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Note

Isolation from urine of 4′-hydroxypropranolol sulfate, a major new propranolol metabolite, by ion-pair extraction

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Sulfate conjugation is a common pathway in the metabolism of many phenolic drugs and drug metabolites [1]. The polar nature of these conjugates makes them difficult to isolate and purify from biological material. This is particularly true, if the metabolite also contains an aliphatic amino group, which renders the molecule zwitter-ion properties. Such a conjugate, 4′-hydroxypropranolol sulfate (HOPS), Fig. 1, was recently identified in urine as a major new propranolol metabolite in both dogs and man [2]. This conjugate was separated from endogenous compounds and other propranolol metabolites by tedious multiple extraction and high-performance liquid chromatographic (HPLC) separation steps.

A previous study of sulfate esters of simple phenols [3], e.g., 2-naphthyl sulfate, has, however, suggested that sulfate conjugates, also of more complex molecules, may be extracted from biological material as ion-pairs, possibly

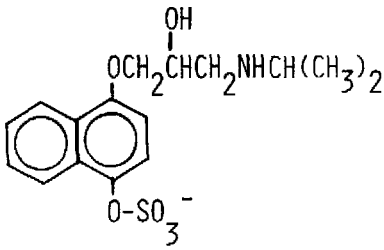

Fig. 1. Chemical structure of 4′-hydroxypropranolol sulfate (HOPS).

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providing a selective and rapid isolation step prior to HPLC separation. Conditions for quantitative extraction of HOPS as an ion-pair with tetrabutylammonium (TBA^+) were therefore investigated and the method applied to determinations of this conjugate in urine. For comparison, the extractability of the corresponding glucuronic acid conjugate, i.e., 4'-hydroxypropranolol glucuronide (HOPG) was also investigated.

EXPERIMENTAL

Chemicals

HOPS and HOPG were isolated and purified from urine of dogs dosed with [4'-^{3}H]propranolol [2, 4]. Their purity was greater than 95% as assessed by HPLC. The specific activity of HOPS was 0.12 μCi/mg and for HOPG 0.07 μCi/mg. Tetrabutylammonium (TBA^+) hydrogen sulfate was obtained from Aldrich (Milwaukee, WI, U.S.A.). The aryl sulfatase (*Aerobacter aerogenes*) was purchased from Sigma (St. Louis, MO, U.S.A.). The chloroform used was of analytical grade and was washed with water prior to its use. The liquid scintillation solution (ACS) was purchased from Amersham (Arlington Heights, IL, U.S.A.).

Instruments

Radioactivity measurements were performed after the addition of 10 ml scintillation cocktail, using a Beckman Model LS-355 liquid scintillation counter.

The HPLC system consisted of a Model 6000 high-pressure pump, a Model U6K injector, a Model 440 UV detector operated at 280 nm (Waters Assoc., Milford, MA, U.S.A.) and a C_{18}-column (5 μm, 25 cm × 4.6 mm I.D., Alltech Assoc., Deerfield, IL, U.S.A.). The mobile phase used was acetonitrile—water (15:85) in 0.01 *M* ammonium acetate.

The gas chromatograph—mass spectrometer was an LKB Model 9000S instrument operated in the electron impact mode at 20 eV, using a glass column (30 cm × 1.5 mm I.D.) packed with 3% OV-1 on Supelcoport 80—100 mesh (Supelco, Bellefonte, PA, U.S.A.).

Extraction constants

A charged organic compound can be transferred from an aqueous phase to an organic phase as an ion-pair. The charged compound is extracted as an association complex with a counter ion, an ion of opposite charge [5, 6]. The extraction of an organic anion, X^-, with a counter ion, TBA^+, can be expressed:

$$TBA^+_{aq} + X^-_{aq} \rightleftharpoons TBA^+X^-_{org} \quad (1)$$

The extraction constant, K_{ex}, can be defined:

$$K_{ex} = \frac{[TBA^+X^-]_{org}}{[TBA^+]_{aq}\,[X^-]_{aq}} \quad (2)$$

$[TBA^+X^-]_{org}$ is the concentration of the ion-pair in the organic phase and

$[TBA^+]_{aq}$ and $[X^-]_{aq}$ are the concentrations of TBA^+ and X^- in the aqueous phase at equilibrium. If no side reactions occur in the system, the distribution ratio, D, for the organic compound, X, can be expressed:

$$D = \frac{[TBA^+X^-]_{org}}{[X^-]_{aq}} = K_{ex} \times [TBA^+]_{aq} \quad (3)$$

or

$$\log D = \log K_{ex} + \log [TBA^+]_{aq} \quad (4)$$

As seen from eqns. 3 and 4, the distribution ratio is determined by the value of K_{ex} as well as the counter ion concentration. K_{ex} is dependent on the nature of the organic phase and the counter ion [5, 6].

