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Simple and selective assay of 4-hydroxymephenytoin in human urine using solid-phase extraction and high-performance liquid chromatography with electrochemical detection and its preliminary application to phenotyping test

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Abstract

A simple and selective HPLC method for the determination of 4-hydroxymephenytoin (4-OH-M) in human urine, using a controlled potential coulometric detector equipped with a dual working electrode cell of fully porous graphite, has been developed. After acid hydrolysis of urine, 4-OH-M and the internal standard (I.S.), 5-hydroxy-1-tetralone, were extracted from urine by means of a Bond Elut Certify LRC column. The extracts were chromatographed on a reversed-phase μBondapak C_{18} column using methanol–50 m*M* KH_2PO_4 (pH 4.0) (30:70, v/v) as the mobile phase at a flow-rate of 1.0 ml/min. Electrochemical detection at applied potential of 800 mV resulted in a limit of quantitation of 0.76 μg/ml. The method showed a satisfactory sensitivity, precision, accuracy, recovery and selectivity. The present method was applied to the phenotyping test in thirteen Japanese healthy volunteers who received an oral 100-mg racemic mephenytoin. The phenotypes determined by the present method were found to be in agreement with those obtained with the reported customary assay based on gas chromatography.

Keywords: 4-Hydroxymephenytoin; Electrochemical detection; Phenotyping

1. Introduction

An antiepileptic drug, mephenytoin (M), is used clinically as a racemate. The metabolism of M has been shown to be highly stereoselective in humans [1]. Only the *S*-enantiomer undergoes hydroxylation to form 4-hydroxymephenytoin (4-OH-M) (Fig. 1). The polymorphic metabolism of M has been widely investigated, and individuals are classified to either poor metabolizers (PMs) or extensive metabolizers (EMs) [1–4]. The incidence of PMs is ca. 3% in

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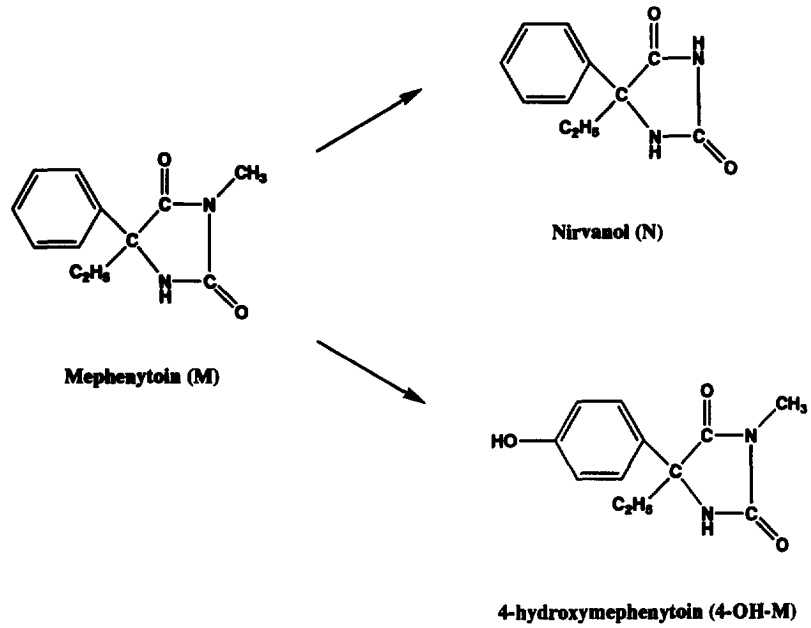

Fig. 1. Chemical structures of mephenytoin (M), 4-hydroxymephenytoin (4-OH-M) and nirvanol (N) and metabolic pathways of M.

Caucasians [4], but as high as 23% in Japanese population [3]. The metabolism of several clinically important drugs such as diazepam, proguanil and omeprazole has been demonstrated to cosegregate with this hydroxylation polymorphism [2].

