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Simultaneous determination of nefiracetam and its metabolites by high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatographic assay was developed to simultaneously quantitate nefiracetam (NEF), a novel nootropic agent, and its three known oxidized metabolites (N-[(2,6-dimethylphenylcarbamoyl)methyl]succinamic acid (5-COOH-NEF), 4-hydroxy-NEF and 5-hydroxy-NEF) in human serum and urine. The quantitative procedure was based on solid-phase extraction with Sep-Pak C_{18} and ultraviolet detection at 210 nm. The calibration curves of NEF and the metabolites were linear over a wide range of concentrations (0.5–21.5 nmol/ml for NEF and 0.4–9.5 nmol/ml for metabolites in serum and 4–86 nmol/ml for NEF and 8–190 nmol/ml for metabolites in urine). Intra- and inter-day assay coefficients of variation for the compounds were less than 10%. The limit of detection was 0.1 nmol/ml for NEF, 5-COOH-NEF and 4-hydroxy-NEF, and 0.2 nmol/ml for 5-hydroxy-NEF in both serum and urine. This method is applicable for the determination of NEF and its metabolites in human serum and urine with satisfactory accuracy and precision.

INTRODUCTION

Nefiracetam [N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl)acetamide, NEF, I, Fig. 1], a cyclic derivative of γ-aminobutyric acid (GABA), is now under clinical investigation as a new nootropic agent. In pharmacological studies, compound I has shown anti-amnestic and antihypox-

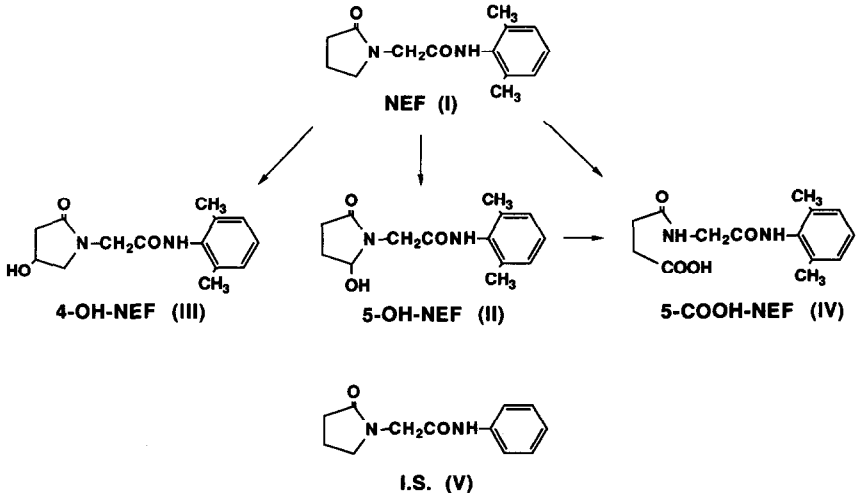

Fig. 1. Structures of NEF (I), 5-OH-NEF (II), 4-OH-NEF (III), 5-COOH-NEF (IV) and the internal standard (V).

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ic effects and also potentiated learning acquisition [1–5]. The metabolic disposition of I has previously been studied in experimental animals and humans. In rats, dogs and monkeys, ^{14}C-labelled I administered orally (30 mg/kg) was well absorbed and widely distributed to tissues, with more than 80% of administered radioactivity excreted in the urine within 24 h. The compound was extensively metabolized in animals. More than twenty metabolites were observed in serum, urine and tissues [6]. In phase I studies using healthy volunteers, serum concentrations of I after single oral administrations increased rapidly and dose-dependently, with times to peak concentration (T_{max}) of 1–2 h and elimination half-lives of 3.3–5.9 h. Less than 10% of the administered dose was excreted in the urine in its unchanged form [7,8]; several oxidized and conjugated metabolites have been found present in urine [9], indicating that I is also extensively metabolized in humans. The major metabolites in serum and urine were suggested to be 5-hydroxy-NEF (5-OH-NEF, II), 4-hydroxy-NEF (4-OH-NEF, III) and N-[(2,6-dimethylphenylcarbamoyl)methyl]succinamic acid (5-COOH-NEF, IV) in both animals and humans (Fig. 1) [6,9]. Consequently, it is important to investigate the detailed metabolic fate of I and to clarify the pharmacokinetics of I and its metabolites in humans for the sake of safety and efficacy in clinical use.

