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ROUTINE DETERMINATION OF HYDROXYPHENYTOIN IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AN AUTOMATIC COLUMN-SWITCHING TECHNIQUE

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SUMMARY

A high-performance liquid chromatographic method for the determination of the main phenytoin metabolite, hydroxyphenytoin, in the urine of epileptic patients is described. The use of an automated column-switching technique greatly simplifies the pretreatment steps. Thereby, both time and chemicals are saved. The possibility of error arising during the several pretreatment steps is considerably reduced. Following acid hydrolysis of the hydroxyphenytoin glucuronic acid conjugate the sample is diluted with water and after centrifugation is injected onto the pre-column. After washing for a short time with water, the substances which were absorbed on the head of the pre-column were backflushed with water—acetonitrile as eluent onto the analytical column. Separation is achieved by gradient elution using an ODS reversed-phase column with a particle size of 5 μm.

INTRODUCTION

The determination of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) in the urine of patients treated with the anticonvulsant drug phenytoin (5,5-diphenylhydantoin, DPH) offers a means of examining the elimination of this drug as well as assessing whether or not the drug has been taken by the patient.

High-performance liquid chromatography (HPLC) is, next to gas—liquid chromatography, of prime value for carrying out such analyses, because derivatization of drugs can be dispensed with using HPLC. Several HPLC methods for the estimation of p-HPPH in body fluids have, therefore, already been described [1—11]. These methods, however, have some disadvantages.

Using ion-exchange columns the samples (serum) can be injected directly into the column [2]. However, comparing the results using modified silica gel the quality of separation is poor (band broadening) and it takes a very

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long time to carry out a single analysis. The other methods require a number of different steps in preparing the sample. For the determination of p-HPPH in urine these include at least the following steps: (1) Hydrolysis of the sample by heating with concentrated hydrochloric acid or enzymatic cleavage of the p-HPPH glucuronide. (2) Neutralisation of the hydrolysate. (3) Extraction of the drugs and metabolites with an organic solvent. (4) Centrifugation. (5) Evaporating off the organic phase. (6) Taking up the residue with methanol or with the mobile phase of the subsequent chromatographic separation.

METHODS

In recent years it appeared that the use of various column-switching techniques for organic trace analysis is becoming the method of choice in all fields of chemistry [12—14]. In 1981 Roth et al. [15] described a fully automated HPLC method for use in clinical chemistry. Sample pretreatment and chromatographic analysis are combined in one step. Thus, the body fluid (plasma, urine, or saliva) is injected directly onto a pre-column, and protein and other serum constituents are washed through the pre-column with a purge liquid. Following the washing step the retained drugs are carried over with an eluent using the back-flush technique, to the analytical column where the substances are separated.

Time required for analysis can be saved using two pre-columns which are charged alternately. An exact scheme needed for the connection of the valves is given in ref. 15.

Until recently, users of column-switching techniques had to construct their own systems. Since last year systems are commercially available which offer a complete range of possible ways of switching columns, e.g. in the wash-through, pre-separation or back-flush mode, and alternating stages for applying the sample and chromatographic separation. An example of such a construction is given in ref. 16, in which the arrangement is shown in greater detail. This system was used in developing the method as described in this paper.

In previous publications concentrated hydrochloric acid was used for the non-enzymatic hydrolysis of the glucuronide [3, 5, 6, 8—10]. However, to avoid possible corrosive effects of chloride ions in acidic medium on the steel parts of the HPLC apparatus (especially on the inner walls of the analytical columns) a non-oxidizing mineral acid was sought to replace hydrochloric acid.

Sulphuric acid (98%), phosphoric acid (85%) and perchloric acid (70%) were each mixed in a 1:1 ratio with urine and further processed under the conditions given in the experimental section. Only the addition of perchloric acid gave values which were comparable to those obtained with hydrochloric acid. The results of the analyses of 68 patient samples using, in each case, hydrochloric acid and perchloric acid, gave a coefficient of correlation of $r = 0.997$.

A further difficulty in determining p-HPPH in urine is that most of the patients examined here are treated with a mixture of various anticonvulsant drugs which, in the first place, very frequently includes phenobarbital (PB).

Thus a separation column must be found whose specificity enables a sharp separation to be made between p-HPPH, PB and m-HPPH which likewise occurs in significant amounts in the urine of patients. Errors in analysis of up to 40% could otherwise arise, especially with samples containing a low concentration of p-HPPH and a comparatively high concentration of PB.

