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Note

Separation of the enantiomers of intact sulfate conjugates of adrenergic drugs by high-performance liquid chromatography after chiral derivatization

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A recent study on the stereochemistry of propranolol metabolism in man [1] suggested that sulfate conjugation of its main active metabolite, 4'-hydroxypropranolol (HOP), to 4'-hydroxypropranolol sulfate (HOPS, Fig. 1A) is stereoselective. This was assessed by isolation and enzymatic hydrolysis of HOPS and determination of the enantiomeric composition of HOP either by gas chromatography—mass spectrometry using a stable isotope approach [1] or by silica gel high-performance liquid chromatography (HPLC), the latter after chiral derivatization with (+)-1-phenylethyl isocyanate (PEI) [2]. Enzymatic hydrolysis of HOPS to the unstable HOP was, however, a tedious and at low levels unpredictable procedure. For studies of the biochemical mechanism(s) of the stereoselective sulfoconjugation of HOP as well as of other adrenergic drugs, separation and quantitation of the enantiomers of intact sulfate conjugates is essential.

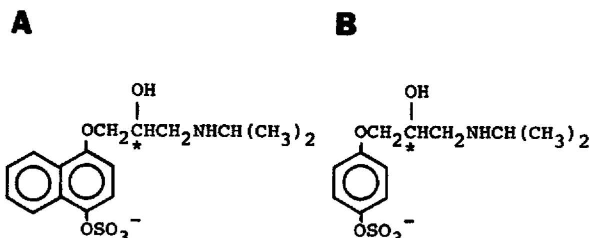

Fig. 1. Chemical structures of (A) 4'-hydroxypropranolol sulfate (HOPS) and (B) prenalterol sulfate.

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Chiral derivatization with PEI failed to give adequate separation of the HOPS enantiomers both on silica gel and reversed-phase HPLC. In this paper we describe the chiral derivatization of intact HOPS with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and separation of the resulting diastereomers by reversed-phase HPLC. This reagent has been shown to separate the enantiomers of amino acids [3, 4], catecholamines [5] and several classes of basic drugs [6—8]. This method was shown to be useful for plasma determinations of the enantiomers of HOPS in man during chronic propranolol therapy. Application to prenalterol sulfate (Fig. 1B), a conjugate of a selective β_1-receptor agonist [9], suggests that this derivatization technique may be useful for the separation of the enantiomers of intact sulfate conjugates of many other adrenergic drugs.

MATERIALS AND METHODS

Materials

GITC was synthesized from α-acetobromoglucose (Sigma, St. Louis, MO, U.S.A.) and silver thiocyanate (Pfaltz and Bauer, Stamford, CT, U.S.A.) [3]. The reagent, a white crystalline solid, was stable for months when stored refrigerated in a desiccator. HOP hydrochloride was prepared as previously described [10] as was HOPS [11]. Prenalterol sulfate was a gift from Hässle (Mölndal, Sweden). All solvents were glass-distilled (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Triethylamine was purchased from Aldrich (Milwaukee, WI, U.S.A.).

Chiral derivatization

Methanol solutions of synthetic HOPS and prenalterol sulfate, 0.1—5 μg, were evaporated to dryness under nitrogen. A 50-μl volume of 0.4% triethylamine in acetonitrile—water (50:50) and 50 μl of 0.06 *M* GITC in acetonitrile were added to the dried samples. After brief mixing, the capped tube was left at room temperature for 5—10 min. Samples of the reaction mixture were injected into the chromatographic column.

Chromatographic conditions

The HPLC column (25 cm × 4.6 mm) was a 5-μm Spherisorb ODS from Alltech Assoc. (Deerfield, IL, U.S.A.). The mobile phase was acetonitrile—methanol—water—acetic acid (35:5:59:1 for HOPS and 30:5:64:1 for prenalterol sulfate) in 0.05 *M* ammonium acetate (pH 4) at a flow-rate of 1 ml/min.

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.).

Isolation of HOPS from plasma

Plasma samples (2 ml) from a hypertensive patient, treated with 80 mg propranolol (Inderal) every 6 h, were extracted for HOPS by an ion-pair procedure (chloroform and tetrabutylammonium at pH 12) [12]. HOPS isolated this way (100% extraction) was purified by reversed-phase HPLC using acetonitrile—water (15:85) in 0.01 *M* ammonium acetate (pH 6.5) as

the mobile phase [12]. The peak corresponding to HOPS was collected and after freeze-drying derivatized with GITC as above.

