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Simultaneous analysis of anthranilic acid derivatives in pharmaceuticals and human urine by high-performance liquid chromatography with isocratic elution

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Abstract

A high-performance liquid chromatographic (HPLC) method for simultaneous determination of mefenamic acid (MFA), flufenamic acid (FFA) and tolfenamic acid (TFA) is presented for application to pharmaceuticals and human urine. Isocratic reversed-phase HPLC was employed for quantitative analysis using *tetra*-pentylammonium bromide (TPAB) as an ion-pair reagent. Urine samples were purified by solid-phase extraction using a silica-based strong anion-exchanger, Bond-Elut SAX cartridge. The HPLC assay was carried out using a Wakosil ODS $5C_{18}$ column (5 μm, 150×4.6 mm I.D.). The mobile phase consisted of 1.9 g of TPAB dissolved in 1 l of a mixture of acetic acid–sodium acetate buffer solution, pH 5.0, and acetonitrile (11:9, v/v). The calibration curves of MFA, FFA and TFA showed good linearity in the concentration range of 33–167 μg/ml with a wavelength of 280 nm for pharmaceuticals, and in the low concentration range (1.7–30.1 μg/ml) with a wavelength of 230 nm for biological fluids. The correlation coefficients were better than 0.9999 in all cases. The lower limits of detection (defined as a signal-to-noise ratio of about 3) were approximately 2 ng for MFA, 3.5 ng for FFA and 2.5 ng for TFA. The procedure described here is rapid, simple, selective and is suitable for routine analysis of pharmaceuticals and pharmacokinetic studies in human urine samples. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Anthranilic acid derivatives; Mefenamic acid; Flufenamic acid; Tolfenamic acid

1. Introduction

Mefenamic acid (MFA) [*N*-(2,3-xylyl) anthranilic acid], flufenamic acid (FFA) [*N*-(α,α,α-trifluoro-*m*-tolyl) anthranilic acid] and tolfenamic acid (TFA) [*N*-(3-chloro-*o*-tolyl) anthranilic acid] are non-steroidal anti-inflammatory drugs (Fig. 1) with analgesic and antipyretic properties [1]. These compounds are used in the treatment of osteoarthritis, rheumatoid arthritis and other painful musculoskeletal disorders. Fenamates inhibit cyclooxygenase and thus exert their anti-inflammatory activities by inhibition of prostaglandin synthesis [2].

Several high-performance liquid chromatographic (HPLC) methods have been published for the individual determination of three fenamates in pharmaceutical preparations [3–5] and biological fluids, serum and urine samples [4–11]. There have been

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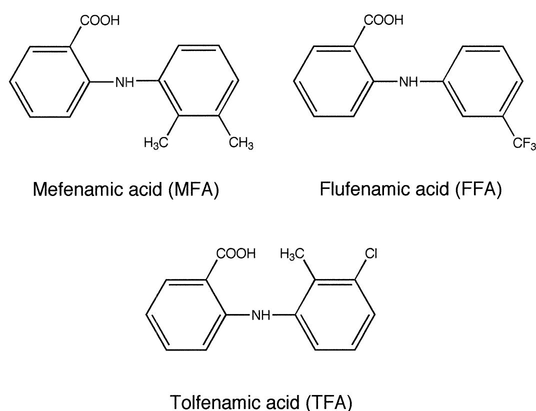

Fig. 1. Structures and abbreviations of three fenamates used in this study.

few reports concerning the simultaneous analysis of anthranillic acid derivatives. HPLC methods have been proposed for the determination of MFA and FFA with electrochemical detection [12], MFA, FFA and TFA using a gradient elution system with UV detection [13], MFA, FFA, TFA and diclofenac with conversion into methylphthalimide derivatives [14]. However, there have been no reports concerning the simultaneous determination of MFA, FFA and TFA by isocratic HPLC method.

Ion-pair chromatography is a powerful tool for the separation of ionized and ionizable compounds by HPLC. Normally, retention and subsequent separation of solutes can be achieved by addition to the eluent of a lipophilic reagent with the opposite charge to that of the solute ion. Thus, *tetra*-pentylammonium bromide (TPAB) is a useful ion-pair reagent [15,16] for the simultaneous analysis of three fenamates by HPLC.

