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Note

Silica gel high-performance liquid chromatography for the simultaneous determination of propranolol and 4-hydroxypropranolol enantiomers after chiral derivatization

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Propranolol (Inderal®) is used clinically as a racemate of (+)- and (—)-propranolol with the (—)-enantiomer being responsible for most of the drug's antihypertensive and other cardiovascular actions [1, 2]. The kinetics and metabolism of the enantiomers differ considerably in both animals [3—6] and man [7—12]. It is therefore essential to be able to analyze individually the enantiomers of both parent drug and its main metabolites.

Practically useful approaches for separation of the enantiomers of propranolol include stable isotope-labeled pseudoracemates with gas chromatographic—mass spectrometric (GC—MS) analysis [3—6, 10—12] and high-performance liquid chromatography (HPLC) after chiral derivatization [7—9, 13—16]. The chiral reagent first used in the HPLC approach was N-trifluoroacetyl-(—)-prolyl chloride [13, 14]. The prolyl reagent can, however, racemize during storage [14, 17, 18]. An alternative, more stable, reagent appears to be (+)-1-phenylethyl isocyanate [(+)-PEI] [15]. Although all of the HPLC methods give good separation of the enantiomers of propranolol and appear to be useful for analysis of biological samples, these methods have not been used for simultaneous separation of the enantiomers of propranolol metabolites. This would be of clinical importance in particular for 4-hydroxypropranolol (4-HOP). This metabolite has biological actions similar to those of propranolol [19, 20], actions that should be dependent on the stereochemical composition of this metabolite.

In the present communication we describe the simultaneous determination of the enantiomers of both propranolol and 4-HOP by HPLC after derivatization with (+)-PEI. The method was applied to determinations of the stereochemical composition of conjugates of propranolol and 4-HOP in human urine.

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MATERIALS AND METHODS

Reagents

R-(+)-1-Phenylethyl isocyanate [(+)-PEI] was purchased from Aldrich (Milwaukee, WI, U.S.A.) and propranolol hydrochloride from Sigma (St. Louis, MO, U.S.A.). 4-Hydroxypropranolol (4-HOP) hydrochloride was prepared as previously described [20] as were the pure enantiomers of propranolol [6, 21] and 1-(α-naphthoxy)-2,3-propylene glycol [22]. Glusulase was obtained from Endo Labs. (Garden City, NY, U.S.A.). All solvents were glass-distilled from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Glass-distilled water was used for all aqueous reagents.

Chiral derivatization

The free bases of 4-HOP and propranolol (10 to 60 μg of each) were isolated by extraction with 10 ml ethyl acetate from 1 ml of a pH 9.6 carbonate buffer. After transfer to a 15-ml silanized conical centrifuge tube the ethyl acetate was evaporated to dryness and the residue dissolved in 0.5 ml of chloroform. (+)-PEI (5 μl) was added and the tube left for 15 min at room temperature tightly capped. 0.1 *M* Hydrochloric acid (10 ml) was then added and the tube was shaken on a reciprocating shaker for 10 min. After centrifugation the aqueous layer was aspirated off. The chloroform was evaporated to dryness by a stream of nitrogen. The residue was redissolved in 50 μl of mobile phase. A 15-μl sample was injected into the HPLC instrument.

Human urinary metabolites

24-h Urine collections were made from normal volunteers, each receiving a single oral 80-mg dose of propranolol (Inderal).

Glucuronic acid conjugates of propranolol and 4-HOP. Samples (2 ml) were treated with 100 μl of glusulase at pH 4.7 (90 min at 50°C) [23, 24]. After extraction at pH 9.6 with ethyl acetate [25], the extract was derivatized with (+)-PEI as above.

Sulfate conjugate of 4-HOP. Samples (2 ml) were extracted by an ion-pair procedure (chloroform and tetrabutylammonium at pH 12) [26]. The extracted sulfate conjugate was purified by reversed-phase HPLC. Following treatment with glusulase and extraction at pH 9.6 with ethyl acetate, 4-HOP was derivatized as above.

Instrumentation

HPLC. The HPLC system consisted of a Model 6000 high-pressure pump, a Model U6K injector, and a Model 440 UV detector with a 313-nm filter from Waters Assoc. (Milford, MA, U.S.A.). HPLC columns (25 cm × 4.6 mm) were either a 5-μm C_{18} or a 10-μm silica from Alltech Assoc. (Deerfield, IL, U.S.A.). The mobile phases were methanol—water (70:30) and methanol—chloroform (1.2:100), respectively. All solvents were filtered and degassed. The flow-rate was 1 ml/min.

