Journal of Chromatography, 422 (1987) 205–215
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3867

COLUMN LIQUID CHROMATOGRAPHIC DETERMINATION OF HYDROLYSED BOPINDOLOL, IN THE PICOGRAM PER MILLILITRE RANGE IN PLASMA, USING CARTRIDGE EXTRACTION AND DUAL ELECTROCHEMICAL DETECTION

H. HUMBERT* and J. DENOUEL

Sandoz Laboratories, Pharmaceutical Research Centre, B.P. 313, 92506 Rueil-Malmaison Cedex (France)

and

H.P. KELLER

Biopharmaceutical Department, Pharmaceutical Development, Sandoz Ltd., CH-4002 Basle (Switzerland)

(First received April 22nd, 1987; revised manuscript received June 30th, 1987)

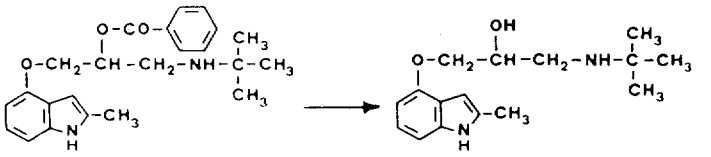
SUMMARY

A highly sensitive and specific column liquid chromatographic assay with electrochemical detection was developed for hydrolysed bopindolol, an active metabolite of bopindolol (Sandonorm®) in human plasma. The pre-chromatographic sample preparation involved Extrelut® column clean-up followed by liquid extraction of the organic extract into dilute acetic acid. Separation was on a Nucleosil ODS 3-μm column at 40°C, with a phosphate buffer–methanol mobile phase. Detection was performed at +450 mV with an ESA electrochemical detector. Mepindolol was used as internal standard and quantitation was based on peak-area ratios. Total analysis time was 14 min per sample. The recovery rate of the assay was at least 70% for both compounds. A detection limit as low as 25 pg/ml, starting with 1 ml of plasma, was achieved. The day-to-day reproducibility and accuracy, checked with quality-control samples, demonstrated the reliability of this assay used by different analysts, on different chromatographic systems and over a long period of time.

INTRODUCTION

Bopindolol [Sandonorm®, 4-(benzoyloxy-3-*tert.*-butylaminopropyl)-2-methylindole hydrogenmalonate, Fig. 1] is a potent and specific β-adrenoceptor antagonist with partial agonist activity [1]. In human clinical trials, this drug has demonstrated more prolonged action per unit dose than other β-blocking drugs

0378-4347/87/$03.50



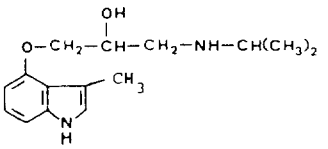


Fig. 1. Structures of bopindolol, its hydrolysed metabolite and mepindolol, the internal standard.

[2]. Bopindolol is very rapidly metabolized to hydrolysed bopindolol [4-(2-hydroxy-3-*tert.*-butylaminopropyl)-2-methylindole, Fig. 1], a major active metabolite.

Very sensitive methods are needed for the study of the pharmacokinetics of this highly active and long-acting compound. The first published method for the determination of hydrolysed bopindolol (Hyd-Bop) in plasma was a high-performance liquid chromatographic (HPLC) assay with fluorimetric detection [3]. A detection limit of 0.5 ng/ml was reported, insufficient for monitoring the plasma concentration up to 24 h after a single oral administration of a therapeutic dose (1 mg). More recently, HPLC with electrochemical detection (ED) or a radioreceptor assay (RRA) have been used in various studies [4–6]. With these methods, concentrations as low as 0.5 and 0.025 ng/ml, respectively, were measured. A good correlation between HPLC and the RRA was obtained for concentrations above 0.5 ng/ml [4,6]. The sensitivity of the RRA is good, but this method is not always easy to use in all laboratories. Moreover, the specificity of the RRA was demonstrated for concentrations above 0.5 ng/ml only [4]. The purpose of the present study was, therefore, to develop a specific and more sensitive HPLC–ED method for the assay of Hyd-Bop in pharmacokinetic investigations.

