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Determination of 3-methylflavone-8-carboxylic acid, the main metabolite of flavoxate, in human urine by capillary electrophoresis with direct injection

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ABSTRACT

The effects of tetraalkylammonium salts and sodium dodecyl sulphate on the migration behaviour of human urinary components and other negatively charged or neutral solutes were investigated. The sulphate acted mainly on hydrophobic and positively charged substances, whereas the ammonium salts acted mainly on negatively charged solutes. By choosing the components of the eluent carefully, the free and conjugate forms of 3-methylflavone-8-carboxylic acid (MFA) in human urine, the major metabolites of flavoxate, could be simultaneously determined without pretreatment, using fenprofen as an internal standard. The calibration curve of MFA was linear in the range 1–50 μg/ml and the detection limit was 0.2 μg/ml, which covered the urine levels encountered in pharmacokinetic studies. The intra-day and inter-day precisions of the method, expressed as the relative standard deviation, were less than 2 and 3%, respectively. This method was successfully applied to an excretion study of MFA in eight healthy volunteers, and the results were in agreement with data in the literature obtained by gas chromatography.

INTRODUCTION

Flavoxate (F), *i.e.* the 2-(1-piperidino)ethyl ester of 3-methylflavone-8-carboxylic acid (MFA), is a smooth-muscle antispasmodic drug with selective action on the pelvic organs [1]. It is rapidly metabolized into MFA and 2-(1-piperidino)ethanol, so excretion studies of F can be performed by monitoring MFA [2,3]. Several assay methods for the determination of MFA in biological fluids have been reported, including radiometric assay [4,5] and gas chromatographic (GC) assay [2,3]. However, the radiometric method lacks specificity and the specific GC method involves extraction and methylation of samples before injection.

The new analytical technique of capillary electrophoresis (CE), initiated in the early 1980s [6,7], has shown great potential for bioanalysis with high separation efficiencies and short analysis times [8–16]. The direct determination of pharmaceuticals in human plasma [10,13,14], serum [15], saliva [15] and urine samples [16] have been reported, although the extraction of body fluids is necessary in most of the reported CE bioanalyses [8–12]

In this paper, the determination of MFA in

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human urine by direct injection is described. Detailed investigation of the components of the mobile phase led to complete separation of the free and conjugate forms of MFA from the endogenous components of urine and from each other in direct injection mode. The urine samples could be directly injected without extraction, so the procedure of analysis was greatly simplified and pharmacokinetic data could be obtained more directly. This assay method was successfully applied to an excretion study of MFA in eight healthy volunteers.

EXPERIMENTAL

Chemicals and drugs

Flavoxate hydrochloride (F · HCl) in tablet form was produced by Recordati (Milan, Italy). MFA was prepared by hydrolysis of F · HCl and recrystallized three times with methanol. It was identified by electrophoretic comparison with a stored aqueous solution of F · HCl. F is known to degrade to MFA in aqueous solution [17]. The purity of the prepared MFA was higher than 98% by CE examination. Fenprofen (FPF) was produced by Shanghai No. 17 pharmaceutical factory (Shanghai, China). The structures of F, MFA and FPF are shown in Fig. 1. All the other chemicals were of analytical-reagent grade.

Capillary electrophoresis

CE was carried out on a Bio-Rad HPE-100 capillary electrophoresis system (Bio-Rad, Richmond, CA, USA) with an uncoated fused-silica capillary (35 cm × 50 μm I.D.). The on-column UV detection wavelength was 245 nm. Electrokinetic injections were performed at 2 kV for 10 s from the positive end. The analytical voltage was 10 kV. The CE data in this paper were obtained from three consecutive runs, and the run-to-run relative standard deviations (R.S.D.) of the migration times and peak-area ratios were less than 2%.

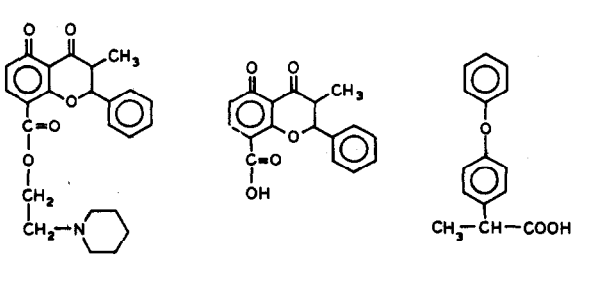

Fig. 1. Structures of flavoxate (F), 3-methylflavone-8-carboxylic acid (MFA) and fenprofen (FPF).