Attempts using a μBondapak RP-18 column (10 μm), which was used in most of the earlier publications [3—8], gave the desired separation only on gradually increasing the gradient. Consequently the time needed for each analysis (elution of the internal standard MPPH takes about 36 min) is relatively high.

This separation problem could not be solved even using a μBondapak alkylphenyl column (10 μm) with which good separations could be obtained in analyzing flavour components, preservatives and drug residues [17—21].

In further tests only silica gels modified with ODS phases and with particle size of 5 μm were used (Hypersil, Zorbax, Spherisorb, Nucleosil) out of which Zorbax-ODS gave the best separations (see Fig. 1).

In addition, an attempt was made to reduce the time of analysis by substitut-

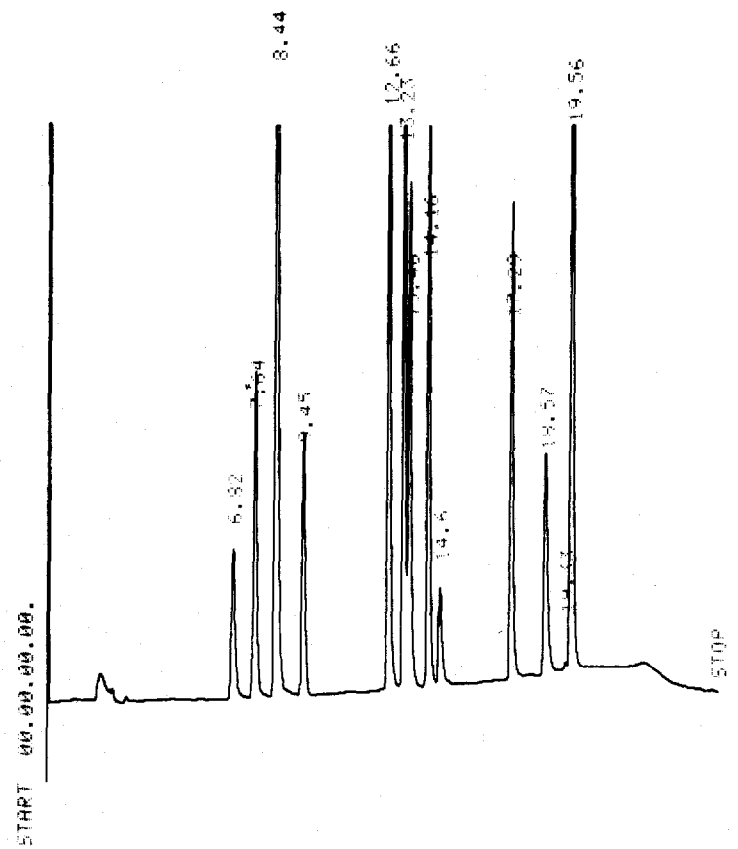

Fig. 1. Chromatogram of a methanolic stock solution of antiepileptic drugs, metabolites and internal standards. Retention times (min): PEMA 6.82, ET 7.64, HPB 8.44, PRI 9.45, p-HPPH 12.66, PB 13.23, m-HPPH 13.46, N-DES 14.16, C-EP 14.60, DPH 17.29, CBZ 18.57, MPPH 19.56.

