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Quantitative Determination of Forphenicinol and Its Metabolites in Human Serum and Urine by Gas Chromatography/Mass Spectrometry with Selected Ion Monitoring

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A simple and sensitive method for the determination of forphenicinol (L-(3-hydroxy-4-hydroxymethylphenyl)glycine) and its metabolites (M-1—M-5) in human serum and urine has been developed by means of gas chromatography-mass spectrometry with selected ion monitoring.

Forphenicinol (FPL) and M-2 (3-hydroxy-4-hydroxymethylbenzylamine) were quantified at the same time as the trimethylsilyl (TMS) derivatives. M-1 (α-(3-hydroxy-4-hydroxymethylphenyl)-α-oxoacetic acid) was methoximated, and then analyzed simultaneously with M-3 (3-hydroxy-4-hydroxymethylbenzoic acid) and M-4 (hydroxyterephthalic acid) after trimethylsilylation. On the other hand, M-5 (L-*N*-acetyl(3-hydroxy-4-hydroxymethylphenyl)glycine) was hydrolyzed enzymatically with acylase to convert it into FPL and determined as the TMS derivative.

In serum, the calibration range for FPL was 0.02—5 μg/ml. As for urine, the calibration curves were linear in the ranges of 0.04—10 μg/ml for FPL, 0.1—10 μg/ml for M-1, M-3, M-4, and M-5, and 0.4—100 μg/ml for M-2.

The metabolic fate of FPL in man was investigated after a single oral dose (50 mg) of FPL to each of five healthy volunteers. In the serum, unchanged FPL was found but no metabolites were detected. The serum FPL levels were followed and analyzed pharmacokinetically. In the urine, unchanged FPL and its five metabolites (M-1—M-5) were detected and quantified; M-2 and M-5 were the major urinary metabolites.

Keywords——forphenicinol; metabolite; gas chromatography-mass spectrometry; selected ion monitoring; acylase; human serum; human urine; pharmacokinetics

Introduction

Forphenicinol (FPL) is a low-molecular-weight immuno-modifier[1-3] which was synthesized from forphenicine (L-(4-formyl-3-hydroxyphenyl)glycine) by Umezawa *et al.*[4,5] The metabolic fate of FPL has been studied in experimental animals (rat,[6,7] rabbit, and dog[8]) and man,[9] and it was found that the metabolites in animals (in addition to unchanged FPL) were M-1, M-2, M-3, M-4, and M-5 (Fig. 1), while those in man were FPL, M-2, and M-3.

Quantitative studies have been carried out by using radioimmunoassay (RIA), or high-performance liquid chromatography (HPLC). The RIA technique has high sensitivity, but is of limited value because of the poor specificity (metabolite M-2 is significantly cross reactive). On the other hand, the reversed-phase HPLC method cannot readily separate the six metabolites simultaneously from the peaks originating from biological substances, and it is not sufficiently sensitive. Thus, a new method which has high sensitivity and specificity was required for pharmacokinetic and bioavailability studies of FPL in man. We have developed a quantitative method, based on gas chromatography-mass spectrometry with selected ion monitoring (GC/MS/SIM).

This method has been successfully applied to establish the metabolic fate of FPL in

healthy male volunteers. The serum concentrations of unchanged FPL could be quantified as late as 24 h post administration, and furthermore, the urinary metabolites were determined quantitatively.

Experimental

Standards and Reagents——FPL and standard samples of the metabolites, M-1, M-2, M-3, M-4, and M-5 (Fig. 1) were synthesized in our laboratory. DL-*m*-Hydroxyphenylglycine and 4-hydroxy-3-hydroxymethylbenzoic acid were also synthesized. *O*-Methylhydroxylamine hydrochloride was purchased from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan), and bis (trimethylsilyl) trifluoroacetamide (BSTFA) was from Gasukuro Kogyo Co., Ltd. (Tokyo, Japan). Acylase Amano was from Amano Pharmaceutical Co., Ltd. (Nagoya, Japan).

Instrumentation——A JEOL JMS-DX 300 gas chromatograph-mass spectrometer connected on-line with a JEOL JMA-DA 5000 data system was used. The samples were introduced into the ion source through a coiled glass column and a glass enricher. The instrument was operated in the electron impact (EI) mode. Measurement conditions for each metabolite were as described below.

