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MASS FRAGMENTOGRAPHIC DETERMINATION OF DIPHENYLHYDANTOIN AND ITS MAIN METABOLITE, 5-(4-HYDROXYPHENYL)-5-PHENYLHYDANTOIN, IN HUMAN PLASMA

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SUMMARY

A method is described for the mass fragmentographic determination of diphenylhydantoin and its main metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin (4-OH-DPH), in human plasma as their dimethyl and trimethyl derivatives, respectively. The derivatives are formed by using the recently described extractive alkylation technique. Pentadeuterated 4-OH-DPH is used as the internal standard. Following acidic hydrolysis of the plasma sample, conjugated 4-OH-DPH and, indirectly, the dihydrodiol metabolite, 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin, are measured. Using 100-μl plasma samples, the lower limit of detection is about 10 ng/ml (0.03 nmole/ml).

INTRODUCTION

The major metabolite of diphenylhydantoin (DPH) in man and rats is 5-(4-hydroxyphenyl)-5-phenylhydantoin (4-OH-DPH)[1], which is found in the urine mainly conjugated with glucuronic acid[2]. In dogs, *meta*-hydroxylation to (3-OH-DPH) represents the major pathway[3].

The elimination of DPH is dose-dependent[4–6] and two hypotheses have been proposed in order to explain this dependence. The first is that the elimination is characterized by saturation or Michaelis–Menten kinetics[7], and the other is product inhibition of DHP metabolism by 4-OH-DPH[8,9]. The evaluation of these hypotheses in man requires the measurement of the plasma concentration of 4-OH-DPH.

A colorimetric method has been described by Dill *et al.*[10] but it is not sensitive enough to measure unconjugated 4-OH-DPH in plasma. The gas chromatographic (GC) measurement of 4-OH-DPH using derivatization with either trimethylsilyl groups[11] or flash methylation[12] has been described. When a flame ionization detector is used, these procedures are not sensitive enough to detect unconjugated 4-OH-DPH

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in plasma. Recently, Albert *et al.*[13] described a high-pressure liquid chromatographic procedure for separating and measuring 4-OH-DPH in plasma, but this method requires the administration of radioactively labelled DPH[14]. This paper describes the application of extractive alkylation[15] in the mass fragmentographic determination of DPH, 3-OH-DPH and 4-OH-DPH in plasma.

MATERIALS AND METHODS

Reference drugs

DPH, 4-OH-DPH, 3-OH-DPH, 5-(3,4-dihydroxyphenyl)-5-phenylhydantoin (3,4-diOH-DPH), 5-(4-hydroxy-3-methoxyphenyl)-5-phenylhydantoin (3-CH_3O,4--OH-DPH) and 5-(2,4-dihydroxyphenyl)-5-phenylhydantoin (2,4-diOH-DPH) were gifts from Parke, Davis & Co. (Detroit, Mich., U.S.A.). The internal standard, 5-(4-methylphenyl)-5-phenylhydantoin (4-CH_3-DPH), was purchased from Aldrich (Beerse, Belgium). The dihydrodiol metabolite of DPH [5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin] was isolated and purified from the urine of rats fed with 0.2% of DPH in the diet[16].

Chemicals

5,5'-[4-^{14}C]Diphenylhydantoin (50.5 mCi/mmole) was purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.) and 4-OH-[4-^{14}C]DPH was synthesized from potassium [^{14}C]cyanide according to the method of Henze and Isbell[17,18]. Tetrabutylammonium hydrogen sulphate (TBA-HSO_4) was purchased from Lab Kemi (Stockholm, Sweden). The TBA-HSO_4 was prepared as a 1 *M* solution in 0.2 *N* sodium hydroxide and extracted twice with methylene dichloride. The extracted TBA-HSO_4 solution resulted in a lower base-line in the chromatograms when an ng on a gas chromatograph. Trimethylanilinium hydroxide (TMAH) was prepared as described by Brochmann-Hanssen and Oke[19]. The external standard, 9-bromophenanthrene, was purchased from Lab Kemi.

All other chemicals were of reagent grade.

