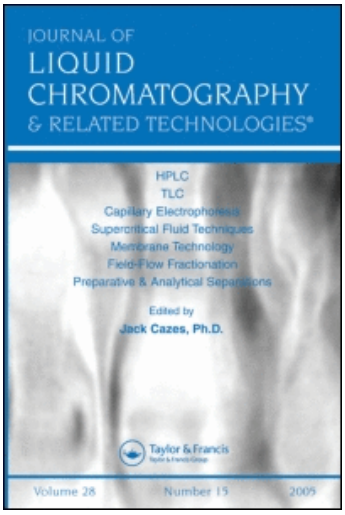

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Rapid Assay for the Determination of Tolfenamic Acid in Pharmaceutical Preparations and Biological Fluids by High-Performance Liquid Chromatography

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RAPID ASSAY FOR THE DETERMINATION OF *TOLFENAMIC ACID* IN PHARMACEUTICAL PREPARATIONS AND BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An improved reversed-phase High-Performance Liquid Chromatographic (HPLC) method using UV detection, at 282 nm, is described for the determination of tolfenamic acid in the presence of caffeine, as internal standard, in pharmaceutical preparations and biological fluids. Sample analyses are performed with a Lichrosorb-RP18, 10 μm, 250x4 mmI.D., column using acetate buffer, (pH 4.6 and constant ionic strength 0.05 M) methanol (18:82) as eluent, at a flow rate of 1.9 ml/min. The retention time is 1.44 min for caffeine and 2.62 min for tolfenamic acid. The absolute detection limit is 0.5 ng in the presence and 0.9 ng in the absence of internal standard and linearity is observed up to 100 ng injected. The method involves the use of solid-

phase extraction for sample clean-up and subsequent separation of tolfenamic acid and internal standard from endogenous interfering compounds on a reversed-phase column. The method outlined in this paper is applied to the determination of tolfenamic acid in pharmaceutical formulations (capsules, suspension) and biological fluids (blood serum and urine). The method can be readily utilised for clinical pharmacokinetic studies.

INTRODUCTION

In recent years considerable interest has been shown in the analysis of drugs in pharmaceutical formulations and biological fluids[1-5]. Tolfenamic acid, N-(2-methyl-3-chlorophenyl)-anthranilic acid (fig. 1) is a nonsteroidal powerful anti-inflammatory agent. It has remarkable analgesic and antipyretic properties. Tolfenamic acid, like other fenamates, has an inhibitory action on prostaglandins as effectively as indomethacin and in a considerably lower concentration than acetylsalicylic acid. The clinical efficacy of tolfenamic acid has been documented in the treatment of rheumatic diseases, dysmenorrhea and migraine. This drug is remarkably non-irritative towards the gastric mucosa, is well tolerated by patients and shows good results in clinical studies[6-12].

The literature on the determination of tolfenamic acid is limited and there is a necessity of developing techniques for the determination of tolfenamic acid in pharmaceutical formulations and samples of biological interest.

During these days High-Performance Liquid Chromatography (HPLC) is being used as a powerful and acceptable technique, because of limited interferences, advantages in speed, ease and sensitivity. This technique offers the analyst the opportunity to obtain qualitative and quantitative results in a few minutes.

This paper presents a simple, sensitive and fast method for the analysis of tolfenamic acid by isocratic HPLC in the presence of caffeine as internal standard, in capsules, suspension, blood serum and urine samples.

EXPERIMENTAL

Apparatus and Chemicals: A high-performance liquid chromatograph consisted of a ternary gradient pump Spectra Physics, model SP8800 with a Rheodyne 7125

injection valve is used. This valve is equipped with a 10 µl loop. The detector used is a Spectra Physics, Spectra Chrom 100 variable wavelength UV-Vis spectrophotometer. The analytical column is 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.