Urine sample preparation for direct injection

A 1-ml volume of urine was added to 1 ml of stock internal standard solution (6 mg/ml FPF in 0.02 *M* phosphate buffer, pH 7), then diluted to 10 ml with 0.02 *M* phosphate buffer (pH 7). The sample solutions were filtered through 0.45-μm disposable filters (Millipore, Bedford, MA, USA) prior to injection.

Collection of urine specimens

For an excretion study of MFA, eight healthy male volunteers were chosen under the following protocol: 21–25 years old, 55–70 kg body weight, and with normal liver and kidney functions and electrocardiograms confirmed by recent examination. Urine specimens were collected at 0–0.5, 0.5–1, 1–1.5, 1.5–2.5, 2.5–3.5, 3.5–5, 5–7, 7–9 and 9–12 h after oral administration of one F · HCl tablet (200 mg of F · HCl). After measurement of the volume of each urine specimen, 10-ml aliquots were stored at 277 K until determination.

RESULTS AND DISCUSSION

CE studies of urine samples

Whether the urine samples can be directly injected for CE analysis depends on the capillary wall and the mobile phase. A few studies have been carried out on the characteristics of capillary walls [18], and more studies phase additives [19] to improve the resolution. In the analyses of plasma, serum and saliva samples by CE with direct injection, sodium dodecyl sulphate (SDS) was frequently used as an additive to remove background interference, and the proteins in these body fluids were solubilized by SDS micelles and then eluted after the drug [13–15]. However, SDS could hardly affect the migration

behaviour of urinary components (see Fig. 5, below).

To improve the resolution of urinary components and MFA, various additives were tested, including surfactants, organic solvents, metal ions and complexing reagents. The results show that tetraalkylammonium (TAA) salts could improve the resolution satisfactorily. Typical electropherograms of a blank human urine specimen and the blank urine spiked with MFA and FPF are shown in Fig. 2, obtained by direct injection using tetramethylammonium bromide (TMAB) as an additive to a phosphate buffer (pH 7). MFA and FPF (internal standard) were completely separated from urinary components and each other. There are four large peaks in the blank urine, symbolized by U1, U2, U3 and U4, respectively.

The effect of the alkyl groups in TAA salts on the migration behaviour of urinary components and other ionic or neutral substances is shown in

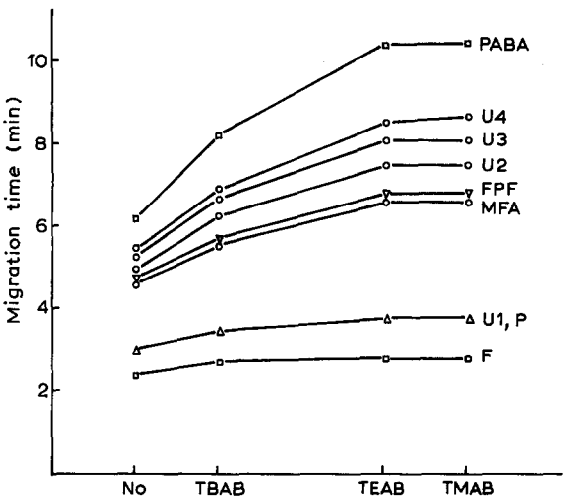

Fig. 3. Effect of tetraalkylammonium salts on the migration behaviour of urinary components and other ionic or neutral solutes. Mobile phase, 0.02 *M* phosphate solution (pH 7) containing 0.01 *M* tetrabutylammonium bromide (TBAB) or tetraethylammonium bromide (TEAB) or TMAB. Other conditions and symbols as in Fig. 2.