Deuterated internal standards

5-(4-Hydroxy-3,5-dideuterophenyl)-5-phenylhydantoin (4-OH-DPH-d_2). 4-OH-DPH was stirred with an excess of bromine in chloroform at room temperature. This reaction was followed by gas chromatography of the trimethyl derivatives obtained by injecting a mixture of TMAH and a portion of the reaction mixture. The reaction was complete after 28 h and only 5-(4-hydroxy-3,5-dibromophenyl)-5-phenylhydantoin could be detected. Its identity was established by combined gas chromatography–mass spectrometry (GC–MS). The crude dibromo compound dissolved in methanol-O-d was stirred with deuterium gas in the presence of palladium on carbon. The exchange of bromine for deuterium was monitored by GC. After 6 h, the reaction was stopped, the catalyst was removed by filtration and the reaction mixture was evaporated to dryness. The residue, 4-OH-DPH-d_2, was characterized by GC–MS (Table I) and thin-layer chromatography (TLC).

5-(4-Hydroxyphenyl)-5-(2,3,4,5,6-pentadeuterophenyl)hydantoin (4-OH-DPH-d_5). Benzoic acid-d_5 was reacted with oxalyl chloride in benzene to give benzoyl chloride-d_5[20]. Condensation of benzoyl chloride-d_5 with anisole under Friedel–Crafts

conditions produced 4-methoxybenzophenone-d_5[21], which, on prolonged heating with aluminium chloride, gave 4-hydroxybenzophenone-d_5[22]. 4-OH-DPH-d_5 was obtained when 4-hydroxybenzophenone-d_5 was subjected to reaction with ammonium carbonate and potassium cyanide in fused acetamide[17]. The product was characterized by GC–MS (Table I) and TLC.

Gas chromatography

A Varian 1400 gas chromatograph was used with a flame ionization detector (FID) and a 1.2 m × 1.4 mm glass column packed with 3% OV-17 on Gas-Chrom Q (80–100 mesh). The temperature of the injector was 290°, the column 225° and the detector 250°.

Mass spectrometry

An LKB 9000 combined gas chromatograph–mass spectrometer was used. The flash heater temperature was 270°. The glass column (1.2 m × 2 mm) contained 3% OV-17 on Gas-Chrom Q (80–100 mesh); the column temperature was either 250° or 270°. For mass fragmentography, a multiple ion detector (MID) was used. The following *m/e* settings were used for the methyl derivatives: 280 (DPH), 310 (3-OH-DPH and 4-OH-DPH), 312 (4-OH-DPH-d_2), 315 (4-OH-DPH-d_5), 340 (3,4-diOH-DPH, 2,4-diOH-DPH and 3-CH_3O,4-OH-DPH) and 342 (dihydrodiol-DPH). The ionization voltage was 20 eV.

Extraction procedure

Unconjugated 4-OH-DPH. The following procedure was developed for the measurement of unconjugated 4-OH-DPH in plasma by the mass fragmentographic method. In a small disposable glass tube, a 0.1-ml plasma sample was buffered with 0.05 ml of 3 *M* Tris–hydrochloric acid, pH 7.5, and 0.1 ml of internal standard (10 μg/ml of 4-OH-DPH-d_5)was added. The solution was extracted with 1.5 ml of methyl isobutyl ketone (water-saturated) for 10 min. After centrifugation, the organic phase was removed with a Pasteur pipette and transferred in to a tube containing 0.2 ml of 1 *N* sodium hydroxide solution. The tubes were shaken for 10 min and centrifuged. The organic phase was removed and discarded. To the aqueous phase, 0.05 ml of 1 *M* TBA-HSO_4 (in 0.2 *N* sodium hydroxide solution) was added, followed by 1.5 ml of methylene dichloride (containing 0.1 ml of methyl iodide). The tubes were shaken mechanically for 30 min. Following centrifugation, the lower, organic phase was carefully removed and transferred in to a clean, conical-tipped, test-tube. The solvent was removed either under a gentle flow of nitrogen with the tubes in a sand-bath (30–35°) or by placing the tubes in a hood overnight at room temperature. The residue was dissolved in 0.05 ml of methanol and 1–4 μl were injected into the LKB 9000 instrument for GC–MS.