Analysis of FPL, M-2, and M-5: A 1 m × 3 mm i.d. glass column packed with 3% OV-7 on Shimalite W (80—100 mesh, Wako Pure Chemical Ind., Ltd.) was used. The flow rate of the carrier gas (helium) was 40 ml/min. The temperature of the column oven was maintained at 205 °C for 1.0 min and then programmed to 240 °C at the rate of 32 °C/min. The injection port and separator temperatures were 270 °C. The ionization energy and the ionization current were 70 eV and 0.3 mA, respectively.

Analysis of M-1, M-3, and M-4: A 2 m × 3 mm i.d. glass column packed with 1.5% OV-17 on Shimalite W (80—100 mesh, Wako Pure Chemical Ind., Ltd.) was used. The flow rate of the carrier gas was 45 ml/min. The temperature of the column oven was programmed from 220 to 252 °C at the rate of 8 °C/min. The temperatures of the injection port and the separator were 300 and 280 °C, respectively. The ionization energy and the ionization current were 70 eV and 0.3 mA, respectively.

Preparation of Serum Samples——Sample were prepared for SIM analysis for each metabolite as described below. FPL and M-2: To 0.50 ml of serum sample, 0.10 ml of *m*-hydroxyphenylglycine (internal standard, 100 μg/ml) solution, 0.4 ml of water, and 2 ml of ethanol were added. After thorough mixing, the sample was centrifuged at 2000 rpm for 15 min. The supernatant was percolated through a column (10 cm × 8 mm i.d.) of CG-120 (Amberlite, Type I, 100—200 mesh, H^+ form) resin. The column was washed stepwise with 5 ml portions of water–ethanol (1 : 2, v/v), (1 : 1, v/v), (2 : 1, v/v), and finally with 5 ml of water. The metabolites and the internal standard were eluted with 4 ml of 3 N NH_4OH, and the effluent was lyophilized. The residue was dissolved in 0.5 ml of water. A 0.05 ml aliquot was transferred into a glass tube (2 cm × 5 mm i.d.), evaporated and dried *in vacuo*. Then 0.08 ml of BSTFA–pyridine (10 : 3, v/v) was added to the residue, and the tube was sealed with a rubber cap, and heated at 80 °C for 1 h. A 1 μl aliquot of the reaction mixture was injected into the GC/MS apparatus.

M-1, M-3, and M-4: To 0.50 ml of serum sample, 0.10 ml of 4-hydroxy-3-hydroxymethylbenzoic acid (internal standard, 100 μg/ml) solution and 0.10 ml of 2 M aqueous solution of *O*-methylhydroxylamine hydrochloride were added. They were mixed, and reacted at room temperature for 30 min, then adjusted to pH 1 with 1 N HCl. After centrifugation at 2000 rpm for 15 min, the supernatant was extracted three times with 1 ml of ethyl acetate–isopropanol (10 : 1, v/v). The organic layer was evaporated to dryness, and the residue was dissolved in 0.3 ml of methanol. The solution thus obtained was transferred into a glass tube (2 cm × 5 mm i.d.), then dried and trimethylsilylated with 50 μl of BSTFA–pyridine (10 : 3, v/v) at 80 °C for 1 h. A 1 μl aliquot of the reaction mixture was injected into the GC/ MS.

M-5: The pH of 0.50 ml of serum was adjusted to pH 1 with 1 N HCl. The sample was centrifuged at 2000 rpm for

