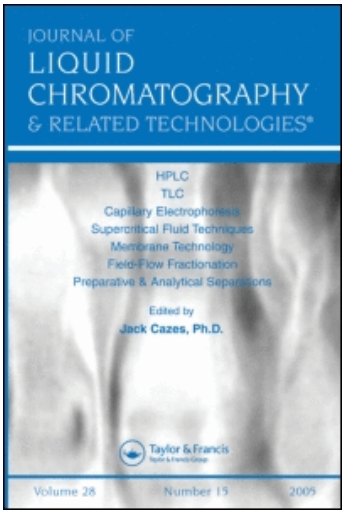

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Comparison of a GC Method and Two HPLC Methods for the Determination of p-Hydroxyphenytoin in Urine

U. Juergens[a]; T. May[a]; B. Rambeck[a]
[a] Department of Biochemistry, Gesellschaft fuer Epilepsieforschung Bethel, Bielefeld-13, Federal Republic of Germany

To cite this Article Juergens, U. , May, T. and Rambeck, B.(1986) 'Comparison of a GC Method and Two HPLC Methods for the Determination of p-Hydroxyphenytoin in Urine', Journal of Liquid Chromatography & Related Technologies, 9: 13, 2921 — 2936
To link to this Article: DOI: 10.1080/01483918608074159
URL: http://dx.doi.org/10.1080/01483918608074159

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(13), 2921-2936 (1986)

COMPARISON OF A GC METHOD AND TWO HPLC METHODS FOR THE DETERMINATION OF p-HYDROXYPHENYTOIN IN URINE

U. Juergens, T. May, and B. Rambeck

Department of Biochemistry
Gesellschaft fuer Epilepsieforschung
Bethel, Maraweg 13
D-4800 Bielefeld-13
Federal Republic of Germany

ABSTRACT

Three chromatographic methods for determining p-hydroxyphenytoin (p-HPT) in urine were compared: (1) GC with derivatisation of the samples, (2) HPLC after extraction with ethyl acetate and (3) HPLC using a column switching system for direct injection of samples. In all three methods the p-HPT glucuronides were first hydrolysed using concentrated mineral acid at boiling point. For method (1) the acidic hydrolysate was adjusted to pH 7-8.5. Benzene-tetrahydrofuran was used for extraction of p-HPT. The extract was evaporated to dryness, taken up in trimethylaniliniumhydroxide and injected. For method (2) the acidic hydrolysate was buffered with tri-sodium phosphate. An aliquot of the buffered solution was extracted with ethyl acetate. The extract was evaporated to dryness, taken up in methanol and injected. For method (3) the hydrolysate was diluted with water/acetonitrile (9:1), centrifuged and directly injected onto the pre-column for the sample washing step.

 0148-3919/86/0913-2921$3.50/0

INTRODUCTION

The determination of p-hydroxyphenytoin (p-HPT) in urine enables the physician to check on the compliance of epileptic patients on phenytoin (PT) therapy [1].

Compliance - the extent to which a patient takes the prescribed medication - can hardly be judged alone from the PT concentrations in serum. Even several comparable PT concentrations are, of themselves, not a proof of reliable drug intake. This is because some patients consistantly forget to take a certain dose, e.g. the midday dose. As an alternative, and complementary, to the determination of the serum concentration, the estimation of the main metabolite of PT, p-HPT, in the urine offers a means of monitoring compliance [1]. This is possible because PT is mainly excreted in the urine as p-HPT glucuronide and only to a very small extent as PT itself or other metabolites.

A number of articles concerned with the analysis of p-HPT in urine using high-performance liquid chromatography (HPLC) have been published in recent years [2-9]. Chow et al. [6] used an enzymatic method for splitting of the p-HPT glucuronide. The other authors describe hydrolysis at boiling point with mineral acid.

Kieselguhr was used by SATO et al. [9] for the extraction of p-HPT from the reaction solutions. The substances adsorbed on the kieselguhr were eluted with diethylether and chloroform. Liquid-liquid extraction using a water immiscible solvent was described in the other seven papers. Kabra and Marton [2] used a mixture of ethyl

acetate and dichloroethane. Inaba and Brien [3], Dykeman and Ecobichon [5] and Chow et al. [6] used ethyl acetate, Hermansson and Karlen [4], and Sawchuk and Cartier [8] diethy lether, and Couri et al. [7] a mixture of chloroform and 2-propanol. The analysis of p-HPT in urine was routinely carried out in our laboratory using gas chromatography (GC) [1].

