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GAS CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF DOPAMINE AND NOREPINEPHRINE METABOLITES

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SUMMARY

A new gas chromatographic method, using only flame ionization detection which can determine nanogram quantities of homovanillic acid, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylethyleneglycol and 3,4-dihydroxyphenylethyleneglycol in the same reaction, is described. These compounds are treated with diazoethane and *n*-butylboronic acid. Homovanillic acid and 3,4-dihydroxyphenylacetic acid are converted to their ethyl esters while 3-methoxy-4-hydroxyphenylethyleneglycol and 3,4-dihydroxyphenylethyleneglycol form cyclic boronates and are thus assayed. This method is quantitative, highly specific and sensitive. It has been applied to the analysis of these compounds in urine.

INTRODUCTION

3-Methoxy-4-hydroxyphenylethyleneglycol (MHPG) and 3,4-dihydroxyphenylethyleneglycol (DHPG) are metabolites of norepinephrine. The usefulness of the determination of MHPG as an index of central noradrenergic activity has been illustrated by Karoum et al. [1] and De Met and Halaris [2]. Homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) are the principal metabolites of dopamine (DA). It has been suggested that the concentration of DA and its metabolites in plasma might reflect their concentration in brain and therefore, serve as an index of central dopaminergic activity [3]. Recent studies on affective disorders in humans have compared the concentrations of neurotransmitters and their metabolites in urine and cerebrospinal fluid of such patients with normal controls in an attempt to identify the neurochemical abnormalities in conditions like depression, mania and schizophrenia

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[4, 5]. In view of our interest in neurochemical characterization of a clinically well-identified group of childhood schizophrenia patients described by Cantor et al. [6], we have sought to develop methods for the simultaneous determination of these metabolites in a single sample of a body fluid. Fluorimetric [7, 8], liquid chromatographic [9], gas—liquid chromatographic [6, 10] as well as mass fragmentographic [11] methods are available for the determination of these metabolites. Fluorimetric methods involve prior separation using ion-exchange resins and are time consuming. Mass fragmentometry and liquid chromatography require special equipment. Available gas—liquid chromatographic methods for the simultaneous determination of all catecholamine metabolites require the use of electron-capture detectors.

We report here a very simple, specific and highly sensitive gas chromatographic method using flame ionization detection for the simultaneous determination of HVA, DOPAC, MHPG and DHPG. Reaction of these compounds with specific reagents such as *n*-butylboronic acid and diazoethane results in formation of volatile derivatives. *n*-Butylboronic acid is very selective for the 1,2-diol group. The selectivity of diazoethane for acid and phenol functions is well known. Furthermore, various solvent extraction operations which contribute to low recoveries in other procedures are completely eliminated resulting in high yields and a simplified procedure.

EXPERIMENTAL

Materials

n-Butylboronic acid, HVA, DOPAC, 2,2-dimethoxypropane, bis(MHPG) piperazine salt and DHPG were purchased from Sigma (St. Louis, MO, U.S.A.). Ethyl acetate, diethyl ether and methanol were obtained from Fischer Scientific (Fair Lawn, NJ, U.S.A.) and were 99.9 mole% pure and used without further purification. 70% Aqueous ethylamine, sodium hydroxide, *p*-toluenesulfonyl chloride and potassium nitrite were purchased from Aldrich (Milwaukee, WI, U.S.A.). 3% OV-101 on 100—200 mesh Gas-Chrom Q was obtained from Applied Science Labs. (Pennsylvania, PA, U.S.A.), Glusulase, a mixture of β-glucuronidase and arylsulfatase, was supplied by Calbiochem (La Jolla, CA, U.S.A.).

Methods

The infrared (IR) spectra were obtained on a Perkin-Elmer Model 337 spectrophotometer as Nujol mulls or neat liquid films. Nuclear magnetic resonance (NMR) spectra were recorded with a Varian Model 56/60A spectrometer in deuterochloroform, using tetramethylsilane as an internal standard. Mass spectra were obtained on a Finnegan 1015 quadruple mass spectrometer. Melting points were determined on a precalibrated Thermopan apparatus.