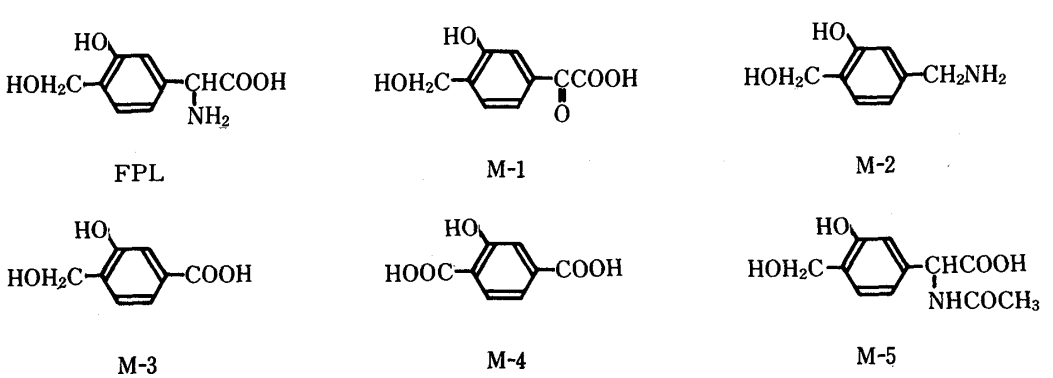

Fig. 1. Structures of FPL and Its Metabolites

15 min, and the supernatant was extracted three times with 1 ml of ethyl acetate–isopropanol (10 : 1, v/v). The organic layer was evaporated, and the residue was dissolved in 1.2 ml of 0.04 M phosphate buffer (pH 7.8) containing 0.60 mg of acylase and 1.2×10^{-4} mmol of $Co(II)Cl_2$. After incubation at 37 °C for 18 h, the reaction mixture was lyophilized and reconstituted with 0.5 ml of water. Then 0.10 ml of 100 μg/ml aqueous solution of *m*-hydroxyphenylglycine (internal standard) and 0.5 N HCl were added to adjust the pH to 2, and the mixture was washed with 1 ml of ethyl acetate twice. A 0.1 ml aliquot of the aqueous layer was taken to dryness and trimethylsilylated with 50 μl of BSTFA–pyridine (10 : 3, v/v) at 80 °C for 1 h. A 1 μl aliquot was subjected to GC/MS.

Preparation of Urine Samples——Urine samples were treated as described above. A 0.25 ml aliquot of urine was used for the analysis of FPL and M-2, and 1.00 ml for the other metabolites.

Calibration Curves——Control serum or urine was spiked with several concentrations of FPL or its metabolites and processed in the same way as described above. Calibration data were obtained by measuring the ratio of the peak area of FPL or its metabolites to that of the internal standard, and the calibration line was calculated by least-squares regression of the drug concentrations (*X*-values) and the peak area ratios (*Y*-values).

Precision and Accuracy——Appropriate amounts of FPL or its metabolites were added to control serum or urine. From five determinations of two concentrations, the accuracy (defined as the difference between the observed mean value and the true value) and the precision (defined as the relative standard deviation) were calculated.

Recovery——Control serum or urine was spiked with appropriate quantities of FPL and applied to a CG-120 column as described above. The internal standard was added and the mixture was trimethylsilylated. On the other hand, control serum or urine was applied to a CG-120 column, then FPL and the internal standard were added prior to trimethylsilylation. The peak area ratio of FPL to the internal standard in the former was divided by that in the latter. The recovery of the other metabolites was measured in the same way.

Administered Samples——A 50 mg capsule of FPL was administered to each of five healthy male volunteers. Blood samples collected at several time points were allowed to stand for 30 min at room temperature, then the serum was separated and frozen at −20 °C until analysis. The urine samples were collected at 12 h intervals. After measurement of their volume, the samples were frozen at −20 °C until analysis.

Results and Discussion

Derivatization

FPL, M-2, M-3, and M-4 reacted with BSTFA to give stable TMS derivatives without formation of by-products. Since M-1 is an α-keto acid, GC/MS analysis after trimethylsilylation was impossible because of decomposition. Thus, the carbonyl group of M-1 was converted to the methoxime group with *O*-methylhydroxylamine before trimethylsilylation, and a stable derivative was produced.

M-5 reacted with BSTFA to yield two products, *i.e.*, a tris-TMS and a tetrakis-TMS derivative. No reaction conditions giving a high yield of either product were found. We therefore attempted to hydrolyze the acetylamino group of M-5 to regenerate FPL by using acylase as a catalyst. FPL was formed in more than 80% yield.

Mass Spectra

The EI MS of the TMS derivative of FPL, M-2, M-3, M-4, DL-*m*-hydroxyphenylglycine and 4-hydroxy-3-hydroxymethylbenzoic acid, and that of the methoxime (MO) and TMS derivative of M-1 are illustrated in Figs. 2 and 3.