Direct injection of diluted urine [6,7,9,12] or liquid–liquid extraction as a sample clean-up procedure [8,11] is frequently used for determination of fenamates in biological fluids by HPLC. Direct injection methods, however, lack sensitivity and removal of interfering substances is insufficient. For the liquid–liquid extraction procedure, complex manipulations, e.g. back extraction and/or some derivatization techniques induced to increase the sensitivity, are often carried out to avoid interference from endogeneous substances. For this purpose, we established a sample clean-up procedure using solid-phase extraction by a silica-based strong anion-exchanger, Bond-Elut SAX cartridge for analysis of three fenamates in human urine samples using HPLC with UV detection.

Here, we describe a simple sensitive, accurate and precise simultaneous analytical procedure for the determination of three fenamates using reversed-phase ion-pair isocratic HPLC. For pharmaceuticals after extraction by solvent, and for urine samples after efficient purification by solid-phase extraction on Bond-Elut SAX cartridges, were directly analyzed by HPLC–UV without prior derivatization. This procedure was used to determine MFA, FFA and TFA in pharmaceutical preparations and human urine.

2. Experimental

2.1. Chemicals and reagents

MFA for determination was of the Japanese Pharmacopoeia quality [17]. FFA and TFA for determination were of Japanese Pharmaceutical Codex quality [18]. MFA contained not less than 99.0% of $C_{15}H_{15}NO_2$ (molecular weight 241.29), when dried. FFA contained not less than 99.0% of $C_{14}H_{10}F_3NO_2$ (molecular weight 281.23), when dried. TFA contained not less than 99.0% of $C_{14}H_{12}ClNO_2$ (molecular weight 261.71), when dried. *n*-Butyl *p*-hydroxybenzoate, TPAB, ethyl acetate and HPLC-grade acetonitrile were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of analytical grade. Acetic acid–sodium acetate buffer solution, pH 5.0, for the mobile phase was a mixture of 1 mol/l sodium acetate solution, 1 mol/l acetic acid solution and water (140:60:800, v/v).

For urine sample preparation, we used Bond-Elut SAX cartridges (3 ml capacity containing 500 mg sorbent; Varian, Harbor City, CA, USA) preconditioned with 5 ml of methanol, 5 ml of ethyl acetate, 5 ml of the HPLC mobile phase and 5 ml of water.

2.2. Apparatus

A Shimadzu (Kyoto, Japan) HPLC system consisting of the following components was used: Model LC-6A pump, a model CTO-2A column oven, a

model SPD-6A UV detector, and a model C-R6A integrator/recorder equipped with a Rheodyne 7125 sample injection valve (20 μl). Column temperature was 40°C and chromatographic separation was carried out on a Wakosil $5C_{18}$ column (5 μm, 150×4.6 mm I.D., Wako Pure Chemical Industries, Osaka, Japan). An ultrasonic bath (model B-42H, Branson Co, Shelton, CT, USA) was used to dissolve the samples in ethanol.

2.3. Standard solutions

Standard stock solutions of MFA, FFA, TFA (1.67 mg/ml) and an internal standard stock solution of *n*-butyl *p*-hydroxybenzoate (1.5 mg/ml) were prepared by dissolving appropriate amounts of respective compounds in ethanol at room temperature.

2.4. Chromatographic conditions

Chromatography was performed under isocratic conditions, at a flow-rate of 1.0 ml/min. The mobile phase consisted of 1.9 g of TPAB dissolved in 1 l of a mixture of acetic acid–sodium acetate buffer solution, pH 5.0, and acetonitrile (11:9, v/v). The solution was filtered and degassed for 10 min in an ultrasonic bath. The column effluent was monitored at 280 nm using a detector range of 0.16 absorbance unit of full scale (aufs) for pharmaceuticals and monitored at 230 nm using a detector range of 0.02 aufs for human urine. The chart speed was 1 mm/min. An aliquot of sample solution (10 μl) was injected onto the analytical column with a manual HPLC injector. The capacity factor (k') was calculated as $(t_r - t_o)/t_o$, where t_r is the retention time of the analyte and t_o is the retention time of void elution determined by the injection of ethanol.

