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DETERMINATION OF MALOTILATE AND ITS METABOLITES IN PLASMA AND URINE

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SUMMARY

A method for the determination of malotilate (I), the corresponding monocarboxylic acid (II) and its decarboxylated product (III) in plasma is described. Plasma was extracted with chloroform spiked with internal standard. The residue, dissolved in methanol, was chromatographed on a reversed-phase column with a mobile phase of 60% acetonitrile and 1% acetic acid in water. The sensitivity limit for I, II and III was 50, 25 and 100 ng/ml of plasma, respectively. Compound I in the same plasma extract was also analysed by gas chromatography—electron-impact mass spectrometry. The base peaks *m/z* 160 for I and *m/z* 162 for internal standard (IV) were monitored; the sensitivity limit for I was 2.5 ng/ml of plasma

The determination of the metabolites of I, II and its conjugate (V), and isopropyl-hydrogen malonate (VI) in urine by high-performance liquid chromatography is also described. The limit of quantification for VI was 2.0 μg/ml, and the overall coefficient of variation of VI was 4.7%. The limit of quantification for II in urine was 0.5 μg/ml and that for V was 1.0 μg/ml as total II (II + V) The overall precision of the method was satisfactory.

The method was used to determine plasma and urine concentrations in four dogs orally dosed with 100, 200 or 400 mg of malotilate.

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INTRODUCTION

Malotilate (I), diisopropyl 1,3-dithiol-2-ylidenemalonate (Fig. 1), was developed as a drug for chronic hepatitis and cirrhosis [1]. After oral administration, malotilate undergoes extensive first-pass metabolism. A major metabolite in plasma is the corresponding monocarboxylic acid (II) (Fig. 1) in humans [2], dogs [3] and rats [4]. Its decarboxylated product (III) (Fig. 1) is found in rat urine and is also a chemical degradation product of II (Fig. 1). In many species, isopropylhydrogen malonate (VI) (Fig. 1) is a major metabolite in urine [2—4]. In humans, the conjugate of II, compound V (Fig. 1), is a major metabolite in urine [2].

As compound II readily degrades to compound III, gas—liquid chromatography and spectrophotometry are not suitable for simultaneous determination. We describe here a high-performance liquid chromatographic (HPLC) method which allows a simple and precise analysis of plasma samples for I, II and III simultaneously, and an analysis of urine samples for V and VI. We also describe a sensitive mass fragmentographic method for I in plasma using the same sample preparation.

This method was used to determine malotilate and its metabolites in plasma and urine of dogs that had received 100, 200 or 400 mg of malotilate orally.

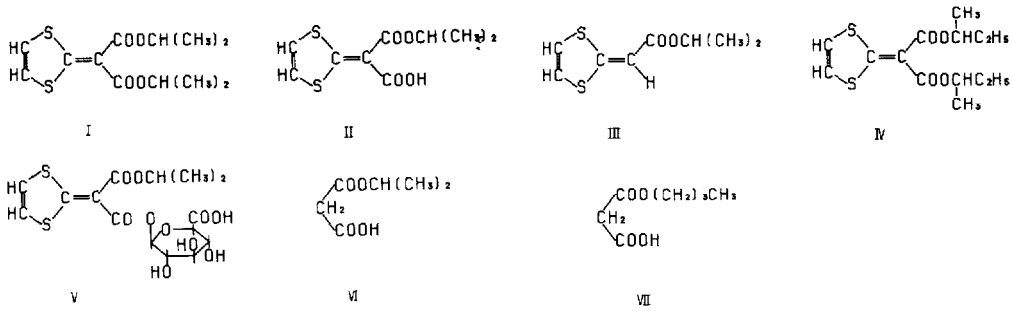


Fig. 1. Chemical structures of malotilate, its metabolites and internal standards.

EXPERIMENTAL

Materials

Malotilate, II, III, VI and internal standard for the plasma assay, di-*sec.*-butyl 1,3-dithiol-2-ylidenemalonate (IV) and *n*-butylhydrogen malonate (VII) for the urine assay, were synthesized at Nihon Nohyaku. *o*-4-Nitrobenzyl-N,N′-diisopropylisourea (NBDI) was obtained from Dohzinkagaku. Other chemicals were obtained commercially (reagent grade) and used without further purification.

The urine sample used for the evaluation of the HPLC assay for V was collected over an 8-h period from a male rabbit (weighing 2.4 kg) that had been given an oral dose of 200 mg of malotilate.