In 1983 we developed an HPLC method using an automatic pre-column switching device for pre-treating the samples [10]. This method has been further improved for routine analyses. The time for one chromatographic separation has been reduced from about 20 to 10 minutes. Care was, however, taken to avoid overlapping of the p-HPT peak with other antiepileptic drugs (AEDs) and their metabolites (MBs) in the chromatograms (see table 1).

MATERIALS

Chemicals

The chemicals p-HPT, m-HPT, p-HPB, and MPPH were obtained from Aldrich, Steinheim (FRG), DIOL from Ciba-Geigy, Basle (Switzerland), and the other AEDs and MBs from Desitin-Werk/Karl Klinke, Hamburg (FRG). Acetonitrile "ChromAR"(R) was obtained from Promochem, Wesel (FRG) and water "for use in HPLC" from Baker Chemicals, Deventer (The Netherlands).

All other chemicals were of analytical reagent grade and were obtained from Merck, Darmstadt (FRG).

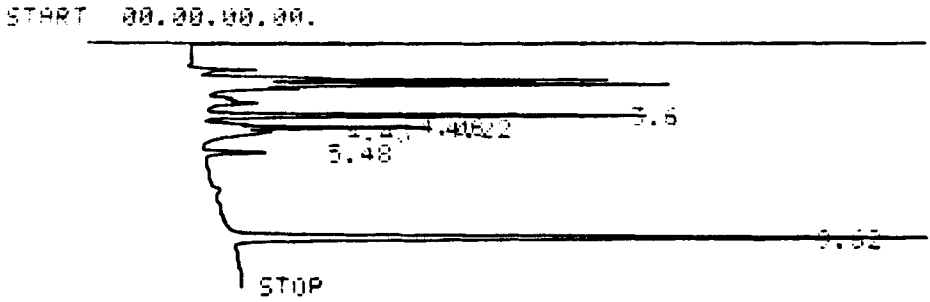

FIGURE 1 HPLC chromatogram of a patient sample with 153 ug/ml p-HPT (column switching method). RT: 3.60 p-HPT, 9.62 MPPH (ISTD).

TABLE 1

Relative Retention Times of Antiepileptic Drugs and Metabolites using the Column Switching Method

RT rel	substance
0.266	p-hydroxyphenobarbital (p-HPB)
0.276	2-ethyl-2-phenyl malonediamide
0.319	ethosuximide
0.328	primidone
0.334	10,11-dihydro-10,11-dihydroxy carbamazepine (DIOL)
0.390	p-hydroxyphenytoin (p-HPT)
0.425	m-hydroxyphenytoin (m-HPT)
0.488	phenobarbital
0.549	N-desmethylmethsuximide
0.572	carbamazepine-10,11-epoxide
0.776	phenytoin (PT)
0.847	carbamazepine
1.000	5-(p-methylphenyl)-5-phenylhydantoin (MPPH)*

* = internal standard (ISTD)

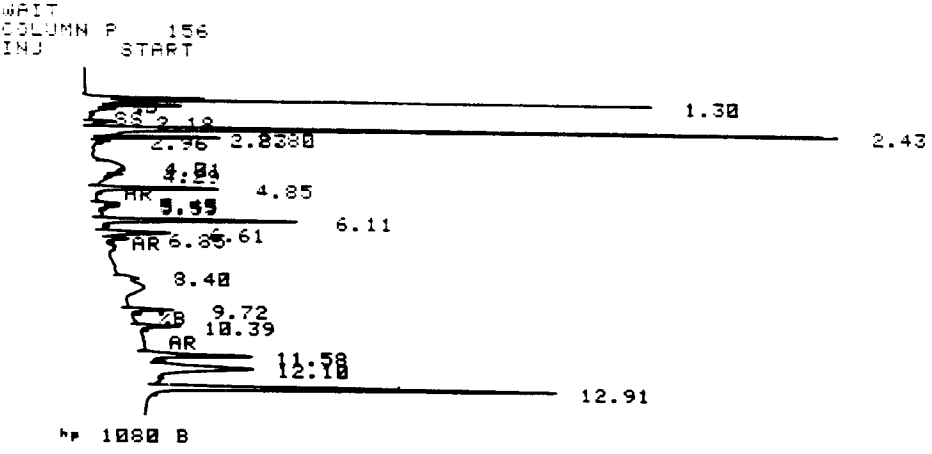

FIGURE 2 HPLC chromatogram of a patient sample with 149 ug/ml p-HPT (ethyl acetate extraction). RT: 6.11 p-HPT, 12.91 MPPH (ISTD).

