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Note

A new internal standard suitable for the gas chromatographic determination of 3-methoxy-4-hydroxyphenylethyleneglycol

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In order to improve the precision of the gas chromatographic (GC) analysis of (3-methoxy-4-hydroxyphenyl)ethane-1,2-diol (3-methoxy-4-hydroxyphenylethyleneglycol, MHPG) a proper internal standard should have a structure as similar as possible to MHPG itself. In this connection in recent procedures for MHPG determination by GC with electron-capture detection (GC—ECD), 3-methoxy-4-hydroxyphenylethanol [1] and the MHPG isomer 3-hydroxy-4-methoxyphenylethyleneglycol [2] have been used as internal standard, but both the former compound to a major extent [3] and the latter compound [4] are shown to be present in urine as endogenous metabolites and do not allow the final MHPG evaluation to be sufficiently accurate without additional adjustments.

Synthetic internal standards that are not so similar to MHPG as the compounds mentioned above, *o*-hydroxyethylresorcinol [5] and tryptophol [6], have been successfully used in other methods for MHPG estimation, again with the GC—ECD technique. In our experience, anyway, also the less sophisticated flame ionization detection (FID) has been shown to be suitable for GC analysis of urinary MHPG, provided that derivatization selectivity is increased by the use of boronic acids, which are specific reagents for

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diol groups [7]. In our method, phenanthrene was used as reference compound added only before injection, but the assay precision was shown to be rather low.

In this connection a synthetic non-endogenous internal standard containing both MHPG functional groups, phenolic and glycolic ones, was indispensable to improve the reproducibility of the GC—FID method without any loss in specificity. The lack of such a compound has been stated to be cumbersome also in a recent MHPG determination by high-performance liquid chromatography (HPLC) carried out with fluorescence detection [8].

A substance with the above-mentioned features not being available, our aim was to obtain it from inexpensive reagents by means of an easy synthesis. For this purpose the MHPG homologue 3-(3-methoxy-4-hydroxyphenyl)propane-1,2-diol (3-methoxy-4-hydroxybenzylethyleneglycol, MHBG) was obtained in the simple pathway shown in Fig. 1 from 3-(3-methoxy-4-hydroxyphenyl)-propene (eugenol, Eu).

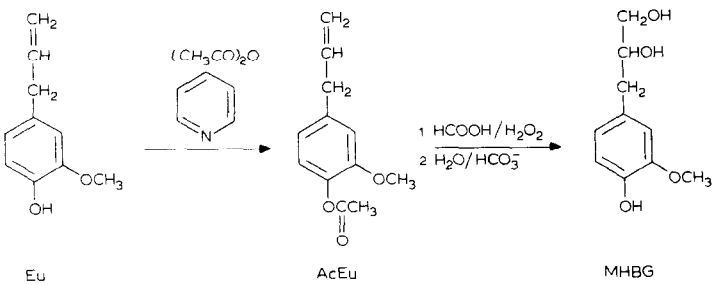

Fig. 1. Scheme of the synthesis of MHBG from Eu.

MATERIALS AND METHODS

The solvents and inorganic and organic reagents used were purchased from Carlo Erba (Milan, Italy); Eu and piperazine were from Fluka (Buchs, Switzerland).

MHBG synthesis

In the first step the Eu phenolic group was protected as acetate: Eu (10 ml) was kept overnight at room temperature in acetic anhydride and pyridine (20 ml each). The reaction mixture was poured into crushed ice and extracted with diethyl ether. The organic phase, washed with 1 *N* HCl and 5% $NaHCO_3$, was dried and evaporated to give acetyl-Eu (AcEu).

In the second step the glycol moiety was introduced by stirring at room temperature AcEu (1 g) with 98% formic acid (5 ml) and 35% hydrogen peroxide (0.8 ml). The reaction was complete within 2 h, as shown by the disappearance of the AcEu spot in thin-layer chromatographic analysis carried out on silica gel 60 F_{254} (0.2 mm) (Merck, Darmstadt, G.F.R.) (mobile phase, toluene—ethyl acetate (8:2); detection, UV lamp at 254 nm). The glycol monoformates so obtained were directly hydrolyzed by diluting the reaction mixture

with water (20 ml), adding $NaHCO_3$ to pH 8 and heating for 3 h at 80°C. During this treatment both formyl and acetyl groups were released from MHBG. The solution was then extracted with ethyl acetate which was finally evaporated.

The oily residue containing MHBG was dissolved in acetone—hexane (2:1) mixture and a saturated solution of piperazine in the same mixture was added to give as precipitate the bis-MHBG-piperazine salt, finally recrystallized in the same solvent mixture (m.p. 106°C). Elemental analysis calculated for $C_{24}H_{38}O_8N_2$: C, 59.74; H, 7.94; N, 5.81. Found: C, 60.25; H, 8.02; N, 5.76. Overall yields ranged from 25% to 30%.