Computations and statistical treatment of the data were performed using a PC Vip 220. Tolfenamic acid anhydrous (powder Ph. Eur.) was supplied from ELPEN, Athens. Caffeine was obtained from BDH Chemicals Ltd; (Poole, England). Both reagents were used as provided without further purification. All standard solutions of these compounds were prepared by dissolving the appropriate amounts in methanol. Buffer components were analytical reagent grade, obtained from Merck (Darmstadt, Germany). HPLC-grade methanol was also from Merck. All other reagents used were also analytical reagent grade and glass-distilled water was used throughout.

Chromatographic Conditions: The mobile phase used was a mixture of acetate buffer[13] (pH 4.6 and constant ionic strength 0.05)-methanol (18:82) and was selected through a number of other mobile phases on the basis of their relative polarities and low absorption at the wave length used. The pressure was 2100 psi at a flow rate of 1.9 ml/min, and the detection was performed at 282 nm with a sensitivity setting of 0.002 Absorbance Units Full Scale (AUFS). All separations were performed at room temperature. The above mentioned conditions were selected among several ones examined, as shown in Table 1, since they were found to be the most suitable for a good separation of the analytes.

System suitability: The Lichrosorb RP-18, 10 µm reversed phase analytical column was equilibrated with the eluting solvent system used, at a flow rate of 1.9 ml/min. After a stable baseline was achieved, the standard and the sample solutions were injected onto the column. The two peaks appeared over the increased retention time. The resolution factor, R_t, was calculated between the two peaks and found to be 2.95 signifying a complete separation[14-15]. This is illustrated in Figure 1.

The relative standard deviations of eight replicate injections of three standards 1, 2 and 3 ppm were found to be 11.9%, 4.3% and 2.3% respectively. These results are given in Table 2.

Table 1. High-Performance Liquid Chromatographic conditions examined in the Present Study.

Eluent (A:B)	Flow Rate (ml/min)	Retention Time		Sensitivity (AUFS)
		Caffeine (min)	Tolfenamic acid (min)	
70:30	1.0	10.98	ND	0.005
50:50	1.0	4.23	ND	0.002
30:70	2.0	NA	6.41	0.005
24:76	1.7	NA	4.89	0.002
24:76	1.8	1.61	5.58	0.001
22:78	1.4	NA	4.97	0.002
22:78	1.6	1.77	4.13	0.001
22:78	1.7	NA	4.09	0.002
22:78	1.8	1.56	3.87	0.002
20:80	1.2	2.36	4.95	0.002
20:80	1.6	1.75	3.32	0.001
20:80	2.0	1.36	2.63	0.002
18:82	1.6	1.71	3.03	0.001
18:82	1.8	1.55	2.75	0.005
18:82	1.9	1.44	2.62	0.002

A = Aqueous acetate buffer (pH 4.6 and constant ionic strength 0.05 M)

