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Note

Improved high-performance liquid chromatographic method for the simultaneous determination of propranolol and 4-hydroxypropranolol in plasma with fluorescence detection

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4-Hydroxypropranolol (4-HOP) has received increased attention recently because of its pharmacokinetic and pharmacodynamic significance [1–5]. We propose an improved method for the simultaneous measurement of 4-HOP and propranolol in plasma by high-performance liquid chromatography (HPLC) with fluorescence detection, which has several advantages over previously published methods [6–12] with respect to stability of 4-HOP, sensitivity and simplicity of use. This makes it a useful method for application in clinical and analytical laboratories.

EXPERIMENTAL

Reagents and apparatus

Propranolol hydrochloride was obtained from Ayerst Labs. (New York, NY, U.S.A.), 4-HOP hydrochloride and 4-methylpropranolol (4-MeP) hydrochloride were supplied by Imperial Chemical Industries (Macclesfield, U.K.). UV-grade methanol, acetonitrile and ethyl acetate were purchased from Bodman Chemicals (Doravill, GA, U.S.A.) Glacial acetic acid, L-ascorbic acid, sodium bicarbonate, sodium carbonate, triethylamine and sodium acetate were purchased from Fisher Scientific (Memphis, TN, U.S.A.). All other reagents and chemicals were of analytical grade.

The HPLC instrumentations are described elsewhere [13]. The fluorescence detector was set on an excitation wavelength of 238 nm, emission cut-off filter of 360 nm and a detection sensitivity of 0.2 μÅ.

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Standards

Stock solutions (100 μg/ml) of propranolol, 4-HOP and 4-MeP were prepared in methanol and were stored at −20°C.

On the day of analysis aliquots of 4-HOP and propranolol stock solutions were mixed and diluted to 100 ml with methanol to give a final concentration of 1 μg/ml each. Aliquots (5, 10, 20 and 40 μl) were used to spike 1-ml drug-free plasma samples containing 0.1 ml of 200 mg/ml ascorbic acid solution and 30 μl of 250 ng/ml 4-MeP. This gave a calibration curve ranging between 5 and 40 ng/ml for both 4-HOP and propranolol.

Sample preparation, chromatography, quantitation and detection limits

Aliquots of plasma (1 ml) were pipetted into 20-ml Teflon-lined screw-capped glass round-bottomed centrifuge tubes which contained 0.1 ml of ascorbic acid solution. After the addition of the internal standard solution, 1 ml of carbonate buffer (1 *M*, pH 10.3) and 8 ml of ethyl acetate were added. All tubes, including the standards, were shaken vigorously for 3 min. Following centrifugation for 5 min, 7 ml of the ethyl acetate layer were transferred to a 7-ml vial and evaporated to dryness under a gentle stream of nitrogen in a 50°C waterbath. The residue was then reconstituted in 0.2 ml methanol, vortexed, and 50 μl were injected onto the HPLC system.

Chromatography was carried out using acetonitrile–water–acetic acid–methanol–triethylamine (350:700:10:400:0.05, v/v) (pH 3.4) as eluent at a flow-rate of 2 ml/min.

The peak-height ratio of 4-HOP and propranolol to the internal standard 4-MeP, obtained from the calibration curve, was used to calculate the concentration of 4-HOP and propranolol in the samples, expressed as ng/ml of plasma.

The limits of quantitation are approximately 1 and 2 ng/ml of plasma for 4-HOP and propranolol, respectively.

Stability of 4-HOP and propranolol

In methanol. To assess the stability of the 4-HOP and propranolol stock standard solutions, fresh working standard solutions of 4-HOP and propranolol were prepared from these stock solutions in methanol, and injected directly onto the HPLC system. This procedure was repeated periodically and the results were compared with freshly prepared 4-HOP and propranolol stock solutions prepared at the time of the run. Between injections the stock solutions in methanol were stored at −20°C.