Fast atom bombardment mass spectrometry (FAB-MS). A Finnigan/MAT 212 mass spectrometer with an SS-200 data system was modified for FAB-MS utilizing an Ion-Tek fast atom gun [27]. Samples were analyzed in a glycerol

matrix on a 316 stainless-steel probe tip at ambient temperature using argon bombardment at approximately 8 keV.

RESULTS AND DISCUSSION

The chiral derivatization of 4-HOP with (+)-PEI was performed in chloroform at room temperature as previously described for propranolol [15]. Analysis of the reaction mixture by reversed-phase HPLC after evaporation of the solvent revealed two sets of equal size peaks (Fig. 1A). The first set of peaks, at 13—14 min, was the diastereomer derivatives of 4-HOP after reaction at the secondary amino nitrogen only (mono-PEI derivative, see below), whereas the second set of peaks, at 45—52 min, was assumed to be the di-derivatives after reaction at both the amino and phenolic groups (di-PEI derivative). Very short reaction times produced mainly the mono-PEI derivative, whereas longer reaction times at elevated temperature mainly gave rise to the di-PEI derivative. Under no conditions was it possible to produce a single set of peaks. A complicating factor was the observation that the diastereomers resulting from the PEI derivative of propranolol appeared at 41—46 min, thus overlapping with the di-PEI derivative of 4-HOP. However, when the reaction mixture was shaken with 0.1 *M* hydrochloric acid, the derivative at the phenolic group (carbamate derivative) was hydrolyzed, producing the mono-PEI derivative only (Fig. 1B).

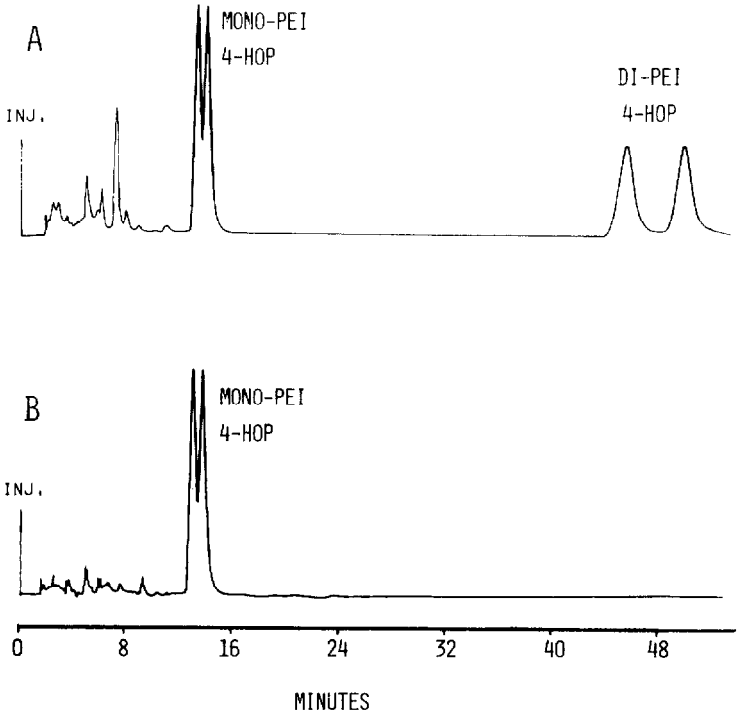


Fig. 1. Reversed-phase HPLC of (+)-PEI diastereomer derivatives of 4-HOP before (A) and after (B) acid-wash. About 5 μg injected at 0.1 a.u.f.s.

The structure of the mono-PEI derivative was confirmed by subjecting the peaks in Fig. 1B to positive-ion FAB-MS. The spectrum in Fig. 2 is consistent with the assigned structure, i.e. a urea-type of derivative. Characteristic ions are the quasimolecular ion at *m/z* 423 $(M+H)^+$ and the fragment ions at *m/z* 276 (loss of the derivatizing group; fragmentation I in Fig. 2) and at *m/z* 263 and 159 (fragmentations II and III). Characteristic of the derivatizing group is the base peak at *m/z* 105 ($[C_6H_5CHCH_3]^+$; fragmentation IV). The derivative is thus analogous to that previously established for propranolol [15].

The identification of the PEI derivatives of the β-receptor blocking drugs by electron-impact MS, rather than by FAB-MS, may be complex. For example, the propranolol derivative gave no molecular ion by this mode of ionization, even after trimethylsilylation [15]. Similarly, the mono-derivative of 4-HOP gave no interpretable spectrum either by direct probe MS or GC—MS after various derivatization techniques and electron-impact conditions.

