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Biotransformation of diethenylbenzenes

IV. A simple high-performance liquid chromatographic method for separation of urinary metabolites of 1,3-diethenylbenzene on analytical and semi-preparative scales

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ABSTRACT

A simple ion-suppression separation on reversed-phase columns, which is applicable for both analytical and semi-preparative work, is described. Six urinary metabolites of 1,3-diethenylbenzene (I), namely 1-(3-ethenylphenyl)-1,2-dihydroxyethane β-D-glucosiduronates (two isomers, II and III), N-acetyl-S-[1-(3-ethenylphenyl)-2-hydroxyethyl]cysteine (IV), N-acetyl-S-[2-(3-ethenylphenyl)-2-hydroxyethyl]cysteine (V), 3-ethenylphenylmandelic acid (VI) and 3-ethenylphenylglyoxylic acid (VII), were isolated (Fig. 1). Four of them, IV–VII, have been identified in our previous work; the two glucosiduronates were identified for the first time by ^{1}H NMR spectroscopy, fast atom bombardment mass spectrometry, and enzymic hydrolysis yielding 1-(3-ethenylphenyl)-1,2-dihydroxyethane as an aglycone. The method was reproducible the concentration range 0.05–5 mg/ml, the coefficient of variation being less than 7% (n = 5). Excretion of II–VI within 24 h in the urine of rats dosed with a single intraperitoneal injection of 100, 300 and 600 mg/kg I was determined quantitatively. The utility of the method is discussed in comparison with gas chromatographic–mass spectrometric techniques used previously.

INTRODUCTION

3-Diethenylbenzene (I, Fig. 1) is a styrene analogue that is used as co-monomer in the production of plastics. Its main urinary metabolites, which can be extracted with ethyl acetate, were isolated and identified in previous part of this study [1]. However, the analytical methods used were not suitable for the detection and determination of polar metabolites, *e.g.* glucosiduronates, which cannot be extracted with organic solvents. The aim of present study was to develop

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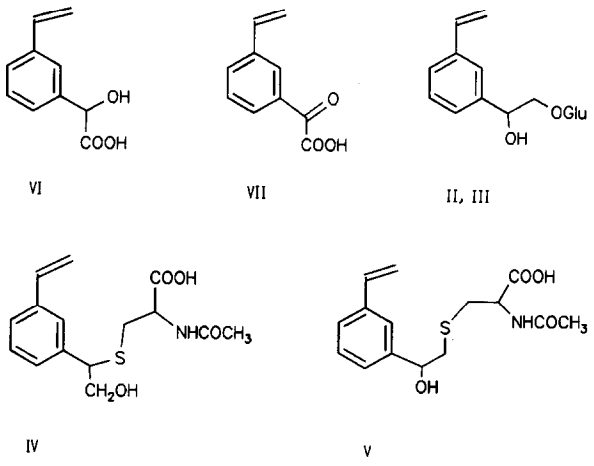

Fig. 1. Structures of the metabolites of 1,3-diethenylbenzene.

a high-performance liquid chromatographic (HPLC) method that would allow a virtually complete metabolic profile of I to be obtained.

EXPERIMENTAL

Chemicals

1,3-Diethenylbenzene (>99.9% pure) was prepared in our laboratory [2]. Analytical-grade methanol and reagent-grade toluene were from Lachema (Brno, Czechoslovakia) and were distilled before use. Water was distilled twice in a glass apparatus. Analytical-grade acetic acid was from Lachema and was used as received. Deuterium oxide, >99.8% deuterium, was from Merck (Darmstadt, Germany). 4,4-Dimethyl-4-silapentane sulphonate was from Janssen Chimica (Beerse, Belgium). Glucuronidase B1 was from Sigma (St. Louis, MO, USA). Silica-Cart C_{18} plastic cartridges were from Tessek (Prague, Czechoslovakia).

Animals

Adult female Wistar rats (Velaz, Prague, Czechoslovakia) weighing 210–260 g were dosed intraperitoneally with a single injection of 100, 300 or 600 mg/kg I in sunflower oil. Controls received sunflower oil only. Individual urine samples were collected 24 h after dosing and stored at −18°C. Animals were handled as described previously in greater detail [3].

Apparatus

Chromatographic separations were performed on a Philips HPLC system consisting of a PU4100 solvent-delivery system, a Rheodyne 7125 sample injector with a 5-μl sample loop for analytical and a 200-μl sample loop for preparative injections, a PU4120 diode-array detector, a PU4110 UV–VIS detector, a PU6030 data-capture unit and a P3202 data station. Thin-layer chromatography (TLC) was performed on precoated glass plates for nano-TLC coated with silica gel 60 (Merck). Plates were developed in a linear developing chamber (Camag, Muttenz, Switzerland).