In frozen plasma (−20°C) using different antioxidants. Experiments were performed to assess the effect of various antioxidants on the stability of 4-HOP and propranolol in plasma. A 73-ml volume of freshly collected drug-free plasma was spiked with 2.4 μg of freshly prepared 4-HOP and 2.65 μg of propranolol. Sets of 15- and 20-ml screw-capped centrifuge tubes, each containing 1 ml of the spiked plasma (32.8 ng 4-HOP and 36.2 ng propranolol) were treated by the addition of one of the following antioxidant agents: no antioxidant agent; 0.1 ml of 20% sodium metabisulfite; 0.1 ml of 20% ascorbic acid, 0.1 ml of 20% sodium bisulfite; 0.1 ml of 20% vitamin E acetate. All tubes were stored at −20°C and assayed in

TABLE I

STABILITY OF 4-HYDROXYPROPRANOLOL (4-HOP) AND PROPRANOLOL (P) IN PLASMA WHEN STORED AT −20°C IN VARIOUS ANTIOXIDANTS

Spiked plasma samples containing 4-HOP (32.8 ng/ml) and propranolol (36.2 ng/ml) were stored at −20°C and measured periodically. Results are expressed as the amount of drug recovered from the plasma sample relative to that measured in fresh spiked plasma extracts. Values represent the mean ± S.D. for three determinations.

Antioxidant agent	Amount recovered (ng/ml)										pH (final extraction)
	0 day		1 week		2 weeks		3 weeks		4 weeks		
	4-HOP	P	4-HOP	P	4-HOP	P	4-HOP	P	4-HOP	P	
No antioxidant	26.6 ± 1.3	35.4 ± 1.0	25.2 ± 0.8	36.0 ± 0.5	24.7 ± 0.5	34.8 ± 1.4	26.2 ± 0.5	35.5 ± 1.0	22.3 ± 1.1	35.0 ± 1.6	9.95
Sodium metabisulfite	8.6 ± 0.5	34.8 ± 1.7	8.4 ± 0.7	34.9 ± 1.0	9.0 ± 0.4	35.9 ± 1.8	10.6 ± 0.5	36.4 ± 1.3	8.8 ± 0.6	35.4 ± 1.1	9.8
Sodium bisulfite	11.7 ± 0.8	37.3 ± 0.2	10.0 ± 0.8	36.8 ± 0.2	9.0 ± 0.1	35.8 ± 0.3	11.8 ± 0.6	37.2 ± 0.8	8.5 ± 0.5	33.8 ± 1.6	9.8
Ascorbic acid	31.9 ± 1.2	36.4 ± 1.4	29.9 ± 0.3	35.9 ± 0.4	32.2 ± 0.5	36.8 ± 0.3	33.9 ± 1.6	36.2 ± 1.7	31.8 ± 0.7	34.5 ± 0.7	9.8
Vitamin E acetate	27.7 ± 0.2	33.5 ± 0.6	25.2 ± 1.3	33.8 ± 0.3	25.0 ± 1.2	33.6 ± 0.7	27.5 ± 1.2	34.2 ± 0.2	27.4 ± 0.4	33.7 ± 0.4	9.95

TABLE II

STABILITY OF 4-HYDROXYPROPRANOLOL (4-HOP) AND PROPRANOLOL (P) IN PLASMA FROM A HUMAN VOLUNTEER WHEN STORED AT −20°C IN THE PRESENCE AND ABSENCE OF ASCORBIC ACID

Plasma from a human volunteer was collected 90 min after a single oral dose of propranolol (160 mg), stored at −20°C and assayed for 4-HOP and propranolol periodically. Results are expressed as the amount of drug recovered from the plasma sample relative to that measured in fresh spiked plasma extracts. Values represent the mean ± S.D. for four determinations.

Antioxidant agent	Amount recovered (ng/ml)											
	0 day		1 week		2 weeks		3 weeks		4 weeks		Mean ± S.D.	
	4-HOP	P	4-HOP	P	4-HOP	P	4-HOP	P	4-HOP	P	4-HOP	P
No antioxidant	24.8 ± 0.5	66.4 ± 1.4	27.1 ± 0.7	66.7 ± 1.3	27.1 ± 0.7	66.2 ± 1.5	27.9 ± 0.5	66.7 ± 1.6	25.6 ± 0.2	69.9 ± 0.9	26.5 ± 1.3	67.2 ± 1.5
Ascorbic acid	29.7 ± 0.3	66.2 ± 0.6	34.4 ± 1.0	69.2 ± 1.7	32.7 ± 0.3	66.7 ± 0.5	31.9 ± 0.2	65.6 ± 0.8	29.5 ± 0.4	69.4 ± 1.0	31.6 ± 2.0	67.4 ± 1.8

triplicate weekly for four weeks, and the results were compared with those of freshly extracted spiked plasma samples.