FPL was converted to its tetrakis-TMS derivative. The molecular ion peak was not observed, but the major ion at *m/z* 368 $[M-117\ (COOTMS)]^+$ was produced by simple cleavage. M-1 was derived to its MO and tris-TMS derivative. The molecular ion $[M]^{+\cdot}$ and the fragment ions corresponding to $[M-15\ (CH_3)]^+$ and $[M-31\ (OCH_3)]^+$ were observed at *m/z* 441, 426, and 410, respectively. In the case of M-2, a tris-TMS derivative was formed. The spectrum showed $[M]^{+\cdot}$, $[M-1\ (H)]^+$, and $[M-103\ (NHTMS+CH_3)]^+$ peaks at *m/z* 369, 368, and 266, respectively. In the MS of a tris-TMS derivative of M-3, $[M]^{+\cdot}$, $[M-15\ (CH_3)]^+$, and $[M-117\ (COOTMS)]^+$ peaks were observed at *m/z* 384, 369, and 267, respectively. A tris-TMS derivative of M-4 gave a MS comprising one major ion at *m/z* 383 $[M-15\ (CH_3)]^+$.

DL-*m*-Hydroxyphenylglycine was derived to its tris-TMS derivative. The spectrum showed peaks at *m/z* 368 and 266 corresponding to $[M-15(CH_3)]^+$ and $[M-117\ (COOTMS)]^+$, respectively. 4-Hydroxy-3-hydroxymethylbenzoic acid is a structural isomer

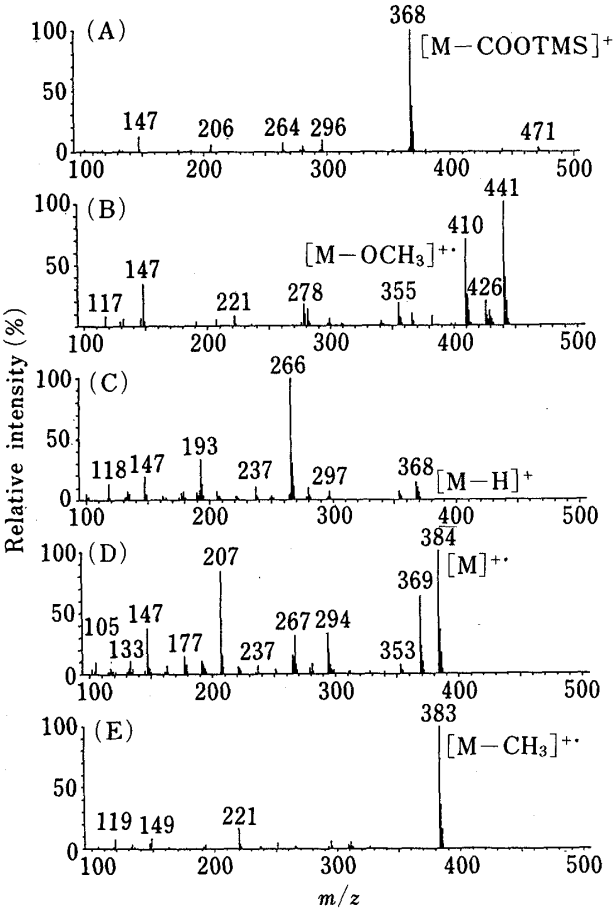

Fig. 2. MS of TMS Derivatives of FPL, M-2, M-3, and M-4, and MO and TMS Derivative of M-1

(A), FPL; (B), M-1; (C), M-2; (D), M-3; (E), M-4.

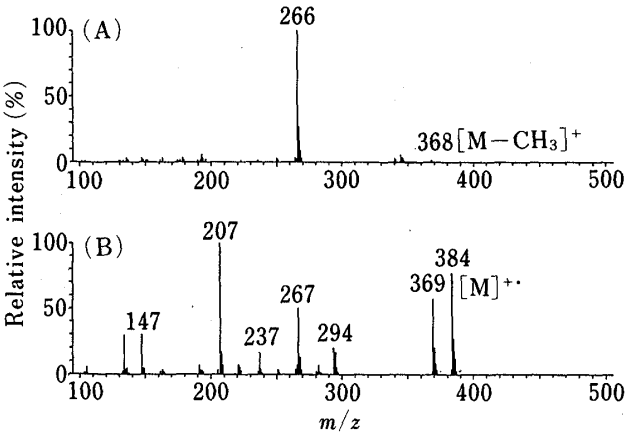

Fig. 3. MS of TMS Derivatives of DL-*m*-Hydroxyphenylglycine and 4-Hydroxy-3-hydroxymethylbenzoic Acid

(A), DL-*m*-hydroxyphenylglycine; (B), 4-hydroxy-3-hydroxymethylbenzoic acid.

of M-3, and the fragmentation pattern of its tris-TMS derivative was similar to that of M-3.

