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Note

Resolution of the seven isomeric ring-hydroxylated propranolols as *tert.*-butyldimethylsilyl derivatives by capillary gas chromatography—mass spectrometry

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Three previously unknown ring-hydroxylated urinary metabolites of the β-adrenergic antagonist drug propranolol were recently identified by Walle et al. [1]. In rats and in man, 2-hydroxypropranolol (2-HO-P), 5-HO-P, and 7-HO-P were found, as well as the previously known 4-HO-P [2—6], which is the major monohydroxylated urinary metabolite of propranolol in both species, and the only hydroxylation product identified in the dog. Briefly, these compounds were identified in the following fashion. Urine samples were treated enzymatically to hydrolyze glucuronide conjugates; then the monohydroxypropranolols were extracted and analyzed as trimethylsilyl (TMS) and trifluoroacetyl (TFA) derivatives by gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS) using packed column techniques (3% OV-1). Identification of the isomers was based upon comparison of retention times and mass spectra with those of derivatized synthetic monohydroxypropranolols [1, 7]. Two derivatization techniques were employed because, under the conditions of the analyses, 2-HO-P and 8-HO-P were unseparated as TMS derivatives, and 4-HO-P and 5-HO-P were unseparated as TFA derivatives. Another complication encountered was that as TFA derivatives the propranolol peak overlapped with the 2-OH-P peak, necessitating the use of selected ion monitoring to distinguish these two compounds. Furthermore, since TFA derivatives partially decompose on columns previously used with TMS derivatives, separate columns had to be used for the two types of derivatives.

The purpose of the work presented here was to develop a simpler technique for separating and identifying the seven isomeric ring-hydroxylated propranolols

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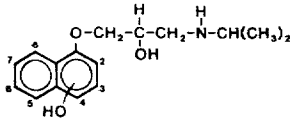
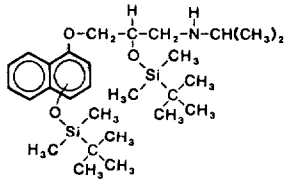
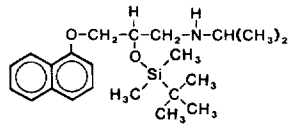
in order to facilitate future studies with these compounds. Such a technique has been developed, using a relatively new silylating reagent, N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), which produces volatile *tert.*-butyldimethylsilyl (TBDMS) derivatives of aliphatic and aromatic hydroxyl groups [8], yielding mono-derivatized propranolol (TBDMS-P) and di-derivatized hydroxypropranolols (TBDMS-HO-P).



Ring-Hydroxylated Propranolol



TBDMS-Hydroxypropranolol



TBDMS-Propranolol

When pyridine is used as a solvent for the derivatization, secondary amines are derivatized very slowly by MTBSTFA [8]; and, with the TBDMS group attached to the aliphatic oxygen which occurs far more rapidly in pyridine [8], derivatization of the secondary amine group is further sterically hindered. No tri-derivatized hydroxypropranolols were observed during the course of this study. (It may be possible to produce the tri-TBDMS derivatives with acetonitrile as the solvent [8—10] but these derivatives would be expected to be less volatile than the di-TBDMS derivatives.) Separations were carried out using glass capillary GC—MS. The applicability of the method was demonstrated by analysis of a urine sample from a rat dosed with (±)-propranolol and identification of the ring-hydroxylated metabolites.

MATERIALS AND METHODS

Chemicals

(±)-Propranolol hydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.). 2-HO-P and 4- through 8-HO-P were synthesized as their hydrochloride salts in these laboratories as previously described by Oatis et al. [7]; 3-HO-P (the least stable isomer) was freshly synthesized as the free base according to the previously described procedure [7]. N-Methyl-N-(*tert.*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) containing 1% *tert.*-butyldimethylsilylchlorosilane was obtained from Regis Chemical (Morton Grove, IL, U.S.A.), and pyridine from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Equipment

Capillary GC—MS analyses were performed using a Varian 3700 gas chromatograph equipped with a split injector. The column, a Grade A, 60 m × 0.25 mm I.D. SP-2100 wall coated open tubular (WCOT) glass capillary obtained from J & W Scientific (Orangeville, CA, U.S.A.), was interfaced through an open-split interface to a Finnigan MAT-212 mass spectrometer equipped with a Spectrosystem SS200 data system. A Fisher Recordall® Series 5000 strip-chart recorder was used during selected ion monitoring in analogue mode.

