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Note

Determination of penbutolol and its hydroxylated metabolite in biological fluids by reversed-phase high-performance liquid chromatography

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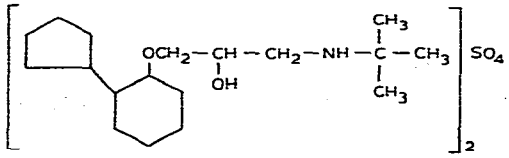
Penbutolol, HOE 893, *d*-(—)-1-*tert*.-butylamino-3-(2-cyclopentylphenoxy)-2-propanol, is a non-cardioselective beta-adrenoceptor blocking agent. Preliminary studies in man have shown that penbutolol (PB) was four [1] to ten [2] times more potent than the other major beta-receptor antagonists and exhibited a long-lasting action. This latter was attributed to the biotransformation of PB in several active metabolites, among which the 4-hydroxy derivative (4-OH-PB) appeared to be the most important [3, 8].



In order to define the role of 4-OH-PB formation in the pharmacological effects of PB in man, it was necessary to possess a method allowing the specific measurement of PB and 4-OH-PB after the administration of a single therapeutic dose of PB.

Since the already published techniques suffered from a lack of either specificity [4,5] or sensitivity [1,6], the aim of the present work was to develop a new method using basic extraction and high-performance liquid chromatographic (HPLC) separation with fluorimetric detection.

The method was applied to the specific measurement of unchanged PB and 4-OH-PB and their conjugates in biological fluids.

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EXPERIMENTAL

Apparatus

An analytical liquid chromatograph (Hewlett-Packard, Model 1081B) was used. The effluent was monitored with the fluorescence-sensitive detector (Kontron LC 770) set at 290 nm excitation wavelength and 330 nm emission wavelength for the detection of 4-OH-PB and propranolol (used as internal standard, IS), then at 278 nm excitation and 310 nm emission for the detection of PB (exictation and emission slits, 4 nm).

The chromatographic column (250 × 4.6 mm I.D.) was packed with Li-Chrosorb RP-8, 10 μm particle size (Merck, Darmstadt, G.F.R.). The working temperature was regulated at 28 ± 0.2°C.

Chemicals and reagents

PB sulfate and 4-OH-PB sulfate (*l*-stereoisomers) were kindly supplied by Hoechst (Frankfurt, G.F.R.). *dl*-Propranolol hydrochloride was a gift of ICI (Mereside, Great Britain).

Diethyl ether and acetonitrile of analytical grade (Merck) were distilled before use.

Solutions of 1 *N* sodium hydroxide and 1 *N* and 0.1 *N* hydrochloric acid were prepared in bidistilled water.

The mobile phase was a mixture of 52% citric acid—sodium citrate buffer 0.1 *M* adjusted to pH 2.85 with 1 *N* hydrochloric acid and 48% acetonitrile and further addition of 1 g/l sodium heptanesulfonate.

The hydrolysis of conjugates was performed in acetate buffer pH 5 (sodium acetate 0.1 *M*) with β-glucuronidase (10,000 U/ml) and sulfatase 0.6 U/ml (Sigma, St. Louis, MO, U.S.A.)

Stock solutions were prepared by dissolving 0.1 mg/ml PB, 4-OH-PB and IS in methanol. They were found to be stable at 4°C for at least three months.

Extraction procedures

Unchanged compounds (procedure I). In a 15-ml glass centrifuge tube 0.5 ml of the IS solution (100 ng/ml) was introduced and evaporated to dryness at 37°C under a gentle stream of nitrogen. To the dry residue, 1 ml of plasma or urine, 0.5 ml of 1 *N* sodium hydroxide and 8 ml of freshly distilled diethyl ether were added. The tube was stoppered and placed on a rotating shaker (60 rpm) for 15 min. After centrifugation (15 min at 1200 *g*) 6.5 ml of the organic layer were transferred into a conical test-tube after passing through a small column (4 mm I.D.) filled with glass-wool to a height of 2 cm, and evaporated to dryness under the conditions already described. A 200-μl volume of mobile phase was added to the extract and a 20-μl aliquot of this solution was injected onto the column by means of an injection loop, at a flow-rate of 1.7 ml/min under a pressure of 38 bars at 28°C.