Plasma sample preparation

To 1.0 ml of plasma were added 1.0 ml of 1 *M* acetate buffer (pH 5.0), 1.0 ml of water and 5.0 ml of chloroform containing 1.0 μg of internal standard

(IV). The extraction mixture was agitated in a horizontal shaker for 10 min. After centrifugation, ca. 4 ml of organic phase were removed and evaporated to dryness. The residue was redissolved in 100 μl of methanol and aliquots of 10 μl were injected into the HPLC system as described below. Aliquots of 2 μl were also analysed by gas chromatography—mass spectrometry (GC—MS).

Urine sample preparation of VI

To 1.0 ml of urine were added 0.5 ml of 1 *M* hydrochloric acid and 5.0 ml of ethyl acetate containing 30 μg of the internal standard (VII). The mixture was shaken for 5 min and centrifuged, and 4.0 ml of the organic phase were pipetted into a centrifuge tube. The ethyl acetate extract was dried with anhydrous sodium sulphate (2 g). After centrifugation, 3.0 ml of the ethyl acetate extract were transferred to another tube. Then 1 ml of NBDI solution (5 mg/ml in ethylene dichloride) was added, and evaporated under a stream of nitrogen at 60°C to a volume of ca. 0.5 ml. The tube was stoppered and placed in a 70°C water-bath for 2 h, and the solvent was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.6 ml of *n*-hexane, and 1 ml of 0.01 *M* hydrochloric acid was added. The contents were vortexed and centrifuged. Then 10-μl aliquots of organic phase were analysed with the HPLC system.

Urine sample preparation of II and V

Aliquots (10 μl) of urine or diluted urine were injected into the HPLC system for assay of II. For assay of V, 0.5 ml of 1 *M* sodium hydroxide were added to 1 ml of urine, and the solution was allowed to stand for 1 h at room temperature. After addition of 0.5 ml of 0.8 *M* hydrochloric acid, aliquots of 10 μl were injected into the HPLC system.

HPLC system

A Hitachi 635 liquid chromatograph was used, equipped with a variable-wavelength UV—VIS detector. A modified stop-flow procedure was used to introduce the samples onto a Nucleosil $5C_{18}$ reversed-phase column (150 × 4.0 mm I.D.). The flow-rate was held constant at 1.0 ml/min.

For the assay of I, II and III in plasma, the mobile phase consisted of 10 ml of acetic acid and 600 ml of acetonitrile diluted to 1000 ml with water, and the effluent was monitored at 360 nm.

For the assay of VI in urine, the mobile phase consisted of 50 ml of acetic acid and 450 ml of methanol diluted to 1000 ml with water; the detector was set at 269 nm.

For the assay of II and V in urine, the mobile phase consisted of 50 ml of acetic acid and 400 ml of acetonitrile diluted to 1000 ml with water, and the effluent was monitored at 360 nm.

Gas chromatography—mass spectrometry

Samples were analysed by a JEOL gas chromatograph and a JEOL JMS-D300 combination instrument equipped with a chemical-ionization (CI) electron-impact (EI) ion source under the following conditions: ionization energy, 70 eV; ionization current, 300 μA; separator temperature, 290°C; ion source temperature, 200°C. The conditions of the gas chromatograph were as follows:

a coiled glass column (1 m × 2 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q (80—100 mesh); injector and column temperatures of 230 and 200°C, respectively; flow-rate of helium as carrier gas, 75 ml/min.

Calculations

Peak height ratios of I, II, III or VI to that of internal standard were calculated, and calibration curves were constructed by plotting the peak height ratios versus concentration of I, II, III or VI (μg/ml) for spiked control plasma and urine samples.

For the urine assay of II, a calibration curve was constructed by plotting the peak height versus concentration of II spiked to control urine samples. For the assay of V, the data were obtained as total II and, from the difference of unhydrolysed and hydrolysed II, the concentration was reported as that of V.

Mass spectrometry

The solvent was evaporated from the eluted VI fraction under a stream of nitrogen, and the residue was redissolved in *n*-hexane. This solution was transferred to a capillary tube for direct insertion into the mass spectrometer. A JEOL JMS-01SG-2 mass spectrometer equipped with an EI ion source was used. Mass spectra were determined under the following conditions: ionizing energy, 75 eV; emission current, 200 μA; accelerating voltage, 10 kV; ion multiplier voltage, 2.5 kV; main slit, 200 μm.