Preparation of N-ethyl-N-nitroso-p-toluenesulfonamide. The method used for the preparation of N-ethyl-N-nitroso-*p*-toluenesulfonamide was similar to that of N-methyl-N-nitroso-*p*-toluenesulfonamide [12]. The following quantities were used: *p*-toluenesulfonyl chloride (320 g), 33% aqueous ethylamine (230 ml), sodium hydroxide (70 g), glacial acetic acid (1750 ml) and potassium nitrite (125 g). Yield 320 g, m.p. 42—43°C.

Preparation of diazoethane. The method used for the preparation of diazoethane was similar to that of diazomethane [12]. The following quantities were used: potassium hydroxide (2.0 g), 95% ethanol (10 ml), water (3.0 ml) and N-ethyl-N-nitroso-*p*-toluene sulfonamide (8.6 g) in diethyl ether (50 ml).

Ethylation of HVA. Homovanillic acid (50 mg) was dissolved in methanol (0.5 ml) and a solution of diazoethane in diethyl ether was added. A brisk evolution of nitrogen was observed and the solution turned colorless. More diazoethane in ether was added and the yellow solution was allowed to stand at 3—5°C for 24 h. The organic solvent was removed on a rotary evaporator and the pale yellow liquid left as residue was identified as ethyl 3-methoxy-4-ethoxyphenylacetate (1) from its spectral data. It showed a single peak when its solution in dimethoxypropane was injected into the gas chromatograph. Yield = 64.0 mg (98%); IR, 1748 cm^{-1}, ester C = O; NMR, δ 1.1—1.7, m, CH_3, 6H; δ 3.6, s, CH_2, 2H; δ 3.9; s, OCH_3, 3H; δ 4.0—4.4, m, CH_2, 4H; δ 6.85, s, aromatic protons, 3H. Mass spectrum, calculated, 238; found M^+ 238.

Ethylation of DOPAC. Ethyl 3,4-diethoxyphenylacetate (2) was prepared from 20 mg of 3,4-dihydroxyphenylacetic acid by using the same procedure as described for the preparations of ethyl 3-methoxy-4-ethoxyphenylacetate. It was a pale yellow liquid (29.1 mg, 97% yield) and its identity was established from its spectral data given below: IR, 1748 cm^{-1}; ester C = O; NMR, the ethyl multiplets for the CH_3 and CH_2 groups occur at δ 1.1—1.7 and 3.9—4.4, respectively. The benzylic singlet resonates at δ 3.55 while the aromatic protons absorb at δ 6.8. Mass spectrum, calculated, 252; found, M^+ 252.

Ethylation of MHPG. Bis(MHPG) piperazine salt (20 mg) was treated with diazoethane as described, for 24 h. The organic solvent was removed on a rotary evaporator and the residue was treated with water (0.5 ml). The aqueous solution was extracted with methylene chloride (5 ml, five times) to remove piperazine. The combined organic layer was dried over anhydrous magnesium sulfate and the organic solvent removed on a rotary evaporator. The residue (17.5 mg, 94% yield) was identified as 3-methoxy-4-ethoxyphenylethyleneglycol (3) from its spectral data: NMR, δ 3.85, s, OCH_3, 3H; δ 1.4, t, CH_3, 3H; δ 4.0, q, CH_2, 2H; δ 3.7—3.4, m, CH_2—CH—, 3H; δ 4.65, broad peak, OH, 2H; δ 6.8, s, aromatic protons, 3H. Mass spectrum, calculated, 212; found, M^+ 212.

Ethylation of DHPG. 3,4-Diethoxyphenylethyleneglycol (4) was prepared from 20 mg of DHPG. The procedure used was the same as described for the preparation of (3). The yield was 25.0 mg (95%). The product was characterized from its spectral data: NMR, δ 1.4, t, CH_3, 6H; δ 4.05, q, CH_2, 4H; δ 3.25—3.75, m, CH_2—CH—; δ 4.6, s (broad), OH, 2H; δ 6.75, s, aromatic protons, 3H. Mass spectrum, calculated, 226; found, M^+ 226.

