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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR LABETALOL IN HUMAN PLASMA USING A PRP-1 COLUMN AND FLUOROMETRIC DETECTION

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SUMMARY

A high-performance liquid chromatographic assay for the determination of labetalol, a novel antihypertensive agent, in human plasma was developed. Reversed-phase separation of labetalol and the internal standard was accomplished on a 150 × 4.1 mm column commercially packed with a spherical (8—12 μm particle size) macroporous co-polymer (PRP-1). Unlike silica-based columns, the unique properties of PRP-1 permit operation at pH extremes. Based on this advantage, a mobile phase which was sufficiently basic (pH 9.5) to optimize the fluorescent yield of analyte and provide the necessary specificity was selected. Detector response (peak area ratio) was linear from 4 to 500 ng/nl. Following a simple extraction procedure, samples were automatically injected and analyzed using microprocessor-controlled equipment. No interferences were observed in the extracts obtained from drug-free plasma which were processed under the conditions described for unchanged drug. The limit of quantitation using 0.5 ml of plasma was validated to 4 ng/ml. The inter-assay precision (coefficient of variation) was less than 4.6% at all concentrations evaluated from 4 to 300 ng/ml. This method is suitable for the routine quantitation of labetalol or its *RR* isomer (dilevalol) in plasma (0—24 h) following the administration of therapeutically effective doses to man.

INTRODUCTION

Labetalol · HCl (I), 5-{1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]-ethyl}salicylamide monohydrochloride (Fig. 1) is an effective antihypertensive agent with both α- and β-adrenoreceptor blocking activity as well as direct vasodilator actions [1]. Disposition studies with radiolabeled (^{3}H) drug in man have shown that labetalol is well absorbed following oral administration but undergoes extensive first-pass metabolism [2]. Peak plasma concentrations of

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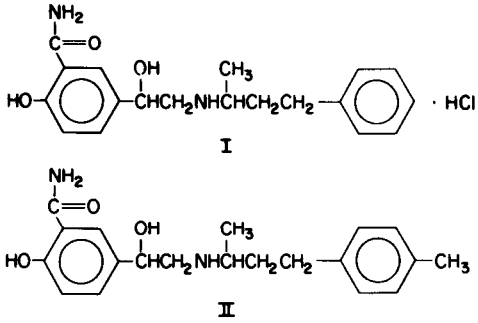

Fig. 1. Structures of labetalol hydrochloride (I) and the internal standard (II).

labetalol after a 200-mg dose were only about 80—100 ng/ml and represented less than 5% of the total plasma radioactivity. For these reasons, analytical methodology which can reproducibly measure nanogram quantities of unchanged labetalol is required to fully characterize the terminal elimination phase in plasma. A selective and sensitive high-performance liquid chromatographic (HPLC) assay for labetalol was therefore developed in our laboratory in order to support a multitude of clinical bioavailability and bioequivalency studies. Several HPLC methods [3—7] for the determination of labetalol in plasma are reported in the literature. Dusci and Hackett [3] described an HPLC method with ultraviolet (UV) detection which was sensitive to 40 ng/ml of labetalol in plasma. This limit of quantitation, however, is unsuitable for detailed pharmacokinetic studies in man following single-dose administrations (per os) of drug. Another more sensitive (10 ng/ml) HPLC assay which employed a UV spectrophotometer (207 nm) was reported by Woodman and Johnson [4]. Although this method requires extensive sample clean-up, significant assay bias (+60%) was observed at the 10 ng/ml concentration in plasma. In a recent publication, Hidalgo and Muir [7] report an HPLC method for labetalol which also used UV detection (216 mm) but with a simplified extraction procedure. Comparable sensitivity was achieved; however, no accuracy or precision estimates at the lower limit of detection (10 ng/ml) were provided.

