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Short Communication

Immunoaffinity isolation of the sulfate conjugate of 4′-hydroxypropranolol from plasma

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ABSTRACT

Selective extraction of sulfate conjugates of basic drugs from biological matrices has been difficult because of their highly polar nature. Immunoaffinity isolation may be the best solution to this analytical problem. This was tested for a model compound, the metabolite 4′-hydroxypropranolol sulfate (HOPS), which was effectively extracted from plasma by a column containing antibodies to the parent drug propranolol. The specificity was very high, giving little interference from the biological material in subsequent high-performance liquid chromatographic analysis with fluorometric detection. The method for HOPS was highly reproducible and provided a sensitivity of 1 ng/ml plasma. The technique was applied to measurements of HOPS in plasma after therapeutic doses of propranolol as well as to the individual enantiomers after chiral derivatization.

INTRODUCTION

The metabolism of phenolic β-hydroxyethylamine drugs in humans is mainly by sulfate conjugation. This includes most of the β-receptor agonists, *e.g.* isoproterenol and more recent analogues [1–6], and the chemically closely related phenolic metabolites of the β-receptor antagonist drugs, *e.g.* propranolol [7,8]. Except for limited studies of albuterol [9] and 4-hydroxypropranolol [10], there is little information on the kinetics of sulfoconjugation of these types of drugs *in vivo*. This can be attributed to the difficulty in isolating these highly polar and ionized conjugates from human plasma and urine. Although ion-pair extraction can achieve good recoveries of such conjugates [9,11], this technique is not selective enough to permit detection of low concentrations in plasma.

Recent studies of sulfate conjugation of phenolic β-hydroxyethylamine drugs *in vitro* have demonstrated that this metabolic reaction can be highly stereoselective [8,12–16] and can potentially lead to dramatic alterations of the enantiomeric composition of these potent chiral drugs in the body. This therapeutically important question needs to be established and further evaluated *in vivo* through both plasma and urine determinations.

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As a more effective and in particular more specific isolation procedure for these conjugates from plasma and urine is needed, the use of immunoaffinity isolation was investigated with the propranolol metabolite 4'-hydroxypropranolol sulfate (HOPS) (I in Fig. 1) as the model compound. This represents an extension and modification of previous work on a radioimmunoassay of propranolol [17].

EXPERIMENTAL

Materials

Immobilized Protein A on agarose was purchased from Pierce (Rockford, IL, USA). Affi-Gel 10 gel was purchased from Bio-Rad Labs. (Richmond, CA, USA). Freund's complete adjuvant, porcine thyroglobulin and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Sigma (St. Louis, MO, USA). [^{35}S]-3'-Phosphoadenosine-5'-phosphosulfate ($PAP^{35}S$) (1.0–1.5 Ci/mmol) was obtained from Du Pont–New England Nuclear (Wilmington, DE, USA). HOPS and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) were synthesized as previously described [18,19]. Phosphate-buffered saline (PBS) consisted of 0.05 *M* potassium dihydrogenphosphate and 0.15 *M* sodium chloride adjusted to pH 7.4 with 1 *M* sodium hydroxide. Sodium azide (0.1%) was used as a bacteriostat in the PBS.

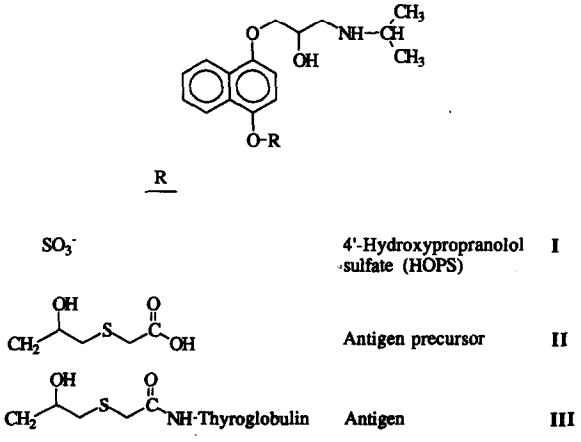

Fig. 1. Chemical structures of 4'-hydroxypropranolol sulfate (HOPS, I), antigen precursor (II) and thyroglobulin antigen (III).

