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Note

Analysis of bopindolol and its active metabolite 18-502 in human plasma by high-performance liquid chromatography

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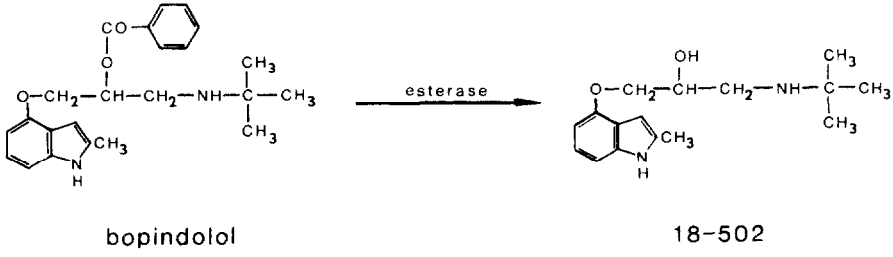
Bopindolol [4-(2-benzoyloxy-3-*tert.*-butylaminopropoxy)-2-methylindole-hydrogenmalonate, LT 31-200] (Fig. 1) is a new, potent and long-acting β-adrenoceptor antagonist [1]. Preliminary clinical trials have shown that the antihypertensive activity of the drug is similar to other currently used β-blocking drugs. This activity, however, is of greater magnitude in regard to blood pressure reduction and duration of action per mg dose than that seen with other β-blocking agents [2].



bopindolol 18-502

Fig. 1. Formulae of bopindolol and its metabolite 18-502.

In vitro pharmacological experiments indicate that bopindolol itself has no β-adrenoceptor blocking activity and that hydrolysis by endogenous esterases to an active metabolite [4-(2-hydroxy-3-*tert.*-butylaminopropoxy)-2-methylindole, 18-502] (Fig. 1) is necessary for activity.

The aim of the present study was to develop an assay to measure the concentration of the active metabolite 18-502 and the parent compound bopindolol in human plasma. The assay is based on previously developed techniques from this laboratory for measuring indoles utilising high-performance liquid chromatography (HPLC) combined with fluorescence detection [3, 4].

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EXPERIMENTAL

Reagents

Bopindolol (LT 31-200, MW 484) and 18-502 (MW 276) were obtained from Sandoz Australia (North Ryde, Australia). Stock solutions (1 mg/ml) were prepared in 0.1 *M* phosphoric acid adjusted to pH 3.0 with sodium hydroxide, and stored at 4°C. Dilutions of the stock solutions in 0.01 *M* phosphate solution pH 3.0 were freshly prepared for each assay and stored on ice. Diethyl ether (analytical grade, Ajax Chemicals, Sydney, Australia) was washed with 1 *M* sodium hydroxide, 1 *M* hydrochloric acid and distilled water before use. Methanol (205 nm liquid chromatography grade) was obtained from Waters Assoc. (Milford, MA, U.S.A.). Water for HPLC was redistilled from alkaline potassium permanganate. Esterase (100 U/mg, Boehringer, Mannheim, G.F.R.) was diluted prior to use to a concentration of 50 U/ml in 0.1 *M* boric acid adjusted to pH 8.0 with sodium hydroxide. All other reagents were of analytical grade.

Chromatographic system

A 5000 series liquid chromatograph fitted with a universal loop injector (Varian Assoc., Palo Alto, CA, U.S.A.) was used with a Zorbax 15 cm × 4.6 mm I.D. column packed with 5 μm cyano-bonded reversed-phase material (Du Pont, Wilmington, DE, U.S.A.). The mobile phase was 0.01 *M* perchloric acid--methanol (11:9) at a flow-rate of 1 ml/min. Detection was by means of a Schoeffel FS-970 fluorimeter (Schoeffel, Westwood, NJ, U.S.A.) fitted with a deuterium arc source. The metabolite 18-502 was detected by excitation at 220 nm and its fluorescence emission selected by a Corning 7-60 glass filter with bandpass 320—400 nm. Retention time of 18-502 was 5.5 min.

Plasma samples

Drug-free venous blood was obtained from healthy human subjects. Blood from patients in a clinical study investigating the pharmacokinetics and therapeutic efficacy of bopindolol was obtained following an oral dose of 1 mg or 8 mg bopindolol. Blood was collected into heparinised tubes on ice and, without delay, centrifuged 10 min at 1000 *g* and 4°C. Plasma was immediately separated and frozen, and stored at −20°C until assayed for 18-502 and bopindolol.

Extraction and HPLC estimation of 18-502

Plasma (1 ml or 2 ml) was placed into 25-ml stoppered glass tubes on ice and 100 μl 2 *M* sodium carbonate per ml plasma and 10 ml diethyl ether added. Extraction was by 2 min mixing using a vortex mixer. The phases were separated by centrifugation for 3 min at 1000 *g* and 4°C. The aqueous phase was then frozen by immersion of the tubes into a dry ice—ethanol mixture and the ethereal extract transferred to 15-ml stoppered glass tubes on ice and containing 200 μl 0.1 *M* phosphate pH 3.0. 18-502 was extracted into the aqueous phase by 2 min mixing using a vortex mixer and the phases separated by centrifugation and freezing as described above. The ethereal phase was aspirated and discarded. Samples and standards were maintained on ice prior to

chromatography. Accurate aliquots (100 μl) of the acidic phase were injected directly on to the HPLC column. A calibration curve was prepared by the simultaneous assay of known amounts of 18-502 in the range 1.25—200 ng/ml added to drug-free or patient control plasma.

