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Note

Determination of carboxybupranolol, the major metabolite of bupranolol, in human plasma by high-performance liquid chromatography

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Bupranolol [1-*tert.*-butylamino-3-(2-chloro-5-methylphenoxy)propan-2-ol, Fig. 1, R = CH_3] is used clinically as a β-adrenoceptor antagonist [1]. In human subjects, oral doses of bupranolol are almost completely excreted as the carboxy metabolite, carboxybupranolol [1-*tert.*-butylamino-3-(2-chloro-5-carboxyphenoxy)propan-2-ol, Fig. 1, R = COOH], which is also the major drug-related component in plasma [2].

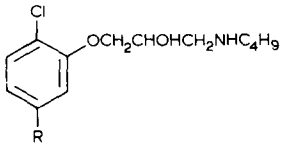

Fig. 1. Chemical structure of bupranolol (R = CH_3) and carboxybupranolol (R = COOH).

After oral doses of 40 mg bupranolol to human subjects, unchanged bupranolol was not detected above a limit of detection of 1 ng/ml using an electron-capture—gas chromatographic procedure [3]. Bupranolol has been detected in lower ng/g concentrations by a high-performance liquid chromatographic—ultraviolet (HPLC—UV) procedure [4] in rat plasma and tissues after administration of a relatively large intravenous dose (1.85 mg/kg). Studies of [^{14}C]bupranolol in both humans and animals indicated extensive first-pass metabolism of the drug and the rapid formation of carboxybupranolol; these results suggested either that bupranolol is pharmacologically active, although present at very low concentrations in plasma, or that (a) metabolite(s) of bupranolol also contributes to the pharmacological activity [2, 3, 5]. Since carboxybupranolol has been identified as the major metabolite of bupranolol in

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both humans and animals [2, 6], and since this compound has some pharmacological activity [7] it may be measured as an indication of the absorption of bupranolol from dose formulations of the drug.

In order to obtain reliable pharmacokinetic and bioavailability data, the method of analysis of carboxybupranolol in plasma must be sufficiently sensitive for the accurate determination of circulating concentrations of the compound. This paper describes an HPLC method for the measurement of carboxybupranolol in plasma over the concentration range 45—623 ng/ml. The assay procedure involves the extraction from plasma of the drug as an ion-pair, followed by chromatography in a reversed-phase mode, coupled with fluorimetric detection. The latter is ideal for carboxybupranolol analysis in that it is sensitive and highly specific thereby minimising the possibility of interference from other bupranolol metabolites, or other drugs administered concurrently with bupranolol.

EXPERIMENTAL

Reagents

Methanol was HPLC grade; sodium lauryl sulphate was Prima reagent grade; chloroform and propan-2-ol were Distol grade. All other reagents were of analytical grade. All solutions of inorganic reagents were prepared in freshly glass-distilled water. Standard solutions of the hydrochloride salt of carboxybupranolol (obtained from Dr. R. Bonn, Pharma-Schwarz GmbH, Monheim, F.R.G.) were prepared at concentrations of 1 mg/ml and 0.01 mg/ml in methanol. Solutions of deschlorocarboxybupranolol [1-*tert.*-butylamino-3-(5-carboxyphenoxy)propan-2-ol] hydrochloride, used as the internal standard for the assay, were made at similar concentrations. All standard solutions were stored in the dark at 4° C.

Sample preparation procedure

Plasma samples (0.1—0.5 ml) were transferred into conical centrifuge tubes and the volume adjusted to 0.5 ml with control plasma; the samples were spiked with internal standard (30 μl, containing 300 ng deschlorocarboxybupranolol hydrochloride). The pH of the samples was adjusted by the addition of sodium dihydrogen orthophosphate buffer (1 *M*, pH 3, 0.5 ml). Sodium dioctyl sulphosuccinate (DSS) (1%, w/v, 1.0 ml) was added as an ion-pairing agent. Samples were extracted by manual shaking for 15 sec with chloroform—propan-2-ol (9:1, v/v, 5 ml). After centrifugation, the organic layer was carefully transferred into another centrifuge tube and was evaporated to dryness at 37° C under a stream of nitrogen. The residue was washed to the bottom of the tube with a small amount of chloroform—propan-2-ol, which was again evaporated to dryness. The residue was mixed on a vortex mixer with mobile phase (50 μl) for 15 sec, after which time the sample was transferred to an autosampler vial. The total sample was injected into the chromatograph.