Preparation of TBDMS derivatives of propranolol and monohydroxypropranolols

A 1—1.5 mg sample of each compound was dissolved in 200 μl of pyridine; 20 μl of MTBSTFA were added, and the reaction mixture was tightly capped and heated at 100°C for 2 h. Derivatization of 8-HO-P is apparently sterically hindered, and requires 1—2 h heating to drive it to the di-derivatized product. The other compounds were more readily derivatized, but all of the individual samples were treated identically. A mixture of propranolol and the seven monohydroxypropranolols (1—1.5 mg of each) was similarly derivatized using 100 μl of pyridine and 120 μl of MTBSTFA; this mixture required heating for 16 h at 100°C to complete the derivatization of 8-HO-P. The derivatives were stable in solution for at least 2 weeks.

Biological sample

The hydroxypropranolol metabolites from a 2-ml aliquot of a 24-h urine collection (total urine volume was 26 ml) from a male Sprague—Dawley rat injected intraperitoneally with 10 mg/kg (±)-propranolol were enzymatically hydrolyzed and extracted according to the method described by Walle et al. [1]. The extracted metabolites were derivatized using 100 μl of pyridine and 20 μl of MTBSTFA with 16 h heating at 100°C.

Capillary GC—MS resolution of propranolol and the monohydroxypropranolols

The TBDMS derivatives of propranolol and the seven isomeric monohydroxypropranolols were resolved under the following conditions: injector temperature 240°C; split ratio 1:350, column temperature programmed from 240 to 255°C at a rate of 0.2°C/min; helium carrier gas average linear velocity 23—25 cm/sec; open-split interface temperature 260°C; line-of-sight temperature 250°C; ion source temperature 240°C; ionization energy 70 eV. During the analyses of synthetic samples the mass spectrometer was scanned repetitively from 30 to 550 a.m.u. at a rate of 3 sec per decade with an interscan time of 1 sec. Selected ion monitoring was used as the detection system during the analysis of the biological sample because of the enhanced sensitivity of this technique. The mass spectrometer was set to monitor *m/z* 217 using a direct-probe sample of TBDMS-derivatized 2-HO-P for calibration. All of the compounds eluted before the final column temperature was reached.

RESULTS

The TBDMS derivatives of propranolol and the hydroxypropranolols (with the exception of 8-HO-P) were readily prepared using MTBSTFA with pyridine as the solvent. 8-HO-P formed the di-derivatized product less rapidly than the other hydroxypropranolols, but was driven to completion with heating at 100°C for up to 16 h. With insufficient heating, two peaks were observed corresponding to mono- and di-derivatized 8-HO-P, with the mono-derivatized product eluting first. With sufficient heating, only the later eluting peak was observed.

Table I presents the elution order and the retention times of the separately derivatized synthetic compounds; the retention times presented have been

TABLE I

ELUTION ORDER OF THE TBDMS DERIVATIVES OF PROPRANOLOL AND THE MONOHYDROXYPROPRANOLOLS ON AN SP-2100 WCOT CAPILLARY COLUMN

The column temperature was programmed from 240 to 255°C at a rate of 0.2°C/min.

Derivatized compound	Adjusted retention time* (min, ± 0.1)	Relative retention time** (± 0.01)
Propranolol	14.7	1.00
8-HO-P	34.4	2.34
2-HO-P	36.1	2.46
3-HO-P	46.1	3.14
7-HO-P	49.0	3.33
4-HO-P	54.1	3.68
6-HO-P	59.4	4.04
5-HO-P	60.0	4.08

*The elution time of a non-retained compound (butane) was subtracted from the absolute retention times to obtain the adjusted retention times.
**Relative to TBDMS-propranolol.