Antigen

The antigen was prepared in this laboratory and consisted of a conjugate of synthetic antigen precursor II [20] covalently bound to porcine thyroglobulin via the carboxyl group as indicated in Fig. 1. To obtain the conjugate, 50 mg of II and 50 mg of porcine thyroglobulin were stirred for 4 h at 0°C in 5 ml of distilled water containing 100 mg of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 100 mg of EDC. The protein gradually precipitated as the reaction proceeded. The mixture was subsequently dialyzed against 4 l of distilled water at 4°C, which was changed twice at 24-h intervals and lyophilized to yield 48 mg of antigen III (Fig. 1).

Antiserum

The antiserum used in this study was raised in New Zealand White rabbits injected intramuscularly at monthly intervals with an emulsion consisting of 1 ml of complete Freund's adjuvant and 0.95 ml of distilled water containing 1 mg of III. Blood was collected by venipuncture and allowed to clot. After centrifugation the serum was removed and stored at −20°C.

Immunoaffinity gel

The polyclonal antibody obtained in rabbit serum must be partially purified prior to immobilization. To this end, 2 ml of serum were diluted with 4 ml of PBS, and the solution was filtered to remove any insoluble material. The filtrate was applied to 5 ml of Protein A agarose contained in a stoppered column with fritted disc, and the mixture was resuspended two to three times over a 20-min incubation period. The liquid was subsequently drained off and the gel washed with 100 ml of PBS followed by 25 ml of distilled water. The bound immunoglobulin G (IgG) fraction was eluted with 15 ml of 1% glacial acetic acid in 0.15 *M* sodium chloride. The pH was immediately raised by addition of 0.2 ml of 2.5 *M* sodium hydroxide and adjusted to 8.0 using small solid portions of sodium carbonate and sodium bicarbonate. An additional 100 mg of sodium bicarbonate was added, and the solution was stored on ice until coupling. A 12.5-ml volume of

Affi-Gel 10 was placed in a 1-in. diameter column with a fritted disc and 250 ml capacity and washed rapidly with ice-cold distilled water (250 ml). The washed gel was transferred to a 50-ml bottle and the IgG solution added. The sealed bottle was rotated at 4°C overnight. Unreacted coupling sites were subsequently capped by addition of 0.1 ml of 10% ethanolamine adjusted to pH 8.0 and rotation at room temperature for 1 h. The suspension was transferred to a column and drained, and the gel washed with 100 ml of distilled water, 100 ml of 50% aqueous acetonitrile, 100 ml of distilled water and finally 100 ml of PBS. The gel was stored under PBS at 4°C.

Sample preparation

For isolation of the sulfate metabolite HOPS from plasma, a small silanized glass column with a fritted disc (10 cm × 1 cm I.D.) containing 2 ml of the immunoaffinity gel was employed. A 0.5-ml aliquot of plasma was diluted with an equal volume of PBS and filtered to remove any insoluble materials. The filtrate was applied to the top of the gel and allowed to incubate for 30 min at room temperature. The gel was washed with 25 ml of PBS followed by 10 ml of distilled water. The void volume was pushed off with a brief pulse of nitrogen to minimize the amount of water in the final eluate. Bound HOPS was eluted with 20 ml of acetonitrile–water (95:5, v/v), and collected in a 50-ml flask. The solvent was removed under reduced pressure (about 400 Torr) at 40°C or under a stream of nitrogen. The dried residue was redissolved in 1 ml of methanol and transferred to a small vial, evaporated under a stream of nitrogen and reconstituted in 0.5 ml of mobile phase for HPLC analysis (see below).

The gel was regenerated by washing with 25 ml of 95% acetonitrile and 15 ml of 1% glacial acetic acid to remove the last traces of analyte, then with 15 ml of distilled water and 20 ml of PBS. The gel was stored at 4°C and was reusable for at least 100 cycles without change in extraction efficiency.

Preparation of ^{35}S-labeled HOPS

Small amounts of radiolabeled HOPS were prepared *in vitro*, using Hep G2 cells, a human hepatoma cell line expressing high levels of phenol sulfotransferases [21], as the enzyme source. The Hep G2 100 000 *g* cytosol was incubated with 5 μM 4'-hydroxypropranolol and 0.4 μM of the cosubstrate PAP^{35}S for 1 h at 37°C [21]. After precipitation of excess PAP^{35}S and protein the HOP^{35}S formed was isolated by reversed-phase high-performance liquid chromatography (HPLC). After lyophilization it was used in recovery studies.