In the NMR spectrum of free MHBG, obtained by extracting the acidic solution of the piperazine salt, the expected signals appeared: Ph-CH_2 (2.67, 2H, d); CH_2OH (3.45, 2H, m); CHOH (3.65, 1H, m); OCH_3 (3.77, 3H, s); aromatic hydrogens (6.78, 3H, m).

The same derivative used in our method for the MHPG determination, acetyl-MHBG-*n*-butaneboronate (Ac-MHBG-BuB), was obtained according to the previously described procedure and analyzed by gas chromatography—mass spectrometry (GC—MS) using a Finnigan Model 3100 instrument with the same GC conditions described below and with an electron energy of 70 eV. As well as for MHPG derivative [9] the mass spectrum showed a low molecular peak (*m/e* 306) and a high peak (95% of *m/e* 138 base peak) corresponding to the fragment from $CH_2{=}C{=}O$ loss (*m/e* 264).

Urine analysis

For the treatment of urine samples, the only difference in comparison with the described method was the addition of MHBG (10 μg) to the urine specimen (15 ml) from the 24-h collections.

GC analysis was carried out on a Dani (Monza, Italy) Model 3600 B apparatus equipped with a glass column (2 m × 2.5 mm I.D.) packed with 3% SP-2100 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The injector and detector temperatures were 260°C and 310°C, respectively. The column temperature was programmed from 220°C to 300°C at 12°C/min. The carrier gas (nitrogen), hydrogen and air flow-rates were 42, 35 and 400 ml/min, respectively.

Calibration curves were obtained by adding different amounts (5—40 μg) of MHPG and constant amounts of MHBG (10 μg) to urine samples and carrying out the whole procedure described elsewhere [7]. The MHPG peak was corrected for its endogenous content.

RESULTS

The methanolic standard solutions of MHBG (0.1 mg/ml as free MHBG) were kept in the refrigerator and showed no content variations up to two weeks.

In the GC conditions used the MHPG retention time relative to MHBG was 0.91 ± 0.06 (n = 20) and the zone of MHBG peak was free from interfering peaks when MHBG was not added to the urine samples, as shown in Fig. 2.

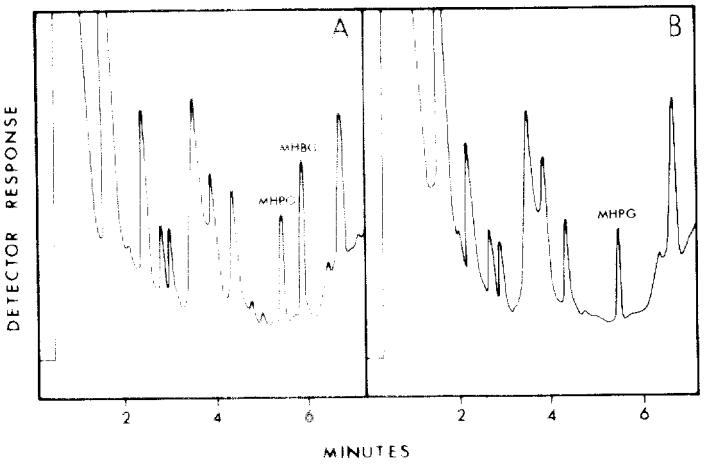

Fig. 2. Typical GC profiles obtained from a urine sample with (A) and without (B) MHBG.

Routine regression lines were obtained for each preparation of MHBG with r values ranging from 0.997 to 0.999. The slope of the standard curves (peak height ratio between MHPG peak and MHBG peak/MHPG amount) was 0.109 ± 0.008 (n = 5).

Within-run precision was estimated by analyzing seven samples of a 24-h urine collection. The MHPG content and the standard deviation was 535 ± 12 μg/l. Day-to-day precision was measured on three urine samples analyzed ten times in a month. The MHPG mean contents and the standard deviations were 415 ± 10 μg/l, 917 ± 19 μg/l and 1228 ± 24 μg/l.

CONCLUSIONS

The data on statistical validation of the use of MHBG clearly showed higher precision in comparison to that obtained with phenanthrene as reference compound.

In conclusion, the use of a "true" internal standard made the MHPG analysis by GC more simple, rapid and reliable. In spite of our experience being restricted to the GC–FID technique, MHBG could be an ideal analytical tool suitable for the evaluation of MHPG in biological fluids by other techniques such as GC–ECD and HPLC with electrochemical or fluorescence detection.

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