adjusted for the void volume of the system. A representative chromatogram of the results obtained when all eight compounds are derivatized as a mixture is presented in Fig. 1, as a mass chromatogram of the sum of *m/z* 72, 217, and 274. Proposed structures of these ionic species are depicted in Fig. 2. A profile essentially identical with that in Fig. 1 was obtained for *m/z* 72 alone, and the same profile minus the propranolol peak was obtained for *m/z* 217 or

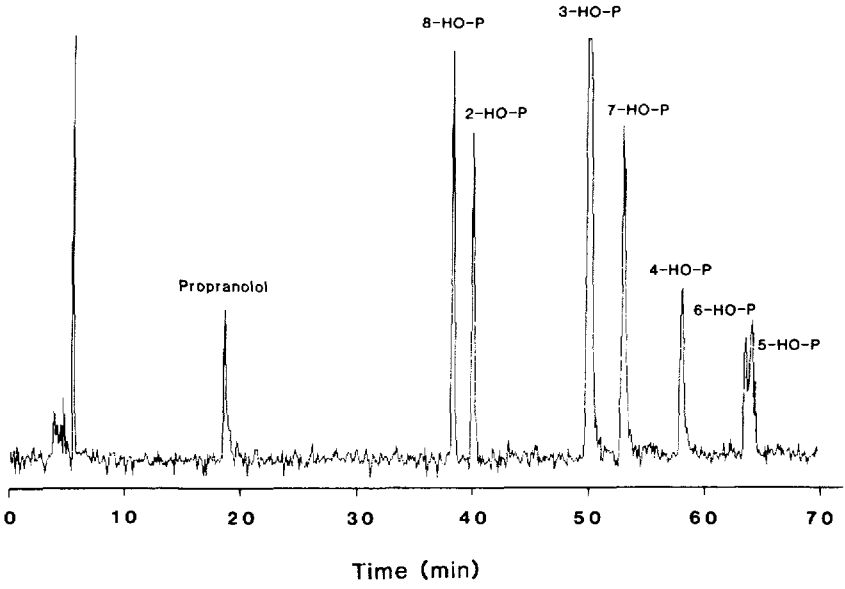


Fig. 1. Capillary GC—MS chromatogram of the TBDMS derivatives of propranolol and ring-hydroxylated propranolols, as a retrospective combined plot of three ionic species, *m/z* 72, 217, and 274 (see Fig. 2). The sample size was 3 μl (0.1 mg). See text for GC—MS parameters.

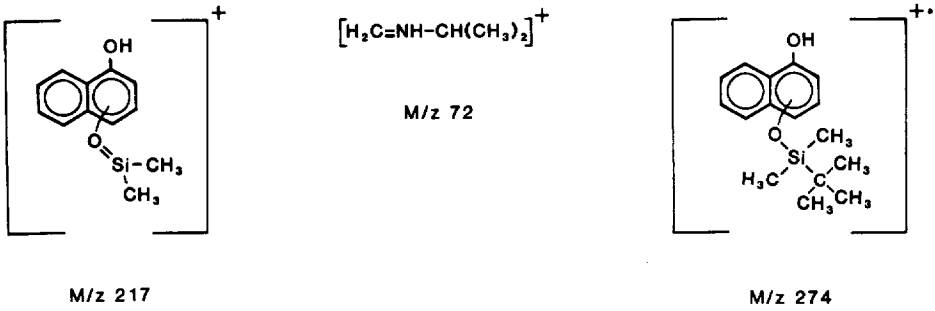


Fig. 2. Proposed structures of the ionic species traced in Fig. 1.