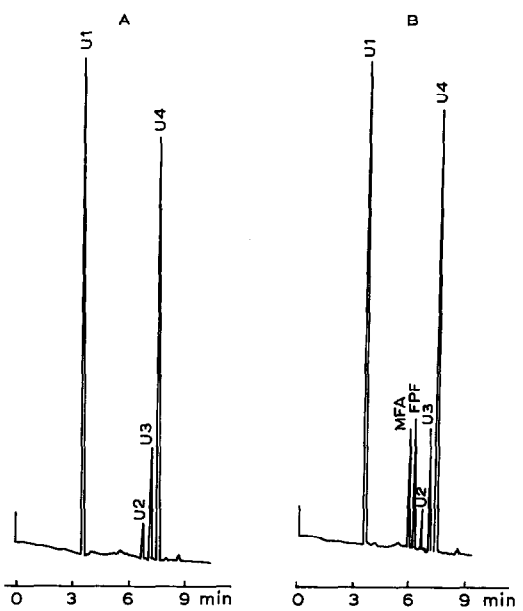

Fig. 2. Electropherograms of (A) a human blank urine and (B) the blank urine spiked with 0.1 mg/ml MFA and 6 mg/ml FPF. The urine samples were directly injected after ten-fold dilution with 0.02 *M* phosphate solution (pH 7). Mobile phase, 0.02 *M* phosphate solution (pH 7) containing 0.01 *M* TMAB and 0.002 *M* SDS. Other conditions are given in Experimental. U1, U2, U3 and U4 are endogeneous compounds of human urine.

Fig. 3. When TAA was not present, MFA, FPF and *p*-aminobenzoic acid (PABA) eluted behind the neutral phenol (P), which showed that they were negatively charged owing to the dissociation of carboxy group at pH 7. F eluted before P, being positively charged owing to the protonation of the piperidino group. Addition of TAA increased the migration times of MFA, FPF, PABA and U2–U4, with TMAB showing the greatest effect.

The effects of different TMAB concentrations are shown in Fig. 4. Migration times of the negatively charged MFA, FPF, PABA and U2–U4 increased with increasing TMAB concentration. However, there was little effect on the neutral P, or on the positively charged F and U1. Nishi and Tsumagari [20] have suggested an ion-pair formation mechanism between positive TAA ions and anionic solutes. TMAB, which has short alkyl chains, would pair more strongly than those TAA with longer alkyl chains owing to the lower steric hindrance. In any case, positive TAA ions could hardly pair with neutral or positive solutes.

The effects of different SDS concentrations are

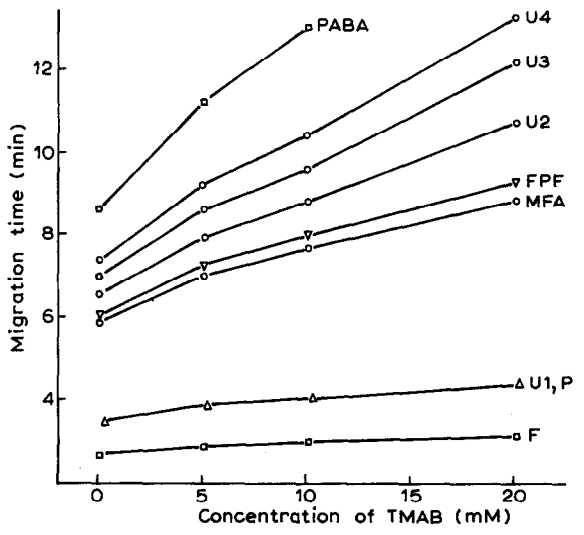

Fig. 4. Effect of TMAB concentrations on the migration behaviour of urinary components and other anionic or neutral substances. Mobile phase, 0.02 *M* phosphate solution (pH 7) containing TMAB. Other conditions and symbols as in Fig. 2.

shown in Fig. 5. The neutral P and positively charged F would be solubilized by SDS and their migration time increased with increasing SDS concentrations. The data of F are not included in Fig. 5 because its migration time becomes too long. Owing to the electrostatic repulsion between the negatively charged SDS and anions, the migration times of the anions, except FPF, varied little with changing SDS concentration. FPF has the additional hydrophobic group of phenyl ether, which may undergo Van der Waals interaction with the hydrophobic part of SDS. Thus its migration time increased remarkably with increasing SDS concentration. Nishi and Tsumagari [20] have also described a similar experimental result that SDS had little effect on the migration times of common anions of cinnamic and hydroxybenzoic acids, but greatly increased the migration time of naproxen anion, which has a hydrophobic naphthalene skeleton. Otherwise, SDS had little effect on the migration times of urinary components.

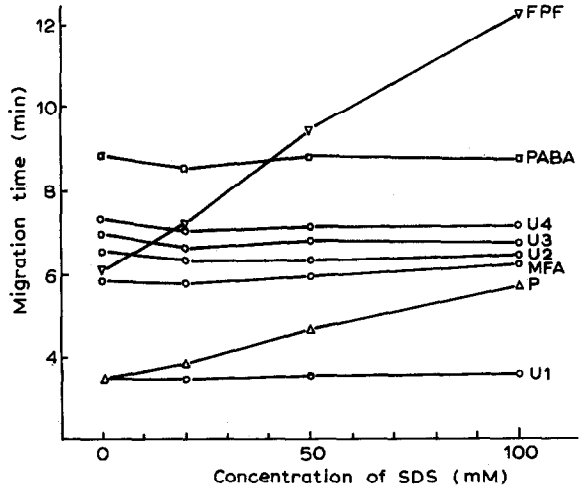

Fig. 5. Effect of SDS concentration on the migration behaviour of urinary components and other anionic or neutral solutes. Mobile phase, 0.02 *M* phosphate solution (pH 7) containing 0.005 *M* TMAB and SDS. Other conditions and symbols as in Fig. 2.