Conjugated 4-OH-DPH. To a 0.05-ml plasma sample, 0.05 ml of internal standard (10 μg/ml of 4-OH-DPH-d_5) and 0.1 ml of 10 *N* hydrochloric acid were added. The tubes were covered with aluminium foil and heated in a water-bath at 95° for 60 min. After cooling, 0.1 ml of sodium hydroxide solution and 0.05 ml of 3 *M* Tris–hydrochloric acid, pH 7.5, were added. The pH of tubes at random was checked with litmus paper so as to ensure adequate adjustment of the pH. The samples were then processed as for unconjugated 4-OH-DPH.

RESULTS AND DISCUSSION

The overall scheme of the procedure used for the determination of DPH and its hydroxylated metabolites in plasma is given in Fig. 1. When methyl isobutyl ketone is used for the extraction of plasma buffered to pH 7.5 with Tris–hydrochloric acid, the recovery of [^{14}C]DPH and 4-OH-[^{14}C]DPH is greater than 99%. Following centrifugation, the organic phase is removed and added to 1 ml of 1 *N* sodium hydroxide and mixed. The recovery of DPH and 4-OH-DPH in the aqueous (sodium hydroxide) phase in this back-extraction step is greater than 99%. The volume and concentration of sodium hydroxide during back-extraction are critical.

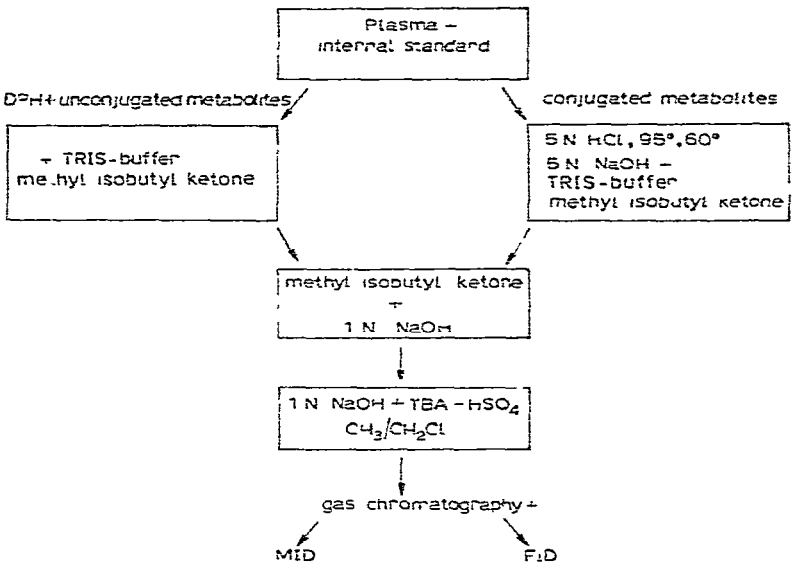

Fig. 1. Scheme for the general procedure.

Extractive alkylation

The extraction of anions from an aqueous alkaline phase as ion pairs with quaternary ammonium ions into an organic phase where the anions are alkylated[15] has been applied recently to analysis of a number of drugs[23–27]. The technique of extractive alkylation was adapted in order to determine the plasma concentration of DPH and its hydroxylated metabolites. The optimal conditions for extractive alkylation were studied by GC–FID. For these studies, DPH, 4-OH-DPH and 4-CH_3-DPH were used in a final volume of 1 ml. The concentration of sodium hydroxide and TBA-HSO_4 and the time of extraction were varied. The organic phase contained 100 μl of methyl iodide in 5 ml of methylene chloride. After the reaction was completed and the methylene chloride had been evaporated, the residue was dissolved in 50 μl of ethyl acetate containing 9-bromophenanthrene as an external standard. The peak heights were compared with the external standard and quantitated using [^{14}C]-DPH and 4-OH-[^{14}C]DPH. The influence of the concentration of TBA-HSO_4 and time on the extractive alkylation of 4-OH-DPH is shown in Fig. 2. The optimal conditions found for the extractive alkylation of 4-OH-DPH were 1 *N* sodium hy-