This paper describes a reversed-phase high-performance liquid chromatographic (HPLC) method for the simultaneous determination of NEF (I) and its three major oxidized metabolites, 5-OH-NEF (II), 4-OH-NEF (III) and 5-COOH-NEF (IV) in human serum and urine.

EXPERIMENTAL

Materials

Compound I was synthesized in the Production Technology Research Laboratories of our company. Derivatives of I (II, III and IV) were synthesized in our research centre as previously described [9]. Acetonitrile and methanol were of HPLC grade. All other chemicals and reagents were of analytical grade.

Synthesis of internal standard

2-(2-Oxo-1-pyrrolidinyl)-N-phenylacetamide (V, Fig. 1) was synthesized in our research centre and used as the internal standard (I.S.) of the assay. Synthesis was as follows: chloroacetyl chloride was added dropwise to a solution of aniline in ethylene dichloride under ice-cooled conditions. The solution was stirred for 1 h, then most of the solvent was removed using a rotary evaporator. The residue was recrystallized from ethanol to give 2-chloro-N-phenylacetamide (VI). Sodium hydride was added dropwise to a solution of 2-oxopyrrolidine in toluene and stirred for *ca.* 4 h. Compound VI was then added to the solution. After stirring for 2 h, the solvent was evaporated under vacuum. The white solid residue was recrystallized from water to give V. Elemental analysis of V was: C, 66.12%; H, 6.62%, N, 12.79%. Calculated for $C_{12}H_{12}N_2O_2$: C, 66.04%; H, 6.47%; N, 12.84%. ^1H NMR (CD_3OD): δ = 2.11 (2H, m, H-4), 2.45 (2H, t, J = 7.9 Hz, H-3), 3.55 (2H, t, J = 7.1 Hz, H-5), 4.12 (2H, s, NCH_2CO), 7.08–7.54 (5H, m, Ar-H). Fast atom bombardment mass spectrometry (FAB-MS): m/z 219, $[M + H]^+$. m.p.: 175–177°C.

Preparation of standards and samples

Stock solutions of I (53.7 and 429.7 nmol/ml), II (84.9 and 849 nmol/ml), III (81.4 and 814 nmol/ml) and IV (94.9 and 949 nmol/ml) were prepared by dissolving weighed samples in methanol. Working solutions of appropriate concentrations were made by diluting each stock solution with methanol. The I.S. sample (451.5 μg/ml) was dissolved in 10% methanol. All solutions were stored at −20°C in the dark.

Serum, standard samples were prepared as follows: appropriate volumes of the standard solutions were added to 1 ml of human blank serum, resulting in nominal concentrations of 0.537–21.485 nmol/ml for I, 0.425–8.490 nmol/ml for II, 0.407–8.140 nmol/ml for III and 0.475–9.490 nmol/ml for IV. Urine standard samples were prepared as follows: to 0.5 ml of human blank urine, the standard solutions were added at different concentrations for each compound; from

4.30 to 85.94 nmol/ml for I, from 8.49 to 169.80 nmol/ml for II, from 8.14 to 162.80 nmol/ml for III and from 9.49 to 189.80 nmol/ml for IV.

Instrumentation and chromatographic conditions

The samples were analysed by an HPLC system consisting of a Model SP8700 ternary solvent-delivery system (Spectra-physics, San Jose, CA, USA), a Model 638-41 variable-wavelength UV detector (Hitachi, Tokyo, Japan) and a Model AS8000 autosampler (Tosoh, Tokyo, Japan). Reversed-phase HPLC separation was carried out with Nucleosil $5C_{18}$ (particle size 5 μm, 200 mm × 4.6 mm I.D., Macherey-Nagel, Düren, Germany). Mobile phase A consisted of acetonitrile–1/60 *M* KH_2PO_4 (pH 4.7) (1:9). Mobile phase B consisted of acetonitrile–water (50:50). The HPLC gradient was programmed as follows (where A + B = 100%): from 0 to 12 min isocratic elution at 100% A; from 12 to 30 min a linear gradient from 0 to 40% B. The HPLC system was operated at ambient temperature with a flow-rate of 1 ml/min. The UV wavelength was set at 210 nm.

