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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENYTOIN AND HYDROXYPHENYTOIN IN HUMAN URINE

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## SUMMARY

Simple and rapid quantitative analysis utilizing high-performance liquid chromatography was performed to determine the concentration of the antiepileptic agent phenytoin, and its main metabolite hydroxyphenytoin, in human urine. For the purposes of simple and rapid determination, the commercially available Extrelut® columns were used with diethyl ether and chloroform as extraction solvents. High-performance liquid chromatography was performed on a LiChrosorb RP-8 column, with a mobile phase of methanol—0.02% ammonium acetate (1:1). The internal standard was 5-(4-methylphenyl)-5-phenylhydantoin.

The method was applied to patients' urine, to examine the influence of concomitant drugs. Also, the results obtained using the commercially available enzymatic immunoassay method were compared with those from the present method, and it was concluded that a simple and rapid microanalysis is possible with a high extraction ratio.

## INTRODUCTION

We have previously reported the extraction of blood phenytoin (DPH) and its main metabolite in humans, hydroxyphenytoin (HDPH), for the purposes of a simple and rapid quantitative analysis utilizing commercially available pre-packed columns, and their quantitative analysis by high-performance liquid chromatography (HPLC) [1].

In the present study, we examined the quantitative analysis of DPH and HDPH in human urine as a follow-up to the previous report. It has been reported that about 75% of the administered DPH is excreted as conjugated glucuronides of HDPH in the urine and that about 5% is excreted as unchanged

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DPH [2]. We have attempted to analyze quantitatively human urinary DPH and HDPH under conditions where combined drug therapy, including DPH, was undertaken for treating epilepsy.

## EXPERIMENTAL

### *Reagents and solutions*

The phenytoin (DPH) used was recrystallized from one standardized according to the Japanese Pharmacopoeia. Hydroxyphenytoin (HDPH) and 5-(4-methylphenyl)-5-phenylhydantoin (internal standard) were the products of Sigma, St. Louis, MO, U.S.A. Diazepam, phenobarbital, carbamazepine, and acetazolamide were those standardized according to the Japansese Pharmacopoeia. Methanol for HPLC was from Wako Junyaku, Co. Ltd., Osaka, Japan, and the other agents were commercially available extra-pure reagents.

Control urine used was the Teck Check™ Control Urine (No. 1) produced by Miles-Sankyo, Tokyo, Japan. The kit for quantitative analysis of DPH was Markit® Phenytoin, a product of Dai Nippon Seiyaku, Osaka, Japan, and the procedure was followed according to the manufacturer's instructions.

### *Conditions for high-performance liquid chromatography*

A Hitachi chromatoprocessor Type 834 was connected to a Hitachi high-performance liquid chromatograph Type 635, and the area under the peak was measured. The column was LiChrosorb RP-8, from Merck, Darmstadt, G.F.R. (5 μm, 150 mm × 4.0 mm I.D., with built-in water jacket, adjusted to 40°C). The detector, Type UV-8 (250 nm), was a product of Toyo Soda Co. Ltd.; a.u.f.s. 0.08—0.16. The mobile phase was methanol—0.02% aqueous ammonium acetate (1:1), at a flow-rate of 0.8 ml/min.

### *Analytical method for DPH and HDPH*

To 1 ml of urine, 200 μl of methanol solutions of DPH (0.3—1.5 mg/ml) and HDPH (0.03—1.5 mg/ml) were added, and 200 μl of a methanol solution of 5-(4-methylphenyl)-5-phenylhydantoin as the internal standard. Then, 20 ml of 0.01 *M* phosphate buffer solution at pH 6.8 were added and the solution was mixed. The solution was transferred to the top of an Extrelut® prepacked column for the extraction of these compounds and was adsorbed. Fifteen minutes later, to elute DPH, HDPH and internal standard, 30 ml of diethyl ether were added, followed by 30 ml of chloroform to elute the compounds.

The clear effluent was dried under reduced pressure at a temperature below 20°C using a concentrator with trap which was cooled to −70°C. After passing nitrogen gas for 1 min, the dried sample was dissolved in 100 μl of methanol, and 5 μl of that solution were injected onto the column. From the chromatogram obtained, the ratio of the areas under the peaks of DPH and HDPH to that of the internal standard was calculated, and the amounts of DPH and HDPH were obtained from the calibration curve prepared beforehand.