2.5. Calibration assay

For pharmaceuticals, 0.5, 1, 1.5, 2, 2.5 ml of MFA, FFA and TFA standard stock solutions were pipetted into 25 ml volumetric flasks, followed by the addition of 1 ml of the internal standard stock solution to the corresponding flasks and then made up with ethanol to the mark. Three fenamates mixed standard solution of 33.4, 66.8, 100.2, 133.6, 167.0 μg/ml were prepared.

For urine samples, 0.5, 1.5, 3, 5, 9 ml of MFA, FFA and TFA standard stock solution were pipetted into 50 ml volumetric flasks, followed by the addition of 3 ml of the internal standard stock solution to the corresponding flasks and then made up with ethanol to the mark. Aliquots of 1 ml of these three fenamates mixed solutions were pipetted into 10 ml volumetric flasks and then made up with ethanol to the mark. Diluted three fenamates mixed standard solutions of 1.67, 5.01, 10.02, 16.7, 30.06 μg/ml were prepared.

An aliquot (10 μl) of each solution was then injected onto the analytical column. All measurements were performed in duplicate for each concentration on five or six different days. The peak areas were measured and the peak area ratios of analyte to internal standard (y) were then plotted against the respective concentration of MFA, FFA and TFA (x). Least square linear regression analysis was used to determine the slope, y-intercept and the correlation coefficients of the standard plots.

2.6. Determination of MFA, FFA and TFA in pharmaceuticals

The contents of 10 capsules of MFA and TFA and 10 tablets of FFA were finely ground and mixed well in a mortar. An accurately weighed powdered sample containing 500 mg (labeled amount) of MFA or 200 mg (labeled amount) of FFA and TFA was transferred to a 200 ml volumetric flask. The volume was adjusted with ethanol and the solution was sonicated for 30 min in an ultrasonic bath. Portions of these solutions were then filtered through a 0.5 μm PTFE filter (Toyo Roshi Kaisha, Tokyo, Japan), respectively. For MFA, 4 ml of the internal standard solution was added to 4 ml of the filtrate and ethanol was added to make the volume up to 100 ml. For FFA and TFA, 2 ml of the internal standard solution was added to 5 ml of the filtrate, then ethanol was added to make the volume up to 50 ml. An aliquot (10 μl) of each solution was injected onto the column. Four different capsules (250 mg/capsule of MFA), one tablets (100 mg/tablet of FFA), and two different capsules (100 mg/capsule of TFA) of commercially available pharmaceuticals were analyzed for statistical evaluation of the assay. Assay precision were assessed by analyzing samples on the same day

(within-day, $n=5$) and on different days (between-day, $n=5$). The precision of the method was calculated as the relative standard deviation (RSD).

2.7. Determination of MFA, FFA and TFA in human urine

2.7.1. Assay precision and accuracy

Drug-free urine samples used in this study were obtained from healthy investigators. They were frozen at −20°C until analytical use.

Assay precision and accuracy were assessed by analyzing samples on the same day (within-day, $n=6$) and on different days (between-day, $n=6$). The accuracy of the procedure was determined by expressing the mean calculated recovery. The recovery was assessed at three concentration levels of 1, 4 and 9.4 μg/ml urine for three fenamates. To 480 μl aliquots of urine was added 20 μl of different concentrations (25, 100 and 234 μg/ml ethanol) of MFA, FFA and TFA mixed standard solution. The samples were then diluted with 500 μl of water and with 1 ml of methanol. Aliquots of 1 ml of diluted methanolic urine samples were applied to pre-conditioned Bond-Elut SAX cartridges. The cartridges were washed with 10 ml of water and eluted with 5 ml of ethyl acetate. These ethyl acetate solutions were evaporated to dryness in a water bath at 40°C. The residues were redissolved in 100 μl of ethanol containing *n*-butyl *p*-hydroxybenzoate (9 μg/ml ethanol) and aliquots of 10 μl were injected onto the chromatograph. Recoveries were determined from the peak-area ratios (MFA, FFA and TFA/internal standard) of extracts with those of standard solutions.