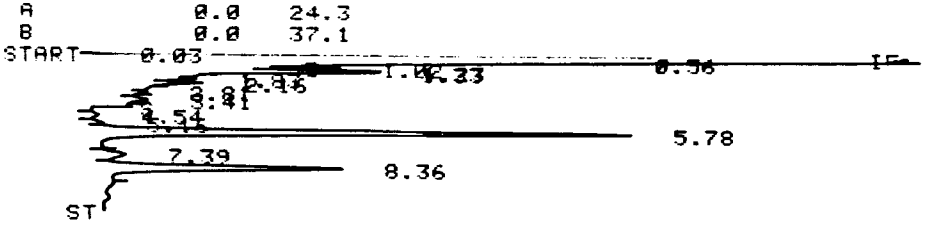

FIGURE 3 GC chromatogram of a patient sample with 203 ug/ml p-HPT. RT: 5.78 MPPH (ISTD), 8.36 p-HPT.

Apparatus

The automatic pipetter/diluter was obtained from Corning/Gilford, Duesseldorf (FRG), the TCS metal block thermostat (ambient temperature to 120°C) from Barkey Labortechnik, Bielefeld (FRG), the Rotixa-K centrifuge from Bender and Hobein, Karlsruhe (FRG), the Vortex mixer/evaporator from Buchler Instruments, Searle Analytic Inc., Fort Lee, NJ (USA). The gas chromatograph, model 5840, fitted with a flame ionization detector and an autosampler, was obtained from Hewlett-Packard, Waldbronn (FRG). The silanized glass columns (length 900 mm, 2.5 mm I.D.) were packed with 3% OV-17 on Chromosorb GHP. The liquid chromatograph HP 1084 B was obtained from Hewlett-Packard, Waldbronn (FRG), the HPLC low-pressure gradient-former 2500, two HPLC constant-flow pumps 600/200, the column-switching module SE-1, the spectro-photometer SP-4, and the printer-plotter-integrator C-R 1B from Gynkotek, Munich (FRG), the autosampler WISP 710 B from Waters/-Millipore, Eschborn (FRG), the analytical HPLC column (length 250 mm, 4.6 mm I.D.), filled with Shandon Hypersil(R) ODS (5 µm), from Grom, Ammerbuch (FRG). The precolumns (length 40 mm, 4.6 mm I.D.) were dry filled with Nucleosil(R) 30-C-18 (30 µm, spherical) from Macherey/-Nagel, Dueren (FRG).

METHODS

Pre-treatment and Storage of the Patient Urines

From each of the urine samples received in our laboratory 500 µl portions were taken immediately, during

constant stirring, and transferred to 10-ml centrifuge tubes. The tubes were closed with screw caps and the samples frozen at -18°C. This procedure was shown to be necessary, because on allowing the urines to stand, considerable precipitation occurs in a short time. An investigation showed that the concentration of p-HPT was considerably higher in the precipitate than in the supernatant fluid [1].

Calibration and Control Samples

Calibration samples were made by adding 50 µl of a stock solution, containing 300 mg p-HPT in 100 ml acetone, to 500 µl of a blank urine. This blank urine was tested by all three methods to be free of interference with the p-HPT analysis.

In order to control the internal laboratory quality a pool urine from patient urines was mixed together and divided into 500 µl portions, in the same way as described for the patient urines. The calibration and control samples were frozen at -18°C.

Analysis of p-HPT by Gas Chromatography

To the thawed patient, calibration and pool urines (500 µl) a quantity of 100 µl internal standard (ISTD) solution (150 mg MPPH in 100 ml acetone) and 500 µl 12 N hydrochloric acide were added. The centrifuge tubes were closed with screw caps, shaken and heated at 100°C for 75 minutes. After cooling, 0.5 ml 12 N NaOH was added and the pH of the mixture was adjusted to 7.0-8.5 with 6 N HCl or 6 N NaOH. These titrated samples were mixed with 1 ml of

3 M Tris buffer (tris(hydroxymethyl)aminomethane with 6 N HCl to pH 7.5). The hydrolyzed p-HPT was extracted with 3 ml benzene/tetrahydrofuran (1:1, vol/vol). After centrifugation, 1 ml of the organic phase was evaporated off at 37°C, and 50 µl trimethyl anilinium hydroxide (0.2 M in methanol) was added. A 3 µl quantity of this solution was injected into the gas chromatograph.