HOPS. Chloroform (2 ml) was shaken for 30 min with 2.0 ml aqueous phase containing $1.39 \cdot 10^{-4}$ *M* TBA^+ and $0.8 \cdot 10^{-5}$ to $6.1 \cdot 10^{-5}$ *M* radioactive HOPS and adjusted to pH 12 with a small volume of 5 *M* sodium hydroxide. After centrifugation, the radioactivity content in 1.5 ml of each phase was measured by liquid scintillation spectrometry for the determination of HOPS. Before the addition of 10 ml of scintillation cocktail the organic phase was evaporated to dryness and reconstituted in methanol and the aqueous phase was acidified. The D values were calculated as the ratio of radioactivity in the organic and aqueous phases for each experiment. The K_{ex} values were calculated from eqn. 4 after finding the TBA^+ concentration remaining in the aqueous phase, $[TBA^+]_{aq}$.

HOPG. The determination of K_{ex} for HOPG was performed as for HOPS but with a higher counter ion concentration, 0.529 *M* TBA^+. The HOPG concentrations used were $4.7—38 \cdot 10^{-5}$ *M*. Due to the high salt concentration, smaller aliquots (0.5 ml) of the aqueous phase were counted.

Extractions from urine and HPLC

For the determination of the extent of extraction 2-ml aliquots of human control urine, to which had been added radioactive HOPS (42 μg per sample) or HOPG (173 μg per sample), 2.0 ml of water and 1.0 ml of 0.1 *M* TBA^+, were shaken for 30 min with 5.0 ml of chloroform after the pH of the aqueous phase was corrected to pH 12 with 5 *M* sodium hydroxide. Following centrifugation, 0.5-ml aliquots of the organic phase and 0.2-ml aliquots of the aqueous phase were counted as above.

Urine collections (0—24 h) from five healthy volunteers were collected after a single oral dose of 80 mg propranolol and 40 μCi of [4′-^{3}H]propranolol. Urine samples (2 ml) were extracted as above, 4 ml of the organic phase were evaporated and dissolved in 200 μl of mobile phase. Aliquots (40 μl) were injected into the HPLC column. The peak with the same retention volume as purified HOPS was collected. An aliquot of this peak was used for radioactivity measurement by liquid scintillation spectrometry. Another aliquot was hydrolyzed with 0.5 units of aryl sulfatase at pH 7.1 (0.05 *M* Tris buffer) under nitrogen at 37°C for 15 h [2]. After extraction at pH 9.6 with ethyl acetate and derivatization with trifluoroacetic anhydride [7] the sample was analyzed by gas chromatography—mass spectrometry (GC—MS).

RESULTS AND DISCUSSION

The ion-pair extraction of HOPS and HOPG from an aqueous phase into chloroform was studied using TBA^+ as the counter ion. The extraction constants (K_{ex}) were determined using fixed concentrations of TBA^+ and a range of HOPS and HOPG concentrations, Table I. A pH of 12 in the aqueous phase was chosen in order to avoid protonation of the amino group of the side-chain (pK_a about 9.5). Log K_{ex} for HOPS was found to be 3.95 ± 0.03 (mean ± S.D.) and for HOPG −0.76 ± 0.06. This difference in K_{ex} of more than four orders of magnitude indicates a much greater lipophilicity of the TBA^+ ion-pair with the sulfate than with the glucuronic acid conjugate.

The relationship between the distribution ratios (D) for HOPS and HOPG and the TBA^+ concentration in the aqueous phase at equilibrium (eqn. 4) is expressed in Fig. 2. At TBA^+ concentrations $> 1.1 \cdot 10^{-2}$ M the log D for HOPS is > 2, whereas at TBA^+ concentrations $< 5.7 \cdot 10^{-2}$ M the log D for HOPG is < -2. Thus, at the TBA^+ concentration range of 1.1 to $5.7 \cdot 10^{-2}$ M (shaded area in Fig. 2) there is quantitative extraction of HOPS without extraction of HOPG. The validity of this observation was tested directly by extraction of human urine spiked with radioactive HOPS or HOPG. A TBA^+ concentration of $2.0 \cdot 10^{-2}$ M and a pH of 12 gave 99% extraction of HOPS and negligibe extraction (< 3%) of HOPG. Doubling of the TBA^+ concentration gave identical results. These findings demonstrate the direct applicability of the ion-pair extraction approach to biological samples.