Butylboronation of 3-methoxy-4-ethoxyphenylethyleneglycol. The method used for the preparation of *n*-butylboronate of 3-methoxy-4-ethoxyphenylethyleneglycol is similar to that of Biondi et al. [13]. 3-Methoxy-4-ethoxyphenylethyleneglycol (1 mg) was dissolved in dimethoxypropane (1 ml) and *n*-butylboronic acid (3 mg) was added to the above solution. The reaction was complete after 30 min at room temperature. A single peak of the cyclic boronate (5) was observed when 1 μl of this solution was injected into the gas chromatograph.

Similar results were obtained when 3-methoxy-4-ethoxyphenylethylene-

glycol and *n*-butylboronic acid were injected directly into the gas chromatograph.

Butylboronation of 3,4-diethoxyphenylethyleneglycol. Cyclic boronate (6) was prepared using the same conditions described for the boronate of 3-methoxy-4-ethoxyphenylethyleneglycol.

Extraction of HVA, DOPAC, MHPG and DHPG from urine. Urine samples were preserved with 0.5 mg sodium metabisulfite per ml urine and stored at −18°C until used. An aliquot (5 ml) from a 24-h pooled sample was adjusted to pH 6.2 with dilute hydrochloric acid or 5 *M* sodium hydroxide solution and glusulase (100 μl) added. The reaction mixture was incubated overnight at 37°C, lyophilized and treated with ethyl acetate containing 2% methanol (30 ml, four times). The combined extract was centrifuged and the supernatant was stripped off the organic solvent on a rotary evaporator under reduced pressure. The thoroughly dried residue was dissolved in methanol (1.5—2.0 ml) and ethereal solution of diazoethane (prepared from 2.0 g of N-ethyl-N-nitroso-*p*-toluene sulfonamide and 15 ml of diethyl ether) added until a persistent yellow solution was obtained. The slight turbidity which appeared in some reactions was cleared by further addition of methanol. The reaction mixture was allowed to stand at 3—4°C in the refrigerator for 24 h. The organic solvent along with any unreacted diazoethane was removed under reduced pressure and the residue dissolved in 0.5 ml of 2,2-dimethoxypropane. A 1-μl aliquot of this solution together with 0.5 μl of *n*-butylboronic acid in dimethoxypropane (3 mg/ml) was injected into the gas chromatograph programmed at 150—190°C as described under Gas—liquid chromatography.

Gas—liquid chromatography

Gas—liquid chromatography was accomplished with a Beckman GC-65 gas chromatograph provided with a flame ionization detector. A U-shaped glass column (1.8 m × 2.0 mm I.D.) packed with 3% OV-101 on 100—200 mesh Gas-Chrom Q was used. The injector, inlet and detector temperatures were 225°C, 250°C and 275°C, respectively. The column temperature was initially set at 150°C for 17 min and then programmed to 190°C at 35°C/min and maintained at 190°C for 13 min. The carrier gas (nitrogen), hydrogen and air flow-rates were 30, 45 and 280 ml/min, respectively. The attenuation range used was 100 × 4.

RESULTS

Diazoethane reacted with HVA, DOPAC, MHPG and DHPG at 3—5°C and gave compounds 1, 2, 3 and 4, respectively. Compounds 3 and 4 reacted further with *n*-butylboronic acid and gave cyclic boronates 5 and 6 (Fig. 1). Cyclic boronates of acetyl-MHPG and -DHPG have been described [13, 14]. Compounds 1, 2, 3 and 4 were characterized fully from their IR, NMR and mass spectral data. The mass spectra of 1 and 3 are shown in Fig. 2. HVA and DOPAC, thus, were determined as esters 1 and 2 while MHPG and DHPG were determined as boronates 5 and 6. The diazoethane reaction forming the above ethyl derivatives was complete in about 24 h as followed by gas chromatography of the reaction mixture. It was further observed that the presence of