B = MeOH

NA = Not Added

ND = Not Detected

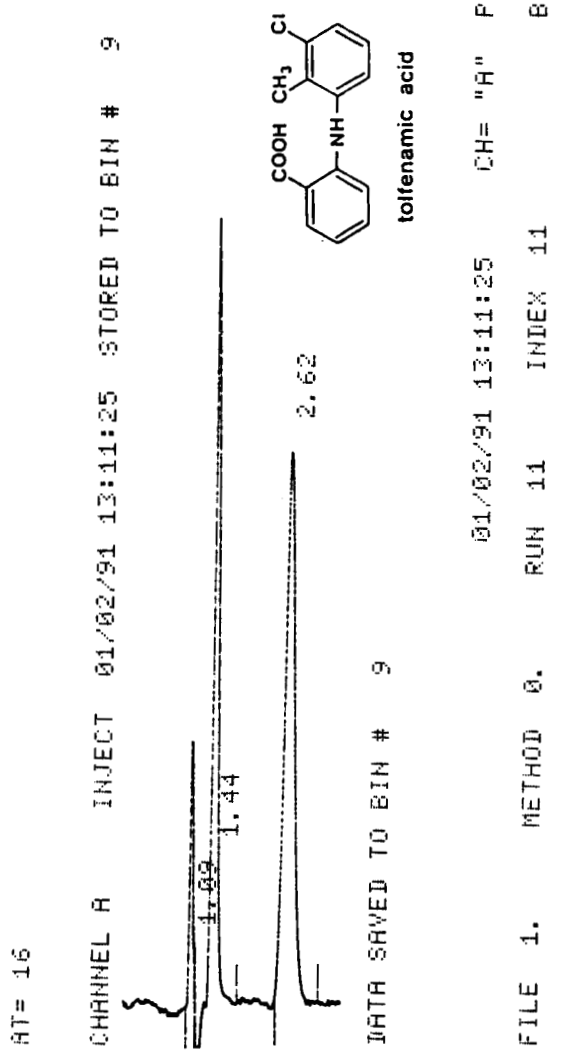

Figure 1. High-Performance Liquid Chromatogram of Tolfenamic acid (2.62) using Caffeine (1.44) as internal standard. Chromatographic conditions are given in text. [Caffeine]= 1 ppm, [Tolfenamic acid]= 2 ppm.

Table 2. *Peak Area ratio of Tolfenamic acid to Caffeine over the concentration range 1-3 ppm in methanolic solutions, equivalent quantity injected 10-30 ng.*

Concentration of Tolfenamic Acid (ppm)	Equivalent Quantity Injected (ng)	Peak Area Ratio of Tolfenacic Acid to Internal Standard Caffeine								Mean Value	SD	RSD (%)
		1	2	3	4	5	6	7	8			
1	10	0.702	0.665	0.911	0.823	0.751	0.710	0.661	0.833	0.757	0.09	11.9
2	20	1.613	1.642	1.580	1.681	1.459	1.539	1.632	1.592	1.592	0.07	4.3
3	30	2.214	2.340	2.250	2.359	2.369	2.319	2.335	2.331	2.315	0.05	2.3

Selectivity: The selectivity of the method was investigated at the retention times of tolfenamic acid and caffeine. No endogenous interferences were found in chromatograms of samples extracted from blood serum and urine. Therefore, the described method can be used in the analysis of tolfenamic acid in these samples without using internal standard.

Detection limit: The detection limit of tolfenamic acid was assessed both in the presence and in the absence of internal standard, caffeine, and was considered to be that quantity producing a signal of a peak height twice the size of background noice. The minimum detectable quantity, expressed in ng injected on the column, was found to be 0.5 ng in the presence and 0.9 ng in the absence of internal standard caffeine.

Standard calibration curves for the determination of Tolfenamic acid in methanol: Calibration curves for the determination of tolfenamic acid were constructed, both in the presence and in the absence of internal standard, caffeine. Tolfenamic acid and caffeine were accurately weighed and dissolved in methanol to give stock solutions of 100 ng/μl each. Standard tolfenamic acid solutions of 0.1, 0.2, 1, 2, 3, 5 and 10 ng/μl were prepared, both with and without caffeine, in 50-ml volumetric flasks, by serially diluting the stock tolfenamic acid solution by a factor of ten. When caffeine was used as internal standard, a 3-ml volume of a 10 ng/μl methanolic caffeine solution, giving a final caffeine concentration of 0.6 ng/μl, was always added to the solutions during dilution. All dilutions were made with methanol. Aliquots of 10 μl of each solution were injected for the analysis. When analyses were performed in the presence of internal standard, the peak area ratios of tolfenamic acid to caffeine were recorded and plotted as a function of tolfenamic acid concentration. In the absence of internal standard, the peak areas of the tolfenamic acid signals were plotted against its concentration. All analyses were repeated eight times and the results were treated statistically.

