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Note

Procedure for the determination of 4-hydroxy-3-methoxyphenylethyleneglycol in urine by gas chromatography with flame-ionization detector

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Measurements of urinary 4-hydroxy-3-methoxyphenylethyleneglycol (HMPG) are of great importance in clinical studies of psychiatric disorders [1, 2] and in the diagnosis of catecholamine-secreting tumours [3]. Recent papers have reported elaborate methods for the determination of HMPG either by gas—liquid chromatography with electron-capture detection (GC—ECD) [4] or by mass fragmentography (MF) [5].

We have devised a selective procedure for the routine determination of urinary HMPG by GC with a flame-ionization detector (GC—FID). This detector is more generally used than the ECD in clinical laboratories. Kahane et al. [6] performed Sharman's [7] preliminary acetylation of HMPG in alkaline solution and finally determined the HMPG by GC—FID of the triacetate derivative. In comparison with this method, we increased the specificity and the rapidity of the HMPG determination.

We previously introduced the use of boronic acids as specific reagents for HMPG bearing a diol group and analysed HMPG as the trimethylsilyl (TMS)—boronate derivative [8]; we then adapted the method described by Sharman [7] to obtain the 4'-acetyl-HMPG—boronates (Ac-HMPG—boronates) available for the MF determination of HMPG [9].

A brief outline of the use of Ac-HMPG—boronates in the analysis of urinary HMPG by GC—FID was given in a communication on the possible clinical applications [10]. However, a more extensive description and discussion of the procedure is necessary, which is the purpose of this paper.

EXPERIMENTAL

Reagents

Bis(4-hydroxy-3-methoxyphenylethyleneglycol) piperazine salt was purchased from Regis, Morton Grove, Ill., U.S.A.; Suc d'*Helix pomatia* containing 10^5 units (Fishman) of β-glucuronidase and 10^6 units (Roy) of aryl sulphatase per millilitre was obtained from Industrie Biologique Française, Genevilliers, France, methaneboronic acid [$MeB(OH)_2$] from Applied Science Labs., State College, Pa., U.S.A., *n*-butaneboronic acid [$BuB(OH)_2$] from Serva, Heidelberg, G.F.R. and 2,2-dimethoxypropane (DMP) from J.T. Baker, Deventer, The Netherlands; all other solvents and inorganic compounds were obtained from Carlo Erba, Milan, Italy.

Apparatus

A Carlo Erba dual-column gas chromatograph (Fractovap Model GV) equipped with a flame-ionization detector was used. The U-shaped glass column (2 m × 2.5 mm I.D.) was packed with 3% OV-101 on 100—200 mesh Gas-Chrom Q (Applied Science Labs.). The injector and detector temperatures were 200° and 260°, respectively. The column temperature was programmed from 150° to 240° at 15°/min and maintained at 240° for 3 min. The carrier gas (nitrogen), hydrogen and air flow-rates were 40, 35 and 390 ml/min, respectively.

Combined GC—mass spectrometry was carried out on a Finnigan Model 3100 instrument with the same gas chromatographic conditions as mentioned above and with an electron energy of 70 eV.

Procedure

The urine sample (15 ml) from the 24-h pool (stored at −18°) was incubated overnight at 37° with Suc d'*Helix pomatia* (0.3 ml) after adjustment of the pH to 6.5 with dilute HCl or NaOH. The sample was acidified to pH 1 with 6 *N* HCl, saturated with NaCl and extracted with three 40-ml portions of ethyl acetate; the combined organic phases were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was dissolved in water (3 ml), treated with acetic anhydride (0.3 ml) and enough powdered $NaHCO_3$ was added to saturate the reaction mixture. The resulting solution was extracted with two 10-ml volumes of dichloromethane; the combined organic phases were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure in a water-bath at 40°. The residue was dissolved in a solution of phenanthrene, which was used as the internal standard, (50 μg/ml) in DMP (0.2 ml). Finally, 1 μl of this solution was injected together with 0.5 μl of $MeB(OH)_2$ or $BuB(OH)_2$ solution in DMP (3 mg/ml) into the gas chromatograph.

