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SIMULTANEOUS DETERMINATION OF PROPRANOLOL AND 4-HYDROXYPROPRANOLOL IN PLASMA BY MASS FRAGMENTOGRAPHY

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SUMMARY

A quantitative method for the simultaneous determination of propranolol and its active metabolite 4-hydroxypropranolol in human plasma is described. Plasma samples are extracted at pH 9.6 with ethyl acetate after the addition of sodium bisulphite and the internal standard oxprenolol. The extracts are derivatized with trifluoroacetic anhydride before separation on a gas chromatograph–mass spectrometer. Detection and quantitation of the trifluoroacetyl derivatives are made by single-ion monitoring. The minimum detectable concentration of propranolol is 1 ng/ml and of 4-hydroxypropranolol 5 ng/ml using 1-ml plasma samples. No interferences from normal plasma constituents or from drugs commonly prescribed together with propranolol were detected.

INTRODUCTION

4-Hydroxypropranolol was first identified by Bond[1] as a major propranolol metabolite in urine of man and several animal species. It is excreted mainly as a glucuronic acid conjugate. Little or no unconjugated metabolite has been detected[1–5].

Fitzgerald and O'Donnell[6] subsequently demonstrated that 4-hydroxypropranolol is an adrenergic beta-blocker in the dog, equipotent to propranolol. A significant contribution of this metabolite to the beta-blocking effects seen after oral doses of propranolol in man has been strongly suggested[7–9].

It has been indicated that 4-hydroxypropranolol reaches similar peak plasma concentrations in man as propranolol after a single oral dose of propranolol, but has a significantly shorter half-life[2]. These findings were based on a radioactive method[2,3] with questionable specificity and have never been confirmed. It was also strongly indicated that this metabolite is formed in man only after oral administration of propranolol[2] and after propranolol injection into the portal vein of the dog[3]. The formation of 4-hydroxypropranolol by rat liver microsomes has been amply demonstrated[10].

The contribution of this metabolite to the pharmacological effects seen after

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propranolol administration eventually has to be evaluated based on urine, plasma and, when possible, tissue concentration determinations after single and repeated oral doses of propranolol using a specific and sensitive technique. This is also true for the bioavailability of this metabolite as effected by different routes of administration.

This paper describes for the first time a method for the simultaneous quantitative determination of propranolol and 4-hydroxypropranolol in human plasma. The method is based on gas chromatography–mass spectrometry, utilizing single-ion monitoring. Conditions for extraction, derivatization, separation and detection are described. Specificity and reproducibility are discussed.

EXPERIMENTAL

Standards and reagents

Propranolol hydrochloride and 4-hydroxypropranolol hydrochloride were kindly supplied by Imperial Chemical Industries (Macclesfield, Cheshire, Great Britain). Oxprenolol hydrochloride was a gift from William S. Merrell Co. (Cincinnati, Ohio, U.S.A.). Standard solutions of these compounds were made up in methanol or in 2% sodium hydrogen sulphite in water. Only freshly prepared standard solutions were used.

Trimethylamine was obtained from Eastman-Kodak (Rochester, N.J., U.S.A.) and trifluoroacetic anhydride from Pierce (Rockford, Ill., U.S.A.). Other reagents, including pesticide-grade ethyl acetate and benzene, were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Aqueous reagents, including 1 *M* carbonate buffer, pH 9.6, and 1 *M* phosphate buffer, pH 6.0, were prepared with glass-distilled water.

Instrument

The instrument used was an LKB 9000 S gas chromatograph–mass spectrometer operated at an ion-source temperature of 290°, an accelerating voltage of 3.5 kV, a trap current of 60 μA, and an electron energy of 18 eV, unless otherwise stated. The column, 150 cm × 2 mm I.D., was made of Pyrex glass and packed with 10% OV-1 on 80–100 mesh re-silanized[11] Chromosorb W-HP. The carrier gas (helium) flow-rate was 20 ml/min. The injector temperature was 260° and the column temperature 230°. The column was conditioned at 280° for 48 h before use.