Extraction procedure

The I.S. solution at 20 and 40 μl was added to 1 ml of serum sample and 0.5 ml of urine sample, respectively. After addition of 1 ml of 1/60 *M* KH_2PO_4, the mixture was applied to a Sep-pak C_{18} cartridge (Waters Assoc., Milford, MA, USA). The cartridge was washed with 1.5 ml of 1/60 *M* KH_2PO_4, and the sample was eluted with 2 ml of 100% methanol. The eluate was evaporated to dryness. The residue was redissolved in 500 μl of mobile phase A; 20 μl of this were then injected into the HPLC system.

Recovery determination

Serum or urine standard samples containing I, II, III and IV were extracted using Sep-Pak C_{18} cartridges. After addition of the I.S., the samples were evaporated and analysed by HPLC. Absolute recoveries of the compounds from serum or urine were assessed by comparing their peak-height ratios to the I.S. obtained after the extraction procedure with those obtained by direct injection of the same amounts of the standard solutions.

Data analysis

The regression line of the peak-height ratios of I or its metabolites to the internal standard (*y*-axis) *versus* serum or urine concentrations (*x*-axis) was calculated by linear least-squares regression analysis, and concentrations in the biological samples were estimated from the regression line.

The accuracy and precision of the method were evaluated over a wide range of I, II, III and IV concentrations in serum and urine. The means, standard deviations and coefficients of variation of concentrations obtained from intra- and inter-day replicates of analysis were calculated.

Serum and urine samples

Blank serum and urine were collected from healthy volunteers who took no medication for at least one week before sampling.

A single 100-mg dose of nefiracetam was orally administered to a healthy volunteer. Serum samples were collected at 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h, and urine samples at 0–2, 2–4, 4–8, 8–12 h after dosing. All samples were stored at −20°C until analysis.

RESULTS AND DISCUSSION

HPLC separation

The composition, pH and concentrations of the buffering ion of the mobile phase were varied to achieve optimum chromatographic conditions. A mobile phase of 1/60 *M* KH_2PO_4 (pH 4.7)–acetonitrile (9:1, v/v) gave optimum resolution of I, its metabolites and the I.S. Fig. 2 shows the effect of the pH of the mobile phase on the retention of these compounds. The capacity factors of I, II, III and V were not sensitive to the pH of the mobile phase. On the other hand, the effect of the pH was most apparent for IV, the most acidic metabolite; when the mobile phase pH was 7.0, IV was less retained in the column, the retention increasing with decreasing pH. The optimum separation of the compounds was ob-

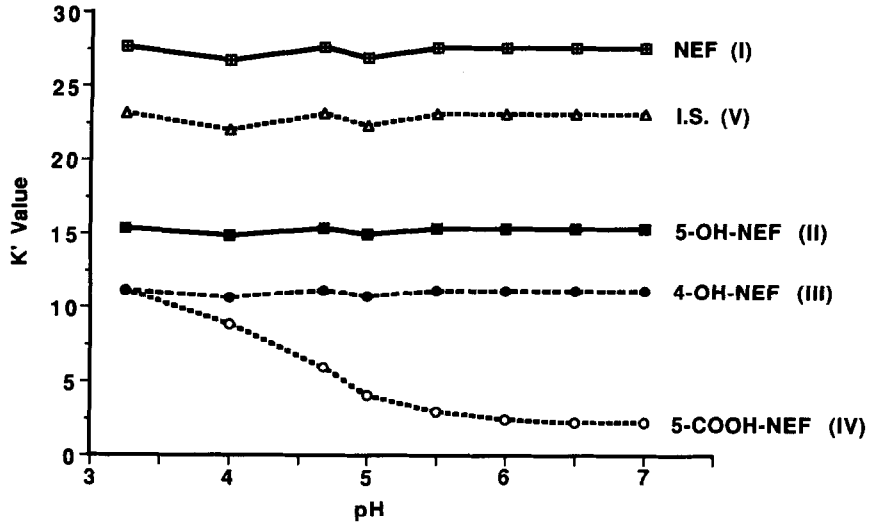

Fig. 2. *K'* values of NEF (I), 5-OH-NEF (II), 4-OH-NEF (III), 5-COOH-NEF (IV) and the internal standard (V) in HPLC.

tained using a mobile phase pH of 4.7.