The injection port and detector temperatures were 250°C and 260°C, respectively. The temperature program was set to give a rise from 230°C to 260°C at a rate of 5°C/min. The carrier gas was nitrogen with a flow of 35 ml/min.

Analysis of p-HPT by HPLC with Ethyl Acetate Extraction

The thawed samples (500 µl) were mixed with 100 ul of the ISTD solution (see above) and with 500 µl perchloric acid (70 Vol%), and heated to 100°C for 30 minutes. After cooling down the pH of the samples was adjusted to pH 2-5 with 7-8 ml of a saturated tri sodium phosphate solution. A 500 µl quantity was taken from the buffered samples and extracted with 3 ml ethyl acetate by shaking for 5 min. After centrifuging for 10 minutes, 1 ml from the organic phase was evaporated to dryness. The residue was dissolved in 100 µl methanol and 5 µl were injected.

Separation was carried out by gradient elution. The mobile phase was a mixture of acetonitrile and a phosphate buffer pH 4 (a solution of 0.01% phosphoric acid was adjusted to pH 4 using a saturated di sodium hydrogenphosphate solution). The gradient program was as follows:

start = 17.5% CH_3CN, min 1.5 = 17.5% CH_3CN%, min 10.0 = 40% CH_3CN. The flow rate was 2.0 ml/min, the column temperature 75°C, and the detector wavelength 215 nm.

Analysis of p-HPT by HPLC using a Column-Switching System

The thawed samples (500 µl) were mixed with 100 µl ISTD solution and 500 µl perchloric acid (see above) and heated to 100°C for 30 minutes. After cooling down the hydrolysate was diluted with 8 ml water/acetonitrile (9:1 v/v) and centrifuged for 10 minutes. From the supernatant fluid 30 µl were injected onto the pre-columns and washed for 2 minutes with 0.01% phosphoric acid (purge flow: 1.0 ml/min). The same purge liquid was used for the direct serum injection [11] and has the advantage of being more stable than pure water. The eluent mixtures for the gradient elution were the same as those used for the separations of the ethyl acetate extracts.
The gradient program was as follows: start = 25% CH_3CN, min 1.0 = 25% CH_3CN, min 8 = 40% CH_3CN. The flow rate was 1.5 ml/min, the column temperature 75°C, and the detector wavelength 205 nm.

RESULTS AND DISCUSSION

Range of Linearity of the three compared Methods

At first it was checked in which concentration range the three methods for the analysis of p-HPT in urines were linear. For this purpose corresponding quantities from 10 to 150 µl of the stock solution (see Methods) were mixed

with 500 µl of a 0.35 M urea solution (to pH 5 with HCl) and analysed by GC and the two HPLC methods. The urea solution was used to avoid interference with endogenous compounds which are found even in blank urines. A normal calibration sample (300 µg p-HPT/ml) was used for these measurements. The theoretical values, the values found, and their correlation are given in Table 2.

The data in Table 2 and the correlation with the theoretical values for the three methods show that there is sufficient linearity in the concentration range tested with the clean urea solution. In spite of the high coefficient of correlation, it is, however, to be noted that large random deviations from the theoretical values can occur in the GC method.

The data, furthermore, indicate a slight tendency to underestimate the higher p-HPT concentrations.

TABLE 2

Check of the Linearity of the three Methods

MSS = microliters of stock solution, THV = theoretical value [µg/ml], EX = ethyl acetate extraction, DI = direct injection with column-switching, X = values found [µg/ml], X% = values found expressed as a percentage related to the theoretical value, r = coefficient of correlation

		GC		EX		DI	
MSS	THV	X	X%	X	X%	X	X%
10	60	74.3	123.8	59.6	99.3	59.0	98.3
25	150	154.4	102.9	150.2	101.1	151.1	100.7
50	300	305.3	101.8	301.8	100.6	300.7	100.2
75	450	459.6	102.1	447.6	99.5	443.1	98.5
100	600	503.3	83.9	596.9	99.5	590.8	98.5
125	750	729.1	97.2	739.7	98.6	744.2	99.2
150	900	886.3	98.5	887.6	98.6	862.2	95.8
r		0.9933		0.9999		0.9996	

In spite of the linearity up to 900 µg/ml p-HPT in clean urea solutions it is recommended that patient urines which contain more than 500 µg/ml p-HPT should be diluted and re-analysed. This is because high p-HPT contents correspond normally to highly concentrated urines (e.g. morning urines). Therefore a base line separation between p-HPT and interfering compounds is not possible in those cases.