Although the peak symmetry of the mono-PEI derivative of 4-HOP in Fig. 1 was quite good, the resolution of the diastereomers (R = 0.62) was inadequate, in particular when one enantiomer was present in considerably lower concentrations than the other. The resolution of the corresponding diastereomers of propranolol at a similar retention time was found to be considerably better (R = 1.5), which is similar to previous observations [15].

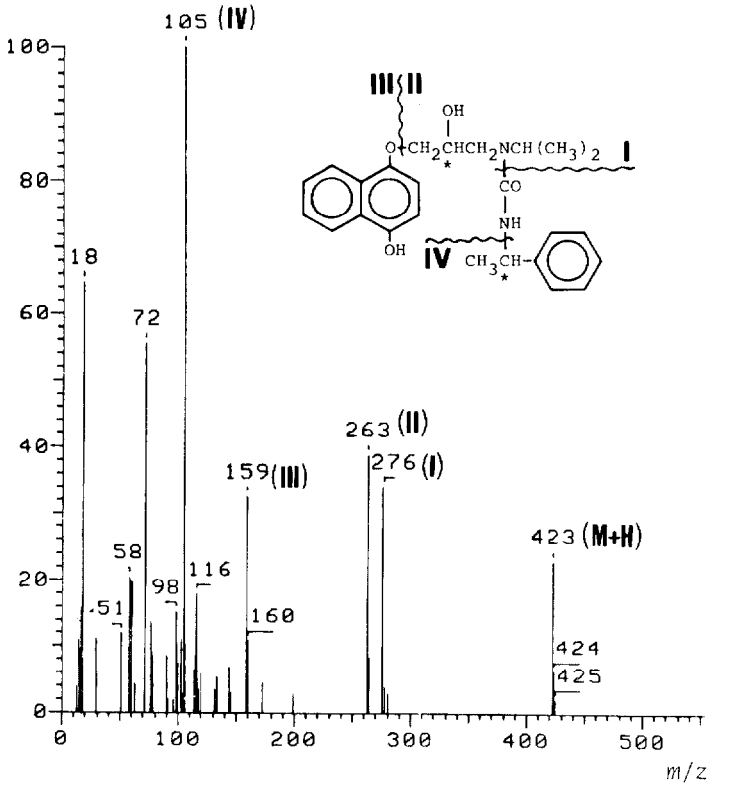
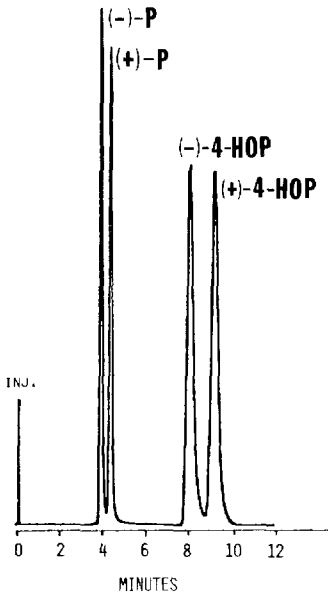


Fig. 2. Positive-ion FAB-MS of the mono-(+)-PEI derivative of 4-HOP. The derivative was collected from the HPLC effluent in Fig. 1, retention time 13—14 min. Roman numerals I to IV indicate sites of fragmentation for ions at *m/z* 276, 263, 159 and 105, respectively.

Manipulation of the mobile phase gave some improvement in the resolution of the 4-HOP diastereomers but only at the expense of a marked prolongation of the retention time, which at 13—14 min already was rather long.

In attempts to improve the resolution normal-phase silica column HPLC was examined. Using this mode of separation the diastereomers of both propranolol and 4-HOP appeared well resolved (see Fig. 3). The resolution of the 4-HOP diastereomers showed a dramatic improvement (R = 1.9) compared to reversed-phase chromatography. The resolution of the propranolol diastereomers (R = 1.5) remained the same. This high degree of resolution was seen in spite of considerably shorter retention times on the silica as compared to the reversed-phase column. The peak symmetry was excellent.