The effect of the buffering ion concentration was also examined. The retention of the compounds was not affected by varying the ion concentration between 1/60 and 1/15 *M*.

The proportion of acetonitrile in the mobile phase drastically affected the retention of I, its metabolites and the I.S. Increasing the percentage of acetonitrile decreased the retention times proportionately, with that of I changing from 60 min to 6 min when the acetonitrile content was increased from 10 to 30%. However, with 30% acetonitrile, IV was not resolved from the endogenous compound. The HPLC gradient was therefore programmed as described in chromatographic conditions for optimum separation of I,

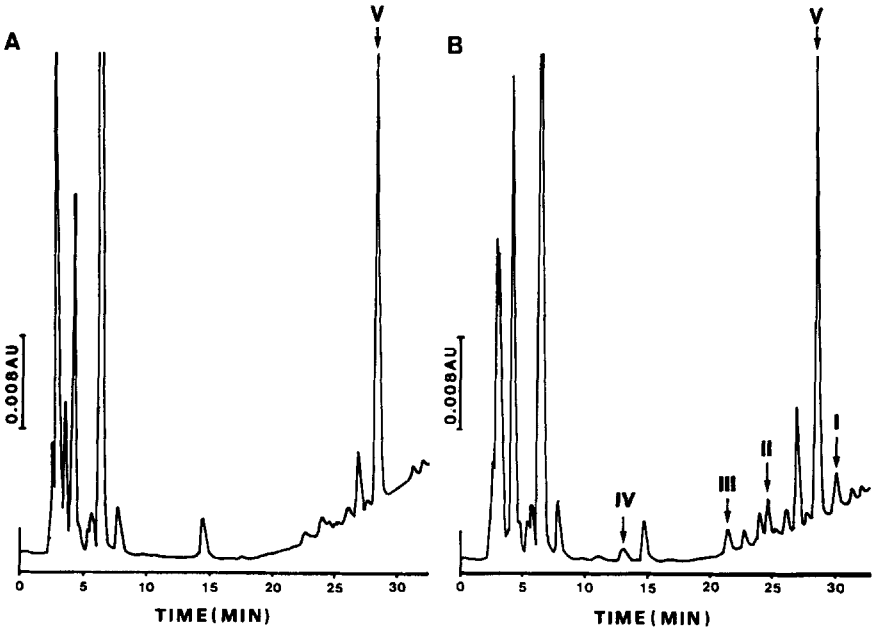

Fig. 3. Typical chromatograms of (A) blank human serum and (B) human serum spiked with I (2.1 nmol/ml), II (1.7 nmol/ml), III (1.6 nmol/ml) and IV (1.9 nmol/ml).