Enzyme hydrolysis of bopindolol

The levels of bopindolol in plasma were determined by enzymatic conversion to 18-502 and subsequent analysis of 18-502 according to the method described above. The amount of bopindolol in the plasma could then be calculated from the difference between 18-502 levels in the enzyme hydrolysed and non-hydrolysed samples.

Plasma (1 ml) was placed into 25-ml stoppered glass tubes on ice, 50 μl (2.5 U) esterase in borate pH 8.0 added and hydrolysis allowed to proceed by incubation for 30 min at 37°C. Reaction was stopped by removal of the tubes on to ice and addition of 10 ml diethyl ether. Sodium carbonate (2 *M*, 100 μl) was then added and extraction of plasma and all subsequent steps of the 18-502 method carried out. Calibration curves of bopindolol and 18-502 were prepared by subjecting known amounts of the respective compounds to the same procedures of enzyme hydrolysis and 18-502 analysis. The bopindolol calibration curve was in the range 12.5—400 ng/ml.

Plasma concentration of 18-502 and bopindolol in human subjects

Plasma concentrations of the active metabolite and parent compound were measured in five informed volunteer patients (three male, two female) with essential hypertension, who were part of a study investigating the therapeutic efficacy and pharmacokinetics of bopindolol. The patients were on no other medication during the study. Bopindolol was administered as oral doses of 1 mg and 8 mg and plasma concentrations were monitored over 8—10 h.

RESULTS AND DISCUSSION

The overall recovery of 18-502 through the assay procedure was 78 ± 7% (mean ± S.D., n = 18). Precautions to keep plasma samples cold during collection and analysis were necessary to minimise losses due to endogenous esterase activity and an instability of 18-502 at room temperature. 18-502 was stable when stored in plasma at −20°C for periods checked up to one month. No hydrolysis of bopindolol to 18-502 when similarly stored was seen; however, at 37°C for 60 min a 5% conversion of bopindolol to 18-502 in plasma occurred, presumably due to the presence of endogenous esterases. 18-502 shows a 20—30% loss in detectable fluorescence when allowed to stand at room temperature in 0.1 *M* phosphoric acid pH 3.0 over periods of up to 6 h. Maintaining samples on ice during analysis reduced this decay to 4%.

18-502 isolated from plasma chromatographed with a retention time of 5.5 min and was resolved from other peaks present in plasma (Fig. 2). The mean assay blank for 18-502 in drug-free and patient control plasma was 0.019 ± 0.094 ng/ml, n = 24.

The calibration curve for the assay related fluorescence (y) expressed as nA of detector current to plasma concentration of 18-502 (x) in ng/ml according

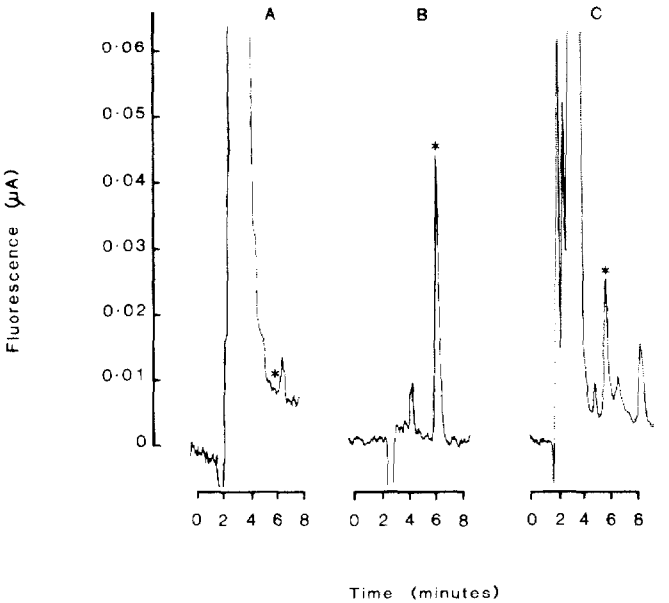

Fig. 2. Chromatographic traces of 18-502: (A) extract of control drug-free plasma shown at 2 × scale; (B) 5 ng 18-502 standard (*); (C) plasma extract from patient No. 3, 2 h following 1 mg bopindolol administered orally, equivalent to 2.6 ng/ml.

to the equation $y = 8.55x + 0.99$ ($r^2 = 0.996$). The calibration curve was linear over the range 0—200 ng/ml.

The precision and accuracy of the method were determined by replicate analysis of known amounts of 18-502 added to 1 ml drug-free plasma. The results are shown in Table I. The regression line for 18-502 added versus 18-502 obtained, corrected for the mean recovery (79%), has the formula $y = 1.057x - 0.509$ with a correlation coefficient of 0.999.