SIM Chromatograms

Selected ions for the SIM measurement are shown in Table I. These ions were chosen because they showed strong peaks at high mass, resulting in very little interference from biological components.

Figure 4 shows a typical SIM chromatogram obtained from serum after the administration of FPL. Figure 5 shows SIM chromatograms of FPL, M-1, M-2, M-3, and M-4 in urine. FPL and M-2 gave symmetrically shaped peaks with retention times of 2.43 and 1.73 min, respectively. On the other hand, the retention times of M-1, M-3, and M-4 were 3.07, 1.55 and 1.92 min, respectively. No interfering peaks were observed in serum or urine.

As for M-5, it was converted to FPL and measured as its TMS derivative. Complete separation of M-5 from FPL was accomplished by ethyl acetate–isopropanol extraction before the hydrolysis reaction.

Calibration Curves

Calibration curves were prepared for the metabolites confirmed in serum or urine. For example, the calibration curve of FPL in serum is shown in Fig. 6. The quantification range, the equation, and the coefficient of correlation for each calibration curve are summarized in Table II.

TABLE I. Selected Ion for Quantitative Measurement by GC/MS

Compound	Derivative	*m/z*	Assignment
FPL	Tetrakis-TMS	368	$[M-COOTMS]^+$
M-1	MO, tris-TMS	410	$[M-OCH_3]^+$
M-2	Tris-TMS	368	$[M-H]^+$
M-3	Tris-TMS	384	$[M]^{+\cdot}$
M-4	Tris-TMS	383	$[M-CH_3]^+$
DL-*m*-Hydroxyphenyl-glycine	Tris-TMS	368	$[M-CH_3]^+$
4-Hydroxy-3-hydroxy-methylbenzoic acid	Tris-TMS	384	$[M]^{+\cdot}$

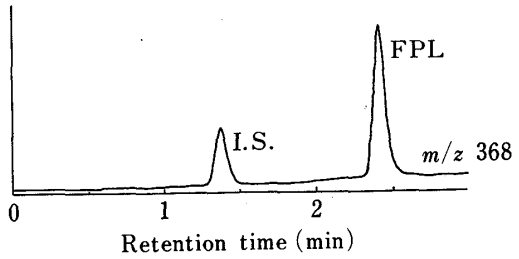

Fig. 4. SIM Chromatogram of Serum after Oral Administration of FPL in Man

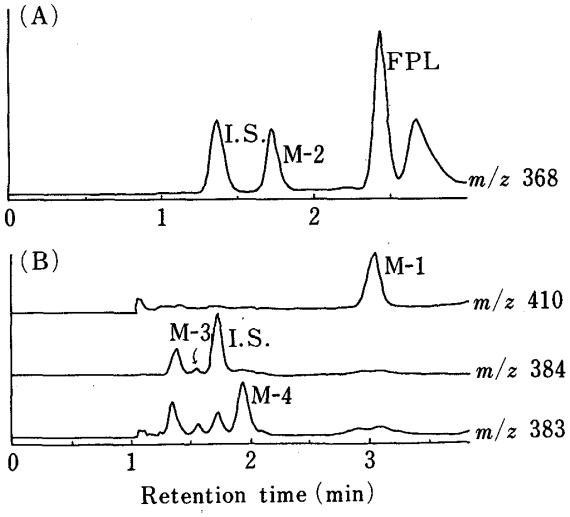

Fig. 5. SIM Chromatograms of Urine after Oral Administration of FPL in Man

(A), simultaneous determination of FPL and M-2; (B), simultaneous determination of M-1, M-3, and M-4.

