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Note

Direct high-performance liquid chromatographic analysis of *p*-hydroxyphenyl-phenylhydantoin glucuronide, the final metabolite of phenytoin, in human serum and urine

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Phenytoin (diphenylhydantoin, DPH) is widely used in the treatment of epilepsy. The compound is hydroxylated, via the arene-oxide mechanism, to *p*-hydroxyphenyl-phenylhydantoin (pHPPH, I), which in turn is conjugated with glucuronic acid (II) (Fig. 1), and to the corresponding racemic mixture of diols (DHPPH) [1]. Metabolite I is a racemic mixture (*R,S*), and it is thought that metabolite II is also a racemic mixture. DPH is measured in blood and plasma of epileptic patients in therapeutic drug monitoring (TDM) programmes by means of high-performance liquid chromatography (HPLC) or fluorescence polarization immunoassays (TDx techniques). Metabolite I is rarely measured in plasma and urine in these TDM programmes; it is present in plasma in low concentrations and in higher concentrations after enzymic deglucuronidation with β-glucuronidase, and the major amount is excreted by the kidneys in the urine. The percentage of the dose excreted in the urine (24

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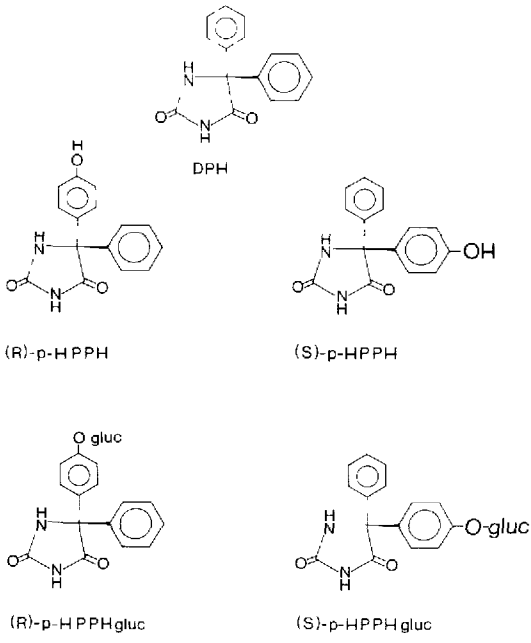

Fig. 1. Structures of phenytoin (DPH) and its metabolites *R*- and *S*-*p*-hydroxyphenyl-phenylhydantoin (p-HPPH, I) and the corresponding glucuronide conjugates (II).

h) as the final metabolite, may be used as an index of DPH oxidation by a cytochrome P450 isoenzyme for each patient [2]. Maguire and co-workers [3,4] reported the HPLC analysis of the racemic mixtures of I and DHPPH after deglucuronidation. I and DHPPH are mainly present in the urine as the *S*-isomer. No method for the direct HPLC measurement of II or of the diol glucuronides has been published. This communication describes the rapid and simple analysis of II in the serum and urine of epileptic patients.

EXPERIMENTAL

Chemicals

(*R,S*)-I was obtained from Aldrich (Milwaukee, WI, U.S.A.). β-Glucuronidase Type BI (from bovine liver), Type VII (from *Escherichia coli*), Type LII (from *Limpets Patella vulgata*) and Type H5 (from *Helix pomatia*) were obtained from Sigma (St. Louis, MO, U.S.A.). The solvents acetonitrile and methanol were of HPLC grade from Fisons (Loughborough, U.K.). All other chemicals were or reagent grade (Merck, Darmstadt, F.R.G.).

Sample preparation of serum I and II

To 0.1 ml of serum in an Eppendorf reaction vessel, 0.1 ml of acetonitrile was added and mixed thoroughly on a vortex mixer. The mixture was centri-

fuged in a Biofuge A (Heraeus Christ, F.R.G.) at 11 000 *g* for 5 min. A 100-μl volume of the clear supernatant was diluted five-fold with water and mixed, and 100 μl were injected onto the column.

Deglucuronidation of II

Serum. To 0.1 ml of serum (pH 7.4) were added 10 μl of a β-glucuronidase solution (Type VII, 20 000 U/ml). The solution was allowed to stand overnight at 37 °C. After incubation, 0.1 ml of acetonitrile was added and the mixture was treated as described above. Standard samples were prepared by adding known amounts of I (0.1–5.0 μg/ml) to blank human serum.

Urine. Four different β-glucuronidase reactions were investigated for establishing the most effective glucuronidase reaction.

(A) To 0.1 ml of urine were added 0.8 ml of 0.2 *M* KH_2PO_4 buffer (pH 5.0) and 0.1 ml of a β-glucuronidase solution (Type BI, 6250 U/ml). The solution was mixed and incubated at 37 °C for 24 h.

