2294	$6.41 \cdot 10^{-3}$	2.79
2	20	1.0209	1.0115	1.0706	0.9605	1.0813	1.0677	1.0583	1.0433	1.0393	0.040	3.86
3	30	1.4619	1.4190	1.4358	1.4821	1.4388	1.5016	1.4973	1.4924	1.4661	0.0318	2.17
Mefenamic Acid												
0.5	5	0.2653	0.2702	0.3066	0.3005	0.2766	0.2823	0.2955	0.2688	0.2833	0.0157	5.54
2	20	1.0694	1.0702	1.0689	1.0746	1.0711	1.0653	1.0703	1.0787	1.0711	$4.01 \cdot 10^{-3}$	0.37
3	30	1.6688	1.6799	1.6695	1.7608	1.6733	1.6660	1.6687	1.6754	1.6716	$4.45 \cdot 10^{-3}$	0.27
Tolfenamic Acid												
0.5	5	0.3378	0.3208	0.3305	0.3216	0.3200	0.3129	0.3178	0.3264	0.3235	$7.84 \cdot 10^{-3}$	2.42
2	20	1.2204	1.2619	1.2731	1.2825	1.2699	1.2515	1.2703	1.2743	1.2630	0.019	1.54
3	30	2.0308	1.9282	1.9224	1.9703	1.9649	1.9588	1.9862	1.9397	1.9627	0.035	1.79

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Calibration Curves for the Simultaneous Determination of Anthranilic Acid Derivatives in Methanol: Calibration curves for the determination of flufenamic acid, mefenamic acid and tolfenamic acid were constructed in the presence of internal standard, caffeine. Flufenamic acid, mefenamic acid, tolfenamic acid and caffeine were accurately weighed and dissolved in methanol to give stock solutions of 100 ng/μl each. Standard flufenamic acid, mefenamic acid and tolfenamic acid solutions of 0.1, 0.2, 0.3, 0.5, 1, 2, 3, 5, 6, 7, 8 and 10 ng/μl were prepared with caffeine, in 50-ml volumetric flasks, by serially diluting the stock flufenamic acid, mefenamic acid and tolfenamic acid solutions by a factor of ten. In the cases where caffeine was used as internal standard, a 5-ml volume of a 10 ng/μl methanolic caffeine solution, giving a final caffeine concentration of 1 ng/μl, was always added to the solutions during dilutions. All dilutions were made with methanol. Aliquots of 10 μl of each solution were injected onto the analytical column. The peak area ratios of flufenamic acid, mefenamic acid and tolfenamic acid to caffeine were recorded and plotted as functions of flufenamic acid, mefenamic acid and tolfenamic acid concentrations. All determinations were repeated eight times and the results were treated statistically.

Determination of Anthranilic Acid Derivatives in Pharmaceutical Preparations: The developed high pressure liquid chromatographic method was applied to the analysis of pharmaceutical formulations such as ointment for flufenamic acid, tablets, suspension and suppositories for mefenamic acid, capsules and suspension for tolfenamic acid.

Analyses of Flufenamic Acid in Pharmaceutical Preparations: A quantity of 461.8 mg of ointment (3g of flufenamic acid per 106.84 g of ointment-labelled amount) was diluted by 50 ml of methanol and sonicated for 5 min in ultrasonic bath. By diluting this solution to 100 ml with methanol a solution of 129.67 ng/μl in flufenamic acid was prepared according to the labelled amount.

An aliquot of 2 ml of this solution and 10 ml of 10 ng/μl caffeine solution were diluted to 100 ml with methanol giving a final concentration of 2.59 ng/μl for flufenamic acid and 1 ng/μl for caffeine.

Aliquots of 10 μl of this solution were injected onto the HPLC column.

Determinations of Mefenamic Acid in Pharmaceutical Preparations: Ten **tablets** of 500 mg (labelled amount) in mefenamic acid were weighed and the average tablet weight was found to be 726.2 mg. The tablets were finely powdered and a portion of 14.5 mg was quantitatively transferred into a 100 ml volumetric flask. The content was diluted to volume with methanol and gave a concentration of 100 ng/μl according to labelled amount of mefenamic acid. From this stock solution, a dilute solution of 1 ng/μl was prepared by sequential dilutions with methanol by a factor of ten. To the last solution caffeine was added as internal standard at the final concentration level of 1 ng/μl.

