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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PINDOLOL IN HUMAN PLASMA

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SUMMARY

A sensitive, simple and highly reliable high-performance liquid chromatographic method using fluorescence detection is reported for the determination of pindolol in plasma. This method involves a single extraction of pindolol from alkalinized plasma into methyl *tert.*-butyl ether followed by a back-extraction into dilute hydrochloric acid. Injection of the dilute acid phase directly onto an octyl (LC-8) bonded-phase column provides the final separation, and detection of pindolol is achieved by monitoring the intrinsic fluorescence of pindolol at 315 nm following excitation at 255 nm. The method is sensitive enough to measure with confidence pindolol plasma concentrations of 2 ng/ml using a 2-ml sample. No internal standard is required. This method has been applied to the analysis of 1500 human plasma samples by two different laboratories.

INTRODUCTION

Pindolol (Visken®), *d,l*-4-(2-hydroxy-3-isopropylaminopropoxy)indole, is a potent β-adrenoceptor-blocking agent and possesses intrinsic sympathomimetic activity [1,2]. Due to the high bioavailability and low first-pass effect of pindolol, as compared to other β-adrenoceptor-blocking agents, a low daily dose is possible [3,4].

Several methods have been reported for the determination of pindolol in human plasma since the first procedure described by Pacha [5]. This non-chromatographic fluorimetric procedure uses an *o*-phthalaldehyde reaction with pindolol, but has limited sensitivity because of background from the biological matrix (ca. 5 ng/ml). More recently, gas–liquid chromatographic (GLC) procedures with electron-capture detection have been reported [6,7]; however, both procedures entail rather lengthy sample preparation followed by derivatization steps. A high-performance liquid chromatographic (HPLC) procedure using fluorescence detection has been reported [8] but involves an additional solvent wash of the

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final extract to remove interferences prior to chromatographing the sample. In addition, there have been reports [9,10] that the detection limit of 2 ng/ml can rarely be attained in routine use. An HPLC method using ultraviolet detection at 264 nm has been proposed [11] with a detection limit of 2 ng/ml using 2 ml of plasma. However, no detailed application of the method was reported. A more recent HPLC method with amperometric detection reports a detection limit of 0.5 ng/ml using a 1-ml plasma sample [9]. The reported problems of maintaining a clean working electrode and stabilizing the detector response before use would discourage the use of this procedure for the analysis of the large numbers of samples associated with clinical studies. Most recently, a thin-layer chromatographic procedure [12] and an HPLC method using ultraviolet detection have been reported [13]. The thin-layer method is limited in its application and while the HPLC method does provide adequate sensitivity (1–2 ng/ml) for monitoring pindolol plasma levels, there appears to be a potential interference problem from either the large solvent front or from some commonly prescribed drugs.

The HPLC procedure with fluorescence detection reported in this present paper is as uncomplicated as any reported procedure while providing sufficient sensitivity for the monitoring of blood levels following single oral therapeutic doses of pindolol. Most importantly, this current procedure has been used routinely for the analysis of pindolol in human plasma samples, demonstrating its reliability and reproducibility.

EXPERIMENTAL

Apparatus

Analyses were performed on an HPLC system consisting of an Altex Model 110A pump (Altex Scientific, Berkeley, CA, U.S.A.), fixed-loop pneumatically activated injection valve (Model AH-CV6-UHPa-N60 with DVI, Valco Instruments, Houston, TX, U.S.A.), Perkin-Elmer column oven (Model LC-100, Perkin-Elmer, Norwalk, CT, U.S.A.) and fluorescence detector for liquid chromatography (Model 650-10LC, xenon lamp source). Peak-height measurements, baseline integrations and calculations were performed by a computer system (HP-1000; Hewlett-Packard, Paramus, NJ, U.S.A.) equipped with a computer automated laboratory system (CALS) software package (Computer Inquiry Systems, Waldwick, NJ, U.S.A.).

Reagents and solvents

Chemicals used were pindolol (Sandoz Pharmaceuticals, East Hanover, NJ, U.S.A.), methyl *tert.*-butyl ether, isopropanol, acetonitrile and phosphoric acid (85%) (all of UV, HPLC grade), sodium hydroxide (50%, w/w) and hydrochloric acid (conc., 38%) (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and glass-distilled water. Human plasma (Sera-Tec Biologicals, North Brunswick, NJ, U.S.A.) was used in the preparation of standards.