HPLC conditions

Aliquots (100–200 μl) of reconstituted eluate from the antibody columns were subjected to HPLC, using a Spherisorb ODS-1, 5 μm, 25 cm × 4.6 mm I.D. column (Phenomenex, Torrance, CA, USA), with a mobile phase of acetonitrile–water (17:83, v/v) in 0.05 *M* ammonium acetate (pH 4.0) at a flow-rate of 1 ml/min. The HPLC system consisted of a Model 6000 high-pressure pump and a Model U6K injector from Waters/Millipore (Milford, MA, USA) and a Model FD-200 fluorometric detector (Spectrovision, Chelmsford, MA, USA) with 290 nm excitation and a 300 nm emission cut-off filter.

Separation of the HOPS enantiomers

The HOPS-containing HPLC fractions of the antibody column eluates were freeze-dried and derivatized for 10 min at room temperature with 30 m*M* GITC in acetonitrile–water (75:25, v/v) and triethylamine (0.2%, v/v) as catalyst [22]. The HOPS diastereomers were separated using the same HPLC system and column as above, with a mobile phase of acetonitrile–methanol–water (35:5:60) in 0.05 *M* ammonium acetate (pH 4) at 1 ml/min.

Patient samples

Serial heparinized blood samples were drawn from two healthy young volunteers (one male and one female) at 0, 0.5, 2, 5, 8 and 12 h after a single oral 80-mg dose of propranolol hydrochloride (Inderal) [23]. Plasma was separated by centrifugation and kept frozen at −80°C until analyzed.

RESULTS AND DISCUSSION

The extraction of HOPS from a 0.5-ml plasma sample by the immunoaffinity column was about 90%, as determined with ^{35}S-labeled tracer, *i.e.* only 10% of applied radioactivity was recovered in the PBS and water washes. However, the elution of HOPS from the column with the most suitable solvent, *i.e.* 95% acetonitrile in water, was very slow, presumably due to its very high affinity for the antibody. Although complete recovery of radiolabeled tracer could be achieved, it required about ten 20-ml washes with this solvent. Pure acetonitrile gave a much more rapid elution but led to deterioration of the column. As a practical compromise, 20 ml of 95% acetonitrile was used, which gave a very reproducible 40% recovery. After evaporation of the solvent and dissolution of the sample residue in 500 μl of mobile phase, 100–200 μl were injected on a reversed-phase HPLC column with fluorometric detection. With a spiked sample containing just 10 ng of HOPS per ml of plasma the chromatogram in Fig. 2 was generated. A single peak with a t_R of 11.0 min was obtained, which is the retention time of HOPS under these conditions. Judging from the chromatogram the specificity of the antibody column appears to be very high. There were no interferences except at about 3–6 min after each injection, as seen in Fig. 2. In order to remove all radioactive HOPS from the column before the next sample, further washes, including with 1% acetic acid, was obligatory.

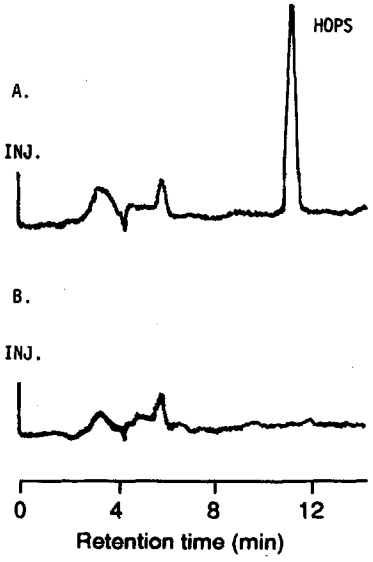

Fig. 2. Reversed-phase HPLC of immunoaffinity column extracts of plasma samples. Plasma samples were (A) blank plasma spiked with HOPS (10 ng/ml) and (B) blank plasma. Detection was made by fluorescence.