Aliquots of 10 μl were analyzed by HPLC.

Suspension, 10 ml, of labelled concentration 50 mg/5 ml,was transferred into a 100 ml volumetric flask and diluted to volume with methanol. According to labelled amount the concentration of this solution was 1000 ng/μl. The solution was sonicated in ultrasonic bath for about 10 min, then a portion was centrifuged at 3500 rpm for 10 min. A volume of 10 ml of the supernatant layer was diluted with methanol to 100 ml. By sequential dilution, with a factor of ten, a final solution of 1 ng/μl in mefenamic acid and caffeine was prepared.

Aliquots of 10 μl were analyzed by HPLC.

Eight **suppositories** of 500 mg labelled amount in mefenamic acid were weighed and the average weight was found to be 2.0789 g. The suppositories were put into a glass beaker and the beaker was incubated at 38°C for 5 min. A quantity of 2.0750g was dissolved by 50 ml methanol and sonicated in ultrasonic bath for 5 min. Then the entire quantity was transferred into a 250 ml volumetric flask diluted to volume with methanol to give a mefenamic acid solution 2000 ng/μl according to labelled amount. From this solution and the caffeine standard solution a final solution of 1 ng/μl in mefenamic acid and caffeine was prepared by sequential dilution.

Aliquots of 10 μl were analyzed by HPLC.

Analyses of Tolfenamic Acid in Pharmaceutical Preparations: Ten hard **gelatin capsules** of 100 mg (labelled amount) in tolfenamic acid were weighed and the average capsule weight was found to be 249.5 mg and 199.6 mg with and without gelatin respectively. The same procedure for capsules of 200 mg (labelled amount)

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in tolfenamic acid gave 363.6 mg and 299.6 mg respectively. The content of the capsules was finely powdered and portions of 19.96 mg and 14.98 mg from the capsules of 100 mg and 200 mg respectively were quantitatively transferred into 100 ml volumetric flasks. The contents were diluted to volume with methanol and gave concentrations of 100 ng/μl according to labelled amount of tolfenamic acid. A volume of 3 ml of these solutions were transferred into 100 ml volumetric flask. To this flask 10 ml of a 10 ng/μl caffeine solution was added and the final solutions were diluted to 100 ml with methanol, thus the resulting solution according to the labelled quantity was 3 ng/μl in tolfenamic acid and 1 ng/μl in caffeine. Aliquots of 10 μl of these solutions were injected onto the HPLC column.

Suspension, 5 ml, of labelled concentration 10 ml/5 ml, was transferred to a 100 ml volumetric flask and diluted to volume with methanol. According to labelled amount the concentration of this solution was 100 ng/μl. The solution was sonicated in ultrasonic bath for about 10 min in a glass beaker, then a portion was centrifuged for 10 min at 3500 rpm. A 10 ml volume of the supernatant layer was diluted with methanol to 100 ml. A volume of 30 ml of this solution and 10 ml of a 10 ng/μl caffeine solution were diluted with methanol in 100 ml volumetric flasks. According to labelled quantity the resulting solution was 3 ng/μl in tolfenamic acid and 1 ng/μl in caffeine. Aliquots of 10 μl were analyzed by HPLC.

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Determination of Anthranilic Acid Derivatives in Biological Fluids, Blood Serum and Urine: Stock flufenamic acid, mefenamic acid and tolfenamic acid methanolic solutions (100 μl) of 0.3, 0.5 1, 2 and 2.5 ng/μl with caffeine as internal standard at a concentration of 1 ng/μl and acetonitrile (80 μl) for protein precipitation were added to the **plasma sample** (40 μl). After vortex mixing for 2 min and centrifugation at 3000 rpm for 5 min, the supernatant was evaporated to remove the solvents under a stream of nitrogen on a water bath at 45°C. The aqueous phases were subsequently treated by solid-phase liquid extraction using C_{18} cartridges (Bond Elut). These phases were slowly forced through the cartridges which were previously conditioned, by passing 3 ml of methanol, and washed with 3 ml of water. The cartridges were fitted in a Vacuum system (Vac Elut) and washed twice with 2 ml of water before the final elution. The anthranilic acid derivatives were eluted with 2.5 ml of methanol. These methanolic solutions were

evaporated to dryness on a water bath under a nitrogen stream at 45°C. The residues were redissolved in 50 µl of methanol and aliquots of 10 µl were injected into the chromatograph.