Conjugated compounds (procedure II). In a 15-ml glass centrifuge tube 0.5 ml of the IS solution (100 ng/ml) was introduced and evaporated to dryness. Then, 1 ml of plasma or urine, 1 ml of acetate buffer and 0.1 ml of β-glucuronidase—sulfatase solution were added. As demonstrated by preliminary experiments, the mixture was kept at 37°C for 48 h to allow full hydro-

lysis. After return to room temperature, 0.5 ml of 1 *N* sodium hydroxide and 8 ml of freshly distilled diethyl ether were added. The tube was stoppered and placed on a rotating shaker (60 rpm) for 15 min. After centrifugation (15 min at 1200 *g*) 6.5 ml of the organic layer were transferred to another tube and extracted with 6 ml of 0.1 *N* hydrochloric acid for 15 min on a rotating shaker (60 rpm). Following centrifugation (10 min at 1200 *g*) 5.5 ml of the acidic aqueous phase were transferred to a new tube containing 0.7 ml of 1 *N* sodium hydroxide and the mixture was extracted with 6 ml of diethyl ether for 15 min on a rotating shaker (60 rpm). After centrifugation (10 min at 1200 *g*), 5 ml of the organic phase were transferred to a 10-ml conical tube and evaporated to dryness. The residue was redissolved in 0.2 ml of mobile phase and 20 µl of this solution were injected into the HPLC system.

Calibration graphs

Standard curves were obtained by adding known amounts of each compound to 1 ml of biological fluids. The samples were carried through the whole procedure and the peak height ratio of PB and 4-OH-PB to IS was plotted against the concentration of the corresponding drug. The concentration of glucuronides was calculated as the difference between the drug concentration found after and before hydrolysis.

RESULTS AND DISCUSSION

Specificity

Under the chromatographic conditions described above the compounds were well separated. The retention times of 4-OH-PB, IS and PB were 3.25, 4.00 and 7.75 min, respectively.

As shown in Fig. 1, neither plasma or urine extraction nor hydrolysis

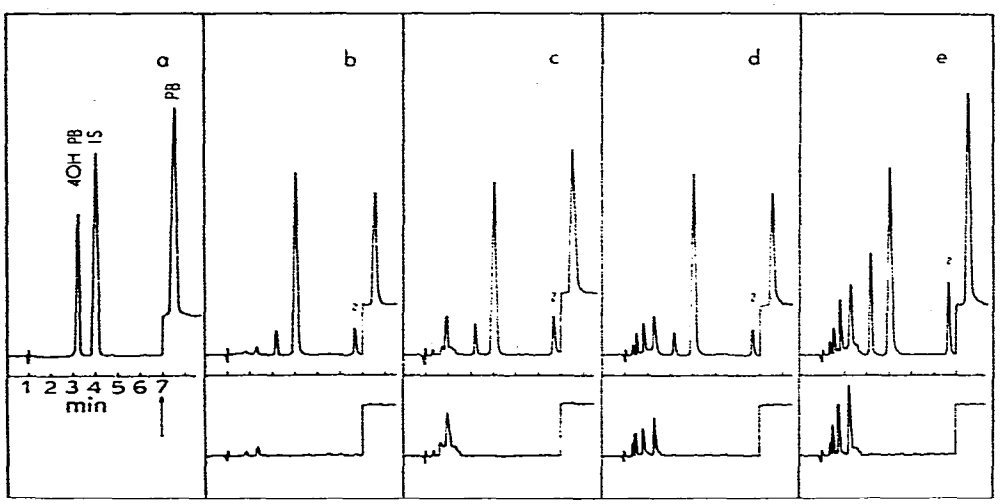

Fig. 1. Chromatograms of a mixture (a) of 4-OH-PB, IS and PB (50 ng each), an extract of plasma sample before (b) and after (c) hydrolysis, and an extract of urine sample before (d) and after (e) hydrolysis. In the lower part are shown the corresponding blanks. The arrow indicates the shift of the baseline due to the change of detection wavelengths.

brought any peaks interfering with those of the compounds measured.