A series of spiked plasma samples with concentrations of HOPS ranging from 1 to 200 ng/ml were then extracted by the antibody column. After reversed-phase HPLC with area measurements of the HOPS peak, the recovery was linear from 5 ng up to at least 200 ng with a correlation coefficient of 0.992. The intra-assay coefficient of variation ranged from 5.9% at 200 ng to 14.6% at 5 ng ($n = 3$ at each of seven concentrations). The inter-assay variability in slope, *i.e.* recovery, was 3.6% ($n = 3$). The accuracy expressed as percentage deviation of mean observed concentration from nominal concentration ranged from 13.9% at 5 ng to 2.5% at 200 ng. The precision and linearity of the method are thus high in spite of the fact that the recovery of HOPS over the whole concentration range was only about 40%. The minimum detectable concentration was about 1 ng/ml (three times background noise level), which to a large extent is dependent on the relatively high fluorescence of HOPS.

This method was applied to serial plasma samples from two subjects who had received a single 80-mg oral dose of propranolol. The oral clearance of propranolol in these subjects had previously been determined to be high in the male subject, J.T. (124 ml/min/kg), and low in the female subject, D.D. (16 ml/min/kg). As can be seen in Fig. 3, the HOPS concentrations were quite high in J.T. and quite low in D.D., as could be expected from the clearance values above. The apparent half-life for HOPS was about 3 h in both subjects, as calculated from a semilogarithmic plot by linear regression of the terminal elimination phase. This represents the first measurements of this very polar metabolite in plasma.

As HOPS formation previously has been shown to be stereoselective both *in vivo* in human urine [10] as well as *in vitro* using human liver and

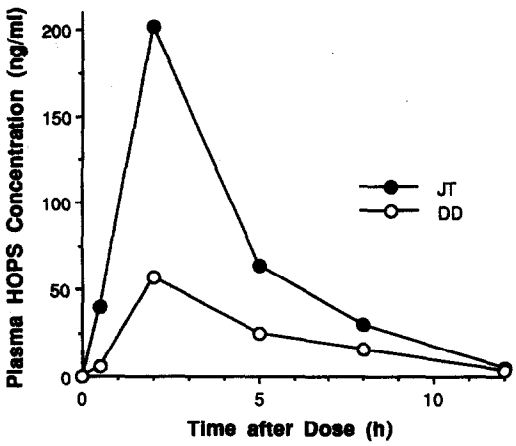

Fig. 3. Plasma concentration–time curves for HOPS in two subjects receiving an 80-mg oral dose of propranolol.

platelet tissue [8,13,14], it was of interest to determine the stereoisomers of this chiral metabolite also in plasma. After elution from the antibody column the extracted HOPS from 2-h samples in both subjects J.T. and D.D. was derivatized with the chiral reagent GITC [22] and the resulting diastereomers were separated by HPLC (Fig. 4). A very clean separation of (+)- and (−)-HOPS was achieved with the only interference at a t_R of 4 min being obtained from the reagent. The (+)-HOPS/(−)-HOPS ratio for the 2-h plasma samples was 3.2 for subject J.T. and 4.3 for subject D.D., which is very similar to previous *in vivo* [10] and *in vitro* [8,13,14] results.

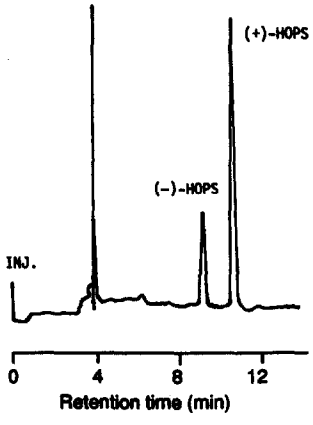

Fig. 4. Reversed-phase HPLC of the enantiomers of HOPS in human plasma 2 h after an 80-mg oral dose of racemic propranolol. Isolation was accomplished by immunoaffinity extraction as in Fig. 2 followed by derivatization with GITC.

In conclusion, by using the proper strategy for hapten design an antigen based on propranolol will produce antiserum with sufficient cross-reactivity for the highly polar HOPS. It can then be effectively employed on a solid-phase support to extract HOPS directly from plasma for quantitation by HPLC with virtually no interferences. This approach was suggested by previous work to develop a specific radioimmunoassay for propranolol [17]. It may be the only effective way to accomplish pharmacokinetic studies of sulfate conjugates of many drugs, in particular of the highly potent β-agonist drugs for which antibody columns for the parent drugs already are available [24,25].

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