As shown in Table II, the calibration curve of FPL in serum (Fig. 6) was linear in the range of 0.02—5 μg/ml. As for urine, the calibration ranges were 0.04—10 μg/ml for FPL, 0.1—10 μg/ml for M-1, M-3, M-4, and M-5, and 0.4—100 μg/ml for M-2. All the coefficients of correlation were 0.996 or above. Satisfactory calibration ranges were obtained in all cases.

Precision, Accuracy, and Recovery

Precision and accuracy in SIM analysis are shown in Table III. Five determinations at high and low concentrations in the calibration range were examined. Added and measured concentrations were in good agreement for all metabolites. The coefficient of variation value (precision) of five runs was satisfactory in every case.

The recovery in the extraction process is shown in Table IV. Mean recoveries of FPL from serum and urine were 102% and 92%, respectively, and those of M-1, M-2, M-3, M-4, and M-5 from urine were 93%, 107%, 103%, 97% and 80%, respectively. These results are considered to be satisfactory.

Administered Samples

By using serum and urine from volunteers before and after oral administration of FPL at a therapeutic dose, the existence of metabolites was investigated by SIM measurement. Only

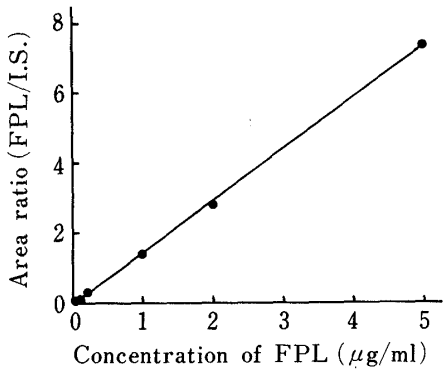

Fig. 6. Calibration Curve for FPL in Serum

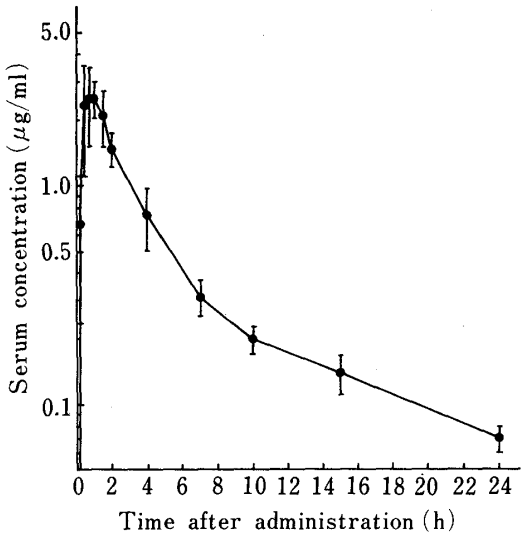

Fig. 7. Serum Concentration of FPL after Oral Administration in Man (FPL, 50 mg/man)

Each value represents the mean ± S.D. ($n=5$).

TABLE II. Linear Regression Analysis for FPL and Its Metabolites in Human Serum and Urine

	Metabolite	Range (μg/ml)	$Y^{a)}=aX^{b)}+b$	r
Serum	FPL	0.02— 5	$Y=1.478X-0.061$	0.9997
Urine	FPL	0.04— 10	$Y=0.763X-0.000_4$	0.9999
	M-1	0.1 — 10	$Y=0.082X-0.007$	0.9990
	M-2	0.4 —100	$Y=0.043X-0.129$	0.996
	M-3	0.1 — 10	$Y=0.201X-0.060$	0.996
	M-4	0.1 — 10	$Y=0.513X+0.030$	0.998
	M-5	0.1 — 10	$Y=1.725X-0.024$	0.9999

a) Y represents the peak area ratio of FPL and its metabolites with respect to the internal standard. *b*) X represents the concentration of FPL and its metabolites.

FPL was observed in the serum post-administration (the detection limits of other metabolites were 0.1 μg/ml for M-1 and M-3, and 0.2 μg/ml for M-2, M-4, and M-5).

Therefore, the serum FPL concentrations were determined quantitatively. Figure 7 shows the time course of the serum FPL levels after oral administration in man. In this case, the method was able to quantify serum levels of less than 1% of C_{max}.