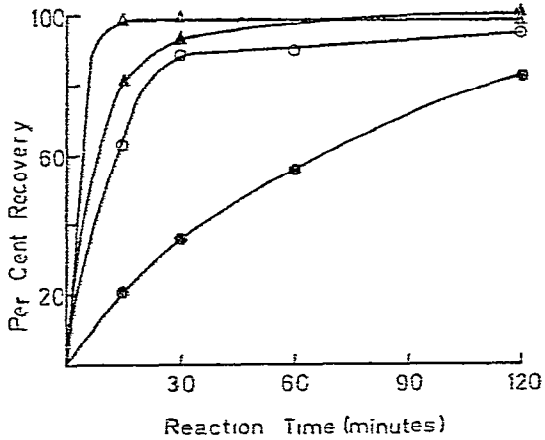

Fig. 2. Influence of concentration of tetrabutylammonium ion on the extractive alkylation of 4-OH-DPH. Concentration of 4-OH-DPH: 25 μg/ml in 1 *N* NaOH. Organic phase: 5 ml of methylene dichloride containing 100 μl of methyl iodide. Temperature: 23°. Concentration of tetrabutylammonium hydrogen sulphate: ●, 0.02; ○, 0.05; ▲, 0.10; △, 0.20 m*M*.

droxide in the presence of 0.2 *M* TBA-HSO_4 using an extraction time of 30 min. The concentration requirements for sodium hydroxide and TBA, and also the time required for extractive alkylation, were greater for 4-OH-DPH than for either DPH or 4-CH_3-DPH. Ethyl iodide could also be used as the alkylating agent, but under the above conditions complete extractive alkylation either required a reaction time of 120 min or the temperature had to be increased to 50° with shorter mixing times (Fig. 3).

The following DPH derivatives were successfully analyzed by extractive alkylation: DPH, 4-CH_3-DPH, 4-OH-DPH, 3-OH-DPH, 2,4-diOH-DPH, 3-CH_3O,4-

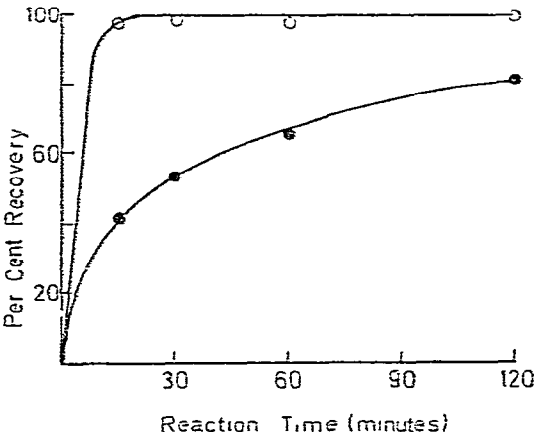

Fig. 3. Influence of reaction temperature on the extractive alkylation of 4-OH-DPH using ethyl iodide. Concentration of 4-OH-DPH: 25 μg/ml in NaOH and 0.2 m*M* tetrabutylammonium ion. Organic phase: 5 ml of methylene chloride containing 100 μl of ethyl iodide. Temperature. ●, 23°, ○, 50°.

OH-DPH, 4-OH, 4'-CH_3-DPH and 4,4'-diOH-DPH. The catechol metabolite, 3,4-diOH-DPH, was degraded under the alkaline conditions used and could not be measured. The dihydrodiol metabolite was not measured under the conditions described.

Standard curves for 4-OH-DPH prepared by using 1 ml of plasma samples and measured by GC–FID are linear from 0.5 to 10 μg/ml and from 10 to 100 μg/ml. The use of GC–FID is not sensitive enough to measure 4-OH-DPH at levels below 0.5 μg/ml without the appearance of interfering peaks.

Mass fragmentographic method

With the use of an MID, the extractive alkylation procedure produces reliable and reproducible results for unconjugated 4-OH-DPH in plasma. In a single day, it is possible to prepare, in duplicate, a standard curve for eight samples plus 40–50 samples from patients (a total of 116 tubes). Further, DPH and 3-OH-DPH can be determined in the same samples. A fragmentogram is shown in Fig. 4 for 3-OH-DPH and 4-OH-DPH added to drug-free control plasma and for a plasma sample from a patient administered DPH. With a column temperature of 270°, 3-OH-DPH and 4-OH-DPH can be separated and measured with injection of samples every 1.5 min. In order to detect DPH also, the temperature of the column can be decreased to 250°, permitting adequate separation of the three compounds. Standard curves for DPH determined by the general procedure are shown in Fig. 5a for concentrations from 0.02 to 0.2 μg/ml and 0.5 to 5 μg/ml. Standard curves for concentrations from 5 to