Meredith et al. [5] and Oosterhuis et al. [6] reported two different procedures for the quantitation of labetalol in plasma which employ HPLC with fluorimetric detection. Both methods share improved lower limits of detection (ca. 1—8 ng/ml) and selectivity. An examination of the method employed by Meredith et al. [5] reveals that detection of drug is accomplished with an acidic mobile phase thus failing to optimize the fluorescence yield of labetalol which is achieved under basic (pH 9—10) conditions [6, 8]. The approach reported by Oosterhuis et al. [6] involved post-column alkalination of the column effluent with buffer in order to enhance the fluorimetric detection of analyte. This, however, was achieved at the expense of an additional pump and associated hardware.

The method detailed herein describes the use of an HPLC column commercially [9] packed with a macroporous co-polymer (PRP-1) for the determination of labetalol in plasma. Unlike silica-based columns, the unique properties of PRP-1 permit operation at pH extremes. Based upon this advantage, a mobile phase was chosen which was sufficiently basic (pH 9.5)

to optimize the fluorimetric detection of labetalol and provide the selectivity necessary for routine determinations of drug at concentrations as low as 4 ng/ml.

EXPERIMENTAL

Apparatus

Analyses were performed on an HPLC system composed of a WISP (Model 710B) automatic injector (Waters Assoc., Milford, MA, U.S.A.), and a Waters pump (M6000A) interfaced with a Waters Model 720 system controller. Detection of analyte was achieved by using an Aminco (Urbana, IL, U.S.A.) Fluoro-Monitor equipped with a round 100-μl flow cell after excitation at 370 nm (Corning 7-51 filter) and emission at 415 nm (Wratten gelatin No. 2A filter). A back-pressure coil constructed with 60 cm × 0.23 mm I.D. stainless-steel tubing was connected to the outlet side of the detector to prevent bubble formation in the cell. The amplified signal from the detector was connected to a recorder and an integrating computer (HP 3357 Lab Automation System, Hewlett-Packard, Palo Alto, CA, U.S.A.) in order to generate real-time chromatographic tracings (10 mV) and to integrate peak areas (0—2 V). The attenuator was maintained at a setting of 10.

Reagents and solvents

Labetalol · HCl and the internal standard (II), 5-{2-[4-(4-methylphenyl)-2-butylamino]-1-hydroxyethyl}salicylamide · HCl, were used as received from the Schering Chemical Distribution Center (Bloomfield, NJ, U.S.A.). All other chemicals except acetonitrile, hexane and methanol (Omni Solv, MCB, Cincinnati, OH, U.S.A.) were reagent grade.

Chromatographic conditions

Chromatography was performed on a 150 × 4.1 mm I.D. stainless-steel column commercially (Hamilton, Reno, NV, U.S.A.) packed with PRP-1, a spherical (10 μm particle size) macroporous poly(styrene-divinylbenzene) sorbent.

Reversed-phase separations were accomplished at ambient temperature using a mobile phase consisting of 0.05 *M* $(NH_4)_2CO_3$—NH_4OH, pH 9.5—acetonitrile—tetrahydrofuran (495:125:40, v/v/v). The solvent mixture was prepared daily, filtered (0.45 μm) and degassed under reduced pressure before use. The flow-rate (1.5 ml/min) generated a back-pressure of approx. 124 bars.

Standard solution preparation

An accurately weighed amount (11.1 mg) of labetalol · HCl was dissolved in 3 ml of methanol and then diluted to 100.0 ml with distilled water. This solution contained 100 μg/ml free base form. A solution of the internal standard was similarly prepared with water in a 100-ml volumetric flask. Subsequent dilutions with distilled water were designed so that the desired amount of both drugs could be conveniently delivered in a 100-μl volume with automatic pipettes.

Detector calibration and standard curve

A standard curve was initially generated after repated (n = 6) injections of standard solutions prepared to contain 4, 20, 50, 100, 200, 400 and 500 ng/ml labetalol and a constant concentration (200 ng/ml) of the internal standard. Data from this multipoint calibration curve were subjected to least-squares fit analysis to determine the best straight-line relationship. Computer-reported estimates of labetalol concentration were based upon the average calculated response factors from triplicate injections of a single concentration point near the mid-point of the standard curve. Calibration was performed using samples containing 100 ng/ml labetalol and 200 ng/ml internal standard.