### *Quantitative analysis of DPH and HDPH in human urine*

The application of the present method to patients' urine was made as fol-

lows. To 1.0 ml of urine was added 1.0 ml of 12 *N* hydrochloric acid and the container was loosely sealed. After boiling at 90°C for 120 min, the solution was cooled, and 1.0 ml of 12 *N* sodium hydroxide was added. After adjusting the pH to 7.0 with 20% sodium hydroxide, the analytical procedure described above was followed. The total amount of HDPH in a free state was calculated after hydrolysis according to the method of Dykeman et al. [3], since most of the HDPH is present as glucuronide conjugates in the urine [4—9].

## RESULTS AND DISCUSSION

Among the reports on the measurement of antiepileptics in urine utilizing HPLC, Kabra and Marton [10], Dykeman and Ecobichon [2], Sawchuk and Cartier [11], and others used μBondapak $C_{18}$ (30 cm × 3.9 mm I.D.) columns. The mobile phases they used were acetonitrile—water (37:63, v/v), methanol—phosphate buffer (0.025 *M*, pH 8.0) (40:60, v/v), and methanol—water (55:45, v/v), respectively, and the measurements were made at a wavelength of 254 nm. However, in these methods, the extraction ratios of DPH and HDPH were 41% and 79%, respectively [11], or the retention time in HPLC of DPH, HDPH and internal standard was long [3]. Therefore, we examined the simple and rapid quantitative analysis of urinary DPH and HDPH following the previously reported simple and rapid method [1] for blood DPH and HDPH in which the extraction ratio was good.

In addition, as the internal standard, we adopted 5-(4-methylphenyl)-5-phenylhydantoin, whose chemical structure is similar to that of DPH, because its retention time does not overlap that of DPH or other combination drugs.

In the next step, we examined the quantitative nature of DPH and HDPH. On the chromatogram of the mixture of DPH and HDPH to which 2 μg of internal standard were added, the ratio of the area under the peak was measured and plotted on the ordinate, which gave the analytical curve. There was good linearity within the range 3.0—15.0 μg for DPH ($Y = 0.340X + 0.027$). For HDPH in 0.3—15.0 μg amounts, all experimental data points are approximately on a straight line ($Y = 0.914X + 0.003$).

At the first step the influence of pH on the extraction ratio with the Extrelut® column was examined using healthy human urine in 1.0 ml of which 120 μg of DPH, 30.2 μg of HDPH, 40 μg of acetazolamide, 80 μg of diazepam, 80 μg of carbamazepine and 1.6 mg of phenobarbital were dissolved.. When comparing the three phosphate buffers of varying pH, the extraction ratio at slightly acidic pH (4.0) was 90.6% for DPH and 104.6% for HDPH. Under slightly basic conditions, pH 9.0, the extraction ratio was 92.9% for DPH and 81.2% for HDPH. In contrast, the extraction ratio was 92.2% for DPH and 95.3% for HDPH at neutral pH adjusted with phosphate buffer of pH 6.8; thus the extraction efficacy is excellent at neutral pH. We decided therefore to make the solution neutral (pH 6.8) at the time of extraction.

Before experiments on the recovery of added DPH and HDPH, the influence of urine components on the HPLC separation when performing the present extraction method was examined. Internal standard was added to the control urine and to healthy human urine and mixed; thereafter, each mixture was transferred to the top of an Extrelut® pre-packed column and extraction

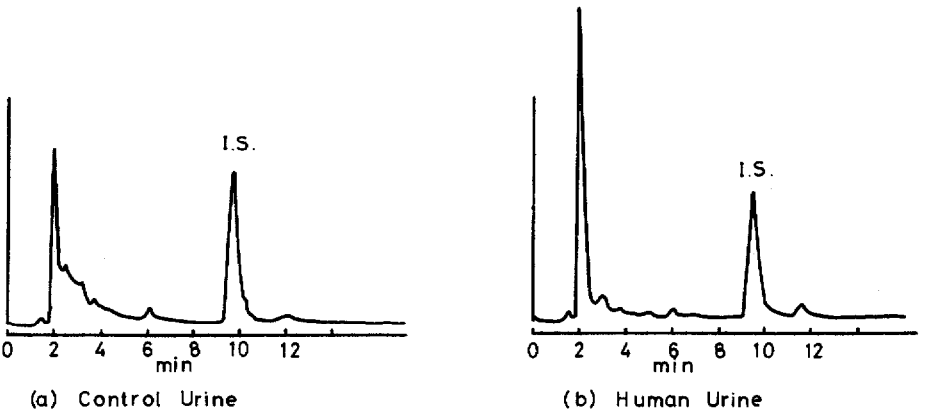


Fig. 1. HPLC of 5-(4-methylphenyl)-5-phenylhydantoin (internal standard, I.S.) added to control urine (a) and healthy human urine (b).