(B) To 0.1 ml of urine were added 0.9 ml of 0.2 *M* NaH_2PO_4 buffer (pH 6.8) and 25 μl of a β-glucuronidase solution (Type VII, 20 000 U/ml). The solution was mixed and incubated at 37 °C for 24 h.

(C) To 0.1 ml of urine were added 0.9 ml of 0.2 *M* KH_2PO_4 buffer (pH 3.8) and 25 μl of a β-glucuronidase solution (Type LII, 25 000 U/ml). The solution was mixed and incubated at 37 °C for 24 h.

(D) To 0.1 ml of urine were added 0.9 ml of 0.2 *M* KH_2PO_4 buffer (pH 5.0) and 10 μl of a β-glucuronidase solution (Type H2, 130 000 U/ml). The solution was mixed and incubated at 37 °C for 24 h.

After incubation the solution was diluted ten-fold with water, and 100 μl were injected onto the C_{18} (5-ODS) column. Standard samples were prepared by adding known amounts of I (10–250 μg/ml) to blank urine.

Sample preparation

Unknown and standard samples were treated as described for the enzymic hydrolysis of II; for the analysis of I, instead of the β-glucuronidase solution, water was added.

Apparatus

A Spectra Physics 3500B liquid chromatograph (Spectra Physics, Eindhoven, The Netherlands) was used, equipped with a Spectroflow 757 variable-wavelength spectrophotometer (Kratos U.S.A., BioApplied Systems, Maarssen, The Netherlands). The detector was connected to a 10-mV recorder (BD7, Kipp and Zonen, Delft, The Netherlands); the chart speed was 1 cm/min. Detection was achieved at 210 nm. The injection volume was 100 μl.

Chromatography

p-Hydroxyphenyl-phenylhydantoin glucuronide (II). Chromatography of II and free I was performed using a stainless-steel column (250 mm×4.6 mm I.D.) packed with Spherisorb 5 ODS (Chrompack, Middelburg, The Netherlands). The column temperature was 21°C.

The eluent was a mixture of H_3PO_4 plus TMACl (36 g of H_3PO_4 and 10 g of tetramethylammonium chloride per litre distilled water)–methanol–acetonitrile–water (100:160:120:620, v/v). The flow-rate was 1.6 ml/min.

(R)(S)-p-Hydroxyphenyl-phenylhydantoin (I). The fraction of I after deglucuronidation was collected for chromatography on a Cyclobond I column (250 mm×4.6 mm I.D.) (Astec, Whippany, NJ, U.S.A.) in order to separate the stereoisomers (*R*)-I and (*S*)-I.

The mobile phase for this separation was water–acetonitrile (80:20, v/v) at a flow-rate of 0.64 ml/min.

Serum and urine samples

Serum samples were taken 3 h after medication, with 24-h urine specimens, from eight healthy epileptic patients (women) undergoing chronic treatment with DPH.

RESULTS

Fig. 2 shows the chromatograms of a serum sample from a patient before and after deglucuronidation: I, II and DPH were present. The capacity factors are 2.4 for II, 6.0 for I and 17.0 for DPH.

Fig. 3 shows the chromatograms of a blank urine sample, a urine sample containing I and II and a urine sample after treatment with β-glucuronidase. II is clearly separated from endogenous compounds excreted and present in the urine. Fig. 4 shows the chromatograms of the reference compounds (*R*)-I and (*S*)-I and the same compounds after deglucuronidation of a urine sample and trapping the HPPH peak in Fig. 2. For this separation the Cyclobond I column was used. The capacity factors are 2.2 for (*R*)-I and 2.4 for (*S*)-I.

Enzymic hydrolysis with β-glucuronidases

Of the four β-glucuronidases tested, Type VII was the most effective. After 30 min 100% of II disappeared from the chromatogram and was converted into I. This 100% conversion was reached for Type BI after 24 h, for Type LII after 24 h and for Type H2 after 2 h. Fig. 5 shows the time course of the deglucuronidation of II by Type BI β-glucuronidase.