EMs and PMs are characterized by determining the urinary *S*-M/*R*-M ratio [5,6] or the hydroxylation index (HI) representing the ratio between the *S*-M dose (half of the administered dose of racemic M) and the recovery of 4-OH-M in the 0–8 h urine (7). The metabolism of M was investigated in normal male subjects after a single oral dose of enantiomerically radiolabeled pseudoracemic M [8], where urinary excretion of M was only 2.9% of the dose administered up to 72 h after drug administration. Wedlund et al. [6] developed a gas chromatographic (GC) method for the separation and determination of the *R*- and *S*-M enantiomers in urine samples using a chiral capillary GC column (Chirasil-Val). Later, a chiral HPLC assay for the determination of M in human urine was developed by employing β-cyclodextrin as a mobile phase additive [9]. Using these reported assays [6,9], however, the quantitation of *S*- and *R*-enantiomers seems rather difficult, because only a trace amount of M is excreted in the 0–8 h urine samples, which contain various interfering endogenous and exogenous substances. It has also been reported that large changes in the *S*-M/*R*-M ratios were observed in some urine samples stored at −20°C for only a few months due to the decomposition of an acid labile metabolite of M, which would lead to inaccurate phenotype assignments [10].

PMs and EMs of M can also be differentiated by the HI [7] as described above. The urinary excretion of 4-OH-M, mainly as the glucuronide conjugate, was rapid, with a cumulative recovery of ca. 46% of the racemic dose within 72 h [8]. The high urinary concentrations of 4-OH-M make it more suitable to determine 4-OH-M in urine for a phenotyping purpose. GC methods have been developed for the determination of 4-OH-M in urine [3,11], which, however, required tedious pre-column derivatization. In addition, although several HPLC methods have been used for the determination of 4-OH-M in urine and other matrices [11–15], they utilized a time-consuming liquid–liquid extraction and measurement of absorbance at 204, 211 or 230 nm. Because of the presence in urine of numerous endogenous substances as well as the possibility that many other drugs that absorb at these wavelengths would exist, there is still considerable interest in developing a new analytical method for the determination of 4-

OH-M in urine, which is simpler and more selective than the commonly employed methods.

HPLC with electrochemical detection (ED) has proven to be a highly sensitive and selective method for determination of trace components in complex biological samples, and the electrochemical detector becomes an important alternative tool to ultraviolet and fluorescence detectors [16]. 4-OH-M is a derivative of phenol, which has been reported to be highly responsive to the electrochemical detector [16,17]. Thus, this paper describes a new simple and selective method for the determination of 4-OH-M in human urine using rapid and convenient solid-phase extraction and HPLC with ED. In addition, the present method was applied to the phenotyping of Japanese healthy volunteers receiving an oral dose of racemic M.

2. Experimental

2.1. Chemicals and reagents

M, 4-OH-M and nirvanol (N) were kindly donated by Professor Grant R. Wilkinson from the Division of Clinical Pharmacology at the Vanderbilt University, Medical Center (Nashville, TN, USA). 5-Hydroxy-1-tetralone used as an internal standard (I.S.) was purchased from Tokyo Kassei Kogyo (Tokyo, Japan). Methanol (MeOH) was an HPLC-grade solvent (Kanto Chemicals, Tokyo, Japan). All other chemicals were of analytical reagent grade and were used without further purification. Purified water from a Milli-Q system (Waters Assoc., Millipore, Milford, MA, USA) was used.

2.2. Instruments and chromatographic conditions

The chromatographic system consisted of an HPLC apparatus (Model LC-10AD, Shimadzu, Kyoto, Japan), a degasser (DEGASYS, DG-1200, Uniflows, Tokyo, Japan), and a reversed-phase μBondapak C_{18} (300×3.9 mm I.D., 10-μm particle size) column (Waters). An ESA Coulochem II 5200 system was used for the ED of the eluting compounds, consisting of a Model 5010 dual analytical cell (positioned at the end of analytical column, detector 1 set at 200 mV, detector 2 set at 800 mV) and a Model 5020 guard cell (set at 900 mV) positioned between the pump and the autosampler. The column temperature was kept at 40°C in a column oven (Model 3510, Senshu Scientific, Tokyo, Japan). Samples of 20 μl were injected automatically to the HPLC system by an autosampler (Model AS-8010, Tosoh, Tokyo, Japan). A mixture of MeOH and 50 m*M* KH_2PO_4 (adjusted to pH 4.0 with phosphoric acid) (70:30, v/v) was used as the mobile phase at a flow-rate of 1.0 ml/min. The mobile phase was degassed in an ultrasonic bath before use.

2.3. Preparation of standard solutions

Stock solutions of standards were prepared by dissolving accurately weighed M, 4-OH-M, N and 5-hydroxy-1-tetralone (I.S.) in MeOH in a volumetric flask. The stock solutions were diluted with water successively to prepare the working standard solutions. Urine standards were prepared at concentrations of 0.76, 1.53, 3.05, 6.11, 12.2, 24.4, 48.9, 97.7, and 195 μg/ml of 4-OH-M.