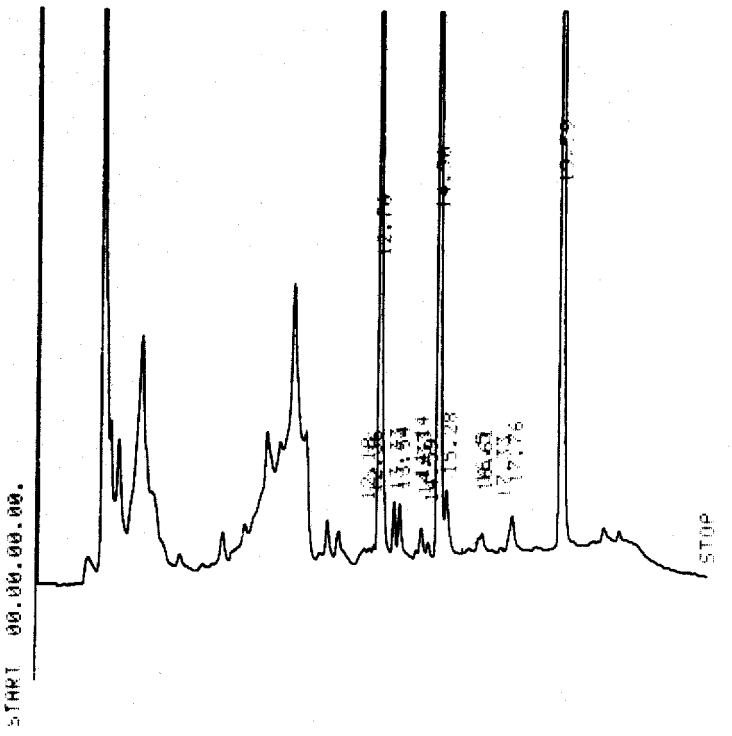

Fig. 2. Chromatogram of a patient sample. Retention times (min): p-HPPH 12.76, PB 13.33, m-HPPH 13.54, HPTH 14.98, MPPH 19.59.

ing the internal standard 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) by 5-(*p*-hydroxyphenyl)-5-(*p*-tolyl)-hydantoin (HPTH). The possible reduction in separation time of about 5 min (see Fig. 2) could not be achieved, because in the time range in which HPTH is eluted carbamazepine-10,11-epoxide (C-EP) and other interfering impurities are also eluted.

EXPERIMENTAL SECTION

Chemicals

Chemicals used were obtained from the following firms: p-HPPH, m-HPPH, MPPH, HPTH, 5-ethyl-5-(*p*-hydroxyphenyl)-barbituric acid (HPB), 2-ethyl-2-phenylmalonediamide (PEMA) and N-desmethylmethsuximide (N-DES) from EGA-Chemie, Steinheim, G.F.R. Phenobarbital (PB) from Bayer, Leverkusen, G.F.R. Carbamazepine (CBZ), carbamazepine-10,11-epoxide (C-EP), primidone (PRI), ethosuximide (ET) and diphenylhydantoin (DPH) from Desitin-Werk/ Carl Klinke, Hamburg, G.F.R.

Acetonitrile "for chromatography" (LiChrosolv) was from Merck, Darmstadt, G.F.R. Water "for use in HPLC" was from Baker Chemicals, Deventer, The Netherlands. All other chemicals were of analytical reagent grade and were obtained from Merck.

Apparatus

The equipment used was obtained from the following firms: Automatic pipetter/diluter from Corning/Gilford, Düsseldorf, G.F.R. TCS metal block thermostat (ambient temperature—120°C) from Barkey Labortechnik, Bielefeld, G.F.R. Rotixa/K centrifuge from Hettich, Tuttlingen, G.F.R. HPLC low-pressure gradient-former 2500, two HPLC constant-flow pumps 600/200, sample preparation Model SE-2, spectrophotometer SP-4, Shimadzu printer-plotter-integrator C-R 1B from Gynkotek, Munich, G.F.R. Autosampler WISP 710 B from Waters, Königstein/Taunus, G.F.R. Analytical column Zorbax-ODS (5 μm, 250 mm × 4.6 mm I.D.) from Du Pont, Frankfurt/Main, G.F.R. Pre-columns LiChrosorb RP-18 (10 μm, 40 mm × 4.6 mm I.D.) from Bischoff-Analysentechnik, Leonberg, G.F.R.

Chromatographic parameters

Temperature of the column: ambient temperature. Injection volume: 30 μl. Detection wavelength: 205 nm. Pre-column: eluent water, flow-rate 1.0 ml/min, duration of washing 200 sec. Analytical column: eluent water—acetonitrile (gradient elution), flow-rate 1.5 ml/min. In order to avoid degassing the solvents, water and acetonitrile were pre-mixed in a ratio 9:1 (v/v) for the container A and in a ratio 4:6 (v/v) for the container B.

The gradient programme was as follows:

Step	A (vol. %)	B (vol. %)	Acetonitrile (vol. %)	Time (min)
1	090	010	15	0.00
2	090	010	15	1.00
3	020	080	50	19.00
4	100	000	10	0.00
5	100	000	10	1.00
6	090	010	15	0.00
7	090	010	15	End

Calibration and control samples, internal standard

From a stock solution containing 300 mg of p-HPPH in 100 ml of acetone, a 50-μl quantity was made up each time to 500 μl using blank urine. This corresponds to 300 μg of p-HPPH per ml of urine in each calibration sample. About 1000 calibration samples, and for purposes of internal quality control 0.5-ml portions of a pooled patients urine, were frozen. These samples can be kept unchanged at about —18°C for long periods of time (at least six months).