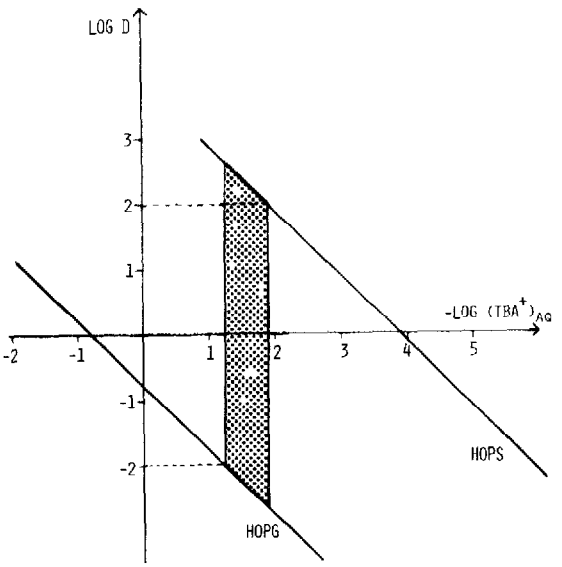
Fig. 3 shows an HPLC tracing of a similar extract of urine from a patient receiving a single 80-mg oral dose of propranolol together with tritium-labeled drug. The shaded peak, which had the same retention volume as purified HOPS, was the only peak containing radioactivity. Although the naphthoxylactic acid metabolite [8, 9] was also extractable as a TBA^+ ion-pair under the conditions

TABLE I

DETERMINATION OF EXTRACTION CONSTANTS, K_{ex}, FOR HOPS AND HOPG

Organic phase: chloroform; aqueous phase: pH 12. C_{X^-} = Initial concentration of X^- in the aqueous phase; C_{TBA^+} = initial concentration of TBA^+ in the aqueous phase; D = distribution ratio for X^-; K_{ex} = extraction constant for X^- as a TBA^+ ion-pair.

Anion X^-	$C_{X^-} \cdot 10^5$	C_{TBA^+}	D	log K_{ex}
HOPS	0.8	$1.39 \cdot 10^{-4}$	1.14	3.93
	1.5	$1.39 \cdot 10^{-4}$	1.11	3.93
	3.0	$1.39 \cdot 10^{-4}$	1.07	3.94
	6.1	$1.39 \cdot 10^{-4}$	1.07	4.00
			Mean ± S.D.	3.95 ± 0.03
HOPG	4.7	0.529	0.080	−0.82
	9.5	0.529	0.116	−0.66
	19	0.529	0.093	−0.76
	38	0.529	0.085	−0.79
			Mean ± S.D.	−0.76 ± 0.06



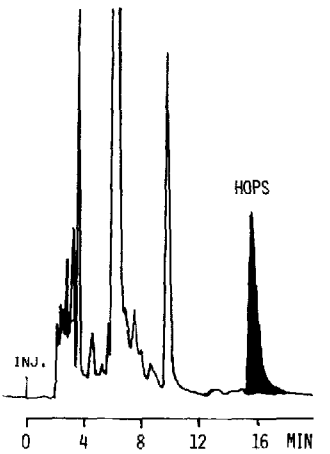


Fig. 2. Distribution ratio as a function of the TBA^+ concentration in the aqueous phase at equilibrium. Log D = log K_{ex} + log $(TBA^+)_{aq}$. Organic phase: chloroform; aqueous phase: pH 12.

Fig. 3. HPLC—UV chromatogram of HOPS after ion-pair extraction into chloroform from human urine following an 80-mg oral dose of propranolol with 40 μCi of [4'-^{3}H]-propranolol. Shaded area contains radioactivity.

used, its elution from the column required pure methanol as the mobile phase. All other peaks in the chromatogram were due to normal urinary constituents, as evidenced by their presence in control urine samples and the absence of radioactivity in these peaks.

The identity of HOPS was further established by hydrolysis of the HPLC peak by bacterial aryl sulfatase and confirmation of the presence of 4'-hydroxypropranolol by GC—MS as previously described [2]. There were no interferences by endogenous compounds or by other propranolol metabolites. It was also shown that HOPS was stable for at least 19 h at the pH of 12 used for the extraction. Analysis of urine samples from five patients demonstrated a high correlation (correlation coefficient 0.993) between the radioactivity content of the HOPS peak and its area measured by UV detection (280 nm), indicating that the method is useful for quantitation of HOPS even without radioactive drug. The relative standard deviations for repeated samples were 3.4% and 4.6% (n = 5), respectively, using radioactivity and UV measurements.

Although HOPG can be extracted as an ion-pair, it would require the use of a larger counter ion, e.g., tetrahexylammonium [3]. HOPG, but not HOPS, can, however, also be determined as the aglycon after direct enzymatic hydrolysis of urine [2, 10].

The ion-pair extraction of a sulfate conjugate with zwitter-ion properties described in this report represents a selective and rapid clean-up step before HPLC separation. It should be useful for the isolation of many other sulfate

conjugates of similar structural complexity prior to chemical characterization and quantitation.

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