NMR spectra were measured on a Bruker AM 400 spectrometer (400 MHz or ^1H) at 25°C in ^2H_2O using sodium 4,4-dimethyl-4-silapentanesulphonate as an internal standard ($\delta = 0$). Fast atom bombardment (FAB) mass spectra were measured on a Jeol DX 303 instrument with DA 5000 data station. Glycerol was used as a matrix and xenon as a bombardment gas.

Analytical procedure

Rat urine was deproteinized by the addition of three times its volume of methanol. Samples were centrifuged at 3000 *g*. Aliquots of the supernatant (5 μl) were injected onto a glass column (150 mm × 3 mm I.D.) packed with Separon SGX C_{18}, particle size 5 μm (Tessek). The mobile phase was methanol–water–aqueous solution of 2% acetic acid (A–B–C). The starting composition of 30% A, 40% B and 30% C was changed linearly to 70% A and 30% C in 20 min, and thereafter held unchanged for another 10 min. The flow-rate was 0.5 ml/min. The detection wavelength was 254 nm.

Isolation of metabolites

Urine from three rats dosed with 600 mg/kg I was pooled. An aliquot of 15 ml was diluted with 45 ml of methanol and centrifuged. The supernatant was concentrated by evaporation under vacuum. Aliquots of 200 μl were injected onto a

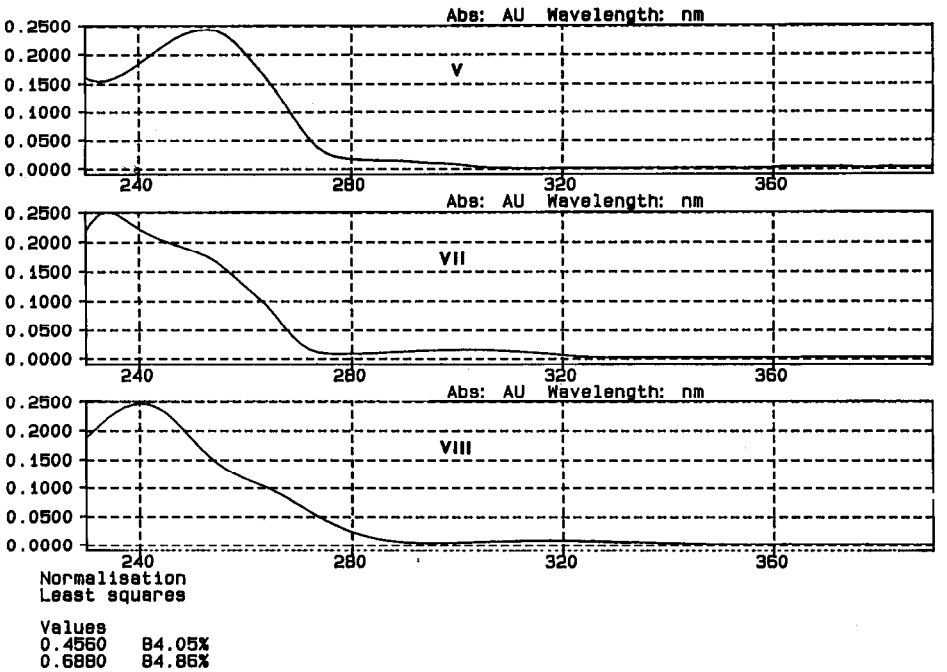

Fig. 2. UV spectra of the main metabolites, obtained from the diode-array detector and corrected for baseline by subtraction. Spectra of metabolites II–VI were closely similar (> 98%), therefore only one representative spectrum is shown here.

stainless-steel column (250 mm × 8 mm I.D.) packed with Separon SGX C_{18}, particle size 7 μm (Tessek). The mobile phase composition and gradient were the same as for analytical separation. The flow-rate was 2.5 ml/min. Fractions containing seven individual metabolites were collected, and the solvents were evaporated under vacuum. To facilitate the evaporation of water and acetic acid, several portions of toluene were added to the solution until all solvents were removed. Solid residues, except the minor metabolite VI, were then re-dissolved in 1–1.5 ml of water and purified by repeated HPLC on the same semi-preparative column using isocratic elution. The mobile phase composition was 40% A, 30% B and 30% C for metabolites II–V and 70% A and 30% C for metabolite VII. After evaporation of solvents, using a rotary vacuum evaporator followed by the removal of remaining volatiles under vacuum of an oil pump, all isolated metabolites were characterized by ^{1}H NMR spectra, UV spectra (Fig. 2) and TLC (Table I). Additionally, the new metabolites II and III were characterized by FAB

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF THE URINARY METABOLITES OF 1,3-DIETHENYLBENZENE

TLC was performed on Merck HPTLC glass plates coated with silica gel 60; the solvent system consisted of (A) 1-butanol–acetic acid–water (4:1:5 v/v), (B) chloroform–ethyl acetate (2:1, v/v) or (C) toluene–acetone (1:1, v/v).