For urine assay a 100-µl volume of flufenamic acid, mefenamic acid and tolfenamic acid stock methanolic solutions 1.5, 2, 2.5, 3 and 5 ng/µl and 400 µl acetonitrile were added to 200 µl of urine sample. After centrifugation for 20 min at 3500 rpm methanol and acetonitrile were removed from the supernatant by evaporation under a stream of nitrogen on a water bath at 40°C. The mixture was purified on a C_{18} cartridge Bond Elut. After washing five times with 2 ml of water, elutions were performed with 2.5 ml of methanol. The methanolic solutions were evaporated to dryness as mentioned above. The residues were reconstituted with 100 µl of methanol and, after mixing, 10 µl were injected onto the analytical column.

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RESULTS AND DISCUSSION

Previous methods for the determination of plasma anthranilic acid derivatives, mainly mefenamic acid, require a large amount of plasma, more than 0.5 ml and a complicate and time consuming liquid-liquid extraction procedure[19-20]. A recent method[21] can be used for the determination only of mefenamic acid in plasma. The method is simple, rapid and sensitive high-performance liquid chromatographic. This method requires minimum pretreatment of the plasma sample (50 µl) and can be successfully used for monitoring only plasma mefenamic acid between sub-the rapeutic and overdose levels. As only small plasma samples can be taken, we propose a new simultaneous reversed-phase gradient HPLC method of anthranilic acid derivatives in blood serum, urine and anti-inflammatory drugs. A very small volume (40 µl) of plasma from which proteins were isolated by acetonitrile precipitation is required. The proteins were also removed from urine samples by employing the same procedure.

To improve the accuracy and precision of the method, at low concentration levels of anthranilic acid derivatives, the samples of biological fluids, after the precipitation of proteins and centrifugation, were subsequently treated by solid-phase liquid extraction and analyzed.

The total chromatographic time for analysis of the anthranilic acid derivatives was less than 6 min, with reproducible retention times under the experimental conditions used.

The linearity of the method was studied and the areas of the chromatographic peaks for flufenamic acid, mefenamic acid and flufenamic acid were related to concentration. The linear regression equations and correlation coefficients were found to be:

STATISTICAL EVALUATION

Samples of Anthranilic Acids Derivatives	Regression Equation	Correlation Coefficient
Methanolic solutions in the Presence of Caffeine		
Flufenamic Acid	$Y=(0.001\pm0.01)+(0.0499\pm0.0004)X$	0.99995
Mefenamic Acid	$Y=(0.008\pm0.02)+(0.0558\pm0.0005)X$	0.99994
Tolfenamic Acid	$Y=(0.01\pm0.03)+(0.0644\pm0.0008)X$	0.99987
Blood Samples		
Flufenamic Acid	$Y=(1.764\cdot10^{-3}\pm4.404\cdot10^{-3})+(0.06045\pm2.785\cdot10^{-4})X$	1.00000
Mefenamic Acid	$Y=(-0.777\cdot10^{-3}\pm4.580\cdot10^{-3})+(0.07389\pm0.29\cdot10^{-3})X$	0.99999
Tolfenamic Acid	$Y=(-0.736\cdot10^{-2}\pm3.105\cdot10^{-2})+(0.0684\pm1.83\cdot10^{-3})X$	0.99995
Urine Samples		
Flufenamic Acid	$Y=(-1.403\cdot10^{3}\pm33.766\cdot10^{3})+(8.52^{3}\pm1.654\cdot10^{3})X$	0.99491
Mefenamic Acid	$Y=(-10.585\cdot10^{3}\pm5.2\cdot10^{2})+(9.705\cdot10^{3}\pm0.81\cdot10^{2})X$	1.00100
Tolfenamic Acid	$Y=(0.441\cdot10^{3}\pm15.5\cdot10^{3})+(13.573\cdot10^{3}\pm0.466\cdot10^{3})X$	0.99993

Where Y = peak area ratio of anthranilic acid derivatives to caffeine for methanolic and blood samples. Peak area for urine samples. X = Concentration of anthranilic acid derivatives in ng/μl.