In frozen plasma (−20°C) from a human volunteer. The stability of 4-HOP and propranolol was also assessed periodically in plasma from a normal human volunteer. In this study aliquots of plasma (1 ml) obtained 90 min after a single oral dose of propranolol (160 mg) were pipetted into 20-ml Teflon-lined screw-capped glass round-bottomed centrifuge tubes which contained no antioxidant agents or 0.1 ml of 20% ascorbic acid solution. All tubes were stored at −20°C and assayed in quadruplicate periodically and compared with freshly extracted spiked plasma samples.

Reconstituting in methanol. The stability of propranolol and 4-HOP, after the ethyl acetate residue was reconstituted in methanol, was assessed. The treatment involved reconstituting the residue in 0.2 ml methanol, injecting a 50-μl aliquot of this solution, storing the remaining solution at −20°C overnight, and re-injecting a 50-μl aliquot. The values obtained were compared to those of freshly extracted samples.

RESULTS AND DISCUSSION

The stability of 4-HOP poses great concern since its quantitative recovery from plasma is critically important. Previous attempts to quantitate this compound in plasma were hindered by its instability. Walle et al. [14] stated that 4-HOP was stable for 7 h in methanol, whereas Pritchard et al. [15] reported that the drug was stable up to one week in methanol. Nation et al. [10] reported similar findings. In the present study a one-, two- and seven-month storage (−20°C) of a freshly prepared stock solution of 4-HOP (100 μg/ml) in methanol yielded 98, 94 and 86%, respectively, of the concentration when compared to a freshly prepared solution. Propranolol was stable over this period of time under the same conditions.

Previous studies showed that 4-HOP was stable in plasma if a 2% solution of the antioxidant, sodium bisulfite [14] or ascorbic acid [16] was present. We conducted a study to establish the relative stability of 4-HOP in plasma in the presence and in the absence of various antioxidants when the samples were stored at −20°C (Table I). Suprisingly, sodium metabisulfite and sodium bisulfite protected the least against 4-HOP oxidation. The concentration fell dramatically. About 70% of the 4-HOP initially present in the plasma was lost on the first day and throughout the study period. In the absence of any antioxidant agents, approximately 20% of the 4-HOP was lost on the first day and throughout the rest of the study period. Vitamin E acetate showed similar results. Ascorbic acid, however, was more effective as a 4-HOP stabilizer. More than 95% of the drug initially present in the plasma was stabilized by the presence of ascorbic acid throughout the study period.

The addition of 20 mg of ascorbic acid to 1-ml plasma samples from a human volunteer who received a dose of propranolol also protected against the loss of 4-HOP for up to four weeks when the samples were kept frozen at −20°C (Table II). The absence of the antioxidant, however, resulted in about 20% loss of 4-

HOP and remained constant throughout the study period under the same experimental conditions.

It was also observed that when the plasma samples were stored at −20°C for three or four weeks and the ascorbic acid added only at the time of analysis, only 80% of the 4-HOP initially present in the plasma was recovered. This indicates that the antioxidant must be added to the plasma prior to storage at −20°C, otherwise the plasma 4-HOP stored without the stabilizer will deteriorate with time.

These results suggest that ascorbic acid is the antioxidant of choice that must be used for protection against 4-HOP oxidation.

We also found that 4-HOP was stable following its extraction from plasma, and the extract kept overnight at −20°C in methanol (96±4% of initial concentration).

Propranolol is stable in plasma kept at −20°C for several weeks with or without the addition of any antioxidants (Tables I and II). It is also stable when extracted in methanol and the extract kept overnight at −20°C.

Various extraction solvents were evaluated using a variety of phase volume ratios and extraction times. Ethyl acetate was found most efficient giving no gel formation at the interface to interfere with phase separation. Blank human plasma extracts yielded no interfering peaks from endogenous plasma. All artifact peaks and baseline variations were complete within 3 min after the injection (Fig. 1A). Propranolol, 4-HOP and the internal standard, 4-MeP, were distinctly separated, and no interference was observed from substances extracted from plasma at the retention times of these three compounds (Fig. 1B). The concentration of these two unconjugated drugs shown in Fig. 1C is about what is observed in clinical samples in patients receiving propranolol (80–160 mg.)