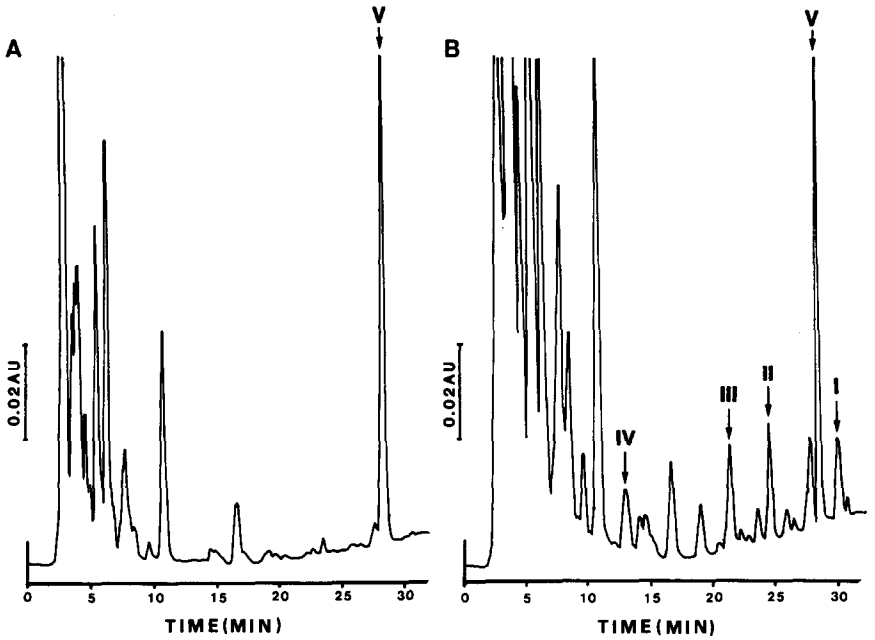

Fig. 4. Typical chromatograms of (A) blank human urine and (B) human urine spiked with I (17.2 nmol/ml), II (34.0 nmol/ml), III (32.6 nmol/ml) and IV (38.0 nmol/ml).

its metabolites and endogenous components.

Figs. 3 and 4 show typical chromatograms of serum and urine samples. The retention times of IV, III, II and I were 12.8, 21.0, 24.2 and 29.6 min, respectively.

Extraction

Several extraction solvents were investigated. Chloroform or 10% methanol in chloroform efficiently extracted I and the I.S., but not the metabolites, particularly IV which was poorly extracted in the solvent mixture even under acidic conditions (*ca.* pH 4); moreover, interference from endogenous materials increased under these conditions. A different extraction procedure using Sep-Pak C_{18} cartridges was therefore examined. When IV spiked to 1 ml of blank serum or 0.5 ml of blank urine was applied to a Sep-Pak C_{18} cartridge and the cartridge was washed with 1 ml of water, the recovery of IV was less than 10%. However, when the sample was applied to the cartridge with 1 ml of the buffer solution (1/15 *M* KH_2PO_4, pH 4.7) and washed with 1.5 ml of this solution, more than 90% of IV was recovered with 2 ml of methanol. The recoveries of I

TABLE I

RECOVERY OF NEF (I), 5-OH-NEF (II), 4-OH-NEF (III) AND 5-COOH-NEF (IV) FROM HUMAN SERUM AND URINE

Compound	Concentration added (nmol/ml)	Recovery (mean ± S.D., n = 5) (%)
Serum		
I	1.074	99.1 ± 7.2
	21.490	99.6 ± 1.8
II	0.849	102.9 ± 4.7
	8.490	99.5 ± 1.7
III	0.814	100.6 ± 4.8
	8.140	100.6 ± 1.6
IV	0.949	99.4 ± 6.7
	9.490	92.7 ± 1.5
Urine		
I	8.60	108.6 ± 12.8
	86.00	105.4 ± 0.8
II	16.98	96.8 ± 1.8
	169.80	97.6 ± 2.9
III	16.28	97.2 ± 2.0
	162.80	98.2 ± 1.4
IV	18.98	98.9 ± 4.6
	189.80	93.4 ± 1.2

TABLE II

CALIBRATION PARAMETERS FOR NEF (I), 5-OH-NEF (II), 4-OH-NEF (III) AND 5-COOH-NEF (IV)

Compound	Concentration (nmol/ml)	Correlation coefficient (r)	Slope	Intercept
Serum				
I	0.537–21.485	0.9998	0.032464	−0.001149
II	0.425–8.490	0.9995	0.022725	0.022487
III	0.407–8.140	0.9997	0.022870	−0.004612
IV	0.475–9.490	0.9998	0.011008	−0.001283
Urine				
I	4.30–85.94	0.9998	0.010105	−0.008534
II	8.49–169.80	0.9999	0.007121	−0.006065
III	8.14–162.80	0.9999	0.006424	−0.018938
IV	9.49–189.80	0.9998	0.003142	−0.002723

and the other metabolites were also over 90% with this procedure (Table I).