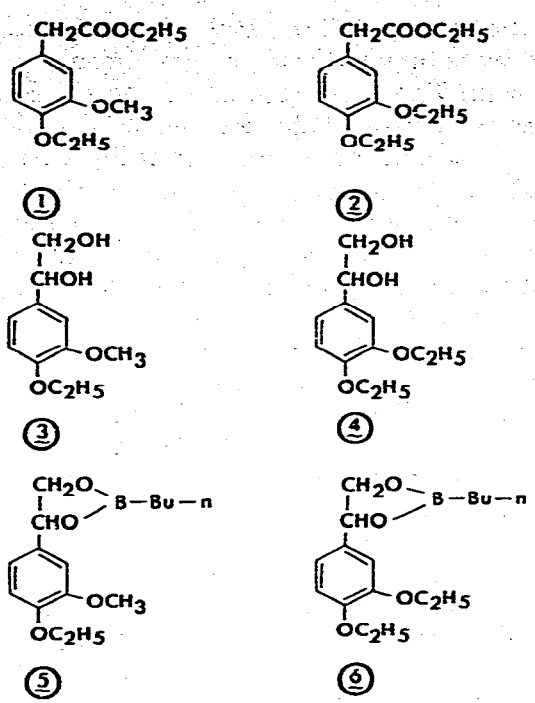

Fig. 1. Formulae of HVA, DOPAC, MHPG and DHPG derivatives.

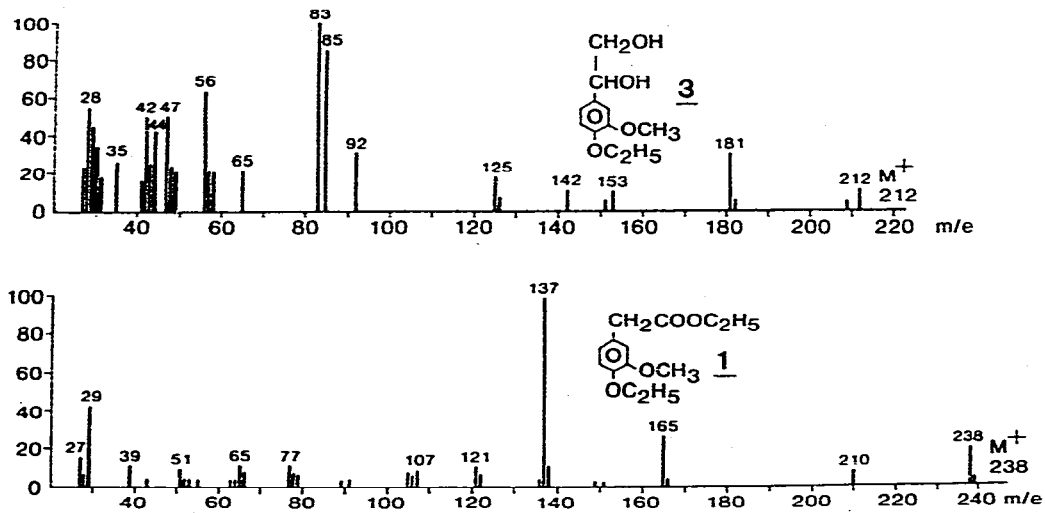

Fig. 2. Mass spectra of ethyl 3-methoxy-4-ethoxyphenylacetate (1) and 3-methoxy-4-ethoxyphenylethylene gycol (3).

large excess of diazoethane and boronic acid in the reaction mixture did not produce any undesirable products.