Recently, we reported a sensitive analytical method for quantitating propranolol in plasma using an excitation wavelength of 228 nm and no emission cut-off filter with a detection limit of 1 ng/ml [13]. We observed, while conducting the present study, that by inserting an emission cut-off filter of 340 nm and excitation wavelength of 228 nm, we were able to improve the sensitivity of propranolol measurement by 24%. In the present study the limits of detection (twice baseline) using 1 ml of plasma were 1 ng/ml for 4-HOP and 2 ng/ml for propranolol when 50 μl of the 200 μl methanol were injected. The detector response was linear for both plasma 4-HOP and propranolol concentrations ranging from 5 to 80 ng/ml.

Analysis of six plasma samples for propranolol and 4-HOP at concentrations of 5, 10, 20 and 40 ng/ml gave a correlation coefficient of 0.99962, a slope of 0.05672 and an intercept of −0.01459 for 4-HOP. Similar concentrations for propranolol gave a correlation coefficient of 0.99966, a slope of 0.02991 and an intercept of 0.00502 (Table III). These parameters were constant over a period of four weeks. Intra-assay coefficients of variation ranged between 1 and 4% for both compounds. Inter-assay coefficients of variation were below 5% for 4-HOP and 4% propranolol over a period of four weeks.

With ethyl acetate as the extraction solvent, the extraction efficiency was 89±2% for 4-HOP and 93±2% for propranolol. This was assessed by comparing

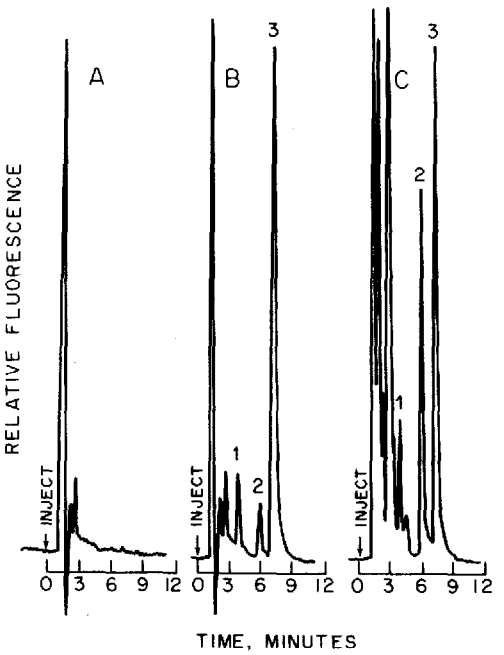

Fig. 1. (A) HPLC profile of the ethyl acetate extract of 1 ml of drug-free human plasma. (B) Chromatogram of an ethyl acetate extract of 1 ml of drug-free human plasma spiked with 8 ng of 4-HOP (1), 8 ng of propranolol (2) and 20 ng of the internal standard, 4-MeP (3). (C) Chromatogram of an ethyl acetate extract of 1 ml of a normal human plasma containing unconjugated drugs 90 min following a single oral dose of propranolol (80 mg): peak 1 = 12.1 ng; peak 2 = 56.2 ng. Detector settings: λ_{ex} = 238 nm, emission cut-off filter = 360 nm; sensitivity range = 0.5 μÅ.

peak heights of 1 ml spiked plasma extracted standards (5, 10, 20 and 40 ng/ml) with those of unextracted standards. Extraction efficiency was not improved when the plasma samples were shaken for 5 or 10 min. Equilibrium was achieved within 3 min.

