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Note

Gas chromatographic determination of the glucuronide of 2-(1-hydroxy-ethyl)-7-(2-hydroxy-3-isopropyl-aminopropoxy)-benzofuran, a metabolite of befunolol, in human urine

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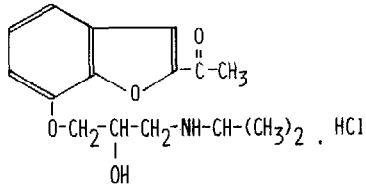
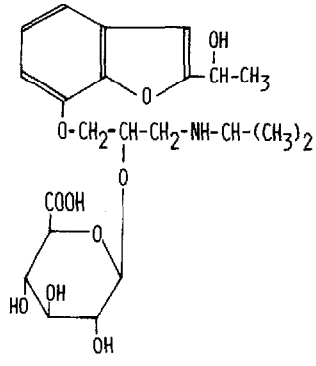
Befunolol (Fig. 1) is a β-adrenergic receptor blocking agent [1]. 2-(1-Hydroxy-ethyl)-7-(2-hydroxy-3-isopropyl-aminopropoxy)-benzofuran (MI) and its glucuronide (MIG, see Fig. 1) have been known as the metabolites of befunolol excreted in animal and human urine [2, 3]. MIG in human urine after oral administration of befunolol has been determined by enzymatic hydrolysis of glucuronide followed by gas chromatography (GC) and gas chromatographic—mass spectrometric (GC—MS) analysis of the released aglycone (MI) [4, 5]. Direct GC methods have been reported for the quantitative determination of glucuronides of thiamphenicol, chloramphenicol [6], oxazepam and lorazepam [7] after methyl-trimethylsilylation, and of trimetozine [8] after permethylation.



befunolol hydrochloride



MIG

Fig. 1. Chemical structures of befunolol and MIG.

In this paper, we describe a GC method for the quantitative determination of MIG after trimethylsilylation. The conditions for pretreatment of urine and for trimethylsilylation of MIG are investigated. The method established is applied to a comparison of the excretion rate of MIG with those of befunolol and MI (unconjugated).

EXPERIMENTAL

Reagents and materials

Ethyl acetate, diethyl ether, methanol (for pesticide residue analysis) and acetic acid (analytical grade) were obtained from Wako (Osaka, Japan). N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and N-(trimethylsilyl)imidazole (SIM) were specially prepared reagents for GC analysis (Nakarai Chemical, Kyoto, Japan). 6-Bromo-2-naphthyl-β-D-glucuronide used as an internal standard (IS) was obtained from Sigma (St. Louis, MO, U.S.A.), and *n*-tetracontane used as an external standard (ES) was from Applied Science Labs. (State College, PA, U.S.A.). IS and ES were used as aqueous solution (40 μg/ml) and chloroform solution (22 μg per 50 μl), respectively.

Amberlite XAD-2 resin (Rohm & Haas, Philadelphia, PA, U.S.A.) was purified as described in the previous paper [8]. QAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) was swollen in distilled water for three days and washed with distilled water.

Authentic MIG was isolated from the urine of dog dosed with befunolol by the method described in the previous paper [3].

Drug administration to volunteers

Two healthy male adults each received an oral dose of a 20-mg befunolol capsule after a 16-h fast. Urine specimens were collected just before and at 1, 2, 3, 4, 5, and 6 h after administration.

Sample preparation

A 2-ml portion of urine was placed on a QAE-Sephadex A-25 column (3 cm × 5 mm I.D.) and eluted with 7 ml of distilled water. The eluate was washed twice with 10 ml of ethyl acetate. The trace of ethyl acetate remaining in the aqueous layer was removed by washing twice with 10 ml of diethyl ether. After complete removal of diethyl ether by evaporation under reduced pressure at room temperature for 10 min, a 1-ml portion of IS solution was added to the aqueous layer and the mixture was placed on an Amberlite XAD-2 column (5 cm × 10 mm I.D.). The column was first washed with 20 ml of distilled water and subsequently eluted with 20 ml of methanol containing 1.5% acetic acid. The latter eluate was collected and evaporated to dryness in a flask under reduced pressure at 40°C. The residue was trimethylsilylated by reaction with 40 μl of SIM and 30 μl of BSTFA for 20 min at room temperature. A 1-μl portion of the reaction mixture was injected into the gas chromatograph.