EXPERIMENTAL

Reagents

All chemicals were of analytical grade. Sodium hydroxide and heptane were obtained from Merck (Darmstadt, F.R.G.). Isoamyl alcohol and methanol were supplied by Carlo-Erba (Milan, Italy). Acetic acid, phosphoric acid and potassium dihydrogenphosphate were purchased from Prolabo (Paris, France). All aqueous solutions were prepared with water purified on a Milli-Q system (Mil-

lipore, Molsheim, France). Potassium dihydrogen phosphate (0.03 *M*) aqueous solution was prepared and adjusted to pH 2.2 with phosphoric acid.

The metabolite Hyd-Bop as its tartrate and the internal standard (I.S.) mepindolol [4-(2-hydroxy-3-isopropylaminopropyl)-2-methylindole sulphate] were obtained from Sandoz (Basle, Switzerland). Stock solutions (0.5 mg/ml) were prepared in methanol and stored at 4°C. Working solutions were prepared daily in 0.1 *M* acetic acid.

Extrelut® R^1 (1 ml) columns were obtained from Merck.

Plasma samples

Drug-free blood was drawn from healthy human subjects. Subject samples were obtained from pharmacokinetic studies performed on healthy volunteers, in accordance with the guidelines of the Tokyo amendment of the declaration of Helsinki. The blood was collected into heparinized tubes and immediately centrifuged at 4°C. Without delay, plasma was separated, frozen and stored at −20°C until use.

Spiked plasma samples for calibration or quality control were prepared from drug-free plasma: 10–50 ml of plasma were spiked with 100 µl of Hyd-Bop solution in 0.1 *M* acetic acid. Depending on their intended use, the samples were stored either at 4°C overnight or at −20°C until analysis.

Extraction

A 1-ml volume of freshly thawed and mixed plasma sample was pipetted into a conical glass tube, to which 50 µl of internal standard solution (80 pg/µl) and 100 µl of sodium hydroxide (1 *M*) were added. The tubes were vortexed for 10 s and then centrifuged at 1400 *g* for 4 min. The supernatant (1 ml) was transferred onto a dry Extrelut column. After 15 min, Hyd-Bop and I.S. were extracted from the column by elution with 6 ml of heptane–isoamyl alcohol (95:5, v/v) in a pointed tube. Then 200 µl of 0.1 *M* acetic acid were added to the organic phase. Hyd-Bop and I.S. were back-extracted by shaking for 15 min. After centrifugation (1400 *g* for 5 min), the organic phase was discarded and 150 µl of the aqueous phase were transferred to a glass vial and capped with a septum. The vial was kept at 4°C. About 30 min before injection of 100 µl into the liquid chromatograph, the vial was put into the automatic sample injector.

Column liquid chromatography

An HPLC Model 1090 (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used. It consisted of two solvent containers, a pump, an automatic sample injector and a thermostatted oven at 40°C containing the analytical column. The separation was performed on a Nucleosil ODS reversed-phase column (8.3 cm × 4.6 mm I.D., particle size 3 µm, SFCC, Gagny, France). The mobile phase was 0.03 *M* potassium dihydrogenphosphate (pH 2.2)–methanol (70:30, v/v). These solutions were filtered through a 0.6-µm filter before use and pumped at a flow-rate of 1 ml/min (ca. 180 bar). All HPLC system connections down-stream of the injector were via 0.13 mm I.D. stainless-steel tubes.