Animal study

The studies were conducted in healthy male beagle dogs, weighing 10.5—13.0 kg. The dogs were fasted for 18 h prior to and 4 h after each received orally 100, 200 or 400 mg of malotilate powder with 20 ml of water. Blood samples were withdrawn from the foreleg vein. Urine samples were collected over 48 h after the administration. Throughout the experiments the animals were not restrained.

RESULTS AND DISCUSSION

HPLC assay of I, II and III in plasma

Typical chromatograms resulting from blank plasma and blank plasma spiked with I, II and III are shown in Fig. 2. Under these chromatographic conditions, I, II, III and internal standard (IV) had retention times of 8, 3, 5 and 15 min, respectively, and were resolved satisfactorily from the peaks due to endogeneous plasma components.

The calibration graphs were linear for samples over the concentration range studied here, 0.05—1.00 μg/ml. The least-squares linear regression line for I had an equation of $y = 4.34x - 0.08$ (y = peak height ratio, I:IV, and x = concentration of I), and those for II and III had equations of $y = 1.43x - 0.03$ and $y = 0.205x + 0.04$, respectively. The correlation coefficients of I, II and III were 0.999, 0.999 and 0.992, respectively ($n = 5$).

The coefficients of variation for intra-assay and inter-assay variability of I, II and III are given in Table I. The results demonstrate the high accuracy and reproducibility of the method.

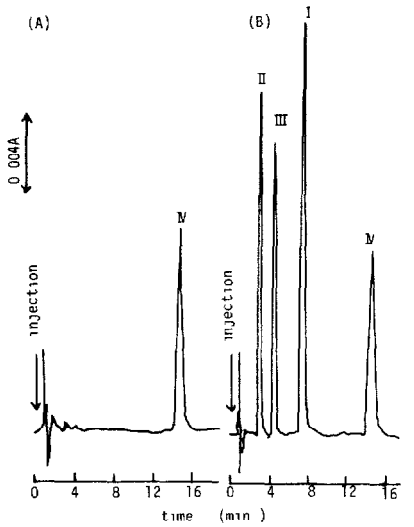


Fig. 2. HPLC chromatograms of (A) blank plasma and (B) blank plasma spiked with I (1.0 μg/ml of plasma), II (0.5 μg/ml of plasma) and III (0.94 μg/ml of plasma). Peak IV = internal standard.

TABLE I

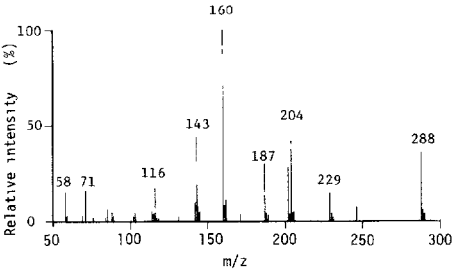
INTRA- AND INTER-ASSAY REPRODUCIBILITY OF HPLC FOR THE DETERMINATION OF I, II and III IN PLASMA

Compound	*Concentration* (μg/ml)	*Coefficient of variation (%)*	
		Intra-assay (n = 5)	Inter-assay (n = 5)
I	0.10	2.3	2.1
II	0.10	4.1	4.2
III	0.10	9.5	7.1

The recoveries of I, II and III from plasma were of the order of 98, 100 and 86%, respectively. The detection limit, defined on the basis of the amount of compound injected that caused an absorption five times greater than the baseline noise, was found to be at 4, 2 and 8 ng, equivalent to 50, 25 and 100 ng/ml plasma for I, II and III, respectively.

GC—MS analysis of I

For GC— MS analysis, compound I and internal standard have a common fragment ion at m/z 160, which was monitored for quantification. The detection limit, defined on the basis of the amount of I injected, was ca. 40 pg. The sample extracts for HPLC assay were also used for GC—MS assay. These samples, however, contained a large amount of internal standard, therefore in addition to monitoring the fragment ion at m/z 160, the corresponding isotope ion at m/z 162 due to S atoms was also monitored for the internal standard. Since the fragment ion at m/z 160 has to S atoms, the intensity ratio of the ion at m/z 160 and that at m/z 162 was ca. 10:1 (Fig. 3).