2.4. Assay procedure

Bond Elut Certify LRC columns (Analytichem International, Harbor City, CA, USA) were activated prior to use by passage of MeOH (2 ml) and 0.1 *M* acetate buffer (pH 7.0) (2 ml).

Human urine (0.1 ml) was diluted with water (0.9 ml). A 0.1-ml aliquot of diluted human urine was transferred into a 14-ml glass test tube, where 12 *M* hydrochloric acid (0.1 ml) was added. The mixture was heated at 100°C for 2 h to perform the acid hydrolysis of the glucuronide to liberate the aglycon [11]. After cooling, the I.S. solution (25 μg/ml) (0.1 ml), water (7.6 ml) and 12 *M* sodium hydroxide (0.1 ml) were added to the acid hydrolysate successively. The resulting mixture was adjusted to pH 7.0 by 1.2 *M* sodium hydroxide and applied to a Bond Elut Certify LRC column (130 mg) using gentle suction. The column was washed with 0.1 *M* acetate buffer (pH 7.0) (1 ml) and dried under full vacuum for 5 min and then washed with hexane–ethyl acetate (95:5, v/v) (2 ml). 4-OH-M and the I.S. were eluted from the column with hexane–ethyl acetate (40:60,

v/v) (4 ml). The eluate was taken to dryness by a centrifugal evaporator (Model EC57C, Sakuma, Tokyo, Japan). The residue was dissolved in mobile phase (1.0 ml) and a 20-μl aliquot was injected onto the HPLC system as described above.

2.5. Calibration curves

The peak area of 4-OH-M was divided by the peak area of the I.S. to obtain the peak-area ratio. The calibration curve for 4-OH-M was constructed from a least-square linear regression of the peak-area ratios of the standards versus the drug concentrations by using a weighting factor of $1/y^2$.

2.6. Recovery

The absolute recovery of 4-OH-M from human urine was estimated by comparing the peak area obtained from injections of standards with those obtained from the injection of extracts of urine samples spiked with known concentrations of 4-OH-M.

2.7. Selectivity

Blank urine samples obtained from six healthy male volunteers were assayed by the procedure as described above to evaluate the selectivity of the method.

2.8. Precision and accuracy

Intra-day precision and accuracy of the method were evaluated by replicate analyses ($n=6$) of the urine standards. Inter-day precision and accuracy were determined by assaying the urine standards on four separate days. The limit of quantitation was chosen to be the concentration of the lowest calibration standard with an acceptable limit of variance (within 20% for both precision and accuracy).

2.9. Preliminary application

Thirteen healthy adults, after having been fully informed, voluntarily participated in this part of the study. After a single oral 100-mg dose of racemic mephenytoin (100 mg per tablet, Methantoin®, Sandoz, Basel, Switzerland), the 0–8 h urine was collected. All procedures were performed in accordance with the ethical standards established by the Institutional Review Board of the Hirosaki University Hospital. The urine samples were stored at −20°C until analysed.

3. Results and discussion

3.1. Hydrodynamic voltammograms

The effect of applied potential on the peak area of 4-OH-M and the I.S. was determined by changing the oxidation potential by 100 or 50 mV increasing from 100 to 900 mV. This was carried out with the mobile phase used for the chromatography of these compounds. The resulting hydrodynamic voltammogram is shown in Fig. 2. The current response was normalized to that at the limiting plateau. The detector gave a linear response up to 800 mV and then showed only a slight increase in response. The applied potentials of electrode 1 and electrode 2 in the analytical cell were set at 200 mV and 800 mV, respectively. Only electrode 2 was used as a detector and electrode 1 was utilized to remove easily oxidizable substances. These oxidation potentials were chosen to minimize the background noise as well as