To ascertain linearity of product formation, 1, 2, 3 and 4 μg respectively of each of HVA, DOPAC, MHPG and DHPG were treated separately with diazoethane as described under Methods. The residues from these reactions were

diluted with 2,2-dimethoxypropane and aliquots containing 100, 200, 300 and 400 ng of each of 1, 2, 3 and 4 produced were injected into the gas chromatograph along with 3 μg of *n*-butylboronic acid. The total volume of each injection was 1.0 μl. These reactions were linear and a plot of the peak areas vs. concentration is shown in Fig. 3. Similar results were obtained when four different mixtures containing 0.4, 0.6, 0.8 and 1.0 μg, respectively, of each of HVA, DOPAC, MHPG and DHPG were reacted with diazoethane and injected into the gas chromatograph with *n*-butylboronic acid as before. The presence of these different components in the reaction mixture did not appear to affect the yield of the final products. The plot for the standards added to a 5-ml sample of pooled urine was also linear from 20 to 80 ng. The separation of 1, 2, 5 and 6 on the gas chromatograph is shown in Fig. 4. The retention times for the derivatives of HVA, DOPAC, MHPG and DHPG were 9.20, 11.06, 21.56 and 22.44 min, respectively, when the column was programmed at 150—190°C as described under Gas—liquid chromatography. The procedure is sensitive enough to determine easily 10—15 ng of each of 1, 2, 3 and 4 in a mixture.

Various attempts at reducing the reaction time in the presence of Lewis acids such as boron trifluoride and anhydrous aluminium chloride as catalyst, met with little success. These reactions gave undesired products along with 1, 2, 3 and 4. The reaction of HVA, DOPAC, MHPG and DHPG was also attempted with diazopropane. The reaction was not complete even after 36 h. This may be due to steric hindrance and/or the lesser reactivity of diazopropane compared to diazomethane or diazoethane.

The precision of the determination as well as the reproducibility of data in the method described here were examined using both a standard mixture of HVA, DOPAC, MHPG and DHPG as well as a pooled sample of urine from normal children.

The average recoveries (n = 20) of the standards (100 ng each) added to a

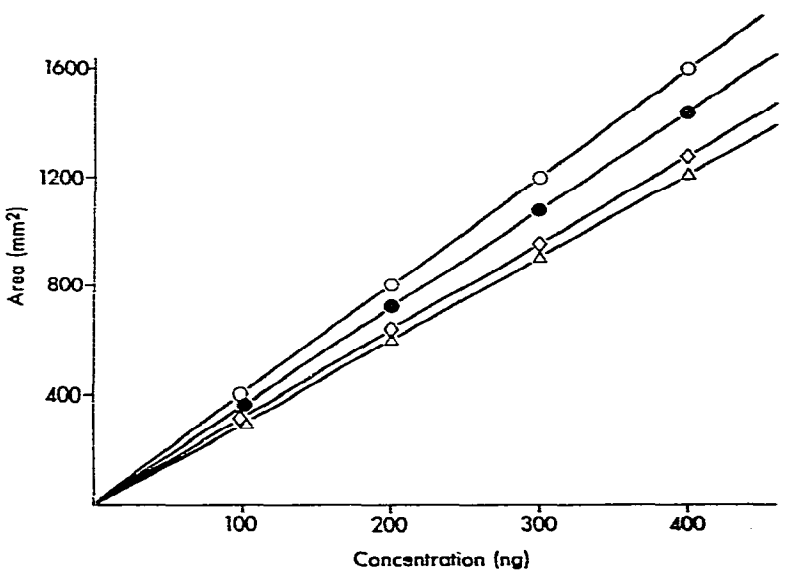

Fig. 3. Plot of peak areas (mm²) vs. concentration to show linearity of reaction. Each dot represents the mean area from 15 experiments. ○, HVA; ●, DOPAC; ◇, MHPG; △, DHPG.