Chromatographic conditions

Reversed-phase separations were accomplished at 60°C on a 250×4.6 mm Supelcosil LC-8 (octyl) 5-μm column (Supelco, Bellefonte, PA, U.S.A.) using a

mobile phase consisting of isopropanol–acetonitrile–0.3% phosphoric acid (1:10:9, v/v/v) at a flow-rate of 2.0 ml/min. The mobile phase was filtered/degassed using a vacuum filter system (Millipore, Bedford, MA, U.S.A.) equipped with a 0.45-μm filter (Nylon 66, Rainin Instrument, Woburn, MA, U.S.A.).

Instrument settings

The fluorescence detector excitation wavelength was set at 255 nm (20 nm bandpass) and the intrinsic fluorescence was monitored at 315 nm (15 nm bandpass). The detector sensitivity was set using a range of 3.

Standard solutions

A stock solution of pindolol was prepared by dissolving 12.5 mg of pindolol in 25 ml of 0.1 *M* hydrochloric acid. Further dilutions of this stock solution were made with glass-distilled water prior to preparing plasma standards containing 5–150 ng/ml pindolol.

Sample preparation

Into glass-stoppered 40-ml centrifuge tubes pipet 2.0 ml of each blank, standard or subject plasma sample. Add, by use of a Repipet® (Labindustries, Berkeley, CA, U.S.A.), 0.5 ml of 2 *M* sodium hydroxide and mix briefly (5 s) using a Maxi-Mix® (Thermolyne, Dubuque, IA, U.S.A.). Using a Repipet, add 6.0 ml of methyl *tert.*-butyl ether, stopper and shake horizontally on an oscillating shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 15 min. Centrifuge at 750–1000 *g* for 5 min and transfer 5.0 ml of the upper organic phase into a second 40-ml glass-stoppered centrifuge tube. To this tube, add 1.0 ml of 0.1 *M* hydrochloric acid, stopper and shake horizontally for 15 min. Centrifuge (750 *g*) for 2 min to separate the phases and aspirate the upper organic phase to waste. Inject a 250-μl aliquot into the chromatograph.

RESULTS

No interfering peaks have been detected in the plasma used for blank standards or from subjects who have been orally dosed with pindolol. Fig. 1 shows a chromatogram of a plasma blank, 10 and 50 ng/ml standards.

Linearity

Daily standardization curves ($n=5$) for pindolol in plasma resulted in a linear concentration–response relationship. Pindolol concentrations of 0, 5, 10, 25, 50, 75, 100 and 150 ng/ml in plasma were used for standardization and regression analysis of the data resulted in mean slope and *y*-intercept values of 5.19 mV/ng/ml and -1.28 mV, respectively. The mean correlation coefficient for these five standardization curves was 0.9982.

Accuracy, precision and reproducibility

The accuracy of the method was evaluated by analyzing plasma samples containing known amounts of pindolol. Using the *t*-value from a one-tailed Student's

TABLE I

VALIDATION OF THE METHOD APPLIED TO PLASMA USING SPIKED SAMPLES

Spiked concentration (ng/ml)	Obtained concentration (ng/ml)										Absolute difference from true value (mean ± S.D.) (ng/ml)	95% Confidence limit of true value (%)
	Day 1 ($n=4$)		Day 2 ($n=4$)		Day 3 ($n=4$)		Day 4 ($n=4$)		Days 1-4 ($n=16$)			
	Mean	C.V (%)	Mean	C.V (%)	Mean	C.V.(%)	Mean	C.V.(%)	Mean	C.V.(%)		
8	7.1	15.5	6.8	11.8	8.3	6 0	7.9	15.2	7 5	13.3	0.9±0.6	±25.0
15	13	7.7	—*		15	6.7	14	7.1	14	7.1**	1.0±1.0	±18.7
25	23	13 0	23	6.1	26	3.8	28	3.6	25	12.0	2.3±1.4	±19.2
50	58	3.4	50	6.0	47	4 3	54	2.3	52	9.6	4.3±3.0	±19.2
75	76	10.5	74	9.5	70	4.3	78	3.8	75	8.1	4.9±3.5	±14.7
100	108	6 5	94	5.4	94	1.1	104	4.8	99	8.5	7.2±3.9	±14 0

*Sample not analyzed on this day.