Metabolite	R_F values		
	A	B[a]	C[a]
II	0.10	–	–
III	0.18	–	–
IV	0.56	0.14	0.43
V	0.61	0.19	0.53
VI	0.26	0.51	0.65
VII	0.90	0.67	0.74

[a] Metabolites were converted into corresponding methyl esters adding an ether solution of diazomethane before spotting on the plate. Metabolites were detected as UV-absorbing spots; also after spraying with naphthoresorcinol solution (II, III) and iodoplatinate reagent (IV, V) [5].

mass spectra and β-D-glucuronidase-catalysed hydrolysis to yield 1-(3-ethenylphenyl)-1,2-dihydroxyethane.

Structure elucidation of new metabolites

Enzymic hydrolysis. A solution of 1 mg of II in 4 ml of acetate buffer (pH 5) was incubated with β-D-glucuronidase B1 (1500 U) for 72 h. Samples (100 μl) were taken after 2, 6, 24, 48 and 72 h. They were treated and analysed as described for urine samples. The decrease of the glucuronide peak was nearly linear as was the increase of the aglycone peak. The rate of hydrolysis was 4.5 ± 2 nmol h^{-1} and 90 ± 1 nmol h^{-1} for II and III, respectively.

1-(3-Ethenylphenyl)-1,2-dihydroxyethane β-D-glucosiduronate, isomer II. ^{1}H NMR (δ values in ppm): CH-1-gluc, 4.27 (d, 7.7 Hz); CH-gluc, 3.39, 3.51 and 3.64 (m); CH_2O, 3.76 (dd, 11.9 Hz and 4.2 Hz) and 3.82 (dd, 11.9 Hz and 7.3 Hz); CHO, 5.05 (dd, 7.3 Hz and 4.0 Hz); $CH_2=CH$, 5.33 (d, 11.1 Hz) and 5.87 (d, 17.7 Hz); $CH_2=CH$, 6.80 (dd, 17.7 Hz and 11.1 Hz); CH-aromatic, 7.35 (d, 7.5 Hz), 7.43 (t, 7.5 Hz), 7.48 (d, 7.6 Hz) and 7.59 (s).

FAB-MS (sodium salt): MH^+ 379, $[MH + Na]^+$ 402, $[M + K]^+$ 417, $[glucosiduronyl\text{-}O\text{-}CH_2]^+$ 223.

1-(3-Ethenylphenyl)-1,2-dihydroxyethane β-D-glucosiduronate, isomer III. CH-1-gluc, 4.57 (d, 7.9 Hz); CH-gluc, 3.43, 3.57 and 3.77 (m); CH_2O, 3.93 (dd, 11 Hz and 3.8 Hz) and 4.16 (dd, 11 Hz and 8.1 Hz); CHO, 5.06 (dd, 8.1 Hz and 3.8 Hz); $CH_2=CH$, 5.41 (d, 11.1 Hz) and 5.95 (d, 17.6 Hz); $CH_2=CH$, 6.88 (dd, 17.6 Hz and 11.1 Hz); CH-aromatic, 7.42 (d, 7.5 Hz), 7.49 (t, 7.6 Hz), 7.55 (d, 7.7 Hz) and 7.61 (s).

FAB-MS (sodium salt): spectrum virtually identical with that of II.

1-(3-Ethenylphenyl)-1,2-dihydroxyethane. An aliquot (2 ml) of the glucuronidase-treated solution of glucosiduronate III, containing less than 3% of unreacted III, was loaded on a 20 mm × 8 mm plastic cartridge packed with Separon SGX C_{18}, particle size 60 μm, which was pre-washed with 2 ml of methanol and 2 ml of water. The cartridge was then washed with 4 ml of water, and the retained material was eluted with 2 ml of methanol. The procedure was repeated three times until all the incubation mixture was worked up. The methanol effluents were pooled and evaporated to dryness under vacuum. The resulting material was sufficiently pure for identification by comparison of its ^{1}H NMR spectrum with that of the closely similar 1-(4-ethenylphenyl)-1,2-dihydroxyethane [3].