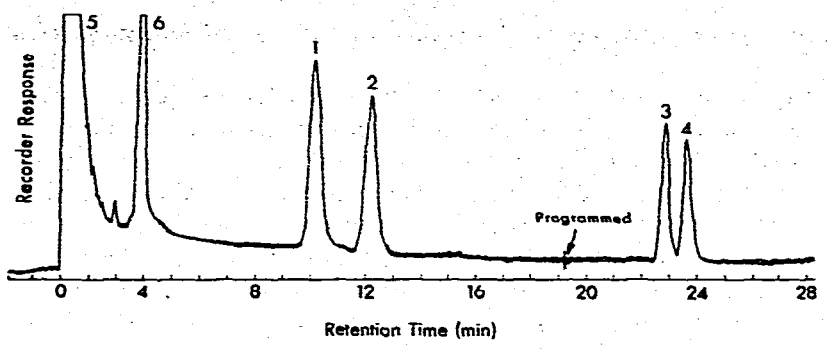

Fig. 4. Gas chromatographic separation of the derivatives of HVA (peak 1, 125 ng), DOPAC (peak 2, 125 ng), MHPG (peak 3, 75 ng), and DHPG (peak 4, 75 ng). Peaks 5 and 6 are of dimethoxypropane and *n*-butylboronic acid. The separation was carried out in a U-shaped glass column packed with 3% OV-101 on 100—200 mesh Gas-Chrom Q. The column temperature was programmed from 150 to 190°C (see text).

5-ml sample of pooled urine prior to ethylation reaction were as follows: HVA, 98.5 ± 1.2%; DOPAC, 96.1 ± 3.4%; MHPG, 97.2 ± 1.8%; and DHPG, 95.4 ± 3.7%. The analysis of the pooled urine sample (n = 20) was as follows: HVA, 1600 ± 82 μg/l; DOPAC, 1355 ± 56 μg/l; and MHPG, 1120 ± 54 μg/l. There was no measurable DHPG in these samples. The average recoveries of HVA, DOPAC, MHPG and DHPG (100 ng each) added to water (5 ml) instead of the urine were also determined according to the above procedure. No significant difference in the recoveries of standards in these two sets of experiments was observed. These results therefore rule out any possible interference from impurities that may be present in the urine extract. A blank run with the enzyme preparation was also carried out to check any interference due to enzyme preparation. No detectable peak with a retention time the same as those of the standards was observed.

DISCUSSION

The difficulties inherent in some of the procedures [15—18] in use for HVA have been reviewed by Sato [19]. The presence of interfering substances in urine results in a high blank in the spectrophotometric procedure for MHPG [20]. Wilk et al. [21] have used a gas chromatographic method for determination of MHPG as the trifluoroacetyl derivative. In this procedure which involves an AG 1-X4 ion-exchange column separation, the recovery of MHPG was only of the order of 50%, as stated by Dekirmenjian and Mass [22]. The gas chromatographic method of Biondi et al. [13] is based on acetylation and subsequent boronation of MHPG. In this procedure both mono- and diacetylated products are formed as shown by our IR and NMR studies of the reaction mixture. Besides, extraction of the aqueous reaction mixture with methylene chloride or ethyl acetate resulted in a yield of 52% of the added MHPG [13]. This method, when applied to DHPG, gave still lower recoveries [14]. In addition multiple acetylated products are formed as per our observations. This procedure can not be applied to a mixture of MHPG, DHPG, HVA and DOPAC as acetic anhydride will form mixed anhydrides with HVA and DOPAC. This

could lead to undesired products with nucleophilic or electrophilic impurities in the urine.

The gas chromatographic method reported here is free of these defects since highly specific reagents such as *n*-butylboronic acid and diazoethane have been used for the preparation of volatile derivatives. Diazoethane reacts only with the phenol and carboxyl functionalities under the conditions employed in this investigation while *n*-butylboronic acid forms cyclic boronates only in the presence of a 1,2-diol group. No side products were observed when these reagents were used in large excess or the reaction periods were extended. More importantly, these reagents allow the simultaneous determination of HVA, DOPAC, MHPG and DHPG from the same sample and in a single reaction. Organic solvent extraction operations, which reduce the yield of the target compounds, are completely eliminated in this procedure. Therefore the yields are quantitative. This method has been applied successfully to the determination of HVA, DOPAC, MHPG and DHPG in the urine of childhood schizophrenic patients [6]. These results will be published elsewhere.

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