GC conditions

An Hitachi 073 gas chromatograph equipped with a flame ionization

detector was used. A U-shaped glass column (0.5 m × 3 mm I.D.) was packed with 1.5% OV-17 on Chromosorb W AW DMCS, 80—100 mesh (Shimadzu, Kyoto, Japan). The column temperature was 230°C, the injection port and detector temperature 260°C; the carrier gas (nitrogen) flow-rate was 70 ml/min.

RESULTS AND DISCUSSION

Pretreatment of urine specimens

The endogenous urinary components were removed as far as possible to avoid interference with subsequent trimethylsilylation and GC separation of MIG and IS. Acidic components were removed by passing through a QAE-Sephadex A-25 column. Recovery of MIG from the column eluted with distilled water was 97.8 ± 0.03% (mean ± S.E.), which was determined by its UV absorbance at 244 nm. The neutral components were removed from the eluate of a QAE-Sephadex A-25 column by washing with ethyl acetate and then diethyl ether, while MIG remained in the aqueous eluate. A trace of organic solvent remaining in the eluate was completely removed by evaporation under reduced pressure, since it interferes with adsorption of glucuronides on an Amberlite XAD-2 column. IS should be added to the aqueous layer at this point, because it is strongly adsorbed on a QAE-Sephadex A-25 column and easily extracted into the organic layer.

The recoveries of MIG and IS from an Amberlite XAD-2 column were examined using methanol containing 0—1.5% acetic acid as an elution solvent. The recovery of MIG was 85.2%, but that of IS was less than 5% when they were eluted with methanol. An acetic acid concentration above 1% in the elution solvent gave almost constant recoveries of MIG (97.5%) and IS (91.7%). From these results, the procedure for the clean-up of urine was settled as described in the Experimental section.

Trimethylsilylation of MIG and IS

The time and temperature dependencies of trimethylsilylation were investigated with varying reaction times between 2 and 60 min and reaction temperatures of 15, 25 and 35°C. *n*-Tetracontane was used as an external standard (ES) to evaluate the effect of these conditions on the yields of MIG and IS derivatives. The peak area ratios of MIG and IS derivatives against ES indicate that trimethylsilylation of MIG and IS was complete within 10 min. These derivatives were stable for at least 60 min at a reaction temperature of 25°C, and the yields of MIG and IS derivatives were independent of temperature (reaction time 20 min). When MIG was trimethylsilylated with SIM alone, a minor secondary peak of MIG was sometimes observed on the chromatogram, which, however, was reduced by the addition of BSTFA.

GC separation

Fig. 2 shows chromatograms of human urine before and after the oral dose of 20 mg of befunolol as a capsule. The TMS derivatives of MIG and IS are clearly separated on the OV-17 column with retention times of 10.9 min and 6.3 min, respectively.