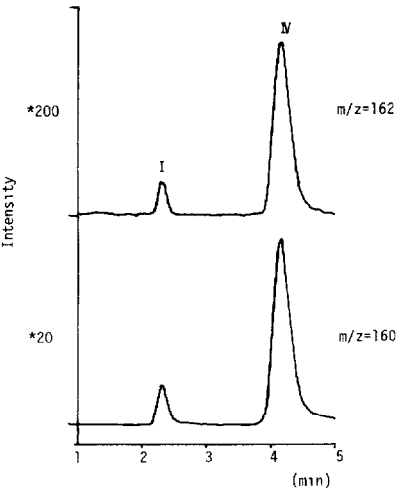


Fig. 3. Electron-impact (70 eV) mass spectrum of malotilate.

Fig. 4. Mass fragmentograms of I (0.8 μg/ml of plasma) and IV (internal standard).

A typical mass fragmentogram resulting from a blank plasma spiked with I, II and III is shown in Fig. 4. The retention times were ca. 2 and 3.3 min for I and internal standard, respectively. The sensitivity limit for I was 2.5 ng/ml of plasma.

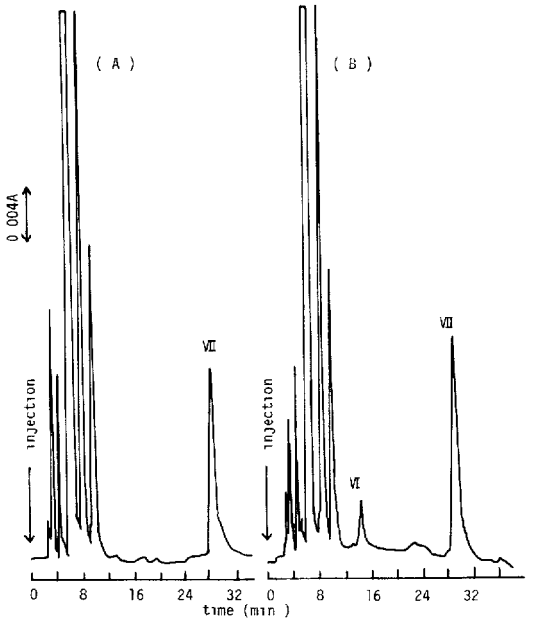
HPLC assay of VI

Fig. 5 shows typical chromatograms from blank urine and blank urine spiked with VI. Under these conditions, VI and the internal standard (VII) had retention times of 15 and 29 min, respectively, and were resolved satisfactorily from the peaks due to endogeneous urine components.

The calibration graph was linear for the samples over the concentration range studied here, 2.5—40 μg/ml. The least-squares linear regression line which represents the best fit of the data for VI had an equation of $y = 0.048x + 0.022$ (y = peak height ratio, VI:VII, and x = concentration of VI). The correlation coefficient was 0.999 ($n = 5$). The coefficients of variation for intra- and inter-assay variability of VI are given in Table II. The results demonstrate the high accuracy and reproducibility of the method.

The detection limit, defined on the basis of the urine concentration of VI that caused an absorption five times greater than the baseline noise, was found to be 2.0 μg/ml. The extraction efficiency was estimated as ca. 60%, but the coefficient of variation of the extraction efficiency was ca. 3%. The overall coefficient of variation of VI was 4.7%. In spite of the low absolute recovery, the overall precision of the method was satisfactory.

Various reaction times were compared to determine the best reaction time for the formation of the *p*-nitrobenzyl derivative; the results indicated that formation was complete within 2 h (Fig. 6). The derivatives of VI and the internal standard (VII) were stable for at least twenty days, if they are protected from light and stored at 5°C.



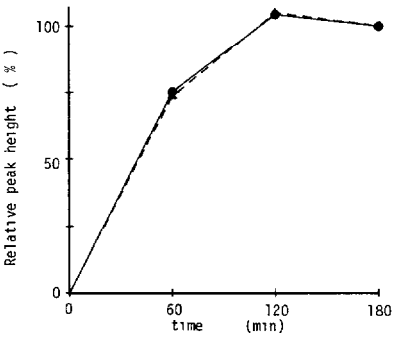


Fig. 5. Chromatograms of (A) blank urine and (B) blank urine spiked with VI (4.8 μg/ml of urine). Peak VII = internal standard.

Fig. 6. Rate of conversion of VI into *p*-nitrobenzyl derivative in ethyl acetate—ethylene dichloride (3:1). Data points: ● = 8.66 μg/ml VI; ▲ = 34.64 μg/ml VI.