The detection was performed with an ESA Model 5100 A (ESA, Bedford, MA,

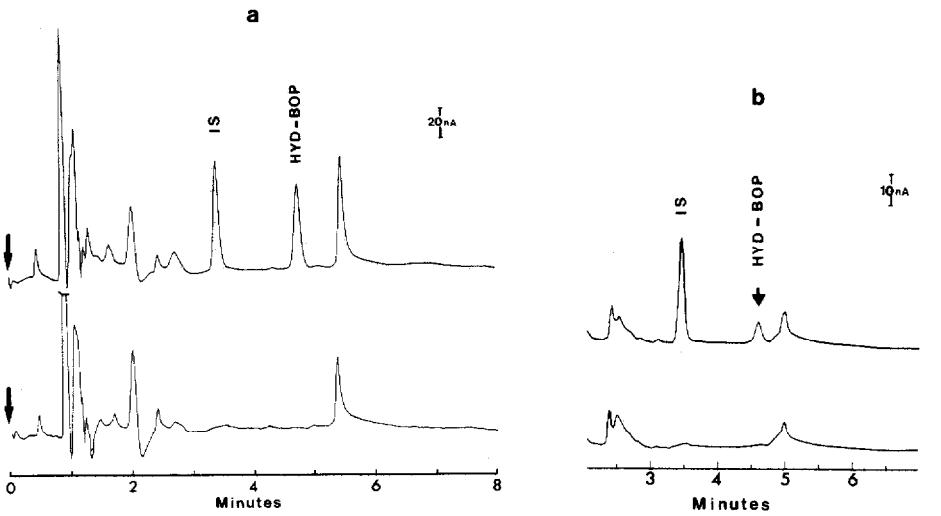


Fig. 2. Representative chromatograms of: (a) extract from plasma samples from a subject before and 6 h (4 ng/ml I.S. and 2.95 ng/ml Hyd-Bop) after oral administration of 2 mg of bopindolol and (b) extract from blank plasma samples with or without 2 ng/ml I.S. and 100 pg/ml Hyd-Bop.

U.S.A.) in the oxidative screen mode. It consists of a 5020 guard cell, a 5011 high-sensitivity dual analytical cell and a command module. The guard cell is situated between the pump and the injector. The applied potentials were +1 V on the guard cell and +160 and +450 mV, respectively, on detectors 1 and 2 of the dual analytical cell. The detector 1 and 2 signals were fed into a dual recorder (Kipp and Zonen, Delft, Netherlands). The detector 2 signal was fed in parallel into a 3357 LAS system (Hewlett-Packard).

Under these conditions, the elution times for I.S. and Hyd-Bop were 3.3 and 4.7 min, respectively. An injection could be performed every 14 min. Typical chromatograms of a blank plasma, spiked or unspiked with Hyd-Bop (100 pg/ml), and of two plasma samples from a patient are shown in Fig. 2.

Quantification

Concentrations of Hyd-Bop were calculated by relating the peak-area ratios of Hyd-Bop and I.S. to a standard calibration curve. This standard curve was obtained by least-squares linear regression over the range of 100–8000 pg/ml Hyd-Bop in plasma. The recovery (%) of Hyd-Bop in plasma was calculated by the ratio of the peak area for spiked samples and of directly injected standard solutions (taking account of the aliquot ratio). The plasma concentrations of Hyd-Bop were given in ng/ml base (0.1 ng/ml = 362 pmol/l). I.S. is expressed below in ng sulphate.

RESULTS AND DISCUSSION

Sample pretreatment

Preliminary tests had shown that it was necessary for all reagents, and in particular the plasma samples, to be at room temperature during the various phases

TABLE I

EXTRACTION YIELD OF HYDROLYSED BOPINDOLOL (Hyd-Bop) AND MEPINDOLOL (I.S.) FROM SPIKED SAMPLES

Compound	Spiked value (ng/ml)	*n*	Extraction yield (%)		Area ratio Hyd-Bop/I.S.	
			Mean	C.V.	Mean	C.V.
Hyd-Bop	0.065	5	79.7	13.6	0.031	2.1
	0.325	14	82.3	8.4	0.119	4.9
	1.300	9	77.5	13.0	0.418	6.6
	3.250	6	81.1	3.8	1.097	1.6
	7.800	3	77.6	3.5	2.550	3.6
I.S.	4.000	9	73.9	8.7		
	2.000	15	74.4	11.2		

of Extrelut extraction. This was to avoid excessive fluctuation in the percentage extracted. It should be noted that the column absorption time of plasma and the elution time may vary from one batch of Extrelut to another. These phenomena have previously been reported for other active principles [7].

Acid-phase back-extraction is justified on two counts: (i) it avoids the need to evaporate the heptane–isoamyl alcohol mixture, which is always a lengthy process when heating is not permissible; (ii) it enhances the selectivity of the extraction in comparison with certain endogenous substances and permits a higher level of sensitivity. Under these conditions, the extraction rate of I.S. ranges from 73.9 to 74.4% and that of Hyd-Bop from 77.5 to 82.3% (see Table I).

For a given concentration, the variable recovery yield was compensated in the assay by use of an internal standard, since I.S. and Hyd-Bop showed very similar behaviour patterns during the extraction phase. These comments are illustrated by the low variability of the area ratio (see Table I). These findings are of particular interest since, for some batches of Extrelut, lower recovery yields have been reported (65%) without any adverse effect on assays performed in the presence of I.S.