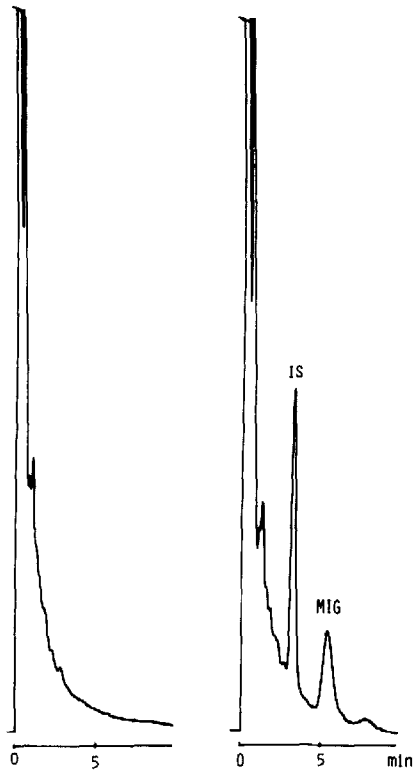


Fig. 2. Chromatograms of urine extracts. Left: normal urine. Right: urine from volunteer A who received a single oral dose of 20 mg of befunolol; MIG = 151 ng (5.3 μg/ml at 6 h after administration)

Calibration graph

The calibration graph of MIG against IS was drawn using 2 ml of control urine spiked with several known amounts of MIG. The calibration graph constructed with peak area ratios of MIG to IS is linear in the range 5—100 μg per 2 ml with a correlation coefficient of 0.998.

The accuracy and precision of the present method are shown in Table I. The average recovery of MIG from spiked urine was 101.1 ± 1.2% and the minimum detectable concentration of MIG was 2.5 μg/ml when 2 ml of human urine were used.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY APPLIED TO SPIKED HUMAN URINE

Amount of MIG added to urine (μg per 2 ml)	Amount of MIG found (μg per 2 ml) (mean of seven experiments)	Precision (coefficient of variation, %)
5	4.99	9.7
10	10.28	9.0
25	24.02	3.3
50	50.83	4.5
100	105.43	4.8

Urinary excretion of MIG

The present method was applied to the determination of MIG in the urine of volunteers receiving 20 mg of befunolol by the oral route. The semi-logarithmic plot for the excretion rate of MIG is shown in Fig. 3. The apparent excretion rate constants of MIG in volunteers A and B calculated from the declining slopes were 0.101 h^{-1} and 0.295 h^{-1}, respectively. On the other hand, befunolol (unconjugated) and MI (unconjugated) were determined by the GC method [4] (minor modification), and the excretion rate constants of befunolol (unconjugated) and MI (unconjugated) in volunteers A and B were 0.401 h^{-1} and 0.464 h^{-1}, and 0.364 h^{-1} and 0.428 h^{-1}, respectively. Comparison of these results shows that the excretion rate constant of MIG is the smallest of all.

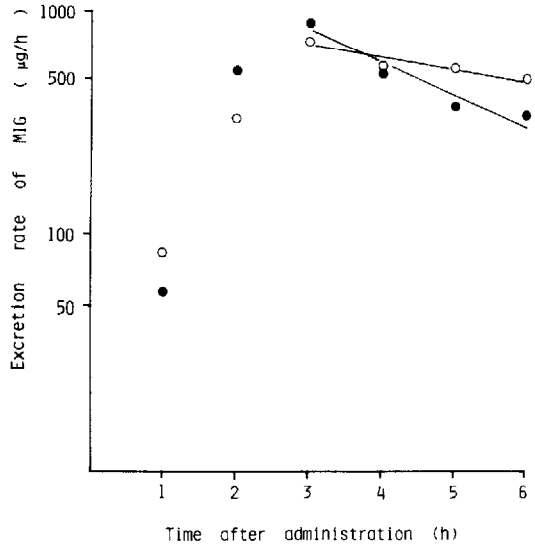


Fig. 3. Urinary excretion of MIG in volunteers after a single oral dose of 20 mg of befunolol. (○) Volunteer A; (●) volunteer B.

CONCLUSION

The present method has advantages over the previous methods in that it avoids the error accompanying enzymatic hydrolysis employed in the GC method, and that it does not need the preparation of monodeutero-MIG (glucuronide of monodeutero-MI) as internal standard and the complicated calculations required in the GC—MS method. The present method is highly sensitive, specific and reproducible, and can be used for the determination of MIG in human urine.

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