U2, U3 and U4 were similar to the common carboxylic acids of MFA and PABA in the dependence of their migration behaviour. This suggests that they are acidic components of human urine without strongly hydrophobic groups. As U1 always coeluted with the neutral P in the absence of SDS, but its migration time was not affected by SDS, it may represent neutral components of human urine with a strongly hydrophilic character.

Optimization of the components of the mobile phase and the internal standard

When the mobile phase contained 0.02 *M* phosphate (pH 7) and 0.1 *M* TMAB, MFA could be fully separated from urinary components. PABA was tried as an internal standard for the quantitation of MFA in human urine, but was not really suitable because its peak was too far from the MFA peak (Fig. 4). In contrast, FPF was too close to MFA for a satisfactory resolution at 0.01 *M* TMAB. The resolution between MFA and FPF could be increased by increasing the TMAB concentration. At 0.02 *M* TMAB the resolution increased to 2, which was perfectly suitable for the quantitative analysis. However, it can be seen from Fig. 5 that SDS could dramatically change the migration behaviour of FPF. The addition of just 0.002 *M* SDS to the phosphate buffer changed the resolution of MFA and

FPF by the same amount as the addition of 0.02 *M* TMBA.

To reduce the effect of Joule heating on the reproducibility, the ionic strength of the mobile phase should be as low as possible. For this reason, we preferred to use 0.02 *M* phosphate solution containing 0.01 *M* TMAB and 0.002 *M* SDS and FPF as the internal standard in the CE assay.

The blank urine specimens from twenty healthy volunteers at different times within one day were analysed, and none exhibited interferences in the determinations of MFA and FPF.

Linearity, limit of quantitation and recovery

According to previous reports [2,3], the pharmacologically interesting MFA concentrations in human urine are in the range 0.01–0.5 mg/ml. In the working sample solutions here, urine specimens would be diluted ten-fold, so the linearity was assessed in the concentration range 1–50 μg/ml for MFA by analysing the standard solutions prepared in 0.02 *M* phosphate buffer (pH 7) containing 0.6 mg/ml FPF as the internal standard. The calibration graph showed excellent linearity, with a correlation coefficient of 0.9999. The detection limit of MFA was *ca.* 0.2 μg/ml at a signal-to-noise ratio of 2. The recovery was examined by analysing six blank urine specimens spiked with MFA over the concentration range 0.05–0.2 mg/ml. The average recovery was 99.9% (R.S.D. 3.4%),. There was little loss in recovery for the CE method with direct injection.

Stability and precision

To test the stability of both the assay and MFA, three samples prepared by dissolving MFA in phosphate buffer (pH 7) or blank urine and stored at 277 K or room temperature (*ca.* 293 K) were analysed over a period of 25 days. Sample solutions were prepared daily under the procedure described in Experimental, and analysed in three consecutive runs in one day. The data in Table I show that there was no evidence of degradation of MFA over a period of 25 days and that the CE assay was stable. The R.S.D. for inter-day and for intra-day assays was less than 3 and 2%, respectively.

Application to excretion studies of MFA

Electropherograms of a urine specimen taken 2.5 h after oral administration of 200 mg of F · HCl to a healthy volunteer are shown in Fig. 6. The electropherogram obtained by direct injection (Fig. 6A) shows that the MFA peak was very small, and a large peak at 4.8 min was detected. After hydrolysis of the urine specimen, the peak at 4.8 min became smaller and the MFA peak became larger (Fig. 6B). Stronger hydrolysis led to the gradual disappearance of the peak at 4.8 min and simultaneous increase of the MFA peak (Fig. 6C), This shows that the peak at 4.8 min should be ascribed to the conjugated form of MFA. According to the literature [2,3], MFA is probably conjugated as the glucuronide.

The resolution in Fig. 6A is sufficient for the

TABLE I

STABILITY OF MFA AND PRECISION OF THE METHOD

The values for each day were from three consecutive runs.