Extraction procedure

An aliquot (0.5 ml) of human plasma was transferred to a 15-ml test tube (125 × 16 mm) fitted with a polytef-lined screw cap. After the addition of 100 ng internal standard which was prepared as an aqueous solution (100 ng per 0.1 ml), each plasma sample was diluted (0.5 ml) with 0.05 *M* Tris buffer (pH 9.0) and extracted with 5.0 ml of ethyl acetate by agitation on an Eberbach (Ann Arbor, MI, U.S.A.) reciprocal shaker for 10 min. Samples were centrifuged (10 min at 1600 *g*) to facilitate separation of the phases. The aqueous portion was frozen in a dry-ice—acetone bath and the organic layer transferred to a clean 15-ml test tube.

Aliquots of hexane (2.0 ml) and 0.05 *M* sulfuric acid (0.5 ml) were added to the ethyl acetate, then shaken and centrifuged as above. The organic layer was discarded and the acidic solution transferred to a disposable polypropylene microcentrifuge tube (1.5 ml). An aliquot (400 μl) of this acidic extract was then automatically injected for analysis by HPLC.

Extraction efficiency

Venous blood was drawn from several human volunteers into heparinized Vacutainers® (Becton-Dickinson, Rutherford, NJ, U.S.A.) and centrifuged at 1600 *g* for 30 min to generate a drug-free plasma pool. The efficiency of extracting drug from human plasma was determined using the following procedure. Labetalol and internal standard were added to aliquots (0.5 ml) of drug-free plasma ($n \geqslant 6$ replicates per group) to achieve labetalol concentrations of 4, 100 and 300 ng/ml with a constant internal standard concentration of 200 ng/ml. Samples were extracted as described above and 400 μl of the acidic layer injected by the WISP.

The recovery of labetalol and internal standard was calculated by comparing the peak area of both compounds from extracted samples with those obtained from the analysis of equivalent amounts of drug injected directly.

Intra-assay accuracy and precision

The precision and accuracy of labetalol quantitation were evaluated in the following manner. Known amounts of drug standards were added to drug-free plasma as described in the previous section and extracted as outlined. In order to calibrate the detector response, extracts from three samples spiked to contain 100 ng/ml labetalol and 200 ng/ml internal standard were injected in triplicate. Quantitation of labetalol concentrations in the remaining samples

was then automatically calculated by the integrating computer using the average (n = 3) internal standard response factor generated at this calibration point.

Inter-assay accuracy and precision

Estimates of inter-assay variability were provided by repeating the accuracy and precision determinations described above for labetalol on two different days. All analytical results within each concentration group were pooled in order to calculate the inter-assay precision (coefficient of variation, C.V.) and accuracy (percentage bias).

Selectivity

Drug-free human plasma specimens were processed and analyzed by HPLC as described above. Chromatograms were examined for the presence of endogenous material which might interfere with the measurement of either labetalol or the internal standard.

Solutions of hydrochlorothiazide, trichloromethiazide and propranolol were injected directly onto the column in order to assess their potential for assay interference.

RESULTS AND DISCUSSION

Chromatography

The retention times for labetalol and the internal standard varied between 4.74 and 5.20 min and 8.53 and 9.6 min, respectively, for three PRP-1 columns which were evaluated for assay suitability. No significant changes in retention time or peak shape were observed for either compound over a three-month period.

Extracts from drug-free human plasma were found to be free of interfering peaks (Fig. 2). Representative chromatograms from plasma spiked with 4 ng/ml