The large time interval (3.75 min) between the IS and PB peaks allowed the change of excitation and emission wavelengths. This change was necessary to take the relative sensitivities of the three compounds into account [5]. On the other hand, the observed baseline shift (indicated by the arrow in Fig. 1) due to this change of wavelengths was not important and did not prevent the measurement of quantities of PB as small as 2 ng/ml of biological fluid when maximal sensitivity was set up.

The chromatograms in Fig. 1 were obtained before and after deconjugation of plasma and urine samples drawn from one subject who had received 6 h previously a single oral dose of 40 mg of PB.

An unidentified peak (?) was detected at a retention time of 7.0 min. Presumably the responsible compound is another metabolite of PB which exists in both the unconjugated and conjugated form. Its structure is currently under investigation.

Linearity

The standard curves (Fig. 2) were checked on plasma and urine samples spiked with different concentrations (from 50 to 1000 ng/ml) of 4-OH-PB and PB and a constant concentration of IS (50 ng/ml). They were expressed as peak height ratios of 4-OH-PB or PB to IS against the concentration of the corresponding compound.

Assuming that injection by the loop system was reproducible, the recoveries were estimated at 69, 88 and 92% for 4-OH-PB, IS and PB, respectively, through procedure I, and at 36, 78 and 80% through procedure II. In the latter case, the decreased recoveries were due to the back-extraction procedures used to remove impurities from the hydrolysis. In particular, this effect was noted for 4-OH-PB which was slightly soluble in the acidic aqueous phase. Consequently, the standard curves for 4-OH-PB obtained during procedures I (Fig. 2a) and II (Fig. 2b) were different.

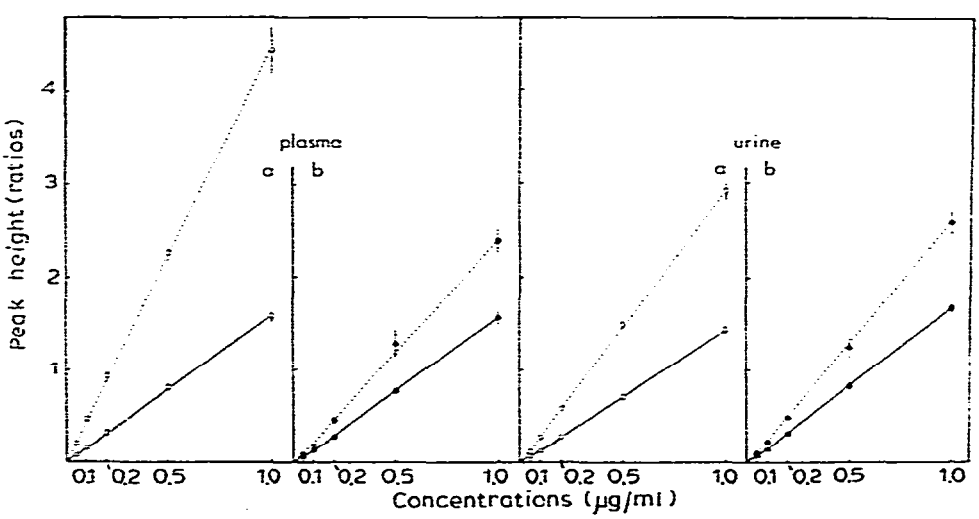

Fig. 2. Standard curves for the determination of 4-OH-PB (- - - -) and PB (——) before (a) and after (b) hydrolysis in plasma or urine. Mean (± S.D.) of six determinations.

Sensitivity

The detection limit in plasma or urine (three times greater than the fluorescence background) was found at 3 and 2 ng/ml for 4-OH-PB and PB, respectively, through procedure I, and at 5 and 2 ng/ml through procedure II. In comparison with other methods [1,4,5] the specificity and the sensitivity were increased. Indeed, the gas chromatographic method used by Giudicelli et al. [1] only allowed the determination of non-conjugated PB in plasma with a sensitivity of 15 ng/ml. Wallner et al. [4] only determined non-conjugated PB in plasma and urine with a fluorimetric method for which the sensitivity was not outlined. The procedures described by Hajdu et al. [5] allowed the determination of both compounds in unconjugated and conjugated form in plasma and urine. However, the lowest detection limit was only 40 ng/ml for PB and 25 ng/ml for 4-OH-PB because direct fluorimetric measurement without chromatographic separation was used.