Derivatization

Trifluoroacetylation was carried out as previously described with trifluoroacetic anhydride, using trimethylamine as the catalyst[12]. To 5–100 ng each of the 4-hydroxypropranolol and propranolol hydrochlorides and 25 ng of oxprenolol hydrochloride in 15-ml tapered centrifuge tubes were added 200 μl of benzene, 25 μl of trimethylamine (1 *M*) in benzene and 50 μl of trifluoroacetic anhydride. After 5, 15, 30 and 60 min at 50° the reaction mixtures were washed with 1 *M* pH 6.0 phosphate buffer[12] and the benzene phase was analyzed. The trifluoroacetylation of propranolol and oxprenolol was complete after 15 min[12]. This was also true for 4-hydroxypropranolol. The stability of the derivatives was tested by re-injecting the samples after they had been stored for 2–7 h at room temperature without separation of the benzene phase from the phosphate buffer.

pK_a for 4-hydroxypropranolol

The pK_a for the phenolic group in 4-hydroxypropranolol was determined by spectrophotometry[13]:

$$pK_a = pH + \log \frac{(D - D_b)}{(D_a - D)}$$

where D is the measured absorbance at a specific pH ($pH = pK_a \pm 0.5$), and D_a and D_b are the molar absorbances for the non-ionized and the ionized forms of the phenol, respectively. All measurements were made at 342 nm, where the ionized form has an absorption maximum and the non-ionized form low absorption. The pK_a was determined to be 9.7 ± 0.1 ($n = 4$).

Standard curves

Standard curve A. Samples containing 5, 10, 25, 50, 100 and 150 ng of propranolol hydrochloride and 4-hydroxypropranolol hydrochloride, respectively, in methanol were prepared in 15-ml tapered centrifuge tubes. Twenty-five nanograms of oxprenolol hydrochloride in methanol were added to each sample before evaporation to dryness at 60° with a gentle stream of nitrogen. After the addition of 200 µl of benzene, the samples were derivatized as described above using a 15-min reaction time.

Standard curves B and C. Two sets of six samples consisting of distilled water (curve B) and blank plasma (curve C) containing 5, 10, 25, 50, 100 and 150 ng of propranolol hydrochloride and 4-hydroxypropranolol hydrochloride, respectively, were prepared in 15-ml centrifuge tubes. Twenty-five nanograms of oxprenolol hydrochloride in water were added to each sample. One milliliter of 1 *M* carbonate buffer, pH 9.6, and 10 ml of ethyl acetate were added to each sample. After gentle shaking for 10 min with the tubes in a horizontal position the tubes were centrifuged at 1500 *g* for 5 min. The ethyl acetate layer was transferred to 15-ml tapered centrifuge tubes and evaporated to dryness at 60° with a gentle stream of nitrogen. After the addition of 200 µl of benzene, the samples were derivatized as above using a 15-min reaction time.

The peak area ratios propranolol/oxprenolol and 4-hydroxypropranolol/oxprenolol were calculated. Propranolol and 4-hydroxypropranolol concentrations, expressed as ng/ml, *versus* area ratios times oxprenolol concentration were plotted.

The effect of sodium hydrogen sulphite on the stability of 4-hydroxypropranolol was investigated. Standard curves B and C eventually included the addition of 20 mg (2%) sodium hydrogen sulphite to each sample prior to extraction. The stability of 4-hydroxypropranolol in methanol was adequate.

All centrifuge tubes were cleaned and silanized as previously described[12].

Quantitative determinations of unknown samples

To 1.0 ml of plasma in a 15-ml round-bottomed centrifuge tube were added 20 mg sodium hydrogen sulphite, 25 ng oxprenolol hydrochloride, 1 ml 1 *M* carbonate buffer, pH 9.6, and 10 ml ethyl acetate. The samples were extracted and derivatized as described above under *Standard curves B and C.* Part of the benzene phase, 2–4 µl, was then injected in the gas chromatograph–mass spectrometer.