Determination of Tolfenamic acid in Pharmaceutical Preparations: The developed high pressure liquid chromatograchic method was applied to the analysis of pharmaceutical formulations such as capsules and suspension.

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) 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. Volumes of 1, 2 and 3 ml of these solutions were transferred into 100 ml volumetric flasks. To these flasks 6 ml of a 10 ng/μl caffeine solution was added and the final solutions were diluted to 100 ml with methanol. Aliquots of 10 μl of these solutions were injected onto the HPLC column.

Suspension, 5 ml, of labelled concentration 10 mg/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. Volumes of 10, 20 and 30 ml of this solution and 6 ml of a 10 ng/μl caffeine solution were diluted with methanol in 100 ml volumetric flasks. According to labelled quantity the resulted solutions were 1, 2 and 3 ng/μl in tolfenamic acid and 0.6 ng/μl in caffeine. Aliquots of 10 μl were analyzed by HPLC.

Determination of Tolfenamic acid in Biological Fluids, Blood Serum and Urine: Stock tolfenamic acid solution (100 μl) of 1, 2, 3, 4 and 5 ng/μl and acetonitrile (100 μl) for protein precipitation were added to the plasma sample (50 μl). After vortex-mixing for 2 min and centrifugation at 3500 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 phase was subsequently treated by solid-phase liquid extraction using C_{18} cartridges (Bond Elut). This phase was slowly forced through the cartridge which was previously conditioned, by passing 3 ml of methanol, and washed with 3 ml of water. The cartridge was fitted in a Vacuum system (Vac Elut) and washed twice with 2 ml of water before the final elution. The tolfenamic acid was eluted with 3 ml of methanol. This methanolic solution was evaporated to dryness on a water bath under a nitrogen stream at 45°C. The residue was redissolved in 100 μl of methanol and aliquots were injected into the chromatograph.

For urine assay a 100-µl volume of tolfenamic acid stock methanolic solution (1, 2, 3, 4 and 5 ng/µl) was added to 1 ml of urine sample. Methanol was removed by evaporation under a stream of nitrogen on a water bath at 45°C. The mixture was purified on a C_{18} cartridge Bond Elut. After washing twice with 2 ml of water, elution was performed with 3 ml of methanol. The methanolic solution was evaporated to dryness as mentioned above. The residue was reconstituted with 100 µl of methanol and, after mixing, 10 µl were injected into the loop.

RESULTS AND DISCUSSION

The retention times of caffeine and tolfenamic acid were found to be reproducible under the experimental conditions used. The average relative standard deviation was less than 0.5%. The mobile phase employed enables a good column performance for long periods of time.

The areas of the chromatographic peaks for both compounds, caffeine and tolfenamic acid, were linearly related to concentration. The linear regression equations and correlation coefficients were found to be:

STATISTICAL EVALUATION

Samples of Tolfenamic acid	Regression Equation	Correlation Coefficient
Methanolic solutions in the Presence of Caffeine.	$Y=(0.01\pm0.02)+(0.0791\pm0.0006)X$	0.9999
Methanolic solutions without Caffeine.	$Y=(2.2\pm1.5)10^3+(9.87\pm0.03)10^3X$	0.9999
Blood.	$Y=(5.2\pm6.0)10^4+(9.6\pm1.8)10^3X$	0.9948
Urine.	$Y=(1.5\pm25.7)10^3+(7.2\pm0.8)10^3X$	0.9983

Where Y = peak area ratio of tolfenamic acid to caffeine for methanolic samples and peak area for the blood and urine samples, X = concentration of tolfenamic acid in ng/µl.

Table 3. Experimental Results for the Determination of Tolfenamic acid in Methanolic Solutions using Caffeine, as Internal Standard, by HPLC.

Compound	Retention time (min)	Added (ng)	Found[a] (ng)
Caffeine	1.44		
Tolfenamic acid	2.62	10	9.4±1.1
		20	20.0±0.9
		30	29.1±0.6

a = average value of eight determinations ± standard deviation

Experimental results for the determination of tolfenamic acid using caffeine as internal standard are laid out in Table 3.