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Table 3. Experimental Results for the Simultaneous Determination of Flufenamic acid, Mefenamic acid and Tolfenamic acid in methanolic solutions using Caffeine as Internal Standard, by Reversed-Phase Gradient HPLC.

Compound	Retention Time (min)	Added (ng)	Found[α] (ng)
Caffeine	3.66		
Flufenamic acid	4.63	5	4.58±0.13
		20	20.81±0.80
		30	29.38±0.65
Mefenamic acid	4.99	5	4.93±0.28
		20	19.06±0.06
		30	29.81±0.08
Tolfenamic acid	5.46	5	4.87±0.12
		20	19.46±0.30
		30	29.96±0.54

α = average value of eight determinations ± standard deviation.

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Linearity was observed up to 70 ng for flufenamic and mefenamic acid and 80 ng for tolfenamic acid.

Experimental results for the simultaneous determination of flufenamic acid, mefenamic acid and tolfenamic acid in the presence of caffeine as internal standard are given in Table 3.

The mean recovery data of flufenamic acid, mefenamic acid and tolfenamic acid were determined by comparing the peak areas obtained from the injection of known quantities of the pure anthranilic acid components, with those obtained from the direct injection of extracted blood serum and urine samples spiked with four different concentrations of anthranilic acid derivatives, in the presence of caffeine as internal standard. The mean percentage recovery of flufenamic acid, mefenamic acid and tolfenamic acid at the various concentrations averaged 95.9%, 95.1% and 96.9% respectively for blood serum analyses. These experimental results are given in Table 4.

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Table 4. Recovery Data for Simultaneous Determination of Anthranilic Acid Derivatives in Blood Serum by Reversed-Phase Gradient HPLC, using Caffeine as Internal Standard.

Anthranilic Acid Derivatives	Sample 1		Sample 2		Sample 3	
	Mean Recovery %	RSD%	Mean Recovery %	RSD%	Mean Recovery %	RSD%
Plasma Flufenamic acid						
Quantity (ng)						
5	92.3±6.9	7.5	98.7±6.7	6.8	95.4±3.9	3.7
10	96.4±2.2	2.3	102.2±0.9	0.9	96.2±3.1	3.2
20	88.8±2.3	2.6	104.9±3.2	3.0	93.1±5.7	6.1
25	96.3±5.2	5.4	90.7±0.6	0.7	96.3±1.8	1.9
Plasma Mefenamic acid						
Quantity (ng)						
5	97.6±10.3	10.5	90.4±0.8	0.9	100.1±2.9	3.0
10	100.6±3.2	3.2	101.7±1.5	1.5	92.8±2.8	2.8
20	86.7±3.1	3.6	85.0±0.2	0.2	92.5±5.6	6.1
25	99.2±2.8	2.9	101.4±0.1	0.1	93.6±4.8	4.5
Plasma Tolfenamic acid						
Quantity (ng)						
5	100.8±2.9	2.8	89.9±4.8	5.3	96.7±2.4	2.3
10	102.5±2.1	2.1	98.3±7.1	7.3	94.6±1.6	1.7
20	99.1±1.7	1.7	100.1±1.77	1.8	95.3±2.7	2.8
25	96.8±3.1	3.2	93.4±5.4	5.8	95.7±2.2	2.3

Mean Recovery = $x \pm (t \cdot SD/\sqrt{n})$/amount added x 100. Where x = mean value for n = 6 determinations at 95% confidence level.

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Table 5. Mean Recovery Data for Simultaneous Analysis of Anthranilic Acid Derivatives in Urine Samples by Reversed-Phase Gradient HPLC.