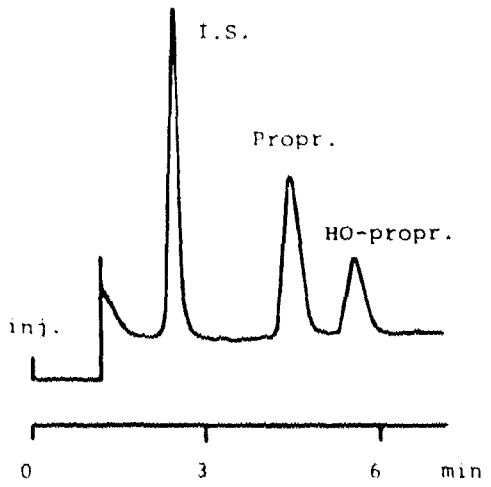

Fig. 1. Total ion-current recording of 500 ng each of tri-TFA 4-hydroxypropranolol and di-TFA propranolol and the internal standard oxprenolol.

A set of three standard samples containing 10, 50 and 100 ng of propranolol hydrochloride and 4-hydroxypropranolol hydrochloride, respectively, per milliliter of blank plasma was run each day together with the unknown samples.

RESULTS AND DISCUSSION

The gas chromatographic separation of the trifluoroacetyl (TFA) derivatives

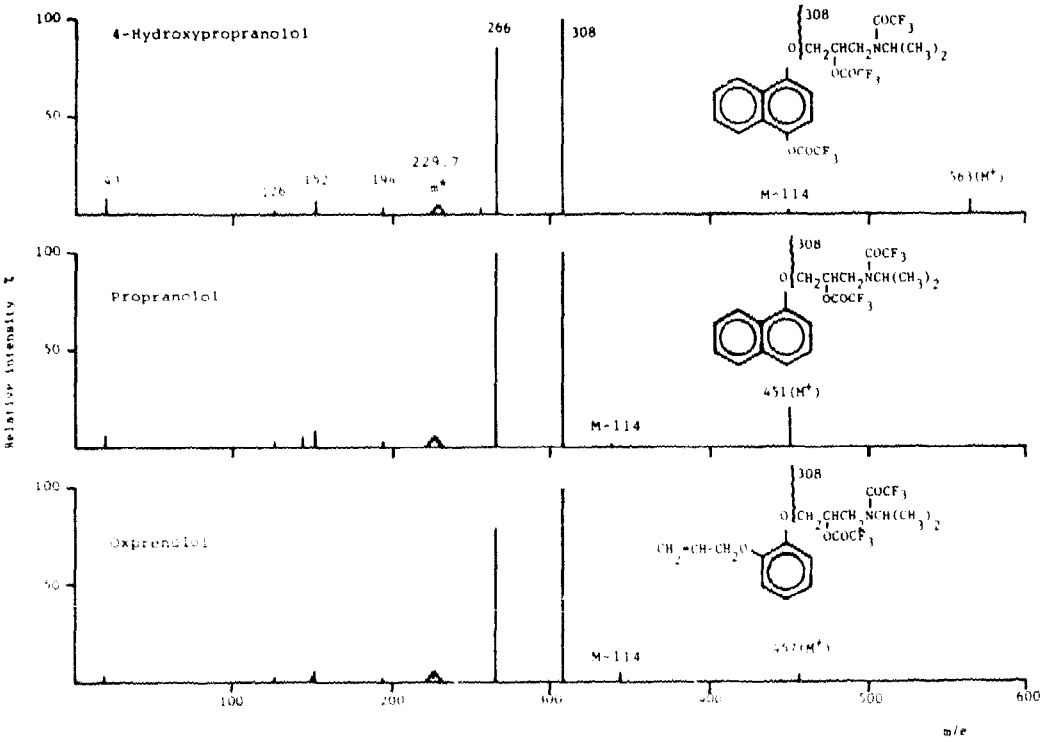

Fig. 2. Low-resolution mass spectra of tri-TFA 4-hydroxypropranolol and di-TFA propranolol and oxprenol. Electron energy, 18 eV. Fragment ions with intensities less than 5% of the base peaks, as well as all isotope peaks, have been excluded for simplicity.

of 4-hydroxypropranolol, propranolol and the internal standard oxprenolol on a 10% OV-1 column is visualized on the total ion-current recording in Fig. 1. Whereas the separation of 4-hydroxypropranolol and propranolol on polar silicon phases such as OV-17 is incomplete ($\alpha < 1.05$[4]), maximum separation is achieved on OV-1 ($\alpha = 1.26$). The high percentage of stationary phase used (10%) minimizes losses by adsorption, in particular losses of the 4-hydroxypropranolol derivative. Complete separation is achieved in 6 min.