The mean percentage recovery of tolfenamic acid was measured by comparing the peak areas obtained from the injection of known quantities of the pure compounds, with those obtained from the direct injection of extracted blood serum and urine samples spiked with five different concentrations of tolfenamic acid. The mean percentage recovery of tolfenamic acid at various concentrations averaged 80.5% for blood serum analyses and 84.2% for urine samples. These results are given in Tables 4 and 5.

The between-day precision and accuracy of the method were assessed by the repeated analyses of methanolic solutions in the presence and in the absence of the internal standard over twelve days. The concentrations of tolfenamic acid ranged from 1 ng/μl to 3 ng/μl. Twelve replicate samples at each of the three concentrations were used in the assessment of the between-day variability. The results are presented in Table 6.

Table 4. Mean Recovery data for Tolfenamic acid assay in Blood serum (n=6).

Plasma Tolfenamic acid quantity (ng)	Sample 1 Mean recovery (%)	RSD (%)	Sample 2 Mean recovery (%)	RSD (%)	Sample 3 Mean recovery (%)	RSD (%)
10	81.8±0.7	8.6	85.0±1.3	13.6	90.6±0.4	4.4
20	72.5±1.2	7.3	102.5±1.7	7.8	82.5±0.9	5.5
30	72.0±1.8	8.0	108.7±1.0	3.1	94.3±0.8	2.7
40	95.5±1.5	3.6	98.0±0.9	2.3	96.0±1.5	3.6
50	80.7±2.5	6.0	98.4±1.6	3.1	86.4±0.5	1.2

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.

Table 5. Mean Recovery data for Tolfenamic acid assay in Urine (n=6).

Urine Tolfenamic acid quantity (ng)	Sample 1 Mean recovery (%)	RSD (%)	Sample 2 Mean recovery (%)	RSD (%)	Sample 3 Mean recovery (%)	RSD (%)
10	92.8±0.4	3.8	89.1±0.3	3.2	89.9±0.5	5.6
20	102.6±0.5	2.6	87.0±1.4	7.4	104.0±1.1	5.3
30	75.7±1.3	5.3	95.0±1.5	4.9	100.8±0.5	1.6
40	76.8±1.5	4.6	97.8±0.4	0.9	100.3±0.8	2.1
50	73.2±1.9	4.9	96.4±1.3	2.5	98.2±0.3	0.5

Table 6. Between-day Precision and Accuracy of Tolfenamic acid assay.

Quantity injected (ng)	In the presence of Internal Standard (n=12) Mean±SD (ng)	RSD (%)	Without Internal Standard (n=12) Mean±SD (ng)	RSD (%)
10	10.19±0.35	3.4	9.86±0.18	1.8
20	20.09±0.39	1.9	20.02±0.24	1.2
30	29.71±0.41	1.4	30.34±0.63	2.1

The peaks on the chromatogram are identified by their retention time. Quantitation was done by comparison of the peak area ratio of tolfenamic acid to caffeine in the unknown sample with those of the standards containing known quantities of tolfenamic acid, extracted and chromatographed in exactly the same way. Both peak areas of tolfenamic acid and peak area ratios were verified to be linearly related to the concentration of tolfenamic acid within the range investigated, 0.1-10.0 ng/μl.

According to the analytical procedure described above, the detection limit of tolfenamic acid, taken as a signal-to-baseline noise ratio of two at asensitivity setting of 0.002 AUFS, was estimated to be 0.5 ng and 0.9 ng in the presence and the absence of the internal standard respectively.

The results of the quantitative determination of tolfenamic acid in pharmaceutical preparations using caffeine as internal standard, by reversed phase HPLC are presented in Table 7.