Linearity and limit of detection

Calibration curves for I and its metabolites ob-

TABLE III

PRECISION AND ACCURACY OF THE ASSAY FOR NEF (I), 5-OH-NEF (II), 4-OH-NEF (III) AND 5-COOH-NEF (IV) IN HUMAN SERUM

Compound	Theoretical concentration (nmol/ml)	Intra-day assay (n = 5)			Inter-day assay (n = 6)		
		Measured concentration (nmol/ml)	C.V. (%)	Accuracy (%)	Measured concentration (nmol/ml)	C.V. (%)	Accuracy (%)
I	0.537	0.506	4.3	−5.8	0.487	4.1	−9.3
	1.074	0.977	2.1	−9.0	0.978	2.2	−8.9
	2.148	2.085	0.6	−2.9	2.149	1.4	0.0
	4.296	4.519	1.4	5.2	4.469	1.4	4.0
	21.485	21.452	1.0	−0.2	21.456	0.0	−0.1
II	0.425	0.467	6.4	9.9	0.420	7.4	−1.2
	0.849	0.911	8.7	7.3	0.888	5.6	4.6
	1.698	1.632	1.8	−3.9	1.644	3.0	−3.2
	3.396	3.324	4.5	−2.1	3.486	2.1	2.7
	8.490	8.524	1.1	0.4	8.483	0.4	−0.1
III	0.407	0.440	2.0	8.1	0.418	5.7	2.7
	0.814	0.787	1.7	−3.3	0.772	2.8	−5.2
	1.628	1.551	1.5	−4.7	1.614	1.3	−0.9
	3.256	3.347	2.0	2.8	3.322	1.7	2.0
	8.140	8.120	1.0	−0.2	8.120	0.2	−0.2
IV	0.475	0.498	1.8	4.8	0.439	3.4	−7.6
	0.949	0.986	3.0	3.9	0.870	3.8	−8.3
	1.898	1.830	1.9	−3.6	1.922	2.7	1.3
	3.796	3.794	1.9	−0.1	3.939	1.5	3.8
	9.490	9.500	1.1	0.1	9.438	0.2	−0.5

TABLE IV

PRECISION AND ACCURACY OF THE ASSAY FOR NEF (I), 5-OH-NEF (II), 4-OH-NEF (III) AND 5-COOH-NEF (IV) IN HUMAN URINE

Compound	Theoretical concentration (nmol/ml)	Intra-day assay (n = 5)			Inter-day assay (n = 6)		
		Measured concentration (nmol/ml)	C.V. (%)	Accuracy (%)	Measured concentration (nmol/ml)	C.V. (%)	Accuracy (%)
I	4.30	4.29	3.5	−0.2	4.46	4.2	3.8
	8.59	8.56	1.4	−0.3	8.63	1.4	0.5
	17.19	16.76	1.6	−2.5	17.11	0.7	−0.5
	34.38	35.01	0.9	1.8	34.18	1.2	−0.6
	85.94	85.77	1.3	−0.2	86.03	0.2	0.1
II	8.49	7.93	1.0	−6.6	8.77	3.7	3.3
	16.98	17.17	0.9	1.1	16.93	1.7	−0.3
	33.96	33.80	1.0	−0.5	33.88	0.6	−0.2
	67.92	68.74	0.7	1.2	67.65	0.6	−0.4
	169.80	169.51	0.8	−0.2	169.91	0.1	0.1
III	8.14	8.65	1.0	6.3	7.92	5.9	−2.7
	16.28	16.49	0.7	1.3	16.55	0.9	1.7
	32.56	31.72	1.7	−2.6	32.41	0.8	−0.5
	65.12	65.12	0.7	0.0	65.26	1.0	0.2
	162.80	162.91	0.6	0.1	162.76	0.1	0.0
IV	9.49	9.59	7.6	1.1	9.80	4.1	3.2
	18.98	18.88	3.7	−0.5	18.38	2.6	−0.12
	37.96	36.96	1.9	−2.6	37.76	1.6	−0.5
	75.92	77.25	0.8	1.8	76.59	1.4	0.9
	189.80	189.47	0.9	−0.2	189.61	0.2	−0.1

tained by the described HPLC method were linear over a wide range of concentrations, with regression coefficients (r) consistently greater than 0.999 (Table II). Based on a 2:1 signal-to-noise ratio, the detection limit of the assay was 0.1 nmol/ml for I, III and IV and 0.2 nmol/ml for II.