For preparing the internal standard solution 150 mg of MPPH are dissolved in 100 ml of acetone.

Preparation of the sample

A 500-μl quantity of urine is pipetted each time into a 10-ml centrifuge tube with a screw cap. To each of these patients samples, as well as to the thawed-out samples for calibration, 100 μl of the internal standard solution and 500 μl of perchloric acid (70%) are added.

The samples are well mixed and heated at 100°C (± 0.5°C) for 30 min in a metal block heater. After cooling the samples are diluted with 3 ml of water. The insoluble constituents of the urine are centrifuged off and 30 µl of the supernatant fluid are injected.

A few hours later a small amount of precipitate is observed in the sample vials which, however, does not disturb the determination.

Lifetime of the column

A detectable increase in pressure of the analytical column was noted after about 800 injections. The filter frit at the head of the column was removed and cleaned in an ultrasonic bath. Apart from this 2 mm of the packing material, which had become coloured, was removed and replaced by a suspension of LiChroprep (40—63 µm, Merck) in isopropanol. In addition to the cleaned metal frit, the head of the column was plugged with a cellulose filter (Knauer, Berlin, G.F.R.). The effectiveness of the column has, following about further 300 injections, hitherto remained unchanged.

The pre-columns were, regardless of the pressure, changed after every 400—500 injections in order to keep contamination of the analytical column down to the smallest amount possible.

RESULTS AND DISCUSSION

To check whether the method described in this paper is suitable for routine laboratory purposes the following analytical parameters were examined: (1) Time required for hydrolysis of the samples. (2) Linearity of the determination. (3) Error in analysis.

The following statistical terms were used for the evaluations (abbreviations in parentheses): number of samples, injections or duplicates (n), maximum value (X_{max}), minimum value (X_{min}), mean value ($\bar{X}$), median (m), standard deviation (s), coefficient of variation (C.V. %), coefficient of correlation (r), mean value of the absolute differences of the duplicates $X-Y$ ($\bar{d}$), mean deviation from the mean value expressed as a percentage $100(X-Y)/(X+Y)$, ($\overline{dX}$ %).

Time for hydrolysis of the samples

Whereas 1 h was apparently needed for the complete hydrolysis of urine samples using concentrated hydrochloric acid [5], 30 min was sufficient time for the complete cleavage of the p-HPPH glucuronide using perchloric acid.

To test the stability of free p-HPPH and of the internal standards MPPH and HPTH under the experimental conditions described above, an aqueous—methanolic solution was treated with increasing boiling time. Thereby a slight decrease in concentration of these three substances even from 60 min onwards was observed. The HPTH constituent was changed the most rapidly, whilst MPPH was the most stable compound.

Linearity of the determination

A patient sample containing more than 500 µg/ml p-HPPH was diluted with blank urine to give percentage concentrations of 80, 50, 20, and 10. The

TABLE I

MEASURED AND CALCULATED VALUES OF THE LINEARITY CONTROL

Diluted sample	X (%), Y (μg/ml p-HPPH)				
	100	80	50	20	10
Measured Y	515.5	420.0	255.5	101.5	51.0
Calculated Y	518.5	414.4	258.3	102.2	50.1

original sample and the dilutions were measured in duplicate (Y) (Table I).

The regression equation of these data is $Y = 5.205X - 1.94$ having a correlation coefficient of $r = 0.9998$. It is thence concluded that the p-HPPH analysis, described in this paper, of the urines of patient samples which come within the usual concentration range is linear.

Analytical error

Deviations on multiple analysis of the same sample. To test what differences arise on preparing samples on the same day, 0.5-ml quantities of a homogeneous pool urine A were taken 53 times and analysed as described above. To evaluate the reproducibility of the p-HPPH determination over several days three samples of a pool urine B were each investigated on six consecutive days, and samples of a pool urine C over several months. The results are summarized in Table II.