From these data, from the assay blank and from the chromatography of 18-502, it can be deduced that the sensitivity of the assay is approximately 0.5 ng/ml.

TABLE I

PRECISION FOR THE 18-502 ASSAY

The values for 18-502 obtained have not been corrected for recovery. In all cases $n = 6$.

18-502 added (ng/ml)	18-502 obtained (ng/ml)		
	Mean ± S.D.	C.V. (%)	Mean recovery (%)
1.25	0.97 ± 0.10	10.2	78
2.5	1.93 ± 0.12	6.3	77
5	3.78 ± 0.12	3.1	76
10	8.00 ± 0.39	4.8	80
20	15.4 ± 0.51	3.3	77
40	33.4 ± 0.86	2.6	84
80	66.7 ± 2.50	3.7	83

It was not possible to measure bopindolol using a direct procedure similar to that for 18-502. Analysis of the excitation and emission spectra of bopindolol showed that it was weakly fluorescent and would therefore not be detectable with adequate sensitivity. By converting bopindolol to 18-502 using enzyme hydrolysis, the necessary sensitivity could be obtained.

Following enzyme hydrolysis, 93% of standard bopindolol could be recovered as 18-502. Increasing enzyme concentration and incubation time did not increase this conversion showing that optimum conditions were being employed and suggesting therefore, that the 7% apparent loss in conversion is probably due to incomplete hydrolysis. The calibration curve of bopindolol enzymatically hydrolysed to 18-502 and calculated as 18-502 equivalents was $y = 7.19x - 22.2$, $r^2 = 0.996$. This was similar to the calibration curve for 18-502 obtained in the same experiments, $y = 7.51x - 7.44$, $r^2 = 0.998$. Under the conditions used for enzyme hydrolysis, there was no significant effect on concentrations of 18-502 present in standards or samples or on recovery (79 ± 8%, $n = 6$) or assay blank (0.008 ± 0.027 ng/ml, $n = 13$). The limit of sensitivity for bopindolol can be calculated to be 1 ng/ml.

Internal standards are not necessary in the 18-502 assay or for the enzyme hydrolysis step because of the good reproducibility and recovery of the procedures.

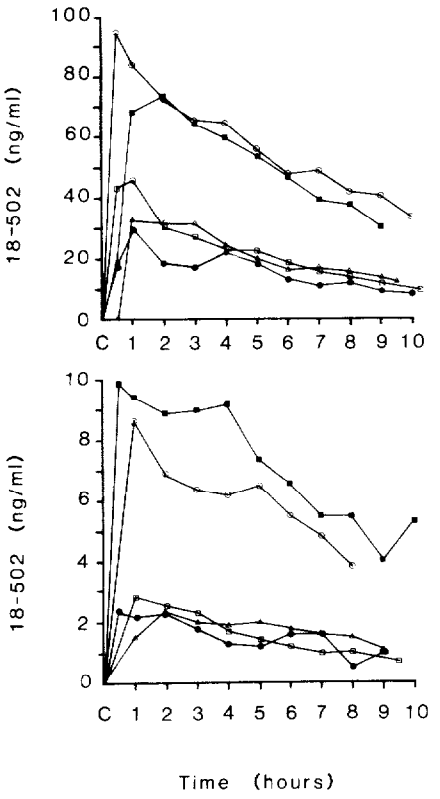

Fig. 3. Plasma levels of 18-502 in five patients with essential hypertension given 1 mg orally (lower panel) and 8 mg orally (upper panel) bopindolol, plotted against time.

Interference from other drugs in the assay was not specifically checked, firstly because the patients studied were on no other medication and secondly because previous experience with similar extraction and HPLC procedures has shown that of the many drugs commonly used in treating hypertension and related cardiovascular disorders, only quinidine and prazosin could be detected [4].

The plasma concentrations of bopindolol and 18-502 were measured in five patients with essential hypertension who were given 1-mg and 8-mg oral doses of bopindolol (Fig. 3). Maximum 18-502 concentrations occurred at 0.5—2 h following administration then declined over the ensuing 8—9 h.

At both doses, 18-502 concentrations in the five patients varied over a three-fold range. Whether this variation represents differences in the bioavailability of 18-502 or in its apparent volume of distribution is not known. At both doses, however, the same two patients (female) had consistently higher plasma concentrations than the three male patients.

No significant difference in 18-502 concentration in enzyme hydrolysed samples compared to non-enzyme hydrolysed samples in the five patients was found, suggesting that bopindolol is rapidly converted to its metabolite following oral administration.

In summary, the HPLC method using fluorescence detection developed for the measurement of 18-502 in plasma and for bopindolol following enzyme hydrolysis to 18-502 shows adequate sensitivity, specificity, precision and accuracy. It has the advantage of being a relatively simple and quick assay. It has been successfully applied to the measurement of 18-502 concentrations in patients with essential hypertension. 18-502 was readily detected following oral administration of therapeutic doses of bopindolol. Bopindolol, however, was not detected suggesting that the pro-drug is rapidly hydrolysed to its active metabolite in vivo.

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