Fig. 2 shows the low-resolution mass spectra of the tri-TFA derivative of 4-hydroxypropranolol and the di-TFA derivatives of propranolol and oxprenolol. The two most intense fragment ions (*m/e* 308 and 266) are characteristic of all three derivatives. The fragment ion at *m/e* 308 comprises the side-chain after cleavage at the ether linkage. The fragment ion at *m/e* 266 indicates loss of isopropylene (42 a.m.u.) from *m/e* 308. This fragmentation pathway is characteristic of all aryloxy beta-blockings drugs[14].

Determination of the signal-to-noise ratio, monitoring each of the *m/e* 308 and 266 ions of the TFA derivatives of 4-hydroxypropranolol, propranolol and oxprenolol in plasma extracts at electron energies ranging from 16 to 70 eV, demonstrated that *m/e* 308 monitoring at an electron energy of 18 eV gives the highest sensitivity and specificity. Typical single-ion recordings (*m/e* 308) of plasma extracts are shown in

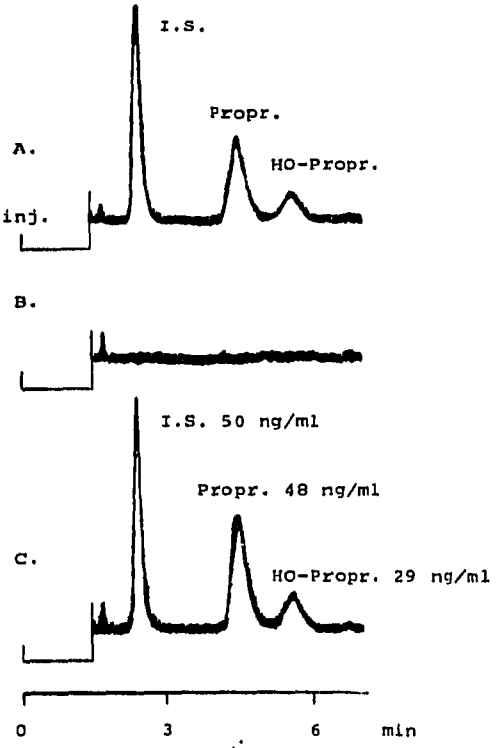

Fig. 3. Single-ion (*m/e* 308) recordings of plasma extracts. A = Plasma from a patient treated with propranolol orally; B = plank plasma; C = blank plasma spiked with known quantities of 4-hydroxypropranolol, propranolol and oxprenolol.

Fig. 3. The center recording (Fig. 3B) is a plasma extract from a normal subject receiving no medication. The bottom recording is the same plasma spiked with known concentrations of propranolol, 4-hydroxypropranolol and oxprenolol. No interference from normal plasma constituents is observed. The amount injected of propranolol was 0.9 ng and that of 4-hydroxypropranolol 0.6 ng. The minimum detectable amount, producing a signal three times the background noise, was for propranolol about 50 pg and for 4-hydroxypropranolol about 200 pg. The top recording in Fig. 3 is a plasma extract obtained from a patient receiving, in addition to propranolol, also digoxin, dicyclomine, chlordiazepoxide and propoxyphene. No interference from these and numerous other drugs commonly prescribed together with propranolol has been observed. It should be noted that the electron capture detection method[12], which has been shown to operate well for determinations of propranolol alone, does not have the specificity required to include 4-hydroxypropranolol.