Reproducibility of the Calibration Samples

Four calibration samples were, as a rule, used for a series (GC or HPLC) of twenty patient samples. Five or six calibration samples were used for longer series. The following statistical parameters were calculated each time within the daily series of calibration samples: the standard deviation (SD), the coefficient of variation (CV%), the mean absolute deviation from the mean value (AD), and the corresponding coefficient of absolute variation (CAV%). The corresponding mean values (and standard deviations) of SD, AD, CV%, and CAV% - namely M(SD), M(CV%), M(AD), and M(CAV%) - for the 22 daily series were then calculated for each of the analytical methods.

The reproducibility of the the GC calibration runs is not as good as that of the HPLC calibration runs, as seen from Table 3. This agrees with the number of outliers which had to be removed from the calculations of the daily series. A value for Q which deviated more than 10% from the mean value of Q in the daily series, was defined as an outlier.

TABLE 3

Reproducibility of the Urine Calibration Samples

Q = quotient of amount ISTD/amount of p-HPT in the calibration samples (all mean values for the 22 series)

method	GC	EX	DI
Q	409.2 ± 45.6	344.6 ± 26.1	310.2 ± 13.3
M(SD)	13.0 ± 5.9	5.1 ± 2.8	3.0 ± 2.3
M(AD)	11.6 ± 5.5	4.4 ± 2.3	2.6 ± 1.8
M(CV%)	3.1 ± 1.3	1.5 ± 0.8	1.0 ± 0.7
M(CAV%)	2.8 ± 1.3	1.3 ± 0.7	0.8 ± 0.6

This was the case in the GC method in 9 out of 88 calibration runs (= 10.2%), in 4 out of 86 runs in the ethyl acetate extraction (= 4.7%), and in 2 out of 89 runs in the direct injection method (= 2.3%). The errors of the direct injection are possibly due to a gas bubble arising in the tube of the dispenser during the production of the calibration samples. Other additional errors, which arise during the extraction procedures, were possible causative factors in the other two methods.

As the reproducibility of the calibration runs on pre-column switching with direct injection of the samples was the best, this method has been chosen as the reference method for comparing the other two methods in the discussion later on.

Precision of the Duplicates

The analysis of p-HPT in the urines of the patients was carried out in duplicates. In order to control the reproducibility of the three methods during the day and

TABLE 4

Precision of the Duplicates in Patient and Pool Urines

N = number of duplicates, M(Δ A) and M(Δ %) [ug/ml]

method	patient urines N	M(Δ A)	MD%	pool urines N	M(Δ %)	MD%
GC	154	14.12	3.54	19	6.03	1.39
EX	154	10.69	2.56	19	2.67	0.63
DI	154	7.62	1.95	19	2.42	0.56

TABLE 5

Reproducibility during the day and from day to day

X = p-HPT concentrations [µg/ml]

method	N	M(X) ± SD(X)	range	CV%
Within-day precision:				
GC	12	204.45 ± 8.40	193.3 - 224.2	4.11
EX	12	216.30 ± 2.20	213.2 - 221.1	1.02
DI	12	219.65 ± 1.61	217.3 - 222.2	0.73
Day-to-day precision:				
GC	19	211.04 ± 13.18	189.4 - 241.1	6.24
EX	19	212.93 ± 5.76	203.7 - 223.2	2.71
DI	19	217.36 ± 8.79	207.7 - 234.9	4.04

from day to day, from the remaining urines of the patients a pool urine was mixed together (see Methods) and also analysed in duplicates. For each double determination the absolute diference of the duplicate Δ A and the percentage difference Δ % in relation to the mean value of the duplicates were calculated. The mean values of Δ A and Δ %, namely M(Δ A) and M(Δ %), are given in table 4.