The results obtained were in good agreement with the labelled amount and no interferences from the excipient were observed in the chromatograms. An example of the chromatographic analysis of a pharmaceutical preparation, suspension, is given in Figure 2.

Table 7. Experimental Results for the Determination of Tolfenamic acid in Pharmaceutical Preparations by Reversed-Phase HPLC, in the Presence of Caffeine as Internal Standard.

Sample	Labelled amount (mg)	Found[a] (mg)	RSD (%)	Analyzed quantity (ng)	Found[b] (ng)	RSD (%)
				10	9.8±0.8	8.0
Capsules	100[c]	100±8	8.3	20	21.2±1.8	8.7
				30	28.8±1.2	4.0
				10	9.0±0.8	8.6
Capsules	200[c]	188±16	8.8	20	19.4±1.7	8.7
				30	28.8±2.3	8.1
				10	10.2±0.6	6.2
Suspension	10[d]	11.2±0.9	8.3	20	23.4±0.8	3.3
				30	34.5±2.7	7.8

a Mean value of 24 determinations ± standard deviation

b Mean value of 8 determinations ± standard deviation, c mg/capsule, d mg/5 ml suspension

The developed method was applied to the analysis of tolfenamic acid in biological fluids, blood serum and urine. Employing the standard addition technique it is possible to determine tolfenamic acid in 50 μl of blood serum and 1 ml of urine samples in under three minutes time. In both cases tolfenamic acid was successfuly determined, employing the procedures described without using internal standard. The chromatograms are given in Figure 3.

In the determination of tolfenamic acid, N-(2-methyl-3-chlorophenyl)-anthranilic acid, there is no interference from flufenamic acid, N-(3-trifluoromethyl-phenyl)-

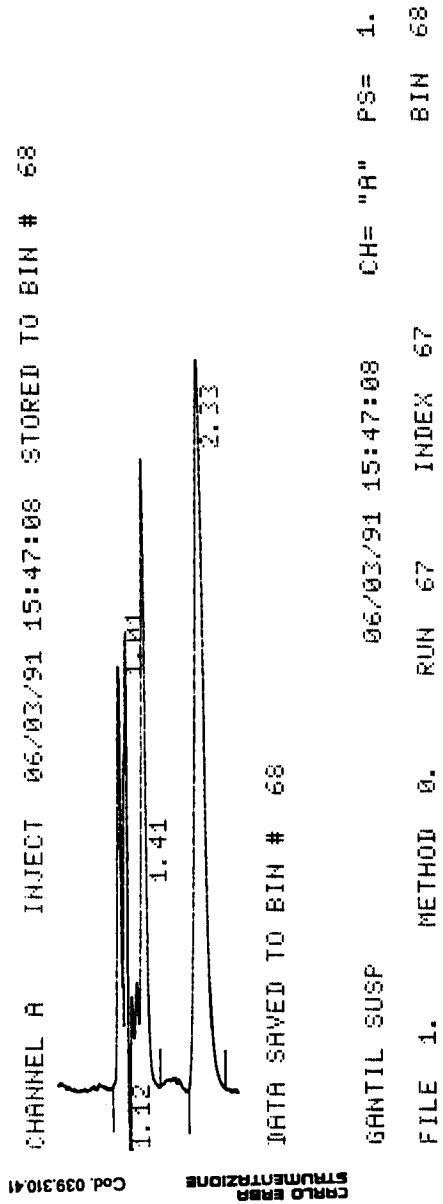

Figure 2. High-Performance Liquid Chromatogram of Tolfenamic acid in Pharmaceutical formulations, suspension. Peaks: 1.01, 1.12 unknown, 1.41 = Caffeine, 2.33 = Tolfenamic acid.