High-performance liquid chromatography

The liquid chromatograph consisted of a Waters M6000A pump (Waters Assoc., Northwich, U.K.) coupled to a Perkin-Elmer 3000 fluorescence detector

(Perkin-Elmer, Beaconsfield, U.K.) operated at an excitation wavelength of 245 nm and an emission wavelength of 330 nm. Injection was via an automatic injector, Waters' Intelligent Sample Processor (WISP™, Waters Assoc.). The column was constructed of stainless steel (25 cm × 0.46 cm I.D.) prepacked with Zorbax® C_8 (mean particle diameter 6 μm, DuPont, Hitchin, U.K.). A pre-column (7 cm × 0.2 cm I.D.) constructed of stainless steel and dry-packed with pellicular Co:Pell® ODS (particle diameter 25—37 μm) (Whatman, Maidstone, U.K.) was installed in front of the analytical column to protect it from contamination.

Chromatography was performed in a reversed-phase mode using an ion-pairing mechanism. The mobile phase consisted of methanol (70%, v/v) in aqueous potassium dihydrogen orthophosphate buffer (0.1%, w/v), containing sodium lauryl sulphate (1%, w/v). The final solution was adjusted to pH 3 with phosphoric acid. The mobile phase was passed through the column at a flow-rate of 2 ml/min.

Chromatograms were recorded using a 3380A computing integrator (Hewlett-Packard, Slough, U.K.).

Under the conditions described, carboxybupranolol had a retention time of 7 min and the internal standard a retention time of 5 min (Fig. 2).

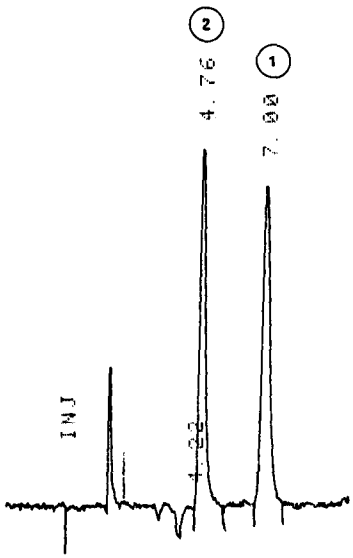

Fig. 2. Chromatogram of reference standards. Chromatographic conditions: column, 25 cm × 0.46 cm I.D., containing Zorbax C_8; mobile phase, 70% (v/v) methanol—aqueous potassium dihydrogen orthophosphate (0.1% w/v) containing sodium lauryl sulphate (1%, w/v), final pH adjusted to 3 with phosphoric acid; flow-rate, 2 ml/min; detector, fluorescence, excitation wavelength 245 nm, emission wavelength 330 nm. Peaks: 1 = carboxybupranolol; 2 = internal standard.

Calibration procedure

The calibration line was constructed from peak area ratio measurements of carboxybupranolol to internal standard against concentration over the concentration range 45—623 ng/ml.

Samples of blank plasma (0.5 ml) were spiked with amounts of carboxy-

bupranolol hydrochloride of 25, 50, 100, 150, 200, 250 and 350 ng per 0.5-ml sample; this was equivalent to concentrations of 45, 89, 178, 267, 356, 445 and 635 ng carboxybupranolol free base per ml. Internal standard (as the hydrochloride) was added into samples at a fixed concentration of 600 ng/ml (300 ng per 0.5 ml). The samples were taken through the extraction procedure described previously.

Studies in humans

Four human subjects were dosed with capsules containing bupranolol (300 mg as the hydrochloride salt), together with 100 ml water, at 1 h following a standardised breakfast. The study was conducted under conditions similar to those previously described [8].