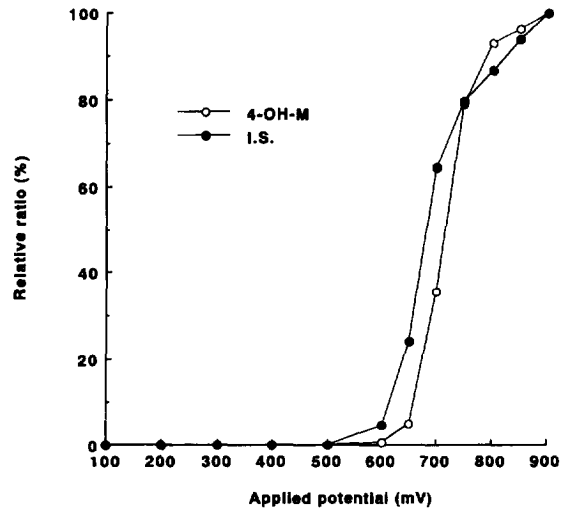

Fig. 2. Hydrodynamic voltammogram of 4-OH-M and 5-hydroxy-1-tetralone, I.S.

the interference of endogenous electroactive compounds with the assay and yet to obtain sufficient sensitivity to detect 4-OH-M in human urine.

3.2. Chromatography and selectivity

Among the several analytical columns tested, a reversed-phase μBondapak C_{18} (300×3.9 mm I.D., 10-μm particle size) column was the most favorable for an efficient separation of 4-OH-M and the I.S. from interfering endogenous substances in human urine. A representative chromatogram of extract from control urine is shown in Fig. 3A. These chromatograms indicated that no endogenous compounds interfered with the detection of 4-OH-M and the I.S. at their retention times. A typical chromatogram of control urine spiked with 4-OH-M and the I.S. is shown in Fig. 3B. 4-OH-M and the I.S. were well-resolved from each other, and the retention times of 4-OH-M and the I.S. were approximately 10 and 17 min, respectively. The overall chromatographic run time was within ca. 20 min. Urine samples collected from six healthy volunteers also showed no interferences with the assay, indicating that the present method had a high selectivity to determine 4-OH-M in human urine.

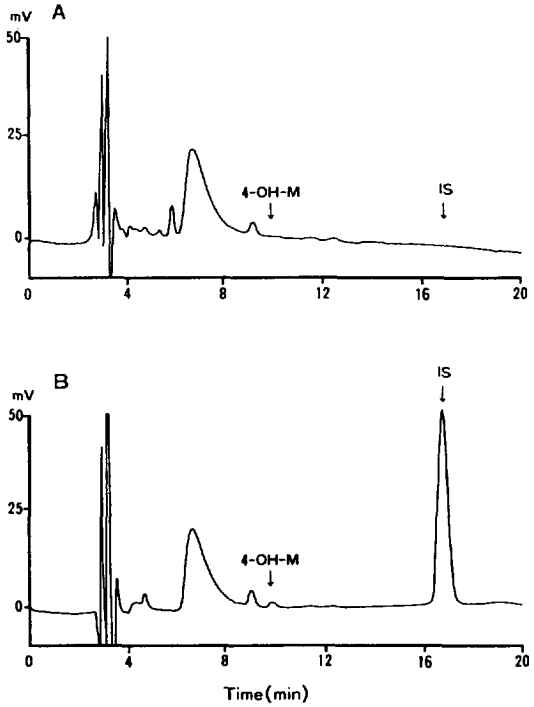

Fig. 3. Representative chromatograms of urine extracts obtained with (A) blank human urine and (B) blank human urine spiked with 4-OH-M (0.76 μg/ml) and the I.S. The chromatograms are shown with a full scale of 50 mV.

The chromatographic conditions as described earlier led to the baseline separation of M, 4-OH-M and N, when the analytes were monitored with a UV detector. On the other hand, only 4-OH-M was highly responsive to the electrochemical detector because of an electroactive phenol group in the molecule, which resulted in a quantitation limit of 0.76 μg/ml by using 0.01 ml of human urine. M and N did not show any response to the electrochemical detector. Thus, the present method proved to be highly selective and sensitive for the detection of 4-OH-M. These results suggest that the present method could also serve as a powerful tool for characterizing the mephenytoin polymorphism in vitro. The smaller amount of liver tissue would be sufficient to measure *S*-mephenytoin 4-hydroxylase (CYP 2C19) activity because of its high sensitivity. Furthermore, the analytical run time could be shortened because M used as a substrate is not detectable at all with electrochemical detector, allowing the subsequent analysis of different samples before the elution of M from the analytical column.