Anthranilic Acid Derivatives	Sample 1		Sample 2		Sample 3	
	Mean Recovery %	RSD%	Mean Recovery %	RSD%	Mean Recovery %	RSD%
Urine Flufenamic acid Quantity (ng)						
15	99.9±2.9	2.9	97.2±1.1	1.2	96.7±3.5	3.6
20	89.6±1.2	1.4	96.0±0.4	0.3	101.5±5.0	5.0
25	102.1±2.0	1.9	97.2±5.1	5.3	99.0±4.6	4.7
30	103.5±4.0	3.8	101.9±4.1	4.0	102.3±1.5	1.5
50	98.8±5.4	5.5	97.4±5.1	5.3	94.0±2.1	4.6
Urine Mefenamic acid Quantity (ng)						
15	98.1±1.8	1.8	100.2±7.8	7.8	98.9±0.7	0.7
20	99.7±0.2	0.2	96.6±5.7	5.9	92.8±2.2	2.4
25	100.2±0.8	0.8	98.9±4.7	4.8	95.7±2.0	2.1
30	97.2±4.4	4.6	102.1±4.2	4.1	99.4±0.8	0.8
50	95.7±6.3	6.5	103.3±2.5	2.4	96.7±2.4	2.5
Urine Tolfenamic acid Quantity (ng)						
15	101.7±3.1	3.0	97.4±1.4	1.5	98.7±1.7	1.8
20	101.8±0.8	0.8	97.7±3.3	3.4	104.6±1.6	1.5
25	102.0±4.8	4.7	98.8±4.3	4.3	97.5±3.3	3.4
30	99.8±1.4	1.4	99.9±2.9	2.9	92.6±1.3	1.4
50	95.1±2.6	2.7	94.0±4.5	4.8	101.0±2.0	2.0

Mean recovery = $x \pm (t\ SD/\sqrt{n})$amount added x 100. Where x = mean value for n = 6 determinations at 95% confidence level.

Table 6. Experimental Results for the Determination of Anthranilic Acid Derivatives in Pharmaceutical Preparations by Gradient Reversed-Phase HPLC in the Presence of Caffeine as Internal Standard.

Samples	Labelled Amount A	Analyzed Quantity B	Found [α]			
			A±SD	RSD (%)	B±SD	RSD (%)
Active Ingredient Flufenamic Acid						
Ointment	3g/106.84g	25.9 ng	3.14±0.33	10.38	27.14±2.84	10.46
Active Ingredient Mefenamic Acid						
Tablets	500 mg	10 ng	577±24	4.17	11.53±0.48	4.17
Suspension	50mg/5ml	11 ng	55.01±5.4	9.80	11.00±1.08	9.80
Suppositories	500mg	10 ng	411±47	11.43	8.22±0.93	11.31
Active Ingredient Tolfenamic Acid						
Capsules 100	100 mg	30 ng	70.03±4.93	7.04	24.74±1.74	7.04
Capsules 200	200 mg	30 ng	174±8.6	4.94	27.02±1.33	4.94
Suspension	10mg/5ml	30 ng	8.78±1.05	11.93	26.33±3.14	11.91

[α]Average value of six determinations ± Standard deviation.

Similar mean percentage recovery data of anthranilic acid derivatives were determined by comparing the respective peak areas from injection of known quantities of the pure compounds, to those taken from the direct injection of extracted urine samples spiked with five different concentrations of anthranilic acid derivatives without caffeine as internal standard. The mean percentage recovery of flufenamic acid, mefenamic acid and tolfenamic acid derivatives, at various concentrations averaged 98,5%, 98,4% and 98.8% respectively for urine samples analyses. These results are laid out in Table 5.

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Table 7. Day-to-day Precision and Accuracy of Flufenamic, acid Mefenamic Acid and Tolfenamic acid assay in the Presence of Internal Standard (n = 10).

Anthranilic Acid Derivative	Quantity injected (ng)	Mean ± SD (ng)	RSD %
Flufenamic acid	5	4.68 ± 0.19	4.04
	20	20.60 ± 0.50	2.44
	30	30.02 ± 0.76	2.54
Mefenamic acid	5	4.79 ± 0.21	4.44
	20	19.28 ± 0.20	1.01
	30	29.53 ± 0.31	1.06
Tolfenamic acid	5	4.90 ± 0.14	2.78
	20	19.52 ± 0.23	1.17
	30	30.40 ± 0.89	2.58

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The obtained method was applied to the analyses of anthranilic acid derivatives in pharmaceutical preparations using caffeine as internal standard. Therefore flufenamic acid was determined in ointment, mefenamic acid was analyzed in tablets, suspension and suppositories and tolfenamic acid was determined in capsules of 100 and 200 and in suspension. The results taken are given in Table 6.