**$n=12$

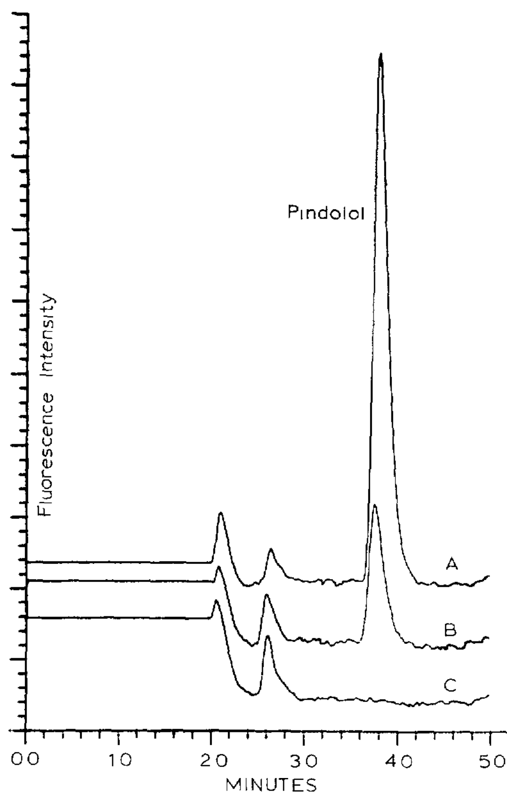

Fig. 1. Representative chromatograms obtained in the analysis of extracted plasma samples containing (A) 50 ng/ml pindolol and (B) 10 ng/ml pindolol; (C) blank plasma.

t-distribution table and the variance of absolute differences between the actual concentrations and the concentrations found (see Table I), the 95% confidence intervals for single determinations of pindolol in plasma were calculated at all concentrations. The results indicate that the result of any single analysis at any concentration would fall within ±25.0% of its true value.

The precision (within-day variability) and reproducibility (day-to-day variability) of the method are also demonstrated by the data in Table I. The coefficients of variation (C.V.) for the within-day variation at any concentration of pindolol in plasma ranged from 1.1 to 15.5% while the day-to-day variation for the same set of data ranged from 7.1 to 13.3%.

Sensitivity

The sensitivity of this method was evaluated by analyzing plasma samples to which pindolol had been added in concentrations near the limit of sensitivity. The results from these analyses are shown in Table II. A detection limit of 2 ng/ml was determined by comparing the coefficients of variation obtained at each concentration in Table II with the coefficients of variation observed in Table I. Method

TABLE II

EVALUATION OF THE DETECTION LIMIT OF PINDOLOL IN PLASMA

Pindolol concentration (ng/ml)	Mean response factor (ng/ml/mV)	Coefficient of variation (%)
1	0.116	91.6
2	0.146	21.6
4	0.123	11.3
8	0.134	0.8
16	0.133	9.8
40	0.123	0.9
80	0.125	2.9

parameters were judged to be consistent down to 2 ng/ml. Concentrations were detected down to 1 ng/ml in plasma; however, the precision and accuracy were regarded as unacceptable.

Selectivity

No interferences from extracted endogenous material or metabolites were observed in the area of pindolol (Fig. 1). Additionally, this procedure has been used for the analysis of propanolol in plasma samples (see Fig. 2) and was found to be free of interferences in the area of pindolol from the other commonly administered drugs shown in Table III.