Before extraction, pH was adjusted to the isoelectric point for 4-hydroxypropranolol, which was indicated to be at pH 9.6; the pK_a for the amino group was assumed to be the same as for propranolol, *viz.* 9.5 (ref. 15), and the pK_a for the phenolic group was determined (see Experimental) to be 9.7. Extraction at pH 9.6 with ethyl acetate using a ratio of organic phase/aqueous phase of 5 appeared to give satisfactory recoveries of both 4-hydroxypropranolol and propranolol and the internal standard oxprenolol; a second extraction yielded only trace quantities of all three compounds.

Fig. 4 shows typical standard curves obtained for 4-hydroxypropranolol and propranolol in human plasma (standard curves C in Experimental). Both curves go

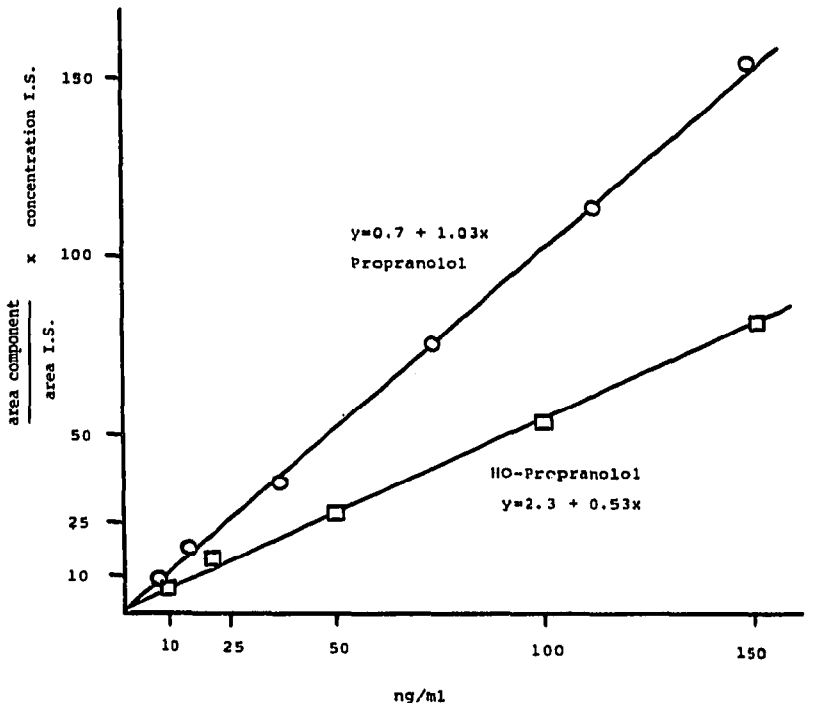

Fig. 4. Standard curves for 4-hydroxypropranolol and propranolol from human plasma. Single-ion (*m/e* 308) recordings. Standard curves C in Experimental.

TABLE I

STANDARD CURVES FOR PROPRANOLOL AND 4-HYDROXYPROPRANOLOL

Conditions: see Experimental. Curve A: no extraction. Curve B: extraction from distilled water. Curve C: extraction from plasma. Correlation coefficients were better than 0.990.

	Curve	$y = a + bx$	
		a	*b*
Propranolol	A	+0.3	1.05
	B	+2.6	1.00
	C	+0.7	1.03
4-Hydroxypropranolol	A	−1.9	0.56
	B	−3.2	0.51
	C	+2.3	0.53

TABLE II

STABILITY OF THE TFA DERIVATIVES

Conditions according to *Standard curves B and C* in Experimental. The TFA derivatives in benzene were left in contact with the pH 6.0 buffer between injections.

Hours after derivatization	*Propranolol (ng/ml)*	*4-Hydroxypropranolol (ng/ml)*
0–1	25.1 ± 1.2 ($n = 6$)	29.9 ± 2.4 ($n = 6$)
2–3	24.0 ± 1.9 ($n = 6$)	28.2 ± 3.3 ($n = 6$)
6–7	25.9 ± 2.3 ($n = 6$)	27.4 ± 2.9 ($n = 6$)

through the origin. The linearity is excellent in the concentration range used (5–150 ng/ml). Minimum detectable concentration of propranolol is about 1 ng/ml and of 4-hydroxypropranolol 5 ng/ml, using 1-ml plasma samples.