During the initial tests it was noted that for some batches of plasma there was a loss of Hyd-Bop and of I.S. when extracts in acid medium were stored at room temperature until injection. Although the behaviour of Hyd-Bop seems to parallel that of I.S. under these conditions, the use of a refrigerated injector at 4°C and storage of the samples at this temperature until use is recommended. These comments are supported by those of Oddie et al. [3].

Column

Various types of analytical column were tested: two with low I.D. [Hypersil ODS 5 μm (100 mm×2.1 mm I.D., Hewlett-Packard) and Sup RS Spherisorb ODS2 5 μm (150 mm×2.1 mm I.D., Prolabo)] and two with regular I.D. [HS3 C_{18} 3 μm (83 mm×4.6 mm I.D., Perkin-Elmer, Norwalk, CA, U.S.A.) and Nu-

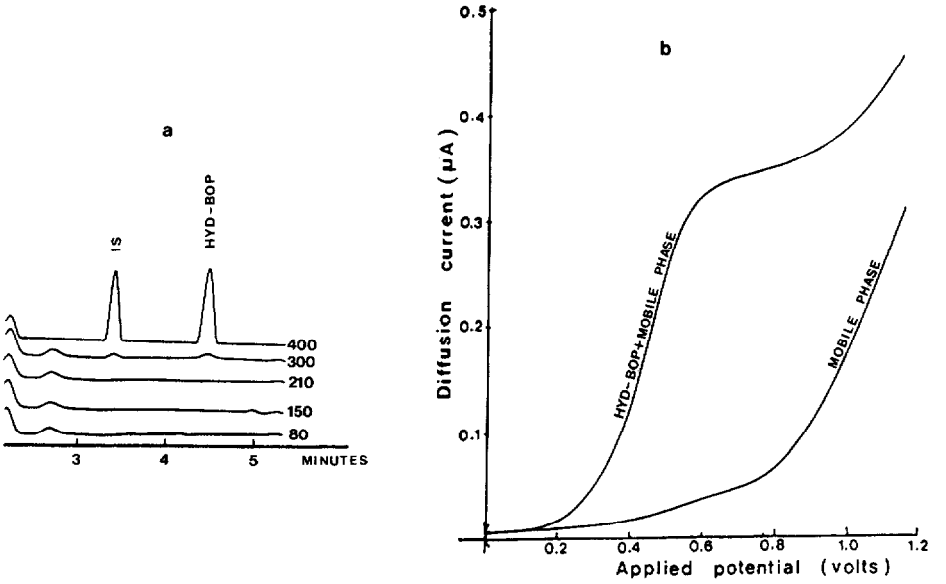


Fig. 3. (a) Chromatogram of I.S. and Hyd-Bop (amounts injected 2 ng of each) recorded at different oxidation potentials (80, 150, 210, 300 and 400 mV) on detector 1 of the dual analytical cell. (b) Scan (oxidation wave) of continuous flow of mobile phase and of mobile phase with Hyd-Bop (20 μg/ml).

cleosil ODS 3 μm (83 mm×4.6 mm I.D., SFCC)]. Nucleosil ODS 3 μm gave the best resolution, with an excellent life-span. Under normal operating conditions, the performance of the column was maintained for ca. 1500 injections and, in some cases, for up to 2500 injections.

Applied potential

The principle of the detector offered the possibility of optimizing the conditions of the electrochemical detection. The potential applied to the guard cell (+1 V) allows the oxidation of all the compounds present in the mobile phase just before the injector. The first detector of the analytical cell was used to oxidize background contamination prior to oxidation of Hyd-Bop and I.S., which were then monitored on detector 2 of the analytical cell. Therefore, the oxidation potential of detector was set at 160 mV, below the potential required for any oxidation of Hyd-Bop and I.S. (Fig. 3a). The oxidation potential of detector 2 was set at +450 mV, which corresponds to the optimum between the maximum oxidation response of Hyd-Bop and the increased of background response of the mobile phase (Fig. 3b).

Day-to-day management of the chromatographic system

The use of an ESA detector provides considerably enhanced reliability and reproducibility of electrochemical detection. However, when very small amounts of the substance are being assayed, some precautions and a certain know-how are required.