The day to day precision and accuracy of the proposed method were assessed by the repeated analyses of methanolic solutions in the presense of the internal standard over ten days. The concentrations of anthranilic acid derivatives ranged from 1 ng/µl to 3 ng/µl. Eight replicate samples at each of the three concentrations were used in the assessment of the between day variability of the method. The results taken of this procedure are laid out in Table 7.

Ten drug-free plasma and urine samples were analyzed for possible interferences from endogenous constituents. From these analyses no background interferences were observed.

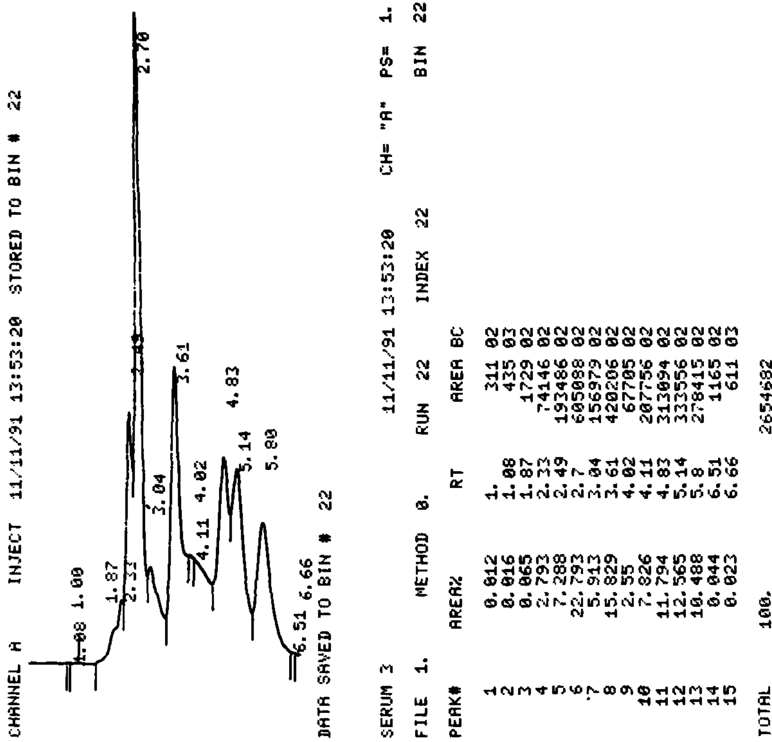

Figure 2. High-Performance Liquid Chromatogram of Anthranilic Acid Derivatives Extracted by Solid Phase Cartridge from Blood Serum. Peaks: (2.49) = Unknown, (2.70) = Unknown, (3.04) = Unknown (3.61) = Caffeine [1 ppm], (4.83) = Flufenamic Acid [1 ppm], (5.14) = Mefenamic Acid [1 ppm] and (5.80) = Tolfenamic Acid [1 ppm].

Using the standard addition technique it is possible to analyze anthranilic acid derivatives in 40 µl of blood serum and 200 µl of urine samples in under six minutes time. Flufenamic acid, mefenamic acid and tolfenamic acid were successfully determined, employing the procedures described using caffeine as internal standard for blood serum and without caffeine for urine samples.

The chromatograms taken are given in figures 2 and 3.