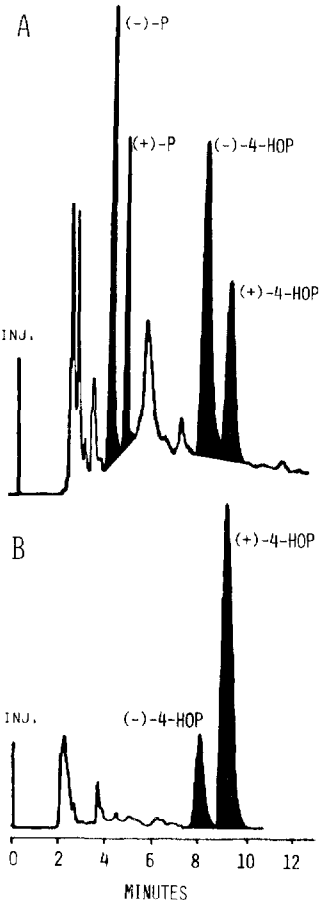


Fig. 3. Normal-phase HPLC of (+)-PEI diastereomer derivatives of propranolol (P) and 4-HOP, respectively, after acid-wash. Peak assignments were made as described in the text.

Fig. 4. Normal-phase HPLC of (+)-PEI diastereomer derivatives of (A) hydrolyzed glucuronic acid conjugates of propranolol (P) and 4-HOP and (B) hydrolyzed sulfate conjugate of 4-HOP. The conjugates were isolated from urine of normal subjects administered 80-mg single oral doses of propranolol. Chromatographic conditions were identical to those in Fig. 3.

The order of elution of the individual diastereomers of propranolol was established by separate derivatization of each optically pure enantiomer. For 4-HOP this was established by derivatization of 4-HOP isolated from urine following separate administration of each optically pure propranolol enantiomer. Both for propranolol and 4-HOP the (—)-enantiomer eluted first (Fig. 3).

The derivatization reaction was then tested for reproducibility. The concentrations of 4-HOP used ranged from 10 to 60 μg per sample. This was the amount of total 4-HOP anticipated to be present in a 2-ml human urine sample after a daily dose of 40—240 mg propranolol. A standard curve for this concentration range, using 1-(α-naphthoxy)-2,3-propylene glycol as internal standard (not derivatized), was linear (r = 0.997). The standard deviation at the midpoint of the curve was 5.9% (n = 6). The ratio of (—)/(+)-4-HOP was 1.00 ± 0.01 (mean ± S.D.; n = 6) using peak area measurements. In addition to making possible a reproducible derivatization, the acid-wash also effectively hydrolyzed and eliminated excess reagent, which could prove important in order to prolong the life of the column. Furthermore, the acid-wash removed most of the peaks early in the chromatogram (cf. Fig. 1). The derivatives were stable for at least several days following the acid-wash.

The method was applied to determinations of the stereochemical composition of the glucuronic acid conjugates of propranolol and 4-HOP as well as the sulfate conjugate of 4-HOP in human urine after 80-mg single oral doses of propranolol. For the glucuronides, urine was enzymatically hydrolyzed with glusulase. This procedure is quantitative for the two glucuronides [23, 24] and does not hydrolyze sulfate conjugates [28]. The aglycons were then extracted at pH 9.6. Following derivatization with (+)-PEI silica column HPLC produced the chromatogram in Fig. 4A. The diastereomers of both propranolol and 4-HOP were well separated from normal biological constituents. The (—)/(+)-enantiomer ratio of 1.47 for propranolol glucuronide and 1.80 for 4-HOP glucuronide, suggesting stereoselective glucuronidation of both propranolol and 4-HOP, agreed well with data recently obtained by administration of a stable isotope-labeled pseudoracemate and GC—MS separation of the enantiomers [12]. The acid-wash following the derivatization as well as the use of a high-wavelength ultraviolet detection (313 nm) both aided considerably in minimizing interferences in the chromatogram. The sulfate conjugate of 4-HOP was isolated intact from urine by HPLC [26, 28], hydrolyzed to 4-HOP by glusulase, extracted at pH 9.6 and derivatized with (+)-PEI as above. Silica column HPLC then produced the very clean chromatogram in Fig. 4B. The (—)/(+)-enantiomer ratio of 0.29 for this metabolite suggests a very large preference for the (+)-enantiomer in sulfate conjugation of 4-HOP. This also agreed well with a stable isotope-labeled approach [12].

Although the sensitivity of this method at present is adequate for determinations in urine, application to nanogram levels in plasma using fluorometric detection is under investigation.

CONCLUSIONS

A previously published method for chiral derivatization and HPLC separation of the enantiomers of propranolol has been extended to include its main

pharmacologically active metabolite, 4-HOP. New features are an acid-wash of the (+)-PEI derivative for consistent derivatization and as a clean-up step and also silica column HPLC for improved separation. Applications to metabolic studies of propranolol in man suggest stereoselectivity in both glucuronidation and sulfation of 4-HOP and in the glucuronidation of propranolol.

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