Reproducibility

Table I shows the intra-assay reproducibility of the technique calculated from six determinations and for two concentrations (50 and 500 ng/ml) of 4-OH-PB and PB in plasma and urine through procedures I and II. The coefficient of variation did not exceed 10.9%.

TABLE I

INTRA-ASSAY REPRODUCIBILITY OF THE METHOD

	Biological fluid	Compound	Added (ng/ml)	Found ± S.D. (ng/ml)	Coefficient of variation (%)
Procedure I	Plasma	4-OH-PB	50	52.2 ± 3.9	7.4
			500	502.2 ± 23.6	4.7
		PB	50	53.3 ± 3.6	6.7
			500	509.3 ± 19.3	3.8
	Urine	4-OH-PB	50	46.6 ± 4.4	9.4
			500	510.1 ± 10.2	2.0
		PB	50	53.5 ± 2.8	5.2
			500	506.9 ± 22.8	4.5
Procedure II	Plasma	4-OH-PB	50	46.5 ± 4.8	10.3
			500	525.0 ± 37.1	7.1
		PB	50	48.4 ± 1.7	3.5
			500	495.6 ± 13.9	2.8
	Urine	4-OH-PB	50	49.2 ± 5.4	10.9
			500	495.8 ± 46.6	9.4
		PB	50	49.9 ± 2.7	5.5
			500	497.0 ± 17.4	3.5

Pharmacokinetic application

In order to assess its validity the method described above was applied to observing the pharmacokinetics of PB and 4-OH-PB in humans.

Fig. 3 represents the plasma concentration—time curves obtained in one patient with severe renal insufficiency (glomerular filtration rate = 18 ml/

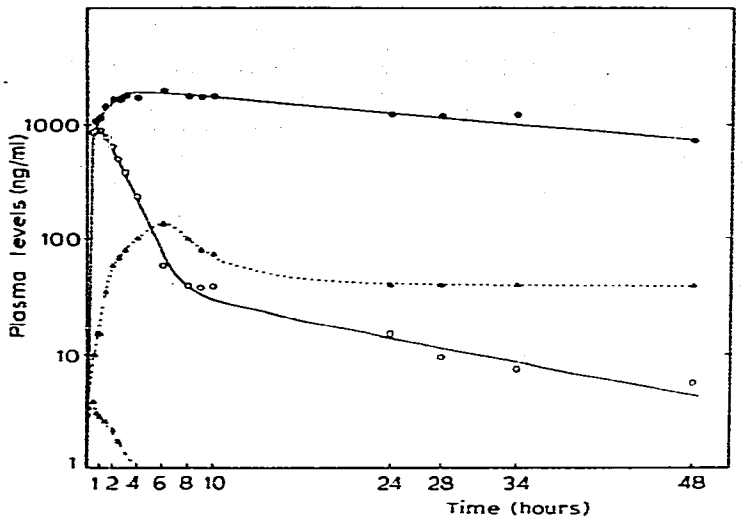

Fig. 3. Plasma levels of PB (——) and 4-OH-PB (- - - - -) before (open symbols) and after hydrolysis (closed symbols) in a patient with renal insufficiency after a single oral administration of 40 mg of PB.

min) who had received a single oral dose of 40 mg of PB.

These results showed that PB was rapidly absorbed and distributed and slowly eliminated, as indicated by the biphasic decreasing part of the corresponding non-conjugated PB plasma level curve. The specificity and the sensitivity of the present analytical method allowed us to follow the apparent elimination phase of PB until 48 h after administration at least. Consequently, a two-compartment open model could be used to describe the pharmacokinetics of PB [7]. Other authors [5] fitted their data (in healthy volunteers) according to a one-compartment open model, but the analytical method used did not allow the measurement of concentrations of PB lower than 40 ng/ml of plasma.

As already known [5], conjugated PB exhibited plasma concentrations far more elevated than those of PB and a very slow elimination rate (Fig. 3). On the other hand, any pharmacological effect could hardly be attributed to the active metabolite 4-OH-PB because of its negligible concentrations.

The present technique appears suitable to describe the pharmacokinetics of PB in humans who have received a single therapeutic dose.

ACKNOWLEDGEMENTS

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