RESULTS AND DISCUSSION

The chiral derivatization of HOPS with GITC was performed in acetonitrile—water (75:25) with 0.2% triethylamine. The presence of both water, to increase the solubility of HOPS, and triethylamine, as a base catalyst, were essential for a rapid and quantitative reaction. These conditions were identical to those used for the derivatization of non-esterified amino acids with GITC [4]. The reaction of GITC with the secondary amino group, resulting in a thiourea derivative, was complete in 5 min with HOPS as a substrate.

A reversed-phase HPLC profile, demonstrating the separation of the (+)- and (—)-enantiomers of synthetic HOPS after chiral derivatization with GITC, is shown in Fig. 2A. It shows complete baseline resolution (R = 2.1) between the enantiomers and an excellent peak symmetry. The order of elution of the enantiomers was established with HOPS isolated from urine of dogs dosed with either (+)- or (—)-propranolol. The peak area ratio (—)-HOPS/(+)-HOPS for the synthetic standard compound was 1.01 ± 0.01 (mean ± S.D.; n = 15). The derivative was stable for at least 24 h.

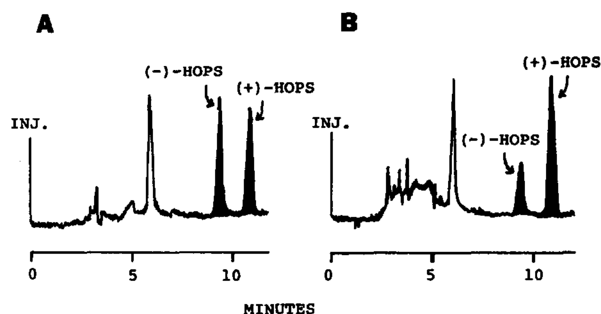

Fig. 2. Reversed-phase HPLC of GITC diastereomer derivatives of (A) synthetic HOPS (250 ng of each enantiomer) and (B) HOPS isolated from human plasma [(—)-HOPS 124 ng/ml and (+)-HOPS 373 ng/ml] at 0.05 a.u.f.s.

The minimum detectable amount of each enantiomer, using 313-nm detection, was about 20 ng. This should, however, be improved by fluorimetric detection. Standard curves (without internal standard) were linear over the range 100—5000 ng derivatized HOPS (correlation coefficients > 0.997). The coefficient of variation at the 1000-ng level was about 5%. The inter-assay variability in slope was less than 7%.

The method was used to determine the plasma concentrations of the enantiomers of HOPS in a patient on chronic therapy with propranolol, 80 mg every 6 h, Fig. 2B. At 2 h after the last propranolol dose the concentration of (+)-HOPS, 373 ng/ml, exceeded that of (—)-HOPS, 124 ng/ml. These concentrations together significantly exceeded the concentration of racemic propranolol measured at the same time, which was 255 ng/ml. The (—)-HOPS/(+)-HOPS ratio of 0.33 is similar to previous findings in urine in man [1, 2]. The method has also been applied to in vitro studies of this conjugation reaction [13].

This chiral derivatization technique was also applied to prenalterol sulfate. The GITC reaction was rapid and quantitative also for this conjugate. The content of acetonitrile in the mobile phase had to be decreased from 35% to 30% to achieve retention volumes similar to those of the HOPS enantiomers, i.e. 8.5 and 10 min. Baseline separation was achieved (R = 2.0). The minimum detectable amount of each enantiomer was 125 ng at 313 nm and 40 ng at 280 nm.

CONCLUSIONS

A method has been described for the separation and quantification of the intact HOPS enantiomers using chiral derivatization with GITC and reversed-phase HPLC. The method is rapid, sensitive and highly reproducible and should be well suited for in vitro studies of the biochemical mechanisms underlying stereoselective sulfation. It should also be suitable for kinetic analyses of the HOPS enantiomers in man, although simplification of the prepurification step would be desirable. Application of this procedure to the separation of the enantiomers of other adrenergic drug sulfates appears feasible.

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