The standard curves obtained from plasma, curves C in Table I, do not significantly differ from the curves obtained from water, curves B, indicating no interference from plasma binding. Neither is there a significant difference if the extraction step is omitted (curves A).

The stability of the derivatives after excess reagent is removed by shaking with the pH 6.0 buffer is good for at least 7 h (*cf.* Table II). It is important to add an adequate amount of buffer to neutralize completely all trifluoroacetic anhydride. If the pH in the buffer solution after shaking falls significantly, the stability of the tri-TFA derivative of 4-hydroxypropranolol rapidly decreases.

TABLE III

EFFECT OF SODIUM HYDROGEN SULPHITE ON THE RECOVERIES OF PROPRANOLOL AND 4-HYDROXYPROPRANOLOL FROM PLASMA

Conditions according to *Standard curves B and C* in Experimental. 20 mg (2%) sodium hydrogen sulphite were added to each 1 ml of plasma before extraction.

	Propranolol (ng/ml)	*4-Hydroxypropranolol (ng/ml)*
Without HSO_3^-	25.6 ± 1.9 ($n = 6$)	18.6 ± 11.9 ($n = 6$)
With HSO_3^-	24.9 ± 1.6 ($n = 6$)	28.4 ± 3.0 ($n = 6$)

As has earlier been emphasized, the low stability of 4-hydroxypropranolol[3] has been one of the limiting factors in the successful analysis of this metabolite, particularly if present in small quantities. The stability decreases rapidly with increasing pH, but even at neutral to acidic pH the stability is low: a 100 ng/ml solution in distilled water (pH about 5.5) in 2 h decreased to about 20 ng/ml and was not detectable after 4 h. However, 4-hydroxypropranolol solutions made up in freshly prepared 2% sodium hydrogen sulphite were, at the 100 ng/ml concentration, quite stable for 7 h but not overnight. 4-Hydroxypropranolol is also quite stable in methanol over a 7-h period and in plasma for at least one week if frozen. The addition of hydrogen sulphite to plasma in the extraction step is important, as shown in Table III. A concentration of about 2% sodium bisulphite (20 mg to each 1-ml plasma sample) prevents the decomposition of 4-hydroxypropranolol.

Daily variations in the slope of the standard curves do occur. In a four-week period, during which twelve standard curves were run, the slopes ranged for propranolol from 0.82–1.17 and for 4-hydroxypropranolol from 0.41–0.68. All curves, however, went through the origin and the correlation coefficients were all high (better than 0.990). For good reproducibility the slopes of the standard curves have to be established every day by running at least three standard samples.

Table IV gives the steady-state plasma concentrations[16] of 4-hydroxypropranolol and propranolol in four patients receiving orally 160 mg propranolol per

TABLE IV

PROPRANOLOL AND 4-HYDROXYPROPRANOLOL IN PLASMA OF PATIENTS CHRONICALLY TREATED WITH PROPRANOLOL

40 mg of propranolol were administered orally every 6 h. Blood samples were drawn 2 h after the morning dose.

Patient	*Propranolol (ng/ml)*	*4-Hydroxypropranolol (ng/ml)*	*Ratio* $\frac{\text{4-hydroxypropranolol}}{\text{propranolol}}$
W.A.	31 ± 5 ($n = 4$)	8 ± 3 ($n = 4$)	0.26
G.P.	45 ± 5 ($n = 3$)	10 ± 5 ($n = 3$)	0.22
H.P.	123 ± 9 ($n = 4$)	25 ± 6 ($n = 4$)	0.20
F.W.	262 ± 11 ($n = 5$)	63 ± 7 ($n = 5$)	0.24

day in addition to numerous other drugs. The reproducibility of the method is satisfactory.

The large variation in plasma propranolol concentrations between the patients is also reflected in large variations in 4-hydroxypropranolol concentrations; the 4-hydroxypropranolol/propranolol ratio is constant. The 4-hydroxypropranolol concentration in these patients is about one fourth of the propranolol concentration. Studies of patients in larger number are currently in progress.

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