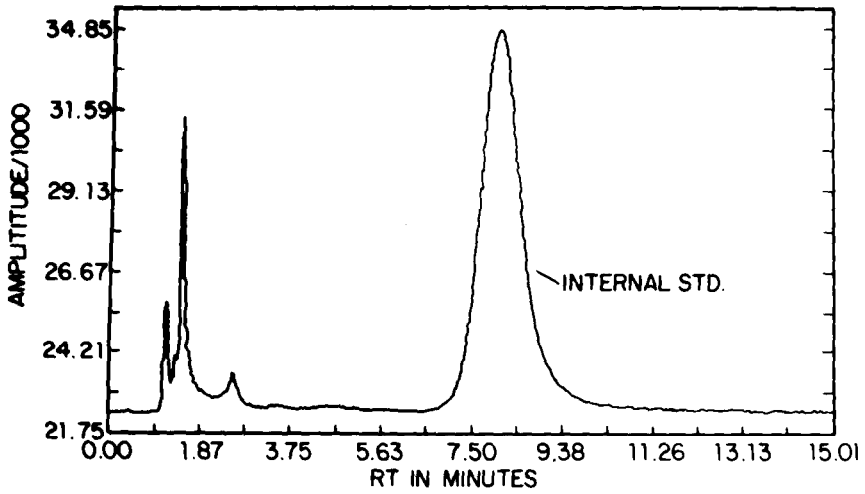

Fig. 2. Computer-reconstructed chromatogram of an extract from drug-free human plasma to which only internal standard (200 ng/ml) was added.

and 100 ng/ml labetalol are shown in Fig. 3. Hydrochlorothiazide, trichloromethiazide and propranolol demonstrated no potential for assay interference.

Detector calibration and standard curves

A standard curve was generated using stock solutions prepared to contain labetalol at concentrations ranging from 4 to 500 ng/ml. The integrated peak area (μV-sec) ratio of labetalol to the internal standard was chosen as the quantitative measure of detector response for each labetalol concentration. Data were then subjected to weighted (1/variance) least-squares fit analysis owing to variance heteroscedasticity in order to determine the best fit straight-line relationship between detector response and labetalol concentration.

Weighted regression of peak area ratio on labetalol concentration yielded a linear ($P > 0.1$) fit of the data with a coefficient of determination (r^2) equal

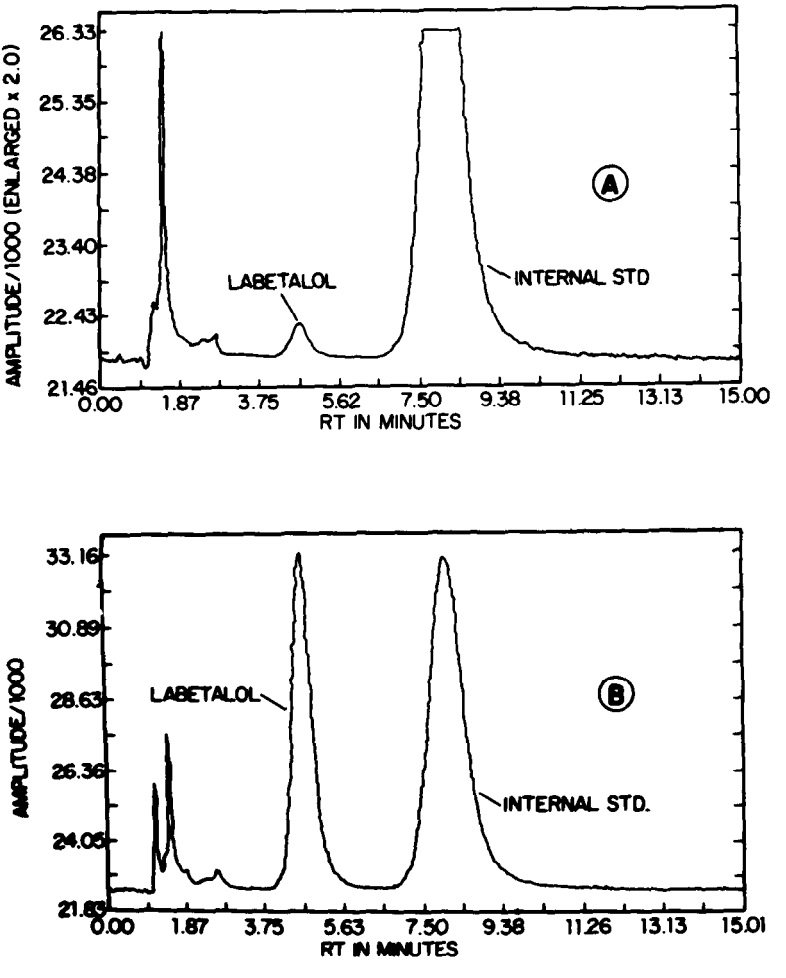

Fig. 3. Computer-reconstructed chromatograms of (A) an extract from plasma spiked to contain 4 ng/ml labetalol and (B) an extract from plasma spiked to contain 100 ng/ml labetalol. Both samples (0.5 ml) were fortified with internal standard to achieve a concentration of 200 ng/ml.