Blood samples were withdrawn by venepuncture into heparinised tubes before dosing and at 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8 and 10 h after dosing. The blood cells were separated by centrifugation and discarded; plasma was stored at −20° C until analysis by the method described.

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each concentration were repeated on six occasions at each point over the calibration range. The precision of the method for the measurement of carboxybupranolol in plasma was indicated by the coefficients of variation of peak area ratios (Table I) which were 21% at 45 ng/ml, 6% at 267 ng/ml and 5% at 623 ng/ml.

TABLE I

BETWEEN-ASSAY PRECISION MEASUREMENTS OF CARBOXYBUPRANOLOL IN PLASMA

Concentration of carboxybupranolol (ng/ml)	Peak area ratio $\left(\frac{\text{carboxybupranolol}}{\text{internal standard}}\right)$						Mean (± S.D.)	Coefficient of variation (%)
45	0.12	0.12	0.18	0.16	0.14	0.12	0.14 (0.03)	21
89	0.30	0.32	0.23	0.36	0.26	0.21	0.28 (0.06)	21
178	0.47	0.54	0.47	0.52	0.55	0.44	0.50 (0.04)	8
267	0.83	0.71	0.81	0.79	0.85	0.82	0.80 (0.05)	6
356	1.02	1.03	1.10	0.96	1.13	1.05	1.05 (0.06)	6
445	1.16	1.19	1.26	1.15	1.15	1.17	1.18 (0.04)	3
623	1.69	1.81	1.87	1.69	1.70	1.71	1.75 (0.08)	5

Accuracy

The calibration line for the measurement of carboxybupranolol in plasma was constructed over the range 45—623 ng/ml; six replicate extractions were made at each concentration over the range. The plot of peak area ratio against concentration was linear ($Y = a + bX$, where $a = 0.031565$ and $b = 0.002732$) where Y is the peak area ratio and X is the concentration of carboxybupranolol free base (ng/ml). The accuracy of the method as indicated by the standard

error of the fitted least-squares regression line, i.e. using the calibration line to estimate the concentrations of carboxybupranolol in plasma, was ± 23 ng/ml.

Recovery

The recovery (extraction efficiency) of internal standard (600 ng/ml) from plasma (0.5 ml) was determined by comparison of peak area ratio measurements of internal standard to carboxybupranolol of standards taken through the extraction procedure, to those injected into the chromatograph without extraction. The mean recovery of internal standard was 79 ± 6% S.D. (n = 6).