of these compounds was made (blank). The results of HPLC after using this procedure are shown in Fig. 1. As is seen in the chromatogram, a peak was observed at the position of HDPH (3.2 min) in the control urine, whereas the influence of this peak was small in the human urine. Therefore, for control urine, the ratio of this peak to the internal standard was subtracted, and the remainder taken as the amount of HDPH. As a result of this procedure, both DPH and HDPH were separated from human urine without interference by urine components.

We then performed experiments on the recovery of DPH and HDPH added to water, control urine and healthy human urine. As shown in Table I, the recovery was 98.5—107.7% for DPH in the range of 3.0—15.0 μg, and 89.2—107.6% for HDPH in the range 0.41—12.00 μg. On the other hand, the recovery for healthy human urine to which several comedicated drugs were added, was 92.2—93.8% for DPH and 95.3—118.9% for HDPH. Therefore, the results were reasonably satisfactory though the results for HDPH against water were slightly low. On the basis of these results, the quantitative analysis of DPH and HDPH was considered to become possible.

The limits for reliable quantitation of DPH and HDPH were 1.0 μg for DPH and 0.4 μg for HDPH.

The next examination concerned the influence of other antiepileptic agents administered concomitantly with DPH on the quantitative analysis of DPH and HDPH. Acetazolamide, phenobarbital, carbamazepine and diazepam were tested as the concomitant drugs. They were added to healthy human urine to which DPH, HDPH and internal standard were already added, and extraction was made using the Extrelut® column. It was found that DPH and HDPH were both separated by HPLC without interference from the added drugs (Fig. 2).

In the next step, to examine the possible application to the clinical field, the influence of the concomitant drugs on the amounts of DPH and HDPH excreted in the urine was examined using patients admitted to the hospital. Similarly to the quantitative analysis of DPH and HDPH described previously, the simultaneous analysis of acetazolamide, phenobarbital, carbamazepine

TABLE I

ANALYTICAL RECOVERIES OF DPH AND HDPH ADDED TO WATER, CONTROL URINE AND HEALTHY HUMAN URINE

| | HDPH | | | | DPH | | | |
|---|---|---|---|---|---|---|---|---|
| | Added (μg) | Found (μg) | Recovery (%) | C.V. (%) | Added (μg) | Found (μg) | Recovery (%) | C.V. (%) |
| Water | 0.61 | 0.54 ± 0.07 | 89.2 ± 11.2 | 12.6 | 6.0 | 6.4 ± 0.1 | 106.1 ± 1.4 | 1.4 |
| | 0.81 | 0.76 ± 0.03 | 93.7 ± 4.1 | 4.4 | 8.0 | 8.6 ± 0.1 | 107.7 ± 0.8 | 0.7 |
| Control urine | 0.41 | 0.36 ± 0.02 | 91.1 ± 4.7 | 5.2 | 6.0 | 6.2 ± 0.1 | 102.6 ± 0.9 | 0.9 |
| | 1.01 | 1.02 ± 0.02 | 101.8 ± 1.2 | 1.2 | 8.0 | 8.2 ± 0.2 | 101.9 ± 2.2 | 2.1 |
| Healthy human urine | 0.91 | 0.95 ± 0.05 | 104.0 ± 5.2 | 5.0 | 3.0 | 3.0 ± 0.2 | 101.7 ± 5.2 | 5.1 |
| | 2.12 | 2.13 ± 0.06 | 100.2 ± 2.8 | 2.8 | 6.0 | 6.1 ± 0.1 | 101.4 ± 2.5 | 2.4 |
| | 3.03 | 3.26 ± 0.06 | 107.6 ± 2.1 | 2.0 | 12.0 | 12.3 ± 0.1 | 102.8 ± 0.6 | 0.6 |
| | 12.00 | 12.50 ± 0.42 | 104.2 ± 3.5 | 3.4 | 15.0 | 14.8 ± 0.6 | 98.5 ± 3.9 | 4.0 |
| Healthy human urine* | 0.61 | 0.72 ± 0.04 | 118.9 ± 6.4 | 5.4 | 3.0 | 2.8 ± 0.1 | 93.8 ± 2.6 | 2.8 |
| | 1.52 | 1.44 ± 0.03 | 95.3 ± 2.3 | 2.4 | 6.0 | 5.5 ± 0.1 | 92.2 ± 1.2 | 1.3 |
| | 2.12 | 2.18 ± 0.03 | 102.9 ± 1.5 | 1.4 | 9.0 | 8.3 ± 0.1 | 92.2 ± 0.6 | 0.7 |
| | 3.00 | 3.35 ± 0.06 | 111.7 ± 2.3 | 2.1 | 12.0 | 11.2 ± 0.1 | 93.5 ± 0.1 | 0.7 |
| | 7.33 | 8.27 ± 0.03 | 112.8 ± 0.4 | 0.4 | | | | |
| | 14.66 | 16.10 ± 0.42 | 109.8 ± 2.9 | 2.6 | | | | |