2.7.2. Analysis of biological samples

For practical application of our method, urine was collected 0–4 h after a single oral administration of 500 mg of MFA or 0–4 h after a single oral administration of 100 mg of TFA. A 500 μl portion of each urine sample was directly used to determine MFA and TFA. The urine samples were diluted fourfold for determination of MFA glucuronides and used directly for determination of TFA glucuronides. MFA and TFA glucuronides were determined as MFA and TFA after alkaline hydrolysis. Alkaline hydrolysis was achieved by adding 250 μl of 0.5 mol/l sodium hydroxide solution to 0.5 ml of urine, and standing at ambient temperature for 1 h [10]. The mixture was neutralized with 250 μl of 0.5 mol/l hydrochloric acid, and diluted with 1 ml of methanol. A 1 ml portion of deconjugated urine sample was used to determine MFA or TFA as described above.

3. Results and discussion

3.1. Chromatography

The objectives of this study were to establish an HPLC method for the rapid and accurate simultaneous determination of MFA, TFA and FFA in pharmaceuticals and biological fluids. In our preliminary study, a mobile phase consisting of acetate buffer and acetonitrile mixture using TPAB as an ion-pair reagent was the most suitable for the separation of three fenamates by reversed-phase HPLC with UV detection. The UV absorption spectra of the three fenamates used in this study exhibited similar wavelengths of maximum absorbance. Therefore, we set the detector wavelength to 280 nm for pharmaceuticals and to 230 nm for biological fluids which showed higher sensitivity. The peak intensities of MFA, FFA and TFA at the absorbance of 230 nm were about 1.8-, 1.2- and 1.8-fold higher than those at 280 nm, respectively.

The fenamates were weakly retained on the reversed-phase sorbents when the mobile phase consisted of a mixture of acetic acid–sodium acetate buffer solution, pH 5.0, and acetonitrile (11:9, v/v). They showed reduced resolution, and increased interference from unretained matrix compounds hampered accurate determination. Ion-pair chromatography is particularly useful for the separation and detection of several drugs. For example, it has been applied to some basic drugs, ketotifen [19], homochlorcyclizine [20] and metoprolol [21] by the addition of heptanesulfonate anions to the eluent, and to some acidic drugs such as glycolic acid [22], carminic acid [23] and aromatic sulfonates [24] by addition of alkylammonium cations. Because it provides in appropriate capacity factors for complexes formed with the counter ion. Therefore, to enhance the retention of three fenamates on reversed-phase

HPLC, the hydrophobic ion-pair reagent TPAB was used as a mobile phase additive.

The effects of TPAB concentration in the mobile phase were examined under conditions in which the three fenamates were eluted within 30 min. As shown in Fig. 2, the capacity factor, k', of internal standard, n-butyl p-hydroxybenzoate, was not affected by the addition of TPAB to the eluent, while those of the three fenamates were increased. Some enhancement of the analyte retention was achieved by increasing the TPAB concentration from 1.5 to 7 m*M*. Enhancement of retention was observed due to ion-pairing of the TPAB cations with the negatively charged fenamate to more hydrophobic forms and partially due to inactivation of the residual free silanol on the silica-based C_{18} packing material. Consequently, a TPAB concentration of 5 m*M* in the mobile phase was selected, because the peaks corresponding to the compounds were well separated and were sharp and symmetric, and chromatography could be completed within 25 min. No significant changes in standard retention or column efficiency were observed over long periods (several months).

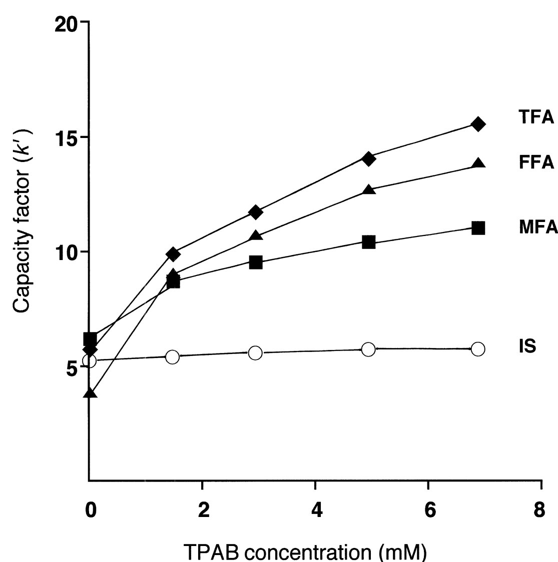

Fig. 2. Effects of TPAB concentration on the capacity factor, k', of MFA, FFA, TFA and n-butyl p-hydroxybenzoate (I.S.). The mobile phase also contained acetic acid–sodium acetate buffer solution (pH 5.0)–acetonitrile (11:9, v/v). The other chromatographic conditions are described in the text.