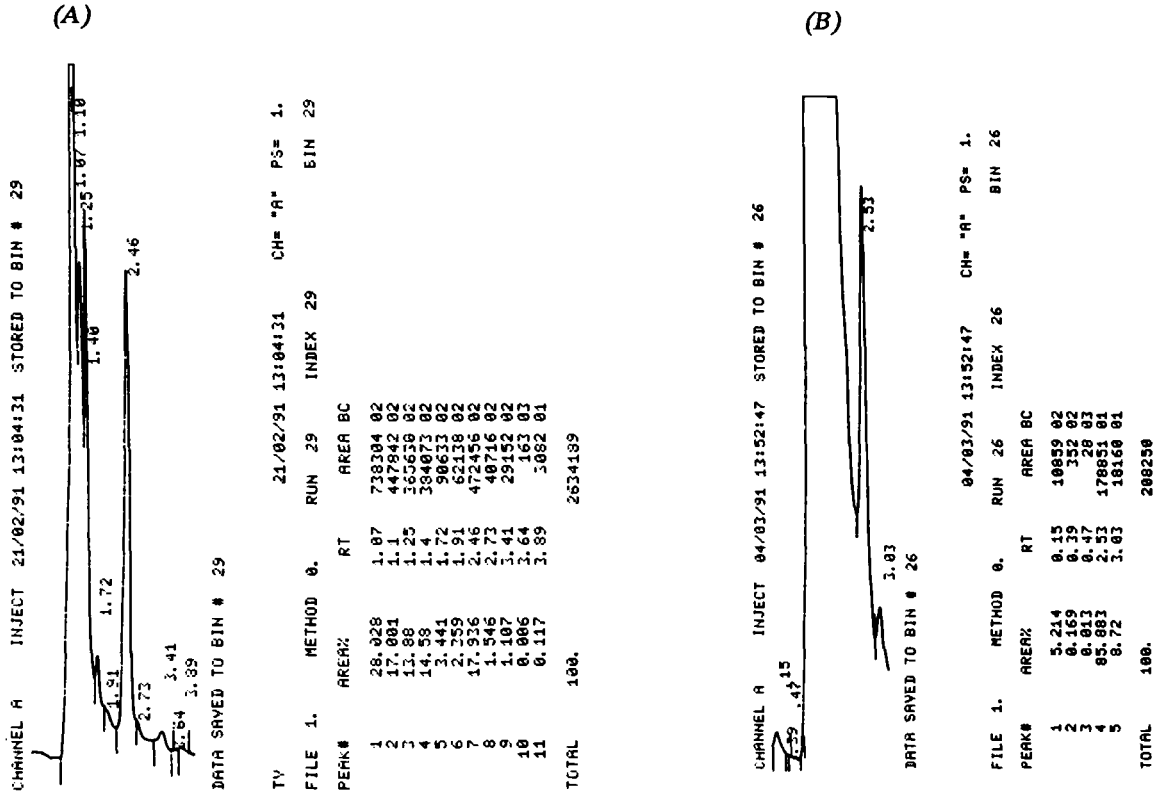

Figure 3. High-Performance Liquid Chromatograms of Tolfenamic acid extracted by solid phase cartridge from Blood Serum (A) and Urine (B) samples. Peaks: 1.40= Caffeine, 2.46, 2.53= Tolfenamic acid. All the other peaks are unknown. Chromatographic conditions as described in text.

anthranilic acid, which has retention time of 1.89 min. Mefenamic acid, N-(2,3-dimethyl-phenyl)-anthranilic acid, with a retention time of 2.65 min, interferes with the analysis of tolfenamic acid using the developed method.

Further work on the HPLC separation and determination of tolfenamic acid, flufenamic acid and mefenamic acid in biological fluids is in progress and results will be published in due time.

CONCLUSION

In conclusion, the present HPLC assay provides high accuracy, precision and specificity. This method is rapid, simple, highly sensitive and possesses good selectivity and reproducibility. This appears to be very useful for the routine analysis of tolfenamic acid in pharmaceutical formulations and biological fluids. The described HPLC procedure requires only 50 μl of blood serum and 1 ml of urine.

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