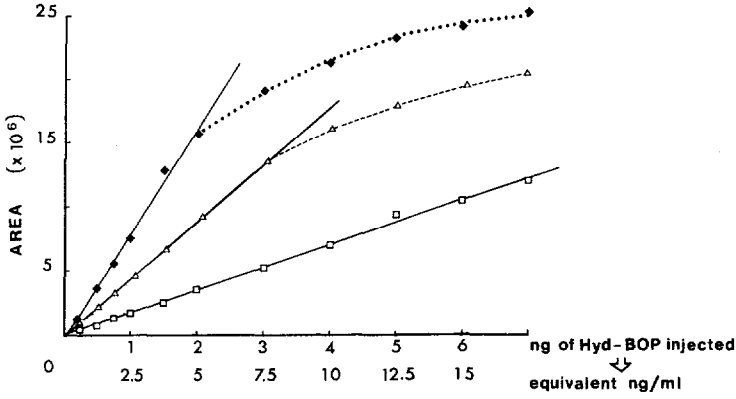


Fig. 4. Response curves obtained for Hyd-Bop (peak area) at different amplifier sensitivities set with the command module of the electrochemical detector: (□) = ×300; (△) = ×700; (♦) = ×1400.

As soon as detector 2 is noisy, or the background is greater than 0.15 μA, or parasite peaks appear, the system must be thoroughly washed to a strict procedure over a period of three days. This procedure was generally performed every two months for a daily use (five days per week) of the system (ca. 1500 injections in 2 months).

When in normal use, the mobile phase is directly recycled as it emerges from the analytical cell into the solvent container, and the system operates with a closed circuit. When the system is not in operation (night-time and weekends, etc.) it is important to leave the pumping system switched on although the flow can be turned down to ca. 0.1 ml/min.

Response detector

The range of linearity of the electrochemical detector depends strongly on the amplifier sensitivity chosen (Fig. 4). The sensitivity setting of the analytical ESA cell was carefully optimized in such a way that the upper limit of linearity corresponded to ca. 8 ng/ml Hyd-Bop. If the sample concentrations exceeded this limit a reduced sample volume was processed or fewer than 100 μl were injected from the final extract.

Calibration curve

The calibration curves in plasma were evaluated with replicate ($n=5$) spiked standard samples over the Hyd-Bop concentration range from 50 pg/ml to 8 ng/ml. A typical least-squares linear regression of the area ratio (y) versus concentration (x) relationship gave $y=270\cdot10^{-3}x+15\cdot10^{-3}$, with a correlation coefficient of 0.996.

The analytical system, even using different columns, showed remarkable stability: the slopes of several calibration lines within 6% (coefficient of variation, C.V.) over one year.

Reproducibility and accuracy

The reproducibility and the accuracy were evaluated from spiked control plasma samples (Table II). The within-day C.V. ranged from 0.8 to 6.7% with an accu-

TABLE II

REPRODUCIBILITY AND ACCURACY OF THE ASSAY OF HYDROLYSED BOPINDOLOL IN PLASMA

From spiked control samples analysed in parallel with unknowns.

Expected value (ng/ml)	*n*	Mean assay value (ng/ml)	C.V. (%)	Accuracy (%)
Within-day (one analyst, one HPLC system)				
0.065	4	0.066	2.2	+1.5
0.130	4	0.129	6.2	−0.8
0.650	4	0.618	1.5	−4.8
1.300	4	1.299	4.2	0.0
2.600	4	2.634	0.8	+1.4
5.200	4	5.142	6.7	−1.0
Day-to-day (four analysts, three HPLC systems over a period of 70 days)				
0.054	24	0.052	82.8	−3.7
0.108	11	0.096	27.7	−11.1
0.350	25	0.358	22.5	+2.3
1.500	9	1.596	12.6	+6.4
3.500	26	3.253	10.6	−7.1
5.000	9	4.918	10.1	−1.6

racy from −4.8 to 1.5% (deviation of the mean concentration from the expected value). The day-to-day variation was assessed with control samples, which were analysed in parallel with unknowns, over a period of 70 days by four different analysts working on three different HPLC systems. The three HPLC systems were all equipped with an ESA electrochemical detector. The other parts were different and respectively based on a Model 1090 Hewlett-Packard system, a Model 320 Gilson system (Gilson, Villiers le Bel, France) and a Model 390 Chromatem system (Touzart et Matignon, Vitry, France). All the data analyses were performed by a Hewlett-Packard 3357 LAS system.