*These results show the values obtained by the addition of 40 μg of acetazolamide, 80 μg of carbamazepine, 80 μg of diazepam and 1.6 mg of phenobarbital as concomitant drugs of DPH and HDPH to 1 ml of healthy human urine. Following extraction and determination procedures were carried out as described, and 5 μl of the extracts (100 μl) were injected for HPLC.

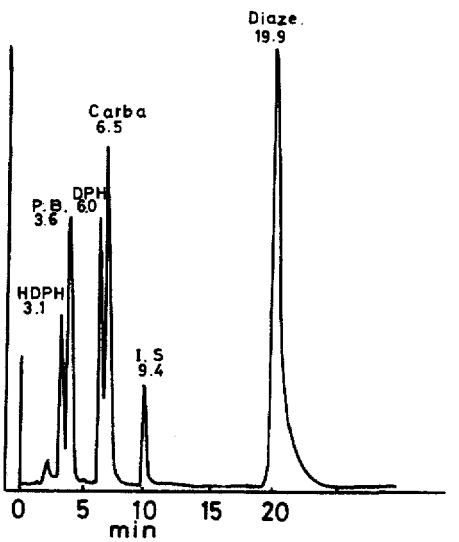


Fig. 2. HPLC of DPH, HDPH, acetazolamide, carbamazepine, diazepam and phenobarbital added to healthy human urine. I.S. = 5-(4-methylphenyl)-5-phenylhydantoin as internal standard. HDPH: 2.1 μg; DPH: 12 μg; I.S.: 2 μg; acetazolamide: 2 μg; carbamazepine (Carba): 4 μg; diazepam (Diaze.): 4 μg; phenobarbital (P.B.): 80 μg.

and diazepam with DPH and HDPH was tried. Firstly, with respect to each drug, the ratio of the area under the peak to that of 2 μg of internal standard was measured and the analytical curves were constructed (acetazolamide: $Y = 3.550X - 0.422$, $r = 0.995$; phenobarbital: $Y = 0.058X - 0.037$, $r = 0.990$; carbamazepine: $Y = 1.447X + 0.011$, $r = 1.000$; diazepam: $Y = 5.259X - 0.503$, $r = 0.990$).

Secondly, experiments on the recovery of the drugs added to water and freshly obtained healthy human urine were carried out. The recovery from healthy human urine was 83.3—88.3% for carbamazepine in the range 1.34—5.35 μg and 78.4—88.0% for diazepam in the range 2.58—10.30 μg, which was satisfactory, and thus the quantitative analysis of these drugs was possible. However, acetazolamide was not extracted by the organic solvent in the Extrelut® column, and was considered to remain adsorbed on the column. In addition, the extraction ratio of phenobarbital was low. Therefore, acetazolamide and phenobarbital did not interfere in the quantitative analysis of DPH and HDPH when phenobarbital was added to human urine in the usual dose.