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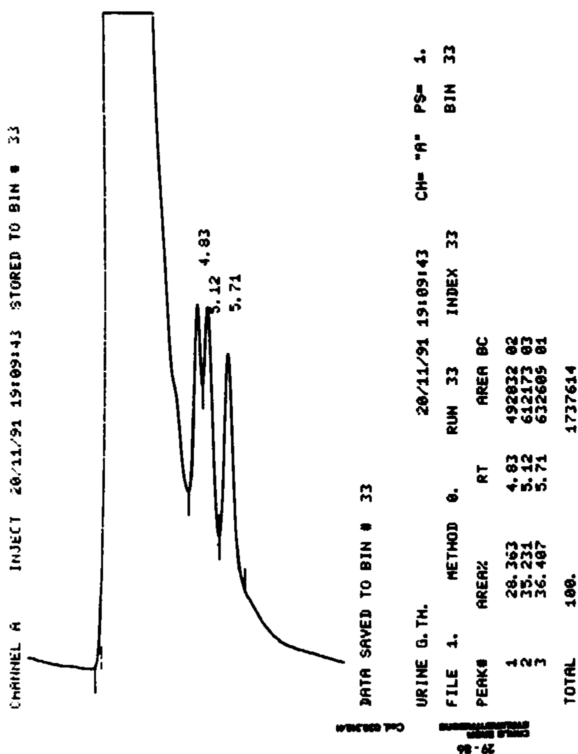

Figure 3. High-Performance Liquid Chromatograms of Anthranilic Acid Derivatives Extracted by Solid Phase Cartridge from Urine Samples. Peaks: (4.83) = Flufenamic Acid [5 ppm], (5.12) = Mefenamic Acid [5 ppm] and (5.71) = Tolfenamic Acid [5 ppm].

The proposed method was applied to the analyses of anthranilic acid derivatives in pharmaceutical preparations such as, ointment, suspension, suppositories and capsules (table 6). A representative chromatogram of the determination of flufenamic acid in ointment is given in figure 4.

The results taken were in good agreement with the labelled amounts of the pharmaceutical preparations and no interferences from the excipients were observed in the chromatograms.

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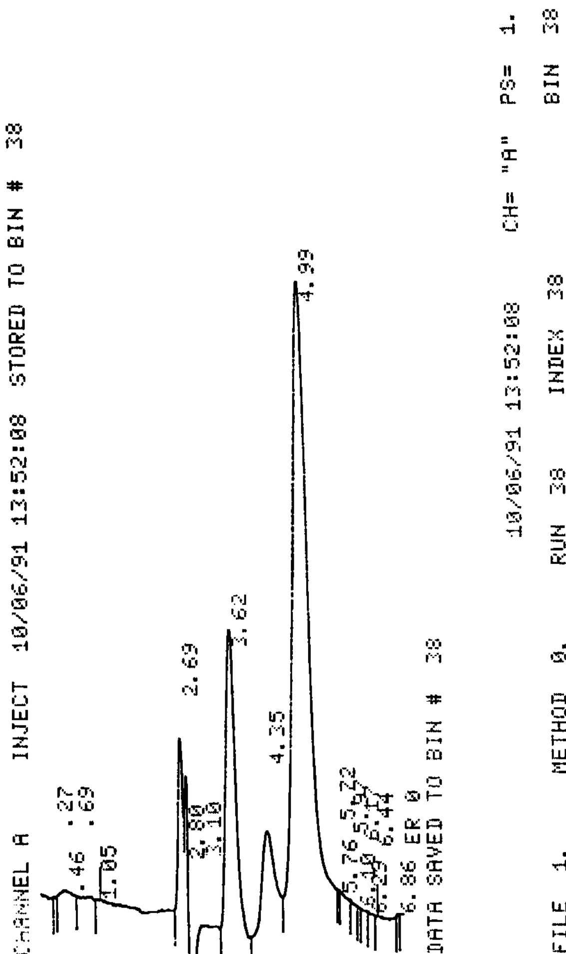

Figure 4. High-performance liquid chromatogram of flufenamic acid in pharmaceutical preparations-ointment. Peaks: (2.69) = Unknown, (3.62) = Caffeine [1 ppm], (4.35) = Unknown, (4.99) = Flufenamic acid [2.59 ppm].

CONCLUSION

In conclusion, the proposed method, requiring only a very small volume of plasma (40 µl) and with rapid and reproducible sample preparation, is very suitable for therapeutic drug monitoring of flufenamic acid mefenamic acid and tolfenamic acid in blood serum. Also because of its high specificity, accuracy, precision and save of time the described simultaneous reversed-phase gradient HPLC procedure appears to be very useful for the routine analyses of anthranilic acid derivatives in 200 µl urine samples. The method is also applicable to analyses of anthranilic acid derivatives in pharmaceutical preparations.

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