The capacity factor of MFA, FFA, TFA and internal standard was 10.7, 12.7, 14.1 and 5.1, respectively.

3.2. Solid-phase extraction of biological samples

Urine data are often required to obtain detailed pharmacokinetic information. To enable the direct determination of anthranilic acid derivatives by HPLC–UV without derivatization, non-selective short-wavelength (230 nm) UV detection was required which resulted in increased interference from endogenous components in urine. However, the selectivity of the HPLC–UV assay for the three fenamates can be enhanced in the sample preparation step by efficient clean-up procedures.

Solid-phase extraction techniques for drug analysis in biological samples would seem to be very useful in routine clinical use prior to HPLC analysis. Although C_{18} silica cartridges have been employed for the extraction of anthranilic acid derivatives [4,5], unknown peaks were observed and specificity of such methods is poor. For comparison, we selected a solid-phase extraction method using Bond-Elut SAX cartridges pre-packed with a silica-based strong anion-exchanger for this investigation. The anion-exchanger provides more selective extraction, compared to the C_{18} phase, as a result of high-energy ionic interactions. In previous studies reports [22,24,25] organic anions were extracted from aqueous samples using disposable columns packed with silica bonded to quaternary ammonium groups (SAX). To test the suitability of the solid-phase extraction of three fenamates in aqueous samples and of the eluting solvent, the extraction was first tested without urine matrix, so that three fenamates could be efficiently retained on Bond-Elut SAX cartridges. This was found to be suitable.

On the basis of our previous experiments, it was expected that higher extraction efficiencies and recovery rates would be obtained by use of ethyl acetate as the eluent. After washing the extraction cartridges with water to remove non-ionic endogenous components, quantitative desorption of three fenamates was obtained by elution of the cartridges with 5 ml of ethyl acetate. Urinary interference was removed by eluting the cartridges loaded urine samples with ethyl acetate, thereby yielding to a high sensitivity and recovery. In addition, the clean-up

method with Bond-Elut SAX cartridges and subsequent evaporation has some excellent features with regard to simplicity and reproducibility compared to many conventional extraction procedures. Therefore, the present method seems to be useful for monitoring three fenamates in clinical practice.

3.3. Analytical performance characteristics

3.3.1. Calibration curves

A linear relationship was established between the peak area ratio and concentration for each standard solution of three fenamates when *n*-butyl *p*-hydroxybenzoate was used as an internal standard. The slopes, intercepts with the y-axis and correlation coefficients after repeated analyses are given in Table 1. For pharmaceuticals, the calibration curves in the concentration range of 33.4–167.0 μg/ml of MFA, FFA and TFA showed good linearity with detector wavelength of 280 nm at a sensitivity of 0.16 aufs. In the low concentration range (1.7–30.1 μg/ml) of three fenamates, the calibration curves were also obtained by shifting the detector wavelength to 230 nm at a sensitivity of 0.02 aufs. The correlation coefficients were better than 0.9999 in all cases. The intercepts with the y-axis were not significantly different from the origin. The instrumental detection limits based on a signal-to-noise ratio of about 3 for standard solutions of MFA, FFA and TFA were 2 ng, 3.5 ng and 2.5 ng, respectively. The lower limits of quantification in human urine were 0.1, 0.15 and 0.1 μg/ml, respectively, using the procedure described above.