Several mobile solvent mixtures were also tested during preliminary studies. The solvent mixture composed of acetonitrile–water–acetic acid–methanol–triethylamine (350:700:10:400:0.05, v/v) provided excellent separation of 4-HOP, propranolol and the internal standard. To further improve sensitivity of the method and to shorten analysis time, the method was modified to include

TABLE III

REPRODUCIBILITY OF 4-HOP AND PROPRANOLOL IN PLASMA

Spiked concentration (ng/ml)	Sample concentration (mean ± S.D., n = 6) (ng/ml)		C.V. (%)	
	4-HOP	Propranolol	4-HOP	Propranolol
5	4.8 ± 0.20	5.0 ± 0.19	4.2	3.8
10	9.9 ± 0.28	9.8 ± 0.30	2.8	3.1
20	19.9 ± 0.62	19.7 ± 0.47	3.1	2.4
40	39.7 ± 0.70	40.1 ± 0.52	1.8	1.3

triethylamine in the mobile system. This provided a total elution time per assay of less than 15 min. Addition of pronethalol (50 μl of 15 μg/ml) under these experimental conditions may be used as an internal standard instead of 4-MeP. It elutes immediately following the 4-HOP peak.

Although various methods for the simultaneous determination of 4-HOP and propranolol in plasma have been published, this method is an improvement over previous methods not only because of its selectivity and sensitivity, but also because of its simplicity. The method does not require back-extraction [10–12], large sample volume [5, 7, 8] and/or more than one detector [6, 9]. Only 1 ml of plasma is required for measuring the unconjugated compounds and as little as 0.25 ml for measuring total drugs according to the method described by Walle et al. [17] with certain modifications. The detection limit of 1 ng/ml for 4-HOP and 2 ng/ml for propranolol can be further reduced if the organic residue is reconstituted in 100 μl instead of 200 μl methanol. This method is suitable for quantitating the drugs in the unconjugated state and after enzymatic hydrolysis, and may be applied for the therapeutic monitoring of these two drugs in chronically treated patients or for use in pharmacokinetic research studies. The results shown in Table III demonstrate the reproducibility of the present method.

Finally, the addition of ascorbic acid to the plasma sample immediately following its separation and storage at −20 °C is essential for protecting against 4-HOP oxidation. Since the stability of 4-HOP is a major problem for any prolonged storage of plasma samples, it is recommended that analysis should be complete within four weeks after collection.

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REFERENCES

1 J.E. Fitzerald and S.R. O'Donnell, Br. J. Pharmacol., 43 (1971) 222.
2 D.J. Coltart and D.G. Shand, Br. Med. J., 3 (1970) 731.
3 C.R. Cleaveland and D.G. Shand, Clin. Pharmacol. Ther., 3 (1972) 181.
4 J.W. Paterson, M.E. Conolly, C.T. Dollery, A. Hayes and R.G. Cooper, Pharmacol. Clin., 2 (1970) 127.
5 T.C. Raghuran, R.P. Koshakji, G.R. Wilkinson and A.J.J. Wood, Clin. Pharmacol. Ther., 36 (1984) 51.
6 W.D. Mason, E.N. Amick and O.H. Weddle, Anal. Lett., 10 (1977) 515.
7 A.M. Taburet, A.A. Taylor, J.R. Mitchell, D.E. Rollins and J.L. Pool, Life Sci., 24 (1979) 209.
8 D.W. Schneck, J.F. Pritchard and A.H. Hayes, Jr., Res. Commun. Chem. Pathol., 24 (1979) 3.
9 G. Tamai, I. Morita, T. Masujima, H. Yoshida and H. Imai, J. Pharm. Sci., 73 (1984) 1825.
10 R.L. Nation, G.W. Peng and W.L. Chiou, J. Chromatogr., 145 (1978) 429.
11 M.T. Rosseel and M.G. Bogaert, J. Pharm. Sci., 70 (1981) 688.
12 M.-W. Lo, B. Sibler and S. Riegelman, J. Chromatogr. Sci., 20 (1982) 126.
13 R.P. Koshakji and A.J.J. Wood, J. Pharm. Sci., 75 (1986) 87.
14 T. Walle, J. Morrison, K. Walle and E. Conradi, J. Chromatogr., 114 (1975) 351.
15 H.F. Pritchard, D.W. Schneck and A.H. Hayes, Jr., J. Chromatogr., 162 (1979) 47.
16 M.-W. Lo and S. Riegelman, J. Chromatogr., 183 (1980) 213.
17 T. Walle, T.C. Fagan, E.C. Conradi, U.K. Walle and T.E. Gaffney, Clin. Pharmacol. Ther., 26 (1979) 167.