Calibration curve

The relationship between the peak height (cm) of II in a urine sample and the corresponding concentration of I ([I], μg/ml) after deglucuronidation of

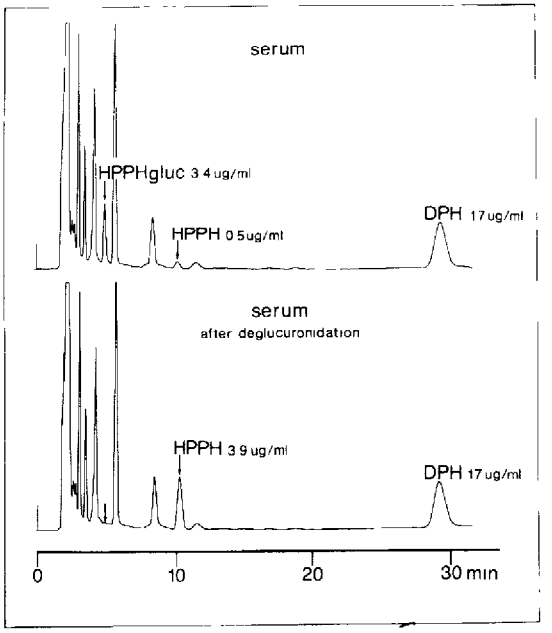

Fig. 2. Chromatograms of a patient serum sample containing II (HPPHgluc), I (HPPH) and DPH before and after deglucuronidation.

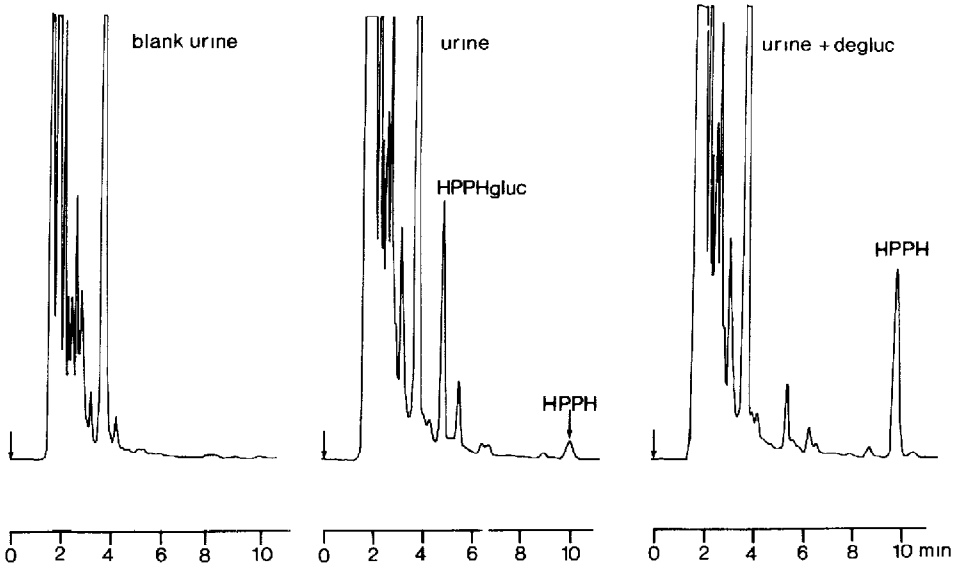

Fig. 3. Chromatograms of a blank urine sample, a patient urine sample containing II (HPPHgluc) and I (HPPH) and the same sample after deglucuronidation

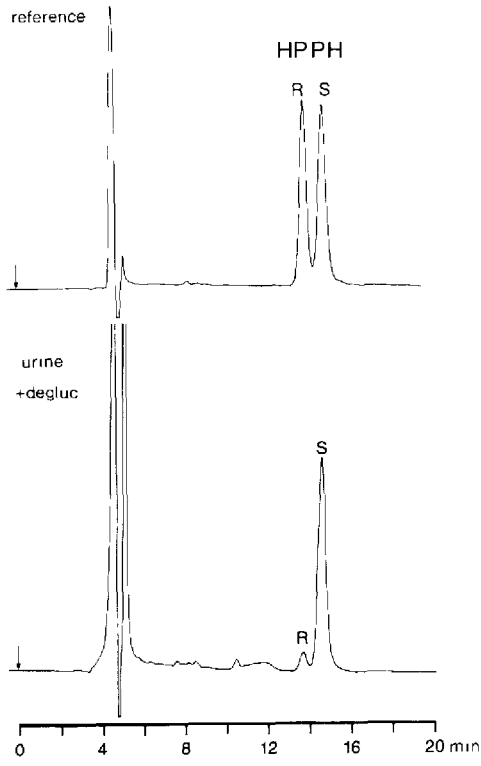

Fig. 4. Chromatograms of reference compounds (*R,S*)-I (HPPH) and of the same compounds after trapping of the I peak in Fig. 2 and deglucuronidation of a patient urine sample.