3.3.2. Assay precision and accuracy for human urine

The reproducibility of the assay for urine samples was determined by recovery experiments. Urine placebos were spiked with three different MFA, FFA and TFA at concentrations at 0.25, 1 and 2.34 μg and subjected to the assay. The recoveries and RSDs after six replicate repeated analyses are given in Table 2. The recoveries of three fenamates from urine samples were greater than 95% at each concentration examined. Assay precision expressed as RSD was better than 4.5%. As can be seen in this table, precision and accuracy of this method hardly depends on the concentration assayed or on the day of the assay. Using this pretreatment technique, it is possible to analyze anthranilic acid derivatives in urine samples within 25 min (Fig. 3). Alkaline hydrolysis is applicable for common hydrolysis conditions of the glucuronide conjugates of drugs in human urine. There were hardly any interfering peaks on the chromatograms during alkaline hydrolysis when drug-free human urine was subjected to the assay. Three fenamates were constantly recovered from urine with or without alkaline treatment. The possibility of interfering peaks was checked by analysis of more than ten different urine blanks.

3.4. Sample analysis

3.4.1. Pharmaceuticals

The specificity of simultaneous analysis of three fenamates with isocratic elution was further confirmed by the results of content uniformity tests of

Table 1
Linear estimation for the calibration curves of anthranilic acid derivatives in the presence of *n*-butyl *p*-hydroxybenzoate as an internal standard

Sample	Compound	Slope	y-Intercept	Correlation coefficient
Pharmaceuticals	MFA	2.41615	−0.03018	0.99995
(n=5)	FFA	2.41153	−0.02515	0.99994
	TFA	2.13563	−0.01798	0.99995
Biological fluids	MFA	31.36946	−0.02558	0.99996
(n=6)	FFA	20.82880	−0.03247	0.99995
	TFA	29.66901	−0.03563	0.99995

Table 2
Within- and between-day precision and accuracy of anthranilic acid derivatives assay for human urine

Nominal concentration (μg/ml)	MFA		FFA		TFA	
	Recovery (%, mean±SD)	RSD (%)	Recovery (%, mean±SD)	RSD (%)	Recovery (%, mean±SD)	RSD (%)
Within-day (n=6)						
1	96.8±4.2	4.4	97.4±3.5	3.6	97.9±3.2	3.3
4	97.4±3.0	3.1	98.0±2.2	2.2	98.5±2.9	2.9
9.4	98.0±3.4	3.5	98.9±3.5	3.5	98.8±3.6	3.6
Between-day (n=6)						
1	98.8±3.1	3.1	97.5±3.6	3.7	98.5±2.1	2.1
4	98.0±2.8	2.9	98.4±3.1	3.2	99.4±3.4	3.4
9.4	98.6±2.6	2.6	98.8±3.2	3.2	98.7±3.0	3.0

MFA, FFA and TFA performed on commercial MFA 1 capsules, FFA 1 tablets and TFA 1 capsules. The average percentages of label claim found in ten capsules or tablets were 100.1±1.1%, 99.3±1.6% and 99.6±2.1%, respectively, in the range of 103.5 to 97.5%. The results of content uniformity tests indicated compliance to specifications of pharmaceuticals and supported the specificity of this method. The reproducibility of the method was assessed by analyzing three fenamates in commercial pharmaceutical preparations on the same day and on different days. The results obtained were in good agreement with labeled amounts of the pharmaceutical preparations (Table 3) and no interference was observed in the chromatograms from the excipients. Within- and between-day precision was better than 3%. The assay results indicated that the proposed analytical method could be used for determination of MFA, FFA and TFA in commercial pharmaceuticals.

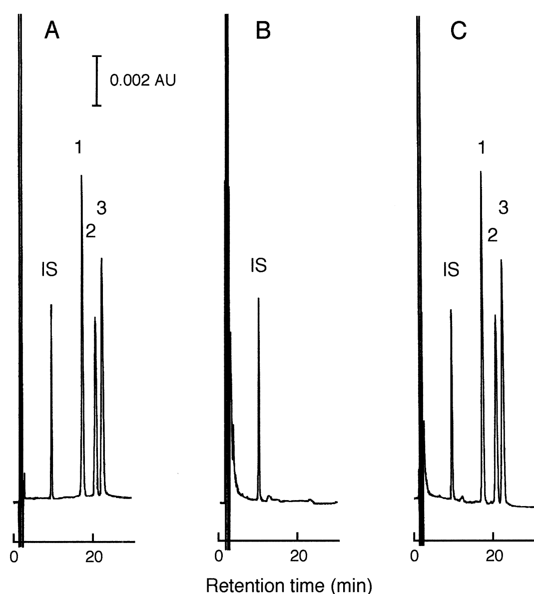

Fig. 3. Chromatograms of (A) standard, (B) drug-free human urine and (C) urine spiked with 4 μg/ml as the final concentration for MFA, FFA and TFA. Chromatographic conditions were described in the text. Peaks: 1=MFA; 2=FFA; 3=TFA; I.S.=n-butyl p-hydroxybenzoate.