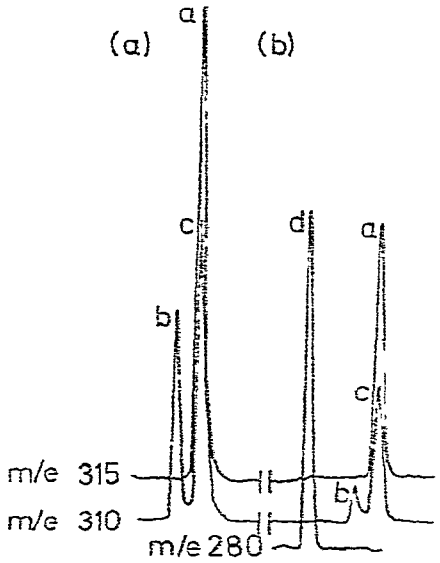

Fig. 4. Mass fragmentogram using 4-OH-DPH-d_5 (peak a) as internal standard. (a) Blank plasma with 0.125 μg of 3-OH-DPH/ml (peak b) and 0.25 μg of 4-OH-DPH/ml (peak c) added; (b) patients' plasma containing 6.6 μg/ml of DPH (peak d), 0.018 μg/ml of 3-OH-DPH (peak b) and 0.171 μg/ml of 4-OH-DPH (peak c).

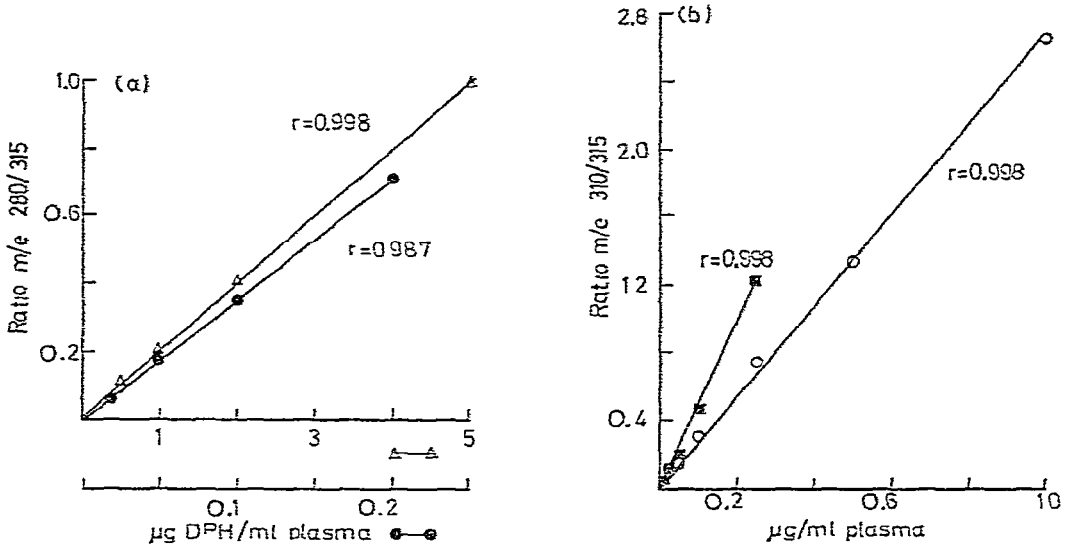

Fig. 5. (a) Standard curves obtained with DPH in human plasma at two concentration ranges. (b) Standard curves for 3-OH-DPH (■) and 4-OH-DPH (○) in human plasma. The internal standard was 4-OH-DPH-d_5, using the general procedure for mass fragmentography.

40 μg/ml are also linear. Fig. 5b shows the standard curve for 4-OH-DPH from 0.01 to 1 μg/ml in plasma and for 3-OH-DPH from 0.01 to 0.25 μg/ml in plasma. The lower limit for detection is about 0.01 μg/ml for DPH or its hydroxylated metabolites. Although the protocol outlines the procedure starting with 100 μl, plasma samples from newborns have been analyzed using 10 μl of capillary plasma[28].