Since the disappearance of FPL from serum showed two phases, the pharmacokinetic parameters were obtained by applying a two-compartment model. Mean values of C_{max}, T_{max}, and area under the serum concentration time curve (AUC) were 2.81 μg/ml, 0.77 h, and 11.0 h·μg/ml, respectively, and the biological half-lives of the α and β phases were 1.08 and 12.6 h, respectivery (Table V).

In the case of urine, FPL, M-1, M-2, M-3, M-4, and M-5 were identified and determined quantitatively. The results of urinary excretion are shown in Table VI. The main metabolites were M-2 and M-5, and their urinary excretions within 24 h were 37.2% and 8.4% of the dose, respectively. The total urinary recovery within 24 h was 54.8% of the dose.

The results obtained in this study suggest that FPL is rapidly absorbed from the gastrointestine, does not suffer any first-pass effect, and is eliminated from the blood into

TABLE III. Precision and Accuracy of Determination of FPL and Its Metabolites in Human Serum and Urine

Metabolite		Concentration (μg/ml)		Accuracy (%)	Precision (%)
		Added	Found ± S.D.[a]		
Serum	FPL	0.10	0.11 ± 0.00$_4$	110	3.6
		2.00	1.97 ± 0.10	99	5.1
Urine	FPL	0.40	0.38 ± 0.02	95	5.3
		4.00	4.20 ± 0.07	105	1.7
	M-1	0.20	0.22 ± 0.02	110	9.1
		5.00	5.18 ± 0.31	104	6.0
	M-2	4.00	4.06 ± 0.18	102	4.4
		40.00	40.17 ± 1.00	100	2.5
	M-3	0.50	0.54 ± 0.03	108	5.6
		5.00	4.94 ± 0.06	99	1.2
	M-4	0.50	0.56 ± 0.02	112	3.6
		5.00	4.90 ± 0.15	98	3.1
	M-5	0.20	0.20 ± 0.03	100	15.0
		5.00	5.11 ± 0.33	102	6.5

a) Each value represents the mean ± S.D. ($n = 5$).

TABLE IV. Recovery of FPL and Its Metabolites from Human Serum and Urine

	Metabolite	Concentration (μg/ml)	Recovery[a] (%)
Serum	FPL	1	102
Urine	FPL	2	92
	M-1	1	93
	M-2	20	107
	M-3	1	103
	M-4	1	97
	M-5	5	80

a) Each value represents the mean ($n = 5$).

TABLE V. Pharmacokinetic Parameters after Oral Administration of FPL at a Dose of 50 mg/man

Subject	T_{max} (h)	C_{max} (μg/ml)	AUC (h·μg/ml)	$T_{1/2\alpha}$ (h)	$T_{1/2\beta}$ (h)
A	0.44	3.22	16.13	1.92	29.53
B	0.80	1.68	8.79	1.22	7.09
C	0.65	3.62	9.98	0.41	6.59
D	0.58	3.11	9.51	1.00	8.38
E	1.40	2.40	10.77	0.84	11.16
Mean ± S.D.	0.77 ± 0.37	2.81 ± 0.77	11.04 ± 2.94	1.08 ± 0.56	12.55 ± 9.66

urine mainly as its decarboxylated metabolite (M-2). The main metabolic changes probably occur in the kidney rather than the liver, as reported in rats.[6,7]

In conclusion, the GC/MS/SIM method described in this paper has adequate sensitivity and specificity, and is suitable for pharmacokinetic and bioavailability studies after administration of FPL at clinical dose levels.

TABLE VI. Urinary Excretion of FPL and Its Metabolites after Oral Administration in Man (FPL, 50 mg/man)

Metabolite	Recovery[a] (% of dose)		
	0—12 h	12—24 h	Total
FPL	2.9±1.2	0.4±0.3	3.4±1.5
M-1	0.9±0.5	0.6±0.3	1.4±0.8
M-2	31.6±8.3	5.6±1.7	37.2±9.8
M-3	2.5±0.6	0.4±0.1	3.0±0.6
M-4	1.2±1.0	0.2±0.2	1.4±1.3
M-5	7.6±4.4	0.8±0.4	8.4±4.1
Total	46.8±8.4	8.0±1.5	54.8±9.9

a) Each value represents the mean±S.D. ($n=5$).

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