Quantitative analysis

Peak areas were calculated as peak height times the width at half-height. Calibration graphs were obtained with standard aliquots (5, 10, 20, 30 and 40 μg) of pure Ac-HMPG, prepared according to Sharman [7]. The peak areas of HMPG derivatives were corrected against that of phenanthrene (10 μg). All of

the test runs on aqueous solutions were carried out both with $MeB(OH)_2$ and with $BuB(OH)_2$ and the final results calculated as the mean of those obtained from the two different reagents.

Recovery

The recoveries were calculated from a comparison of the experimental and theoretical corrected areas for the five different standard amounts (5, 10, 20, 30 and 40 μg) and the mean value was taken.

In order to ascertain the percentage recovery of Ac-HMPG from aqueous solution into dichloromethane, equal aliquots (3 ml) of $NaHCO_3$ saturated solution containing standard amounts (5—40 μg) of Ac-HMPG were extracted and treated according to the described procedure.

To determine the yield of acetylation in the aqueous phase, the above-mentioned recoveries were compared with those obtained from standard amounts (5—40 μg) of pure HMPG in water (3 ml) treated with acetic anhydride, $NaHCO_3$ and then extracted according to the described method.

To check the influence of urine on the recovery throughout the whole procedure, standard aliquots (5—40 μg) of HMPG were added to urine samples from the same pool. Their recoveries were compared with those obtained from the aqueous solutions containing similar amounts of HMPG treated according to the procedure described above.

A blank run with enzyme preparation was carried out in order to check its content of HMPG.

RESULTS

The calibration graphs for Ac-HMPG—methaneboronate (Ac-HMPG—MeB) and Ac-HMPG—butaneboronate (Ac-HMPG—BuB) were linear in the experimented range (5—40 μg) and identical with those for the derivatives obtained by reaction with boronic acids in DMP before injection into the gas chromatograph. The relative responses against phenanthrene were 0.91 ± 0.06 and 0.97 ± 0.03 (mean ± S.D.) (n = 25) for the methyl and butyl derivative, respectively.

Calibration graphs calculated with the peak heights were also linear but with a slightly higher standard deviation.

Fig. 1 shows typical gas chromatograms of the two derivatives obtained from urine samples taken through the described procedure. As the Ac-HMPG—BuB peak appeared to be better separated from neighbouring peaks than the Ac-HMPG—MeB peak, the analyses of biological samples were performed on the butyl derivative. Nevertheless, during test runs with aqueous solutions, the methyl derivative was also used because of its lower retention time relative to the internal standard (0.90 ± 0.05) (n = 50) compared with that of the butyl derivative (1.42 ± 0.13) (n = 50).

The identities of the two derivatives were checked by ensuring that the mass spectra of authentic Ac-HMPG—boronates were identical with those obtained from urine samples. The mass spectra have been reported elsewhere [9].

The recovery of Ac-HMPG was 89.2 ± 5.4 % (n = 10) from the extraction into dichloromethane. The yield of acetylation was 97 ± 2.5 % (n = 10). By