TABLE II

REPRODUCIBILITY OF THE ANALYSES OF POOL URINES

Pool urine	X (μg/ml p-HPPH)						
	n	X_{max}	X_{min}	$\overline{X}$	m	s	C.V. (%)
A	53	244	226	233.1	232	4.34	1.86
B	18	210	195	202.6	204	5.17	2.55
C	59	319	284	299.8	300	9.90	3.30

Reproducibility of the double determinations. Hitherto only the analytical errors due to the variations in preparation of the same samples have been statistically considered. Moreover, the influences which arise due to different substances contained in each patient sample, and which affect each estimation, have not yet been considered.

It is, however, just the samples examined in the course of routine analyses which, seen apart from their content of DPH metabolites, can have a very different substance composition. For this reason it is of particular interest to the analyst to know the degree of reproducibility of each individual value with which he can reckon.

We obtained a measure of the range of error which is to be expected in

TABLE III

REPRODUCIBILITY OF THE DOUBLE DETERMINATIONS

Patient samples	X (μg/ml p-HPPH)					
	n	$\bar{X}_1$	$\bar{X}_2$	r	$\bar{d}$	$\overline{dX}$ (%)
Within-day	216	225.64	224.83	0.998	6.09	1.41
Day-to-day	315	226.86	224.57	0.992	11.61	2.75

the determination method described here by analysing in duplicate patients urine both during the day as well as after several days up to weeks later (frozen samples) (Table III).

CONCLUSIONS

The use of a column-switching technique which combines the separate steps, namely sample purification, concentration, and chromatographic separation, in one process enables a considerable saving both of chemicals and time in carrying out routine laboratory analyses. Following acid hydrolysis of the p-HPPH glucuronic acid conjugate it sufficed to dilute the sample and inject it after centrifugation directly onto the pre-column. It was thereby possible to reduce considerably the number of pretreatment steps mentioned above. Besides this the time required for splitting the p-HPPH glucuronide conjugate is reduced to 30 min, and possible corrosion of the HPLC apparatus by chloride ions in an acidic medium is avoided by replacing hydrochloric acid with perchloric acid.

With a mean value of 1.41 for the percental deviation from the mean value for the double determination within a single day, and of 2.75 for the double determination within a time interval ranging from several days up to weeks (see Table III), the method described is sufficiently reproducible for purposes of routine analyses.

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REFERENCES

1 M.W. Anders and J.P. Latorre, Anal. Chem., 42 (1970) 1430.
2 K.S. Albert, M.R. Hallmark, M.E. Carroll and J.G. Wagner, Chem. Pathol. Pharmacol., 6 (1973) 845.
3 T. Inaba and J.F. Brien, J. Chromatogr., 80 (1973) 161.
4 R.F. Adams and F.L. Vandemark, Clin. Chem., 22 (1976) 25.
5 P.M. Kabra and L.J. Marton, Clin. Chem., 22 (1976) 1672.
6 J. Hermansson and B. Karlén, J. Chromatogr., 130 (1977) 422.

7 J.E. Slonek, G.W. Peng and W.L. Chiou, J. Pharm. Sci., 67 (1978) 1462.
8 R.W. Dykeman and D.J. Ecobichon, J. Chromatogr., 162 (1979) 104.
9 R.J. Sawchuk and L.L. Cartier, Clin. Chem., 26 (1980) 835.
10 D. Couri, C.S. del Villar and P. Toy-Manning, J. Anal. Toxicol., 4 (1980) 227.
11 S.A. Chow, D.M. Charkowski and L.J. Fischer, Life Sci., 27 (1980) 2477.
12 R.E. Kaiser and E. Oelrich, Optimierung in der HPLC, Hüthig-Verlag, Heidelberg, Basel, New York, 1979.
13 H. Hulpke and U. Werthmann, Chromatographia, 12 (1979) 390.
14 T.V. Alfredson, J. Chromatogr., 218 (1981) 715.
15 W. Roth, K. Beschke, R. Jauch, A. Zimmer and F.W. Koss, J. Chromatogr., 222 (1981) 13.
16 H. Riggenmann, LaborPraxis, July/August (1981) 190.
17 U. Jürgens, Deut. Lebensm.-Rundsch., 77 (1981) 93.
18 U. Jürgens, Deut. Lebensm.-Rundsch., 77 (1981) 211.
19 U. Jürgens, Deut. Lebensm.-Rundsch., 78 (1982) 49.
20 U. Jürgens, Z. Lebensm.-Unters. Forsch., 173 (1981) 356.
21 U. Jürgens, Z. Lebensm.-Unters. Forsch., 174 (1982) 208.