^{1}H NMR (D_2O, δ in ppm); CH_2OH, 3.64 (dd, J = 11.8 and 6.8 Hz) and 3.71 (dd, J = 11.6 and 5.1 Hz); CHO, 4.85 (partially obscured by HOD resonance); $CH_2=CH$, 5.28 (d, J = 11.0 Hz) and 5.82 (d, J = 17.7 Hz); $CH_2=CH$, 6.75 (dd, J = 17.7 and 11 Hz); CH-aromatic, 7.27 (d, J = 7.5 Hz), 7.36 (t, J = 7.5 Hz), 7.42 (d, J = 7.5 Hz) and 7.45 (s).

Quantification of metabolites

Isolated metabolite IV was converted into its dicyclohexylammonium salt. Aqueous solutions of the salt were used for calibration. The calibration curve was linear in the range 0.05–1.0 mg/ml (r = 0.9997). Metabolites II–VI were quantified using the molar response of metabolite IV. The dicyclohexylammonium salt of IV was also added to control urine samples to a final concentration of 0.05, 0.5 or 1.0 mg/l IV. These spiked solutions were used for the determination of the recovery.

RESULTS

Separation of the major metabolites of I was achieved by ion-suppression chromatography on a reversed-phase column. The main metabolite peaks (Fig. 3) were isolated on a semi-preparative column. Metabolites IV–VII were known from our previous work, therefore they could be identified by comparison of their ^{1}H NMR spectra with those of their methyl esters [1] (data not shown). The presence of the methyl ester group would not be expected to significantly alter the resonances of the remaining protons in the molecule. Additionally, they were converted into their methyl esters by adding an ether solution of diazomethane so that they could be identified by

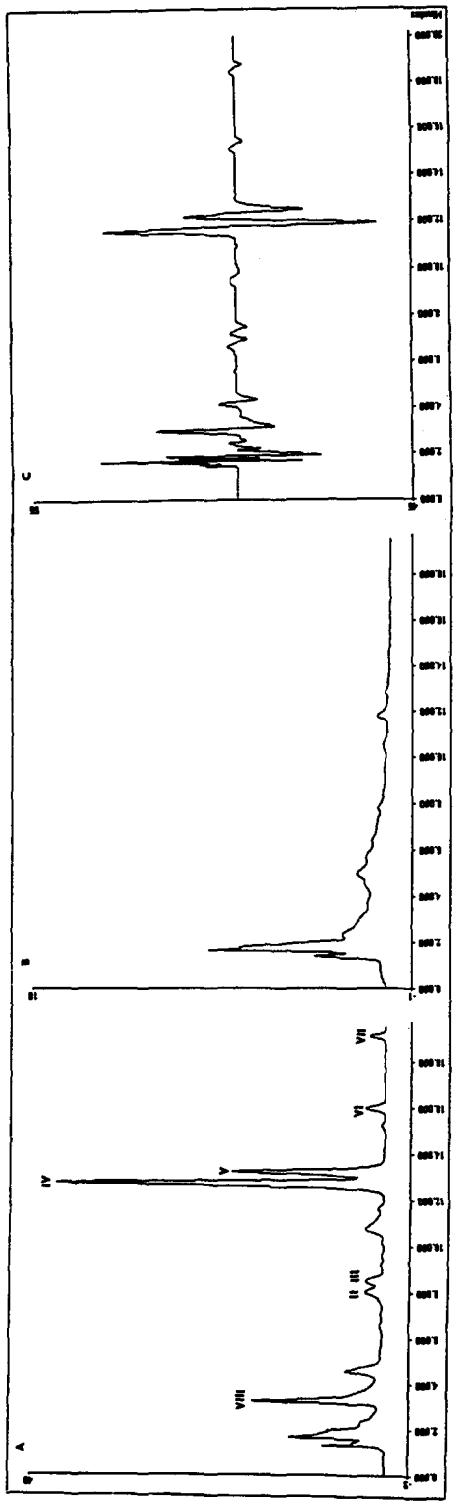

Fig. 3. Chromatograms of urine from exposed rats (A and C) and of control rat urine (B). Chromatograms A and B are absorbances, and C is the first derivative of absorbance at 254 nm. Metabolites were separated on a 150 mm × 3 mm I.D. column packed with 5 μm SGX C_{18} with a linear gradient of methanol (30 to 70% in 20 min) with acetic acid (0.6%).

TABLE II

EXCRETION OF MAIN URINARY METABOLITES

Urine samples were collected 24 h after a single intraperitoneal dose of I in sunflower oil. Values are means ± S.D. (n = 5).