3.4.2. Urine samples

As an example of the application of this method, the urinary excretion of MFA and TFA were investigated in the urine from healthy volunteers after receiving 500 mg of MFA and 100 mg of TFA orally, respectively. Fig. 4 shows the chromatograms obtained in the analysis of 0–4 h urine samples collected after administration. The urinary concentration and intact excretion rates were 0.31 μg/ml and 0.05% of dose for MFA and 0.18 μg/ml and 0.12% of dose for TFA, respectively. On the other hand, the total value (free + conjugate) obtained by deconjugation with alkaline hydrolysis was 7.50 μg/ml and 1.11% of dose for MFA and 1.78 μg/ml and 1.16% of dose for TFA. The present method using a

Table 3
Within- and between-day precision of anthranilic acid derivatives assay for pharmaceutical preparations

Sample	Pharmaceutical forms	Claimed content	Within-day ($n=5$)		Between-day ($n=5$)	
			% of claimed content (mean±SD)	RSD (%)	% of claimed content (mean±SD)	RSD (%)
MFA 1	Capsules	250[a]	100.05±1.06	1.06	99.35±0.61	0.61
2	Capsules	250[a]	100.37±2.31	2.30	100.13±2.52	2.52
3	Capsules	250[a]	99.69±0.92	0.92	100.30±1.20	1.20
4	Capsules	250[a]	99.52±1.55	1.56	99.63±1.66	1.66
FFA 1	Tablets	100[b]	99.34±1.58	1.59	99.03±0.91	0.92
TFA 1	Capsules	100[a]	98.84±1.74	1.76	99.20±1.92	1.94
2	Capsules	100[a]	100.22±1.21	1.21	99.87±1.09	1.09

[a] mg/capsule.
[b] mg/tablet.

Bond-Elut SAX cartridge could be applied to the routine pharmacokinetic study after administration of three fenamates to urine samples.

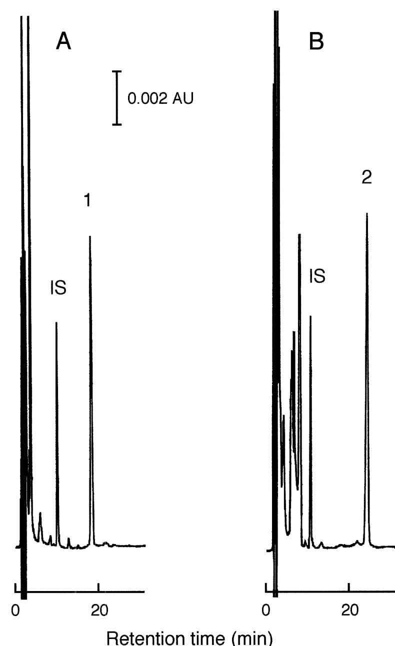

Fig. 4. Chromatograms obtained in the analysis of 0–4 h urine after a single oral administration of (A) 500 mg of MFA and (B) 100 mg of TFA to a healthy volunteer. Chromatographic conditions were described in the text. Peaks: 1=MFA; 2=TFA; I.S.=n-butyl p-hydroxybenzoate.

4. Conclusions

An isocratic HPLC method using TPAB as an ion pair reagent has been developed for the simultaneous analysis of MFA, TFA and FFA in pharmaceuticals and human urine. For pharmaceuticals, the method was applied to commercial capsules and tablets, and was shown to be free of interference from excipients normally used in pharmaceutical formulations. For urine samples, the method could be used to remove endogeneous interference by solid-phase extraction on Bond-Elut SAX cartridge as a sample clean-up procedure, thereby enabling simple manipulation and HPLC with UV analysis as well as achieving a high degree of sensitivity. Due to the minimal sample preparation, and its good precision and accuracy, this method appears to be very useful for the quality control of pharmaceuticals and clinical monitoring.

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