Thirdly, DPH, HDPH and the concomitant drugs carbamazepine and diazepam were analyzed by the present method in the urine of nine patients 25—68 years old of both sexes admitted in the Department of Psychiatry and Department of Neurology who were administered carbamazepine concomitantly with DPH. Most of the main metabolites of HDPH are excreted in the urine as glucuronides [4—9]. Before hydrolyzing the glucuronides, the stability of DPH and HDPH was examined for the hydrolysis conditions by the method of Dykeman and Ecobichon [3]. To 1.0 ml of water, 150 μg of DPH, 60 μg of HDPH and 1.0 ml of 12 *N* hydrochloric acid were added. The mixture was boiled at 90°C for 120 min. The recoveries of DPH and HDPH were 104.3% and 115.2%, respectively. On the other hand, the recoveries without the boiling procedure at 90°C for 120 min were 108.7% and 109.2%, respectively. These data show that DPH and HDPH were stable at 90°C in 6 *N* hydrochloric acid (12 *N* HCl 1.0 ml + $H_2O$ 1.0 ml).

The quantitative analytical method described above was performed after hydrolyzing the urine according to the method of Dykeman and Ecobichon [3]. The chromatograms of the urine of patients after DPH administration are shown in Fig. 3. It was found that the amount of HDPH excreted in the urine was small in the patients concomitantly administered carbamazepine compared to the patients without administered carbamazepine, the difference being statistically significant ($P < 0.05$). With respect to the reason for this, it was speculated first that the amount of HDPH, which is a metabolite of DPH, became smaller because the amount of DPH administered was smaller by about 100—150 mg due to the concomitant administration of carbamazepine, and, secondly, that the oxidation enzyme which converts DPH to HDPH acted increasingly on carbamazepine, leading to a decrease in the effect on DPH, which might result in a decrease in the amount of HDPH. The amount of DPH excreted in the urine was not different between the groups treated with carbamazepine and those without carbamazepine, independent of the dose of DPH. Therefore, it was suggested that the administration of carbamazepine might have some influence on the metabolism of DPH to HDPH.

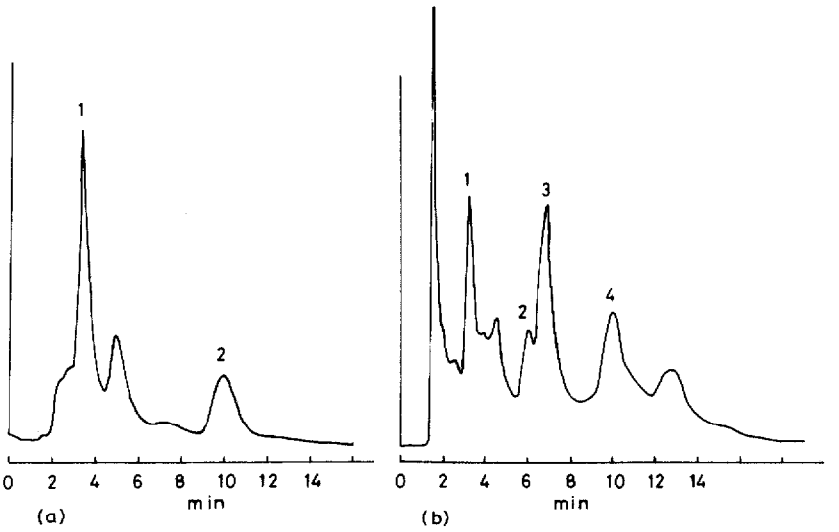


Fig. 3. HPLC of epileptic urine after anticonvulsant administration. (a) 150 mg of DPH were administered a day; peaks: 1 = HDPH, 2 = internal standard. (b) 100 mg of DPH and 600 mg of carbamazepine were administered a day; peaks: 1 = HDPH, 2 = DPH, 3 = carbamazepine, 4 = internal standard.