TABLE II

INTRA- AND INTER-ASSAY REPRODUCIBILITY OF HPLC FOR THE DETERMINATION OF II, TOTAL II (II + V) AND VI IN URINE

Compound	Concentration (μg/ml)	Coefficient of variation (%)	
		Intra-assay	Inter-assay
II	2.98	0.8 (n = 6)	1.7 (n = 5)
	5.95	4.0 (n = 6)	2.3 (n = 5)
Total II	3.68	2.1 (n = 5)	4.8 (n = 5)
	13.18	0.9 (n = 5)	5.2 (n = 5)
VI	9.60	7.2 (n = 6)	9.1 (n = 10)
	19.20	4.7 (n = 8)	4.8 (n = 9)

For identification of the *p*-nitrobenzyl ester of VI, the eluted HPLC fraction of VI was analysed by EI-MS. A satifactory spectrum was obtained (Fig. 7), and the fraction showed the molecular ion m/z 281.

Assay of II and V

For the evaluation of hydrolysis condition of urine samples, the sample from a rabbit given an oral dose of malotilate was used. To 1 ml urine were added 0.2, 0.3, 0.4 or 0.5 ml of 1 *M* sodium hydroxide, and the solution was allowed to stand at room temperature. The mixtures were neutralized with 0.8 *M*

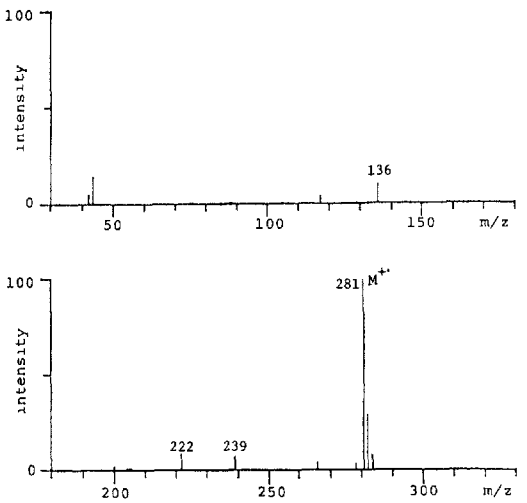


Fig. 7. Electron-impact mass spectrum of the *p*-nitrobenzyl ester of VI

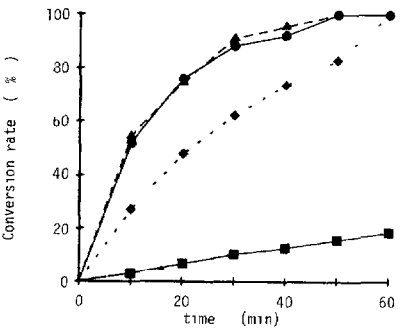


Fig. 8. Rate of hydrolysis of V to II under various alkaline conditions. Data points: ■ = 0.2 ml; ◆ = 0.3 ml, ▲ = 0.4 ml; ● = 0.5 ml of 1 *M* sodium hydroxide.

hydrochloric acid at 10-min intervals and analysed by HPLC. The hydrolysis conditions were thus determined to be 1 h with 0.5 ml of 1 *M* sodium hydroxide (Fig. 8).

Typical chromatograms for II (aqueous standard) and hydrolysed urine sample of a rabbit given an oral dose of malotilate are shown in Fig. 9. Urine components did not interfere, and the retention time of II was 9 min.

The least-squares linear regression line that represents the best fit of the data for II had an equation of $y = 1.09x + 0.4$ (y = peak height, and x = concentration of II). The correlation coefficient was 0.995 ($n = 5$). The coefficients of variation for intra-assay and inter-assay variability of II and total II (II + V) are given in Table II. The results demonstrate the high accuracy and reproducibility of the method.

The detection limit, defined on the basis of the amount of compound injected that caused an absorption five times greater than the baseline noise, was found to be 5 ng, equivalent to 0.5 μg/ml for II and 1 μg/ml for total II (II + V) in urine.

Animal study

The method was applied to pharmacokinetic studies of malotilate in beagle dogs. Four dogs were dosed orally with 100, 200 or 400 mg of malotilate powder. Plasma malotilate concentrations were very low. Compound III was not detected in any plasma samples. Mean concentrations of II in plasma after single oral administration of malotilate are shown in Fig. 10. The area under the curve (AUC), calculated by the trapezoidal rule, was 1.04 (100 mg per dog), 1.90 (200 mg per dog) and 3.91 μg/ml (400 mg per dog).