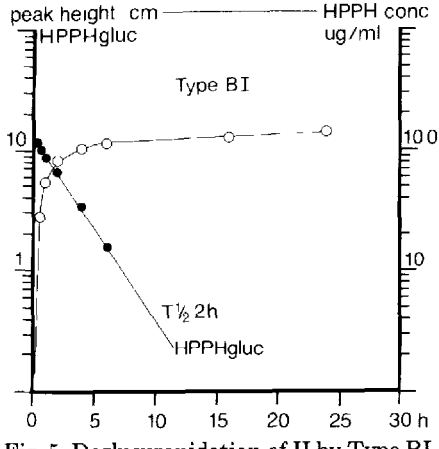

Fig. 5. Deglucuronidation of II by Type BI β-glucuronidase.

the urine sample with Type VII β-glucuronidase is given by the equation:

peak height II = 1.284 [I] − 0.112 (r = 0.999)

The equivalent relationship for a serum sample is given by the equation:

peak height II = 0.066 [I] + 0.446 (r = 0.9981)

Validation

Serum. The inter-day variation for II in serum, expressed as the relative standard deviation (R.S.D.), was 0.7% (n = 8, concentration range 1.35–3.4 μg/ml) and for I it was 2.9% (n = 8, concentration range 0.25–5.0 μg/ml). The

TABLE I

PLASMA CONCENTRATIONS OF DPH, I AND II IN EIGHT PATIENT SAMPLES TAKEN 3 h AFTER ORAL ADMINISTRATION OF DPH

Patient No.	Dosage (mg/day)	Plasma concentration (μg/ml)		
		II	I	DPH
1	400	1.45	0.28	8.9
2	400	2.35	0.36	6.5
3	400	3.40	0.64	17 1
4	300	1.95	0.28	10 3
5	225	1.45	0.28	8.2
6	400	2.35	0.64	28 8
7	660	1.90	0.14	24 8
8	400	1.50	0.21	7.9

TABLE II

URINARY CONCENTRATIONS OF I AND ITS GLUCURONIDE (II) IN 24-h URINE SAMPLES OF PATIENTS

Sample No.	Urinary concentration (μg/ml)		II/I ratio
	II	I	
1	33	8	4.13
2	96	36	2.67
3	144	14	10.29
4	86	8	10.75
5	95	25	3.80
6	56	10	5.60
7[a]	49	6	8.17
8	106	58	1.83

[a]Carbamazepine co-medication.

TABLE III

PERCENTAGE OF THE *R*-ISOMER IN THE TOTAL AMOUNT OF II IN URINE SAMPLES OF EPILEPTIC PATIENTS AFTER TREATMENT WITH DIFFERENT TYPES OF GLUCURONIDASE

Sample No.	Percentage of the *R*-isomer				
	Type BI[a]	Type LII[a]	Type VII[a]	Type H2[a]	Mean ± S.D.
1	7.5	12 6	9 6	8.8	9.6 ± 2 2
2	5.7	6 9	4 2	5.2	5.5 ± 1 1
3	8.1	8 3	7.5	8 1	8.0 ± 0.35
4	9.8	8.8	9.6	9.6	9.5 ± 0 44
5	12.7	13 6	12.4	12.2	12.7 ± 0.62
6	10.3	14.9	9.4	9.4	11.0 ± 2.6
7[b]	17 9	15 3	16.0	16 0	16 3 ± 1.1
8	8.6	9.1	7.5	9.9	8.8 ± 1.0

[a]Glucuronidase.
[b]Carbamazepine co-medication.

intra-day variation in the same concentration range for II, expressed as the R.S.D., was 2.35% and for I it was 4.9%.

Urine. The inter-day variation for II, expressed as the R.S.D., was 2.09% ($n=7$, concentration range 33–144 μg/ml) and for I 2.25% ($n=10$, concentration range 10–225 μg/ml). The intra-day variation for II, expressed as the R.SD., was 4.95% ($n=8$), and for I it was 4.00%.

Patient serum concentrations are shown in Table I. Table II shows the concentrations of I and II in 24-h samples from epileptic patients. Metabolite II is a racemic mixture of *R*- and *S*-isomers. When the urine samples are treated with the four β-glucuronidases, an almost constant percentage of the *R*-isomer is observed, as shown in Table III.

DISCUSSION

Glucuronidation of a drug or its metabolite increases its water solubility and decreases the retention time on reversed-phase HPLC columns. The direct HPLC measurement of drugs, drug metabolites and their corresponding glucuronide conjugates was difficult because of these extreme differences in retention times. The chromatograms in Figs. 2 and 3 show clearly that the metabolites I and its glucuronide (II) are present in the serum and urine of patients on DPH therapy. The measurement of these metabolites can be used in therapeutic drug monitoring as an individual patient index for drug metabolism (Tables II and III). The accessibility of the methodology to measure drug conjugates such as drug glucuronides for HPLC analysis is steadily improving, as witnessed by the series of papers concerning the direct measurement of drug

glucuronides for acetaminophen [5], codeine [6], morphine [7], paracetamol [8], propranolol [9] and sulphadimethoxine [10].

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