Dose (mg/kg)	Percentage of dose excreted in urine within 24 h as				
	II	III	IV	V	VI
100	1.8 ± 0.5	1.2 ± 0.3	44.7 ± 6.5	20.1 ± 2.6	1.55 ± 0.07
300	2.5 ± 1.2	1.8 ± 1.1	25.3 ± 10.2	13.6 ± 6.3	1.42 ± 0.61
600	1.2 ± 0.8	1.2 ± 0.7	12.1 ± 2.3	6.2 ± 1.7	0.46 ± 0.06

comparison of their R_F values in TLC (Table II) with those of authentic samples [1].

^{1}H NMR spectra of isolated peaks II and III are closely similar to each other and consistent with the structure of ether β-glucosiduronates of 1-(3-ethenylphenyl)-1,2-dihydroxyethane, with characteristic glucosiduronyl CH-1 proton signals [4].

Additional evidence for the structure of II and III was obtained from FAB mass spectra and by enzymic cleavage of these metabolites with bovine β-glucuronidase, yielding 1-(3-ethylphenyl)-1,2-dihydroxyethane (IX) as an aglycone. The aglycone contains an asymmetric carbon atom and may therefore exist in two configurations relative to the chiral β-D-glucosiduronyl moiety. On the other hand, two hydroxy groups are present in the aglycone, either of which may be linked to the glucosiduronyl moiety by a β-glycosidic bond. The chemical shifts of CH- and CH_2-protons for II and III are very close, therefore metabolites II and III seem to be diastereomers rather than regioisomers.

A strongly polar metabolite eluting at 4 min was detected (peak VIII on Fig. 3). This metabolite could not be identified unequivocally by the methods used. Its further purification and structure elucidation will be a subject of another study.

The UV spectra of metabolites II–V, which contain the same chromophore, were closely similar and differed from the spectrum of VII (Fig. 2). In contrast, the unidentified polar metabolite shows a UV spectrum similar to but not identical with that of metabolite VII. An assumption was therefore made that the relative HPLC responses of II–V will be nearly equal so that one calibration curve will be sufficient for metabolites II–VI, especially taking into account the laborious isolation of all metabolites in sufficient amount and purity. Therefore, the major metabolite IV was converted into its dicyclohexylammonium salt [6], which could be easily weighed for calibration. The amounts of individual metabolites (except minor metabolite VII and the unidentified compound VIII) excreted within 24 h after exposure to I are shown in Table II. A substantial portion of the dose is excreted in the form of mercapturic acids IV and V.

Peak purity was checked by measuring spectra at the upslope, downslope and apex of each metabolite peak and was greater than 99%. Addi-

TABLE III

PRECISION OF THE METHOD: COEFFICIENTS OF VARIATION FOR INDIVIDUAL METABOLITE PEAKS (n = 5)

Metabolite	C.V. (%)
II	5
III	7
IV	6
V	5
VI	5
VII	5

tionally, the first-derivative chromatogram was taken, and showed a satisfactory purity of all metabolite peaks (Fig. 3). The reproducibility of the method was tested by repeated analysis of the same urine sample. For all metabolites determined, the coefficient of variation (C.V.) was found to be less than or equal to 7% (Table III). The recovery of metabolite IV as 102 ± 5% (mean ± S.D., $n = 3$) at the concentrations 0.05, 0.1 and 1.0 mg/ml. Sample treatment is minimal before injection. It does not include any operation that would be likely to cause a loss of analyte. Therefore, we expect that the recovery would be nearly 100% for all the metabolites unde study.

DISCUSSION

Gas chromatography–mass spectrometry (GC–MS) is at present the most powerful method of the analysis in urine for metabolites of wide range of compounds, as well as for the products of intermediary metabolism [7]. In most cases, GC–MS requires one or more derivatization steps to convert the metabolites of interest into derivatives with better chromatographic characteristics. Each derivatization, however, may lead to the formation of artifacts for certain types of analyte. Thermal degradation in the injector of the gas chromatograph as well as on the column is another possible source of artifacts. In contrast, HPLC analysis of absorbing styrene derivatives does not require derivatization and is free from artifacts arising from derivatization and thermal degradation. GC–MS analysis of rat urine for metabolites of I led to the identification of eight metabolites [1]. The great advantage of this method was its ability to identify even minor metabolites that could not be found in this study. On the other hand, the direct-injection HPLC method reported here led to the detection and isolation of three metabolites (two glucosiduronates and one unidentified polar metabolite) that had not previously been reported, and to the quantification of the major metabolites.

Diode-array detection does not seem to be very suitable for the structural characterization of individual metabolites of I, owing to the close similarity of the spectra of most metabolites bearing the 3-ethenylphenyl moiety. It proved to be very useful, however, for the optimization of chromatographic conditions.

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