The assay procedure described for malotilate and its metabolites in plasma offers easy and rapid sample preparation, selectivity for both drug and metab-

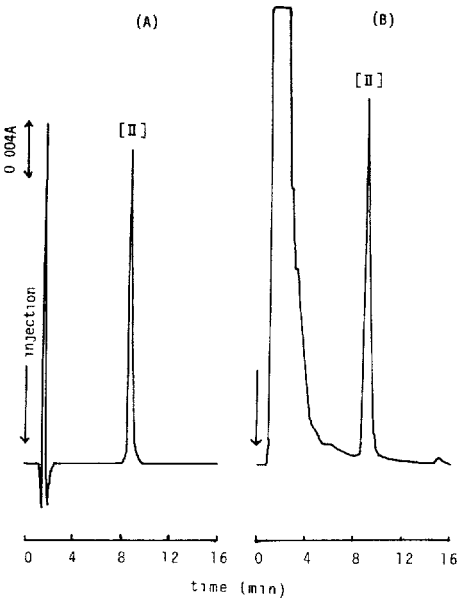


Fig. 9. Chromatograms of (A) standard solution containing 10 μg/ml II and (B) alkali-hydrolysed urine of the rabbit given an oral dose of malotilate.

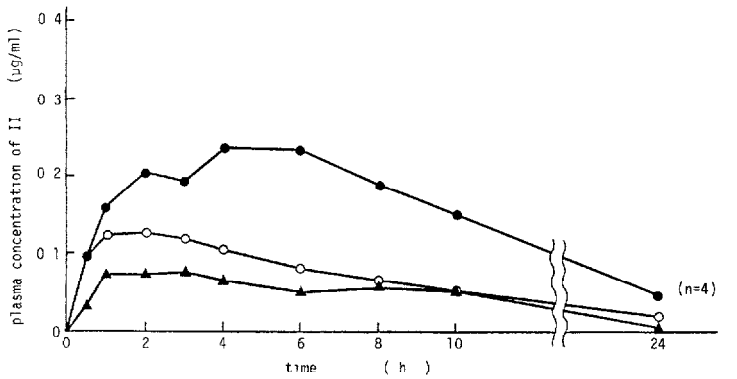


Fig. 10. Average plasma concentration—time curves of II after single oral administration of malotilate to dogs. Data points· ● = 400 mg per dog; ○ = 200 mg per dog; ▲ = 100 mg per dog.

TABLE III

CUMULATIVE URINARY EXCRETION OF TOTAL II AND VI FOLLOWING DOSAGE OF 100, 200 OR 400 mg OF MALOTILATE TO DOGS

Dose (mg)	Cumulative excretion within 48 h (% of dose)	
	Total II (mean ± S.D.)	VI (mean ± S D)
100	0.065 ± 0.047	19.11 ± 3.30
200	0.045 ± 0.064	13.75 ± 8.66
400	0.133 ± 0.079	12.74 ± 12 62

Cumulative urinary excretion data for VI (major metabolite) and other metabolites are presented in Table III. Unconjugated II accounted for less than 0.05% of the dose. Compound VI was excreted over the 48-h collection period and accounted for ca. 15% of the dose. A small amount of V (conjugated II) was excreted in urine, but urinary excretion of II or V was a minor route of malotilate elimination in the dog.

The HPLC method described here for the quantitative determination of II, V and VI in urine is not only convenient but also specific, and its accuracy and sensitivity are satisfactory for most pharmacokinetic studies.

REFERENCES

1 Y. Imaizumi, M. Katoh, T. Sugimoto and T. Kasai, Jap. J. Pharmacol., 32 (1982) 369.
2 H. Hakusui, M. Nakaoka, J. Tanaka, T. Takegoshi, S. Funayama, T. Sugimoto, H. Yasuhara and K. Sakamoto, 102th Annual Meeting of the Pharmaceutical Society of Japan, Osaka, Japan, 1982.
3 N. Takasugi, E. Mafune, S. Yokokawa, T. Tsuchiya and T. Sugimoto, 102th Annual Meeting of the Pharmaceutical Society of Japan, Osaka, Japan, 1982.
4 S. Funayama, N. Murakami, Y. Izawa, T. Suzuki, M. Uchida, K. Tsuchiya, T. Sugimoto, Y. Nambara, M. Suzuki and H. Kitagawa, J. Pharm. Dyn , 6 (1983) s-85.