Sample No.	Solvent[a]	Storage temperature (K)	Determined concentration of MFA (mg/ml)						
				Day 0	Day 2	Day 6	Day 13	Day 25	Inter-day
1	A	277	Mean	0.1004	0.1046	0.1012	0.1032	0.0998	0.1018
			R.S.D. (%)	0.5	0.02	1.1	0.8	0.2	1.9
2	B	277	Mean	0.1083	0.1089	0.1083	0.1083	0.1076	0.1083
			R.S.D. (%)	0.7	1.3	1.1	0.3	0.4	0.5
3	B	293	Mean	0.1083	0.1070	0.1035	0.1098	0.1100	0.1072
			R.S.D. (%)	0.7	1.8	0.8	1.1	1.4	2.5

[a] A = 0.02 *M* phosphate buffer (pH 7); B = human urine.

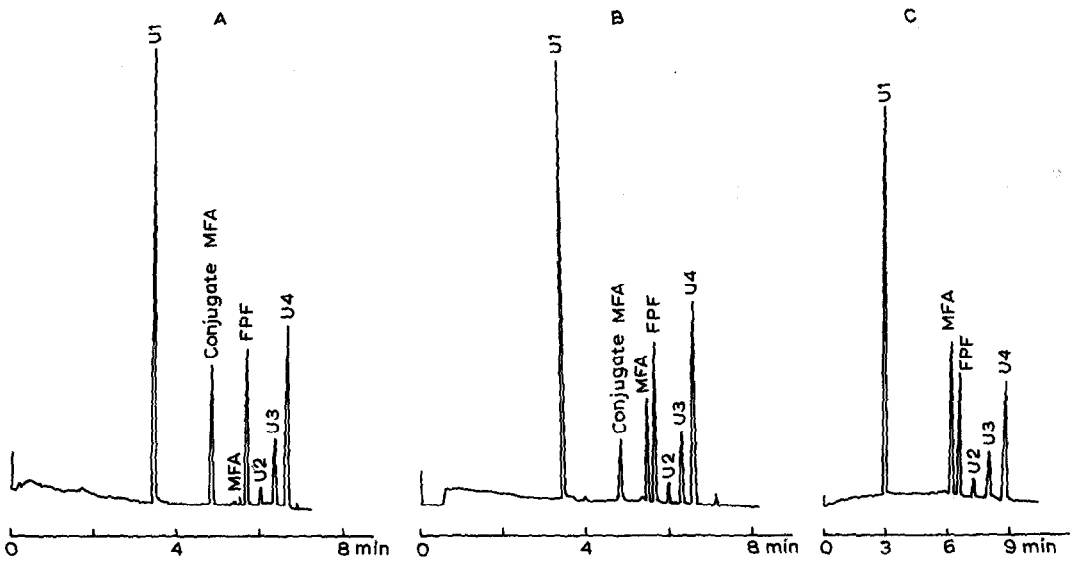

Fig. 6. Electropherograms of a urine specimen taken 2.5 h after oral administration of 200 mg of F · HCl to a healthy volunteer. The urine specimen was (A) directly injected under conditions described in the Experimental, (B) hydrolysed in 0.1 *M* HCl at 373 K for 0.5 h, or (C) hydrolysed in 0.05 *M* NaOH at 373 K for 0.5 h prior the separation. CE conditions and symbols are as in Fig. 2.

simultaneous determination of the concentrations of free and conjugated MFA in human urine with direct injection. The concentration of the conjugated MFA may be calculated approximately by using the calibration factor of the free MFA, owing to the very limited absorption of the glucuronide. The urinary excretion of MFA was obtained by examining the concentrations and the volumes of urine. The urinary excretion rate–time curves of free and conjugated MFA in a healthy volunteer are shown in Fig. 7. It can be seen from Fig. 6A and 7 that MFA is present primarily in the conjugated form in human urine, and that the percentage of the free form in total urinary MFA is only *ca.* 10%. However, both Bertoli *et al.* [2] and Ariga *et al.* [3] reported about half the MFA in human urine to be in the free form, as determined by gas chromatography after extraction, hydrolysis and methylation of urine samples. This difference may be ascribed to the differences in the assay methods or in the races of the subjects.