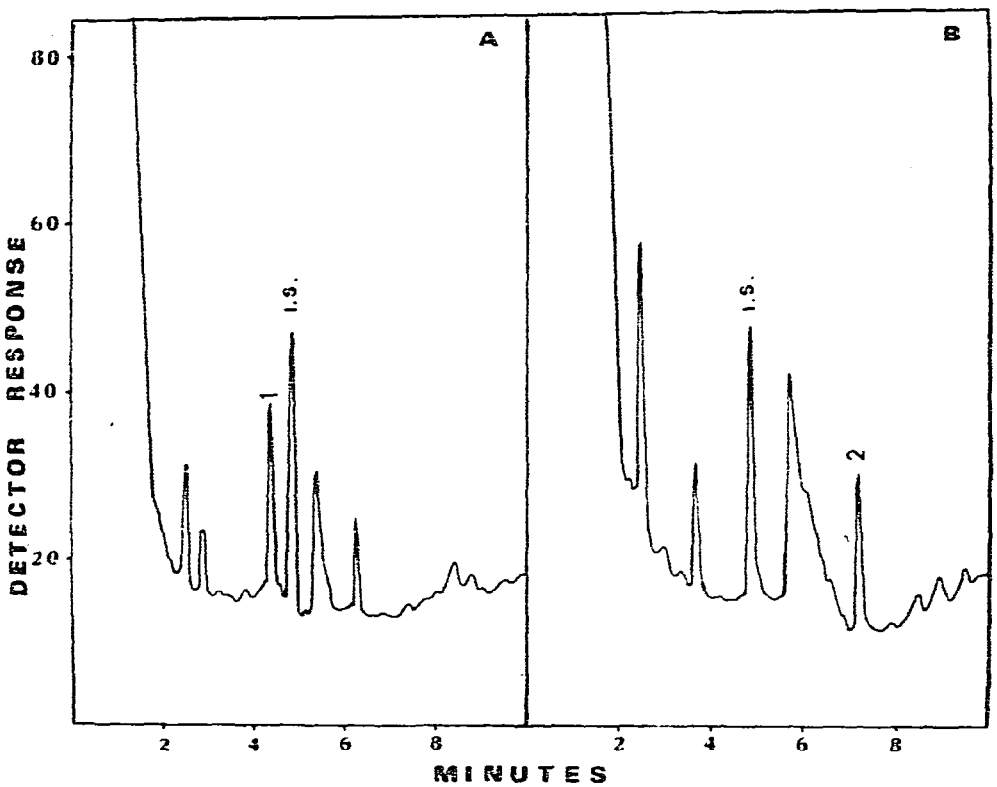

Fig. 1. Typical gas chromatograms of urinary extracts subjected to the complete procedure, using $MeB(OH)_2$ (A) or $BuB(OH)_2$ (B); 1 μl of each sample, containing 0.05 μg of phenanthrene (internal standard), was injected. 1 = Ac-HMPG—MeB, 2 = Ac-HMPG—BuB.

using the whole procedure, the recovery of pure HMPG added to water was 52.1 ± 4.1 % (n = 40).

By adding pure HMPG to urine samples, no significant differences were detected in its recoveries with respect to those obtained from water.

No detectable peak with a retention time the same as that of HMPG was observed when an enzyme preparation was treated according to the described procedure. The amount of HMPG released from the same urine sample was not increased by using a volume of enzyme preparation greater than 0.3 ml. The precipitation of proteins after hydrolysis performed with acetone in preliminary experiments showed no advantages and its use was discontinued.

The precision of the method was tested by evaluating the percentage difference between duplicate samples of urine; the result was 8.15 ± 7.43 % (mean ± S.D.) (n = 20).

The contents of urine samples (15 ml) and the calculated total output of HMPG in 24-h pools ranged between 6.5 and 37.8 μg and between 650 and 1530 μg per 24 h, respectively.

DISCUSSION

In order to make the specificity of an analytical GC method as high as possible, the structural features of the biological compound of interest must be considered when selecting a technique for its separation and the most suitable volatile derivative. In this connection, aqueous phase acetylation and reaction with boronic acids are together very selective for HMPG as it bears both phenolic and glycolic moieties.

With regard to the preliminary treatment of the samples, we used extraction with ethyl acetate from acidified urine, as discussed earlier [11]. In this step, basic compounds were removed from organic extracts. Later, after acetylation in alkaline solution according to the method introduced by Sharman [7], the sample was purified from acidic compounds by extraction into dichloromethane. The final mixture therefore contained only neutral metabolites.

Sharman's GC—ECD procedure was reviewed by Bond [12], who separated HMPG from urine on an anion-exchange resin (AG1-X4) and by O'Keeffe and Brooksbank [13] who purified Ac-HMPG by thin-layer chromatography.

In our work, AG1-X4 resin did not give satisfactory results because, with the larger volumes of urine that we used we did not obtain reproducible chromatographic conditions. On the other hand, Ac-HMPG extracted with dichloromethane did not, in our case, require preliminary purification before gas chromatographic analysis as it did in O'Keeffe's method.

In spite of the lack of sensitivity of the FID itself, the treatment with boronic acids yields derivatives that give a chromatographic profile with fewer and better separated peaks. Hence preliminary purification was not necessary when the urine samples were analyzed. This is due to the specificity of the reagents, which enable part of the sensitivity lost by using an FID instead of an ECD to be regained. In fact, they facilitate accurate quantitative analysis by allowing the GC—FID apparatus to be used successfully at its maximum sensitivity.

Unlike our previous experiments with HMPG—boronates, we introduced the improvement that the reaction with boronic acids can be carried out by direct injection of the reagents into the gas chromatograph.

In conclusion, we can state that the procedure described here is rapid, specific and applicable to the routine analysis of urinary HMPG in clinical studies.

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