3.3. Analytical procedures

Several authors [12,13,15] have described a liquid–liquid extraction of 4-OH-M from acid-hydrolyzed urine samples for the determination of 4-OH-M by HPLC or GC method. The liquid–liquid extraction, however, was time-consuming and not applicable to HPLC with ED because many interfering peaks of endogenous substances appeared on a chromatogram. The present study employs an alternative assay procedure which involves a rapid and convenient solid-phase extraction to assay 4-OH-M. The attempts to use C_8 and C_{18} cartridges as extraction columns were unsuccessful due to the interference from the endogenous substances. We were able to separate 4-OH-M and the I.S. from the interfering endogenous compounds by using Bond

Table 1
Recoveries of 4-OH-M from human urine

Concentration (μg/ml)	Recovery(%) (mean ± S.D., n=6)
6.52	89.9±2.8
32.6	86.2±3.1
163	93.5±1.2

Elut Certify LRC column for solid-phase extraction under the specified conditions, resulting in a simpler and more selective analytical method for the determination of 4-OH-M in human urine.

3.4. Recovery of extraction

The absolute recoveries of 4-OH-M from urine were determined by comparing the peak area of extracted standards with those of injected standards (Table 1). The recoveries from spiked human urine were determined at the concentrations of 6.52, 32.6 and 163 μg/ml in six replicates. The recoveries of 4-OH-M ranged from 86.2 to 93.5%, with that of the I.S. being 63.0±4.7%. Solid-phase extraction has been successfully applied to the extraction of 4-OH-M from human urine.

3.5. Calibration curves

Calibration curves for urine obtained over a one-week period (n=4) were linear over the concentrations examined (0.76–195 μg/ml) and reproducible with mean ± standard deviation values for the constants in the regression equation of y=(29.0±4.03)x+(0.279±0.170). Coefficients of determination (r^2) were always greater than 0.999. The inter-day coefficient of variation (C.V.) of the slope of the calibration curves was 13.9%.

3.6. Precision and accuracy

The intra-day precision and accuracy of the method for human urine were evaluated by analyzing the urine spiked with 4-OH-M at concentrations of 0.76 to 195 μg/ml in six replicates (Table 2). Precision was based on the calculation of the C.V.. An indication of accuracy was based on the calculation of the relative error (R.E.) of the experimentally determined concentration compared to the theoretical one. The C.V.s ranged from 5.1 to 15.2% and the R.E.s ranged from −15.1 to 12.7% at concentrations above 1.53 μg/ml. The limit of quantitation using a 0.01-ml urine sample was set at the concentration of the lowest calibration standard or 0.76 μg/ml of urine with a C.V. of 19.7%, R.E. of 11.7% and the signal-to-noise ratio of at least 8.

The inter-day precision and accuracy were assessed by the analysis of urine samples at the same concentrations as those used for intra-day study on four separate days (Table 3). The C.V.s were less than 15% and the R.E.s ranged from −12.5 to 13.3% at concentrations above 1.53 μg/ml. At the quantitation limit of 0.76 μg/ml, the method showed an acceptable precision and accuracy, i.e., with the C.V. of 6.9% and the R.E. of 6.5%.

These results indicated that the present method has a satisfactory precision, accuracy and reproducibility.

Table 2
Intra-day precision and accuracy for the analysis of 4-OH-M in human urine

Theoretical concentration (μg/ml)	Mean found concentration (μg/ml)	C.V.[a] (%)	R.E.[b] (%)	n
0.76	0.85	19.7	11.7	6
1.53	1.33	11.7	−12.9	6
3.05	2.59	10.6	−15.1	6
6.11	5.56	15.2	−8.9	6
12.2	11.4	10.5	−6.6	6
24.4	23.0	8.6	−5.7	6
48.9	50.2	8.2	2.8	5
97.7	104	5.1	6.1	5
195	220	7.5	12.7	5

[a] C.V. = coefficient of variation.[b] R.E. = relative error.

Table 3
Inter-day precision and accuracy for the analysis of 4-OH-M in human urine

Theoretical concentration (μg/ml)	Mean found concentration (μg/ml)	C.V.[a](%)	R.E.[b](%)	n
0.76	0.81	6.9	6.5	4
1.53	1.47	14.6	−3.6	4
3.05	2.74	7.6	−10.4	4
6.11	5.34	5.7	−12.5	4
12.2	11.0	4.4	−10.2	4
24.4	23.4	5.8	−4.3	4
48.9	47.5	3.4	−2.8	4
97.7	110	4.9	12.5	4
195	221	5.7	13.3	4

[a] C.V. = coefficient of variation. [b] R.E. = relative error.