Under these conditions, the day-to-day C.V. for reproducibility ranged from 10 to 12% for concentrations above 1 ng/ml and to 28% for a concentration as low as 100 pg/ml (Table II). The data also revealed satisfactory accuracy of the assay over the whole concentration range.

Dilution of samples

In view of the non-linear nature of the detector beyond a value that depends on the amplifier sensitivity chosen, experiments were performed in order to validate the assay using a plasma aliquot of less than 1 ml. Tests had demonstrated that it is possible to dilute 0.1–0.5 ml of plasma with sufficient water or plasma blank to make it up to 1 ml before transferance to the Extrelut column. The rest of the extraction process follows the usual procedure.

Dectection limit

Under favourable chromatographic conditions, the concentration of Hyd-Bop in plasma that gave a signal-to-noise ratio of ca. 10 was 25 pg/ml. Under routine conditions, the real limit of determination was mainly influenced by the quality of the samples analysed and was normally ca. 50 pg/ml. Typically, the variability at this level was higher than at higher concentrations, but accuracy was still assured for mean values (Table II). Therefore, samples from kinetic studies with low concentrations from the terminal elimination phase were analysed in replicate.

Selectivity of the assay

Under these experimental conditions, it was not possible to assay bopindolol, because it was completely hydrolysed during the various phases of extraction. However, metabolic studies in humans, following the administration of radioactive bopindolol via both oral and parenteral routes revealed no trace of unchanged drug in the plasma [8]. Hyd-Bop is the main plasma metabolite, and its concentration parallels the activity [4,6].

The high selectivity of the assay was also demonstrated by the interference-free chromatograms usually obtained within the elution window of I.S. and Hyd-Bop for drug-free plasma. The mean false positive value obtained over a period of 3 months without reanalysis from drug-free control plasma from 51 healthy volunteers was 30 pg/ml, with 78% of the values equal to zero.

Interference from other drugs in the assay was not specifically checked, mainly because pharmacokinetic studies were performed on healthy volunteers free of other medication. However, it has been shown that such different molecules as digoxin, phenprocoumon, chlorthalidone and their respective metabolites do not interfere with the assay.

Application of the method

This method has been in use for more than one year for the analysis of ca. 3000 plasma samples from human pharmacokinetic studies. The good level of reproducibility of the assay method was thus demonstrated.

Fig. 5 shows the kinetic profiles obtained for Hyd-Bop after oral administration of 4 mg of bopindolol to four healthy volunteers. For each subject, the two curves show the results of two determinations of Hyd-Bop concentration carried out using the same plasma samples, but performed at different times. The results demonstrate the excellent reproducibility of the assay and the entirely satisfactory storage of the samples for up to eleven months between the two determinations.

As can be seen from the mean curve for fifteen subjects (log–normal plot) shown in Fig. 6, this method also provided close monitoring of the plasma kinetics of Hyd-Bop up to 48 h after the administration of a single dose of 2 mg of bopindolol. Owing to the higher sensitivity an average elimination terminal half-life of ca. 10 h was thus clearly demonstrated for Hyd-Bop, which appears to be approximately twice as long as that previously reported [4,8]. This confirms, more accurately, the long β-phase of elimination previously suggested [6].