The mean recovery of carboxybupranolol from plasma was determined by

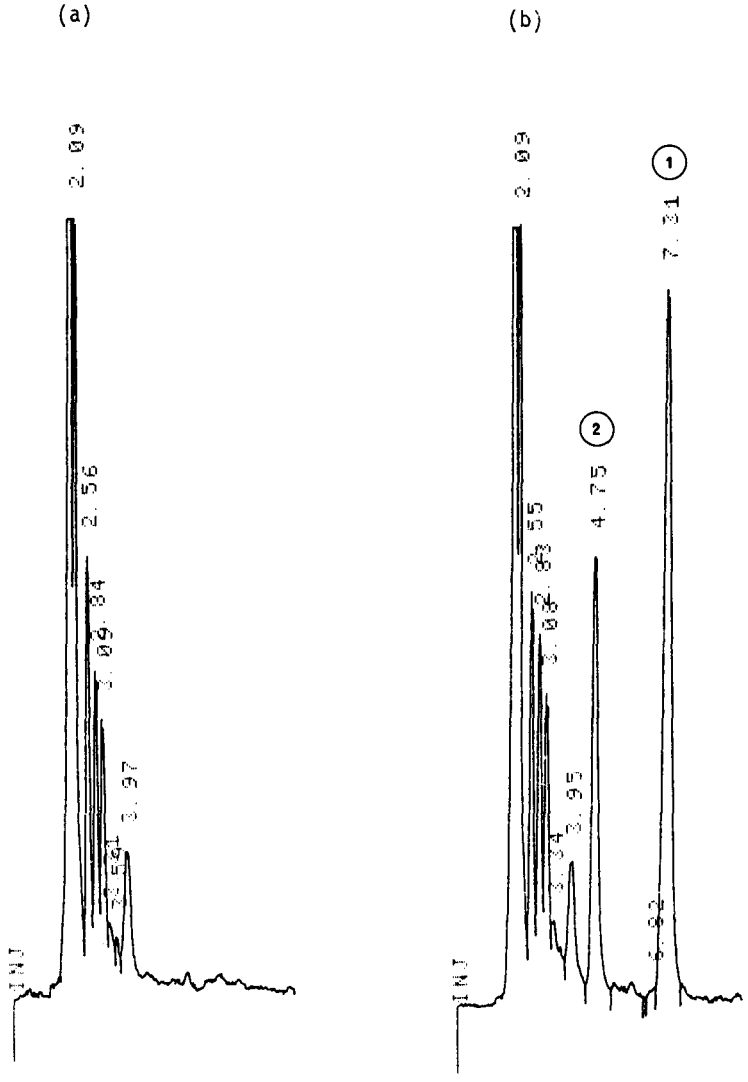

Fig. 3. Chromatograms of (a) pre-dose control plasma and (b) 1-h post-dose plasma containing carboxybupranolol at a concentration of 768 ng/ml. Experimental conditions as for Fig. 2. Peaks: 1 = carboxybupranolol, 2 = internal standard.

comparison of peak area ratios of extracted standards, corrected for 100% recovery of internal standard, to those of non-extracted standards. The mean recovery of carboxybupranolol from plasma over the concentration range 45—356 ng/ml did not differ significantly from 100%.

Stability of carboxybupranolol in plasma

The stability of carboxybupranolol in plasma under the storage conditions used (−20°C) was tested by storing plasma standards at a concentration of 267 ng/ml for nineteen days. Recovery of carboxybupranolol from the stored samples was 95 ± 5% S.D. (n = 6).

Limits of detection

No interfering peaks with retention times similar to either carboxybupranolol or internal standard were present in predose (blank) plasma (Fig. 3). The limit of detection of carboxybupranolol based on the extraction of 0.5 ml plasma, was set by instrumental noise at 20 ng/ml. The reliable limit of accurate measurement based on integrator sensitivity was 45 ng/ml, the lowest datum point on the calibration line.

Selectivity of the analytical method

No peaks interfering with the analysis were present from any control plasma investigated. Samples chromatographed without internal standard showed no interference from metabolites with the same retention time as the internal standard. Bupranolol, desmethylbupranolol and hydroxymethylbupranolol (another less important metabolite) were only weakly fluorescent at the wavelengths employed and did not interfere with either carboxybupranolol or internal standard.

Concentrations of carboxybupranolol

The mean concentrations of carboxybupranolol in the plasma of four volunteers after single oral doses of 300 mg bupranolol (as the hydrochloride

TABLE II

MEAN (± S.D.) CONCENTRATIONS OF CARBOXYBUPRANOLOL (ng/ml) IN THE PLASMA OF FOUR HUMAN SUBJECTS AFTER SINGLE ORAL DOSES OF 300 mg OF BUPRANOLOL HYDROCHLORIDE

Time after dosing (h)	Concentration (± S.D.) (ng/ml)
0.5	236 (143)
0.75	1275 (612)
1	2273 (880)
1.5	4190 (1272)
2	3304 (852)
3	2174 (741)
4	747 (213)
5	308 (88)
6	165 (45)
8	54 (39)
10	<45 (—)

salt) reached a peak of 4190 ng/ml (Table II) at 1.5 h after dosing and thereafter declined to below the limit of accurate measurement at 10 h with a mean half-life of 1.3 h (± 0.33). These concentrations were associated with drug action since concurrent pharmacodynamic measurements indicated that there was a pharmacological effect (inhibition of response to exercise stress) in the healthy volunteers after administration of 300 mg bupranolol.

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