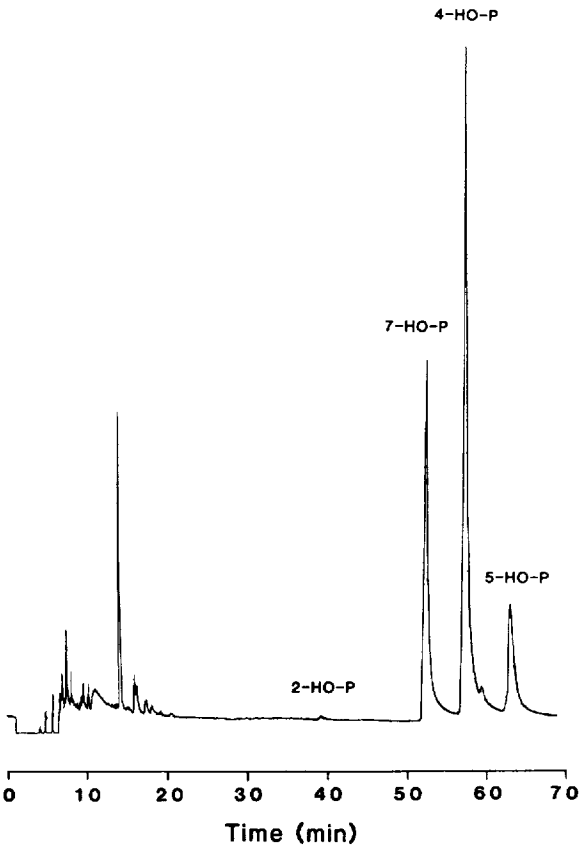


Fig. 3. Selected-ion monitoring (*m/z* 217) capillary GC—MS chromatogram of the ring-hydroxylated metabolites of (±)-propranolol extracted from rat urine. The sample size was 5 μl (10 μg). Peaks are labeled in accordance with the retention times of derivatized synthetic compounds chromatographed on the same day. The apparent peak tailing is due primarily to filtering in the analogue recording electronics. For GC—MS conditions, see text.

274 alone. All three of these ions are abundant in the mass spectra of the hydroxypropranolols, and each is potentially suited for selected ion monitoring. The *m/z* 217 and 274 ions are more specific for the hydroxypropranolols, as the *m/z* 72 ion is subject to interference from smaller molecular weight species likely to be present in a biological sample. Alternatively, the molecular

ion (*m/z* 503) could be monitored to enhance specificity for the hydroxypropranolols, but with a sacrifice in sensitivity.

The TBDMS derivatives of propranolol and the hydroxypropranolols were well resolved on an SP-2100 WCOT capillary column, with the exception of 5- and 6-HO-P, which were separated by only 0.5—0.6 min with an overall retention time of about 1 h. This degree of resolution was obtained only when care was taken to ensure that the initial average linear velocity was only slightly above the optimal average linear velocity for the carrier gas (helium), so that the system would approach maximum efficiency at the higher elution temperatures. Under a given set of flow conditions, capillary GC—MS retention times were reproducible to within ± 0.1 min.

A selected ion monitoring (*m/z* 217) chromatogram of the TBDMS derivatives of the ring-hydroxylated propranolol metabolites extracted from the urine of a rat dosed with (±)-propranolol is presented in Fig. 3. The retention times of the peaks observed from this sample were consistent with the observed retention times of derivatized synthetic 2-, 7-, 4-, and 5-hydroxypropranolol run on the same day. The species identified by these techniques, and their relative proportions, are consistent with the results previously obtained in rats by Walle et al. [1].

DISCUSSION

Propranolol and the seven isomeric ring-hydroxylated propranolols can be separated and reliably identified as TBDMS derivatives using capillary GC—MS techniques. The identification methodology presented here affords significant advantages over the techniques used in the past [1]. The TBDMS derivatives are well-suited for several detection systems, including flame ionization (data not shown), repetitive scanning mass spectrometry, and selected ion monitoring MS. Only one derivatization technique is employed, eliminating the necessity for using two separate columns. TBDMS-propranolol is well separated from the other seven compounds, so that special techniques for distinguishing the parent compound from one of its metabolites are not necessary.

The only major problem encountered using the techniques presented here was the moderate peak overlap between the TBDMS derivatives of 5- and 6-OH-P. In order to ensure reliable identification of these two compounds, care must be taken to ensure that the capillary GC system is operating at near-maximal efficiency. Problems concerning the identity of either or both of these two compounds in a mixture may be overcome by adding a small quantity of derivatized synthetic 5- or 6-HO-P and comparing relative peak heights with and without the added compound. The method presented here should facilitate further studies of ring oxidation metabolic pathways for propranolol, and should also be applicable to isomeric metabolites of related compounds.

ACKNOWLEDGEMENTS

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