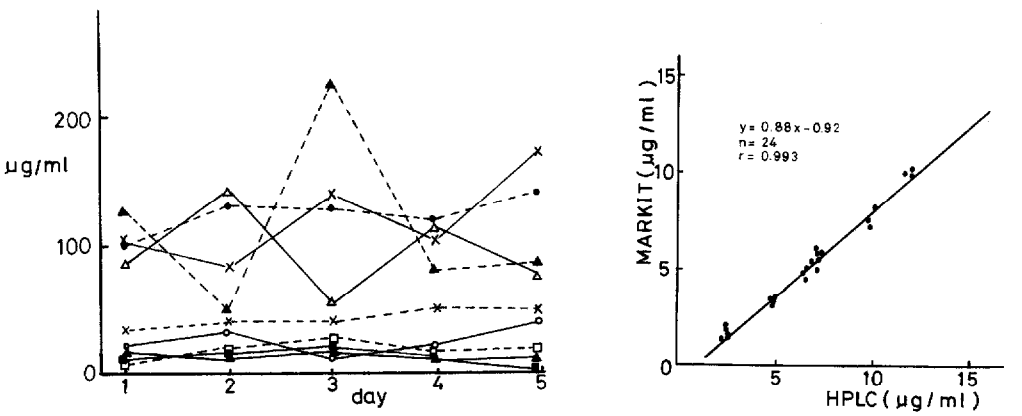


Fig. 4. Concentration of HDPH in urine of epileptics receiving DPH and other drugs. (▲ — — — ▲) DPH 150 mg; (▲———▲) DPH 100 mg, carbamazepine 600 mg; (△———△) DPH 250 mg; (× — — — ×) DPH 250 mg, carbamazepine 300 mg; (×———×) DPH 250 mg; (○———○) DPH 150 mg, carbamazepine 400 mg; (● — — — ●) DPH 200 mg; (□ — — — □) DPH 100 mg, carbamazepine 600 mg; (■———■) DPH 150 mg, carbamazepine 600 mg.

Fig. 5. Correlation between Markit® and HPLC determinations of DPH in human urine.

When 300—600 mg of carbamazepine and 100—250 mg of DPH were administered, the amounts of DPH, HDPH and carbamazepine were 0—229.9 μg/ml, 1.0—50.1 μg/ml, and 0.5—38.1 μg/ml, respectively. When 150—300 mg of DPH were administered without concomitant carbamazepine, the amounts of DPH and HDPH were 0—56.4 μg/ml and 50.5—227.6 μg/ml, respectively (Fig. 4). When comparing the results for the blood concentration of DPH and HDPH we reported previously, carbamazepine administration did not

produce a difference in the blood concentration of HDPH, but produced a clear difference in the amount of HDPH in the urine.

For comparing the present method with the other methods, we used the commercially available DPH assay kit which is based on the enzymatic immunoassay method. At first, the experiments on the recovery of DPH added to healthy human urine with concomitant drugs were performed using the kit and the same sample as for the present method. As a result, the values measured by the kit were slightly lower in the range 2.67—13.19 μg/ml, but an extremely high correlation was observed between the values obtained with the kit and by the present method, the correlation coefficient being $r = 0.993$ (Fig. 5). Therefore, the kit method was considered to be applicable to the quantitative analysis of urinary DPH, and it was applied to the analysis of patients' urine and compared with the results obtained by the present method utilizing HPLC (Table II). However, the DPH value assayed by the present method is slightly higher than that obtained with the kit. This phenomenon can be attributed to the small amount of urinary constituents, as shown at the retention time of DPH (6.0 min) in Fig. 1.

TABLE II

URINARY DPH LEVELS IN PATIENTS TAKING DPH

| No. | Initials | Age | Sex | DPH taken (mg) | Markit® (μg/ml) | Column + HPLC (μg/ml) |
|---|---|---|---|---|---|---|
| 1 | F.S. | 68 | f | 150 | 23.4 | 44.3 |
| 2 | Y.N. | 42 | f | 150 | 13.2 | 23.4 |
| 3 | A.O. | 46 | f | 250 | 17.8 | 29.8 |
| 4 | R.K. | 25 | f | 250 | 25.0 | 49.8 |
| 5 | I.N. | 37 | f | 100 | 5.1 | 17.2 |
| 6 | K.T. | 43 | f | 250 | 23.0 | 56.4 |
| 7 | C.N. | 47 | f | 200 | 14.2 | 27.5 |
| 8 | S.K. | 26 | f | 100 | 0.56 | 0.5 |
| 9 | S.K. | 43 | m | 300 | 11.0 | 11.0 |

From these results, the present method was found to be useful for urine analysis in addition to the measurements of the blood DPH. The advantages of the present method are as follows. Solvent extraction is simply performed by adsorption on the Extrelut® column, and the urinary components can be excluded. Concomitant drugs do not interfere with the measurements, and DPH and HDPH can be analyzed simultaneously; moreover, the quantitative analysis of the concomitant drug carbamazepine can be made simultaneously. In addition, the time for the measurements with HPLC is less than 25 min. Therefore, the present method can be considered to be a simple and rapid method with a high extraction ratio; by this method, microanalysis of an antiepileptic drug, DPH, and its metabolite, HDPH, becomes possible.

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