Preliminary experience with the application of this assay to urine samples in-

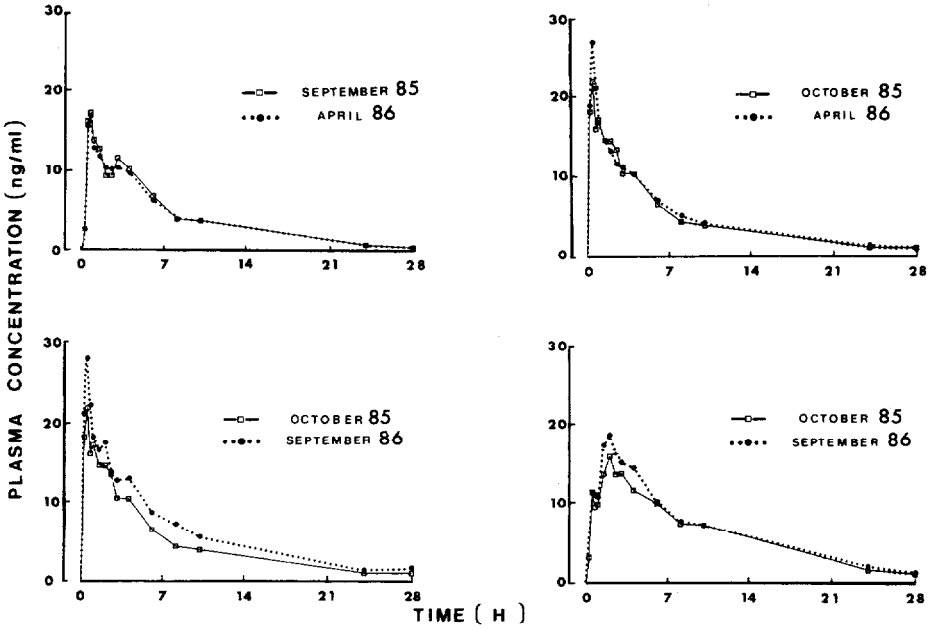


Fig. 5. Example of plasma concentration–time curves observed for four different subjects after a single oral administration of 4 mg of bopindolol. The continuous line represents the first assay of Hyd-Bop and the dotted line the second assay performed on the same plasma samples.

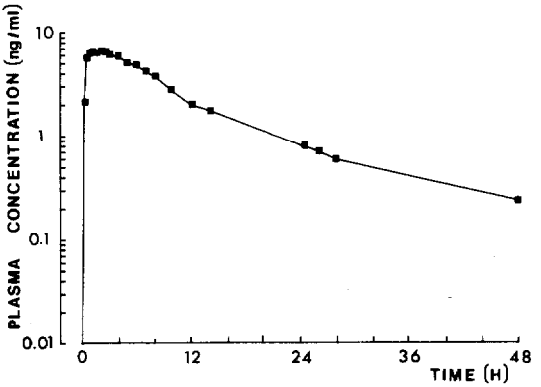


Fig. 6. Mean ($n = 15$) plasma concentrations of Hyd-Bop (log-normal plot) after a single oral administration of 2 mg of bopindolol to healthy volunteers.

dicated that the same method could theoretically be used. However, attempts to include urine samples in pharmacokinetic investigations are hampered by the instability of Hyd-Bop in urine, even when frozen. But the method may be reasonably applied to compliance purposes, especially when the urine samples are stored for short periods only.

CONCLUSION

Compared with the previous HPLC method developed for the assay of Hyd-Bop, which involved fluorimetric detection [3] or electrochemical detection [4,6],

the sensitivity of this new HPLC method is increased by at least a factor 20. The sensitivity is equivalent to that of the RRA [4–6], a method that is known to be highly sensitive. So, this HPLC method offers a real alternative to the RRA when the use of ^{125}I radiolabelled substances is not possible. It could also replace the RRA when the assay must be specific for Hyd-Bop, even if, up to now, good correlation was observed between HPLC and RRA [4,6].

ACKNOWLEDGEMENT

The authors thank Mrs. S. Jilet for secretarial assistance.

REFERENCES

1 R. Berthold, R. Waite and H. Weber, Br. J. Pharmacol., 74 (1981) 829P.
2 W.H. Aellig, Br. J. Clin. Pharmacol., 19 (1985) 775.
3 C.J. Oddie, G.P. Jackman and A. Bobik, J. Chromatogr., 273 (1983) 469.
4 R. Platzer, R.L. Galeazzi, W. Niederberger and J. Rosenthaler, Clin. Pharmacol. Ther., 36 (1984) 5.
5 P. Dayer, T. Leemann, A. Marmy and J. Rosenthaler, Eur. J. Clin. Pharmacol., 28 (1985) 149.
6 W.H. Aellig, E. Nüesch, G. Engel, J. Grevel, W. Niederberger and J. Rosenthaler, Br. J. Clin. Pharmacol., 21 (1986) 45.
7 H. Humbert, J. Denouel, J.P. Chervet, D. Lavene and J.R. Kiechel, J. Chromatogr., 417 (1987) 319.
8 J.P. Baldeck and F. Kalberer, Sandoz, Basle, September 1983, personal communication.