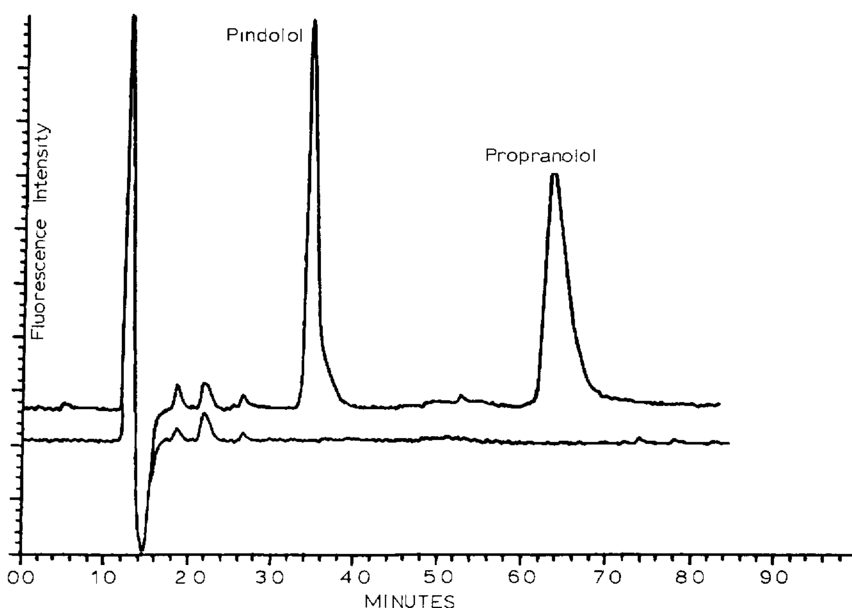

Fig. 2. Representative chromatograms obtained in the analysis of blank plasma (lower trace) and plasma standard containing 150 ng/ml of both pindolol and propanolol (upper trace).

TABLE III

RELATIVE RETENTION TIMES OF OTHER COMMONLY ADMINISTERED DRUGS

Compound	Relative retention time
Pindolol	1.00
Propranolol	1.69
Metoprolol	1.25
Soterenol	0.75
Oxprenolol	1.42
Alprenolol	1.86
Acetaminophen	0.50
Caffeine	0.50
Aspirin	0.57
Thioridazine	No peak observed
Diazepam	No peak observed
Hydrochlorothiazide	No peak observed

Application of the method

Approximately 1500 human plasma samples from several pindolol bioavailability studies have been analyzed using this procedure. In one study, twelve subjects were administered 15 mg of pindolol (Visken) after which serial blood samples were drawn to determine plasma pindolol concentrations. In the majority of cases, for an oral dose of 15 mg, plasma concentrations above the detection limit of the method could be followed for three to five half-lives. The mean pindolol plasma concentrations for the twelve subjects and related pharmacokinetic

TABLE IV

PINDOLOL PLASMA CONCENTRATION AFTER A SINGLE ORAL DOSE OF 15 mg IN TWELVE SUBJECTS

Time (h)	Concentration (mean ± S.D.) (ng/ml)
0.33	28.3 ± 14.3
0.67	54.2 ± 14.3
1	54.6 ± 9.5
2	51.3 ± 14.5
3	40.8 ± 10.1
4	32.2 ± 8.7
6	20.8 ± 9.5
9	11.9 ± 5.3
12	5.6 ± 4.4
16	2.9 ± 2.8
24	0.3 ± 1.0

Area under the curve from 0 to 24 h (mean ± S.D.) 330 ± 104 ng h ml^{-1}
Half-life (mean ± S.D.) 3.3 ± 0.7 h

parameters obtained are listed in Table IV. These observed mean plasma concentrations and pharmacokinetic parameters are in good agreement with previously published data [1,2,4].

DISCUSSION

Although several HPLC and GLC methods are reported for the analysis of pindolol in human plasma, none has demonstrated the combination of simplicity, sensitivity, selectivity, reproducibility and reliability that has been presented here. A simple sample preparation is followed by chromatographic separation, fluorescence detection and data analysis allowing for a sample through-put of up to twelve samples per hour. Moreover, this method has been applied routinely by two separate laboratories demonstrating its reliability.

The sample preparation is much less labour-intensive than that used for the reported GLC procedures [6,7] or the reported HPLC–fluorescence procedure [8] enabling external standardization to be used with no loss in precision and accuracy. The use of fluorescence detection eliminates the problems associated with electrode contamination and the response stabilization time common to amperometric (or electrochemical) detection [10] and provides more selectivity compared to ultraviolet detection [11,13]. The use of elevated column temperature helps to decrease the retention time of pindolol, thereby increasing sample through-put. No significant loss in column life was observed, based upon the use of a single column for the analysis of about 900 samples over a four-week period. Additionally, this procedure has been used for the determination of propranolol and it has been determined that there are no interferences from many other commonly administered substances.

A detection limit of 2 ng/ml is sufficient to monitor plasma levels for three to five half-lives following an oral dose (5–15 mg) of pindolol. Detection limits of < 2 ng/ml can be achieved by increasing the sample size injected on column (500 μl instead of 250 μl) or by decreasing the volume (0.5 ml instead of 1.0 ml) of dilute acid used during the back-extraction step.

In conclusion, the method reported here employs (a) a relatively simple sample preparation, (b) maintenance-free and selective fluorescence detection and (c) sensitivity and reliability under routine use. The utility of the method has been adequately demonstrated with the routine analysis of about 1500 human plasma samples in two separate laboratories.

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