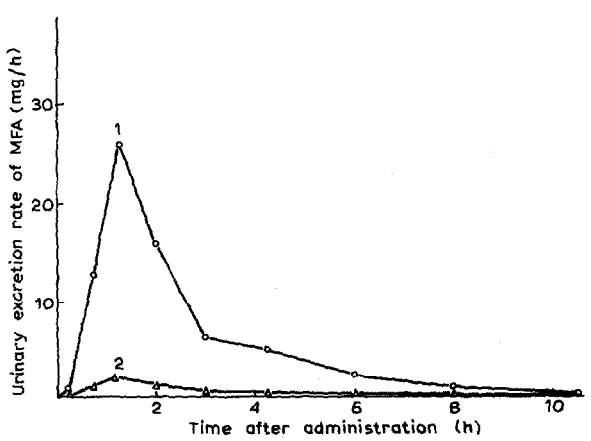

Fig. 7. Urinary excretion rate–time curves of (1) conjugated (calculated on free MFA, $C_{17}H_{12}O_4$) and (2) free MFA following oral administration of 200 mg of F · HCl to a healthy volunteer. Data were obtained by analysing the original urine specimens without hydrolysis.

The urine specimens do not require any pretreatment except dilution, before separation. However, because of the lack of a conjugate MFA reference standard, hydrolysis of the conjugated form to the free form is necessary for the precise determination of the total urinary MFA. Various hydrolysis conditions were explored, including hydrolysis time, temperature, and the concentrations of acid or alkali. The results showed that alkaline hydrolysis were preferable

to the acidic hydrolysis with powerful dissociation at a lower ionic strength. Hydrolysis of conjugated MFA to the free form was complete with 0.05 *M* NaOH, but not quite complete with 0.4 *M* HCl. So we employed the hydrolysis conditions shown in Fig. 6C, under which the conjugated form was not detected in the hydrolysed urine specimens (see Fig. 6C) and the loss of MFA was relatively small. The detailed procedure of the hydrolysis was as follows: 10 μl of 5 *M* NaOH solution were added to 1 ml of urine specimen, then the mixture was placed in a water-bath at 373 K for 0.5 h, cooled, and 10 μl of 5 *M* HCl were added to neutralize the alkali. The hydrolysed specimen was added to 1 ml of internal standard solution, then diluted and filtered as described in Experimental.

The electropherograms of blank urine showed no change after hydrolysis, except that the peak heights of U1–U4 decreased slightly (data not shown). Nine blank urine specimens spiked with MFA were analysed after hydrolysis, and the mean recovery of MFA throughout the sample hydrolysis and CE analysis was 94.6% (R.S.D. 2.7%).

An excretion study of MFA in eight healthy volunteers was performed by analysing the hydrolysed urine specimens. The time-course of the mean urinary excretion rate of MFA is shown in Fig. 8, which shows that the highest rate was 27.5 mg/h for MFA 1–1.5 h after oral administration of 200 mg of F · HCl. The mean total urinary excretion of MFA within 12 h after the administration for the eight volunteers was 79.9 mg (R.S.D. 15%), accounting for 60.6% of the dose. All these are in agreement with the data in the literature [2,3].

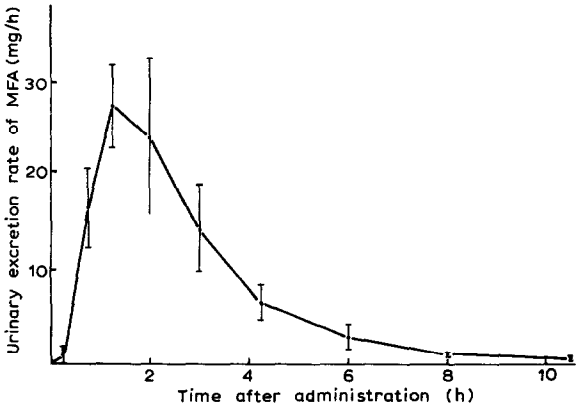

Fig. 8. Time-course of urinary excretion of MFA after oral administration of 200 mg of F · HCl to eight healthy volunteers. The vertical lines on the experimental points (mean values) represent the standard deviations for the eight subjects. Data were obtained by analysing the hydrolysed urine specimens.

CONCLUSION

In CE, SDS mainly affects the migration behaviour of hydrophobic substances and TAA acts mainly on negatively charged solutes. No sample extraction is necessary when TMAB is added to the mobile phase. Both the free and the conjugated forms or MFA in human urine could be simultaneously determined in direct injection mode. Direct injection means that the sample pretreatment procedures in bioanalysis can be greatly simplified, and better pharmacokinetic information can be obtained. It seems clear that CE will be developed before long as another widely used technique in bioanalysis.

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