3.7. Application

The method was then applied to the determination of 4-OH-M in human urine samples obtained from thirteen Japanese healthy male volunteers (seven EMs and six PMs) who received an oral 100-mg (458 μmol) dose of racemic M. Fig. 4 shows the representative chromatograms of 0–8 h urine samples obtained from an EM and a PM. It is apparent that the peak of 4-OH-M in the chromatogram of the PM is almost missing, whereas a well-defined peak of 4-OH-M was observed for the EM. Seven EMs eliminated 39.7±4.6% of the dose of racemic M as 4-OH-M (182±21.1 μmol), whereas six PMs eliminated 0.19±0.18% of the administered dose as 4-OH-M (0.89±0.83 μmol) (Table 4). The urinary excretion data of 4-OH-M determined by the present method correlated significantly ($p<0.0001$) with those obtained with the reported customary GC assay (3) (Fig. 5), indicating that the current HPLC–ED method has a clinical applicability to the phenotyping test with racemic M in humans.

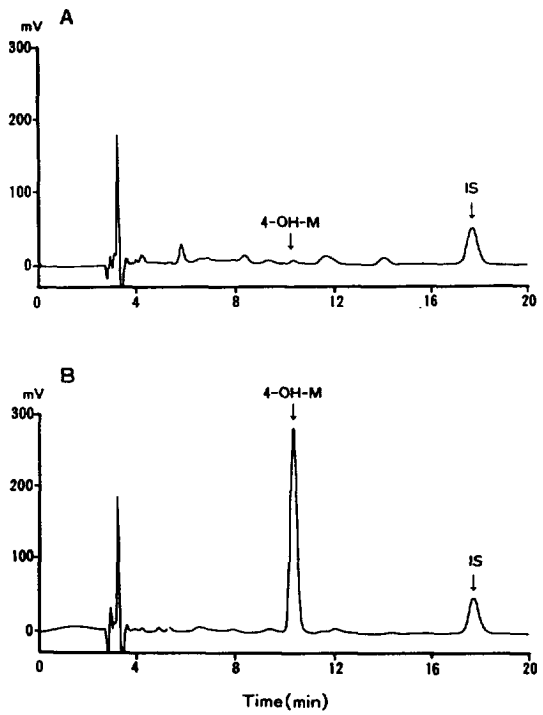

Fig. 4. Representative chromatograms of urine extracts obtained with (A) 0–8 h human urine from a PM (1.29 μg/ml) and (B) 0–8 h human urine from an EM (108 μg/ml). The chromatograms are shown with a full scale of 300 mV.

Table 4
Urine analysis for *S*-mephenytoin polymorphism after a single oral administration of racemic mephenytoin (100 mg) to thirteen healthy male Japanese volunteers

	Extensive metabolizers	Poor metabolizers
Number of subjects	7	6
4-OH-M output (μmol/8-h urine)	182±21.1	0.89±0.83
% of dose eliminated as 4-OH-M	39.7±4.6	0.19±0.18

Mean ± S.D.

Acknowledgments

We thank Professor Grant R. Wilkinson for his generous gift of the analytical reference compounds, mephenytoin, 4-hydroxymephenytoin and nirvanol.

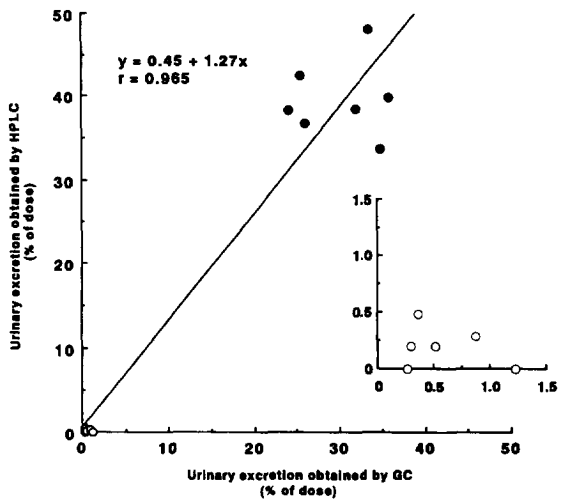

Fig. 5. Correlations of urinary excretion of 4-OH-M determined by the present HPLC method (y-axis) and a GC method (x-axis) reported by Horai et al. [3]. ● = data obtained from the EM group (n =7) and ○ = data obtained from the PM group (n =6) of *S*-mephenytoin 4-hydroxylation. The inset indicates the data from the PM group shown on a different scale.

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