TABLE I

COMPARISON OF MASS SPECTRA OF INTERNAL STANDARDS

4-OH-DPH-d_2		*4-OH-DPH-d_5*	
m/e	*% of base peak*	*m/e*	*% of base peak*
235*	100	233*	100
310	1.6	313	1.0
311	18.1	314	10.0
312	76.2	315	68.8
313	17.8	316	14.3
314	2.9	317	2.0

* Base peak.

Conjugated 4-OH-DPH and dihydrodiol-DPH

The concentration of conjugated 4-OH-DPH is calculated from measurements made after acid hydrolysis of the plasma sample. The present procedure does not measure dihydrodiol-DPH directly. However, with acid hydrolysis, the dihydrodiol metabolite of DPH forms equal amounts of 3-OH-DPH and 4-OH-DPH[16]. The mea-

surement of 3-OH-DPH in the acid-hydrolyzed sample would then measure indirectly the dihydrodiol metabolite. In contrast to the dog, in man 3-OH-DPH does not appear to be a significant metabolite[3]. If 3-OH-DPH is assumed to be absent or an insignificant metabolite in man, then the above calculation should give an indirect measurement of the dihydrodiol metabolite. In support of this assumption, we have detected only small amounts (<20 ng/ml) of unconjugated 3-OH-DPH in the plasma of patients, except uremic patients, where we have observed concentrations of unconjugated 3-OH-DPH in plasma of 20–80 ng/ml. Further, the amount of 3-OH-DPH released during acid hydrolysis in most patients varied between 5–10% of the total 4-OH-DPH measured after acid hydrolysis. Thus, the following calculation was considered sufficiently accurate to determine conjugated 4-OH-DPH. Conjugated 4-OH-DPH was calculated by subtracting both the unconjugated 4-OH-DPH and the amount of 3-OH-DPH released by acid hydrolysis from the total 4-OH-DPH measured after acid hydrolysis. The quantitation of the dihydrodiol-DPH metabolite was calculated as twice the acid-hydrolyzed 3-OH-DPH. Assuming that dihydrodiol-DPH was not present but instead that 3-OH-DPH was conjugated, these calculations would give, at most, a 10% error in the value for conjugated 4-OH-DPH.

Internal standards

Originally, 4-OH-DPH-d_2 was used as the internal standard, but two problems were encountered. Firstly, the deuterium atoms were exchanged during the acid hydrolysis step used in the measurement of conjugated 4-OH-DPH, and thus the 4-OH-DPH-d_2 had to be added after hydrolysis. Secondly, small but significant variations in the day-to-day analysis of the same plasma were observed and occasionally duplicates would be markedly different and, although not evaluated, this effect was felt to be due to exchange of deuterium atoms during the handling of the samples.

As the internal standard 4-OH-DPH-d_5 can be added before acid hydrolysis because deuterium is not exchanged under these conditions, the variance noted in day-to-day runs or within a daily run were significantly decreased. The results in Table II show that a 2.4–6.4% variation was noted in six separate measurements on each of four plasmas using 4-OH-DPH-d_5, whereas the variation was 7.8–16.0% for 4-OH-DPH-d_2.

TABLE II

COMPARISON OF STANDARD DEVIATIONS WITH DIFFERENT INTERNAL STANDARDS

Results are means ± standard deviations for six determinations on plasma samples.

Patient	*4-OH-DPH-d_2 (μg/ml)*	*4-OH-DPH-d_5 (μg/ml)*
1	0.115 ± 0.009	0.150 ± 0.007*
2	0.319 ± 0.029	0.330 ± 0.021
3	0.165 ± 0.026	0.135 ± 0.005*
4	0.269 ± 0.043	0.211 ± 0.005*

* $p < 0.05$ compared with 4-OH-DPH-d_2 when analyzed by Student's *t*-test.

CONCLUSION

A sensitive and specific mass fragmentographic method has been developed for the detection of DPH, unconjugated 3-OH-DPH and 4-OH-DPH in 0.01–0.1-ml samples of plasma. The use of 4-OH-DPH-d_5 as an internal standard gives excellent reproducibility. After acidic hydrolysis of the original plasma sample, analysis by the method described permits the calculation of the concentration of conjugated 4-OH-DPH. In addition, the dihydrodiol-DPH metabolite can be determined indirectly from the 3-OH-DPH peak.

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