Accuracy and precision

The accuracy and precision were evaluated by analysing serum and urine samples spiked with various concentrations, of I, II, III and IV (Tables III and IV). Differences between concentrations added and measured levels were less than 10% in all cases. Coefficients of variation of the five independent samples at each concentration of I and its metabolites in the intra-day assay were less than 9% in the serum samples and less than 8% in the urine samples, and those in the

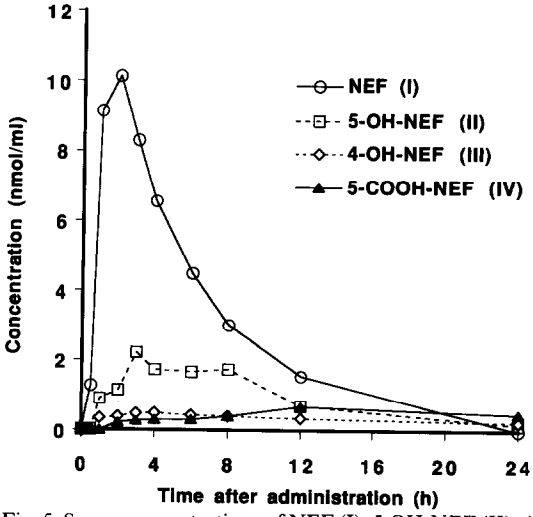

Fig. 5. Serum concentrations of NEF (I), 5-OH-NEF (II), 4-OH-NEF (III) and 5-COOH-NEF (IV) after a single oral administration of 100 mg of I to a healthy male volunteer.

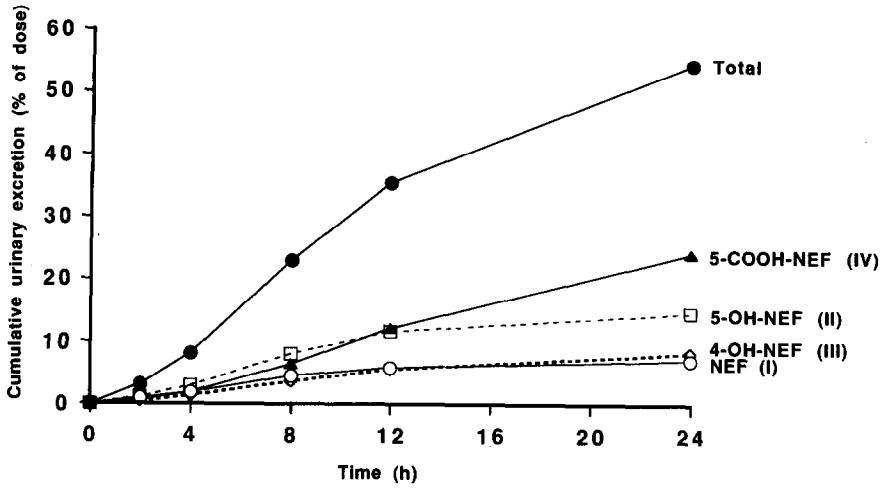

Fig. 6. Cumulative urinary excretion of NEF (I), 5-OH-NEF (II), 4-OH-NEF (III) and 5-COOH-NEF (IV) after a single oral administration of 100 mg of I to a healthy male volunteer.

inter-day assay on six different days were less than 8% in the serum samples and less than 6% in the urine samples.

Application of the method

This method was used to determine the concentrations of I and its metabolites in serum and urine following oral administration of I (100 mg) to a healthy volunteer. The serum concentration–time profile and urinary excretion of I, II, III and IV are shown in Figs. 5 and 6, respectively. An unchanged serum drug concentration of 10.1 nmol/ml was attained 2 h after the administration and thereafter it declined gradually. The peak concentrations of II, III and IV were 2.2, 0.5 and 0.7 nmol/ml, respectively. The cumulative urinary excretion of the unchanged I, II, III and IV were 7.2, 14.7, 8.4 and 24.2%, respectively.

In conclusion, these data demonstrate that this HPLC method is simple, sensitive and reproducible, and is suitable for the simultaneous determination of I and its metabolites in human serum and urine in clinical studies.

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