TABLE 6

Agreement of the three methods compared

Y = b * X + a; X and Y [µg/ml]
Y = p-HPT concentration according to the DI method,
X = p-HPT concentration according to the GC or the EX method, b = slope of the regression line, a = intercept, SEE = standard error of estimate

method	N	M(X) ± SD(X)	b	a	r	SEE
GC	154	210.43 ± 116.66	0.960	12.576	0.989	17.41
EX	154	210.88 ± 123.81	1.060	-7.755	0.995	13.23
DI	154	206.18 ± 116.23		(reference method)		

Reproducibility of the Pool Urine Values

In order to determine the reproducibility during the day, 12 samples of the pool urine for each of the three methods were thawed out and analysed. For the day-to-day control the urine samples were analysed in duplicate in addition to each series of patient samples (see Table 5).

The Agreement of the Results of the three Methods

A linear regression analysis, using the direct injection as the reference method, was carried out in order to check if systematic differences in determining p-HPT occurred between the three methods. The mean values of the patient samples and the results of the regression analyses (see Table 6) show, that the GC as well as the EX method give only negligible deviations from the DI method. In the GC method, nevertheless, a larger random deviation (SEE = 17.41) can be observed than in the EX method (SEE = 13.23).

CONCLUSION

The results of the comparison of the three analytical methods tested show that in principle all three methods are suitable for the routine determination of p-HPT in the urines of patients with phenytoin therapy. In the GC method, nevertheless, more random errors are observed than in the HPLC methods.

The HPLC method in which samples are directly injected into a column switching system, which enables automatic pre-treatment by pre-columns, is superior to the compared methods in two respects: the results are the most accurate and it is simpler to carry out.

The adjustment of the pH is in the GC method the most time consuming step. This is then followed by several sample handling steps. In the case of column switching, on the other hand, the samples can be directly injected into the precolumn after a simple dilution step.

ACKNOWLEDGEMENTS

The authors are greatly indepted to Mr. G.S. Macpherson, B.Sc., M.A., for translating the paper and to Mrs. G. Kunert for skilled technical assistance.

REFERENCES

1. Rambeck B. and May T.; Urinary hydroxyphenytoin/-creatinine ratios as an index of compliance in adult epileptic patients on phenytoin therapy. Ther. Drug Monit. 6, 164-172 (1984).

2. Kabra P.M. and Marton L.J.; High-pressure liquid-chromatographic determination of 5-(4-hydroxyphenyl)-5-phenylhydantoin in human urine. Clin Chem. 22, 1672-1674 (1976).

3. Inaba T. and Brien J.F.; Determination of the major urinary metabolite of diphenylhydantoin by high-performance liquid chromatography. J. Chromatogr. 80, 161-165 (1973).

4. Hermansson J. and Karlen B.; Assay of the major (4-hydroxylated) metabolite of diphenylhydantoin in human urine by reversed-phase high-performance liquid chromatography. J. Chromatogr. 130, 422-425 (1977).

5. Dykeman R.W. and Ecobichon D.J.; Simultaneous determination of phenytoin, phenobarbital and their para-hydroxylated metabolites in urine by reversed-phase high-performance liquid chromatography. J. Chromatogr. 162, 104-109 (1979).

6. Chow S.A., Charkowski D.M. and Fischer L.J.; Separation of phenytoin and its metabolites by high-performance liquid chromatography. Life Sci. 27, 2477-2482 (1980).

7. Couri D., del Villar C.S. and Toy-Manning P.; Simultaneous quantitative assay of phenobarbital, phenytoin, and p-hydroxyphenylphenylhydantoin by HPLC and a comparison with EMIT(R). J. Anal. Toxicol. 4, 227-231 (1980).

8. Sawchuk R.J. and Cartier L.L.; Liquid-chromatographic method for simultaneous determination of phenytoin and 5(-4-hydroxyphenyl)-5-phenylhydantoin in plasma and urine. Clin. Chem. 26, 835-839 (1980).

9. Sato A., Shimada K., Izumo Y. and Sakaguchi T.; High-performance liquid chromatographic determination of phenytoin and hydroxyphenytoin in human urine. J. Chromatogr. 275, 97-105 (1980).

10. Juergens U.; Routine determination of hydroxyphenytoin in urine by high-performance liquid chromatography using an automatic column-switching technique. J. Chromatogr. 275, 335-343 (1983).

11. Juergens U.; Routine determination of eight common anti-epileptic drugs and metabolites by high-performance liquid chromatography using a colmun-switching system for the direct injection of serum samples. J. Chromatogr. 310, 97-106 (1984).