TABLE I

MEAN PERCENTAGE RECOVERY OF LABETALOL AND THE INTERNAL STANDARD FROM HUMAN PLASMA AT VARYING LABETALOL CONCENTRATIONS

n	Labetalol concentration (ng/ml)	Recovery labetalol* (%)	Internal standard concentration (ng/ml)	Recovery internal standard* (%)
8	4	74.8 ± 5.81	200	71.0 ± 5.28
6	100	76.9 ± 5.80	200	70.9 ± 5.04
6	300	74.6 ± 1.73	200	70.4 ± 1.22
		$\bar{X}$ = 75.4 ± 4.81		$\bar{X}$ = 70.8 ± 4.17

*$\bar{X}$ ± S.D.

to 0.998. The *Y*-intercept, which was not significantly different from zero at the 95% confidence interval, was determined to be $8.63 \cdot 10^{-6}$. The slope of the linear response was calculated to be 0.003346 $(ng/ml)^{-1}$.

Extraction efficiency

The average recovery of labetalol from plasma samples to which drug standard had been added at concentrations ranging from 4 to 300 ng/ml was determined to be 75.4 ± 4.81% S.D. (Table I). The internal standard (200 ng/ml) was extracted with a mean efficiency equal to 70.8 ± 4.17% S.D. Statistical analysis by single level ANOVA demonstrated that there were no significant differences ($P > 0.5$) among the mean recoveries for labetalol nor were there significant differences in the recovery of the internal standard as a function of labetalol concentration. These data, therefore, suggest that there is no concentration dependence on extraction efficiency over the range of drug levels in plasma which was investigated.

Intra-assay accuracy and precision

Concentration estimates from plasma spiked to contain 4, 100, and 300 ng/ml labetalol are shown in Table II. The relative accuracy (percentage bias) of

TABLE II

INTRA-ASSAY PRECISION AND ACCURACY OF LABETALOL QUANTITATION FROM HUMAN PLASMA BY HPLC

Theoretical concentration (ng/ml)	*n*	Mean observed concentration (ng/ml)	C.V. (%)	Percentage bias*
4.0	8	3.86	2.97	−3.50
100.0	6	100.11	1.23	0.11
300.0	6	307.44	1.10	2.48
			$\bar{X}$ = 1.77	

*Percentage bias—relative accuracy.

these determinations was found to range from −3.50 to 2.48% with a mean intra-assay precision (C.V.) of ± 1.77%.

Inter-assay accuracy and precision

The inter-assay variability of concentration estimates from plasma spiked with known amounts of drug and processed for labetalol is shown in Table III. These data reflect the variability in labetalol concentration which were determined for each group after extraction and HPLC analysis on two different days. As can be seen in Table III, the inter-assay precision did not exceed ± 4.6% while the relative accuracy of labetalol quantitation from plasma ranged from −3.50 to 0.30% for all concentration groups. These data suggest that the method can reliably quantify labetalol concentrations (4—300 ng/ml) on a day-to-day basis.

TABLE III

INTER-ASSAY PRECISION AND ACCURACY OF LABETALOL QUANTITATION FROM HUMAN PLASMA BY HPLC

Theoretical concentration (ng/ml)	*n*	Mean observed concentration (ng/ml)	C.V. (%)	Percentage bias*
4.0	14	3.86	4.09	−3.50
100.0	12	100.30	1.91	0.30
300.0	12	300.02	4.59	0.01
			$\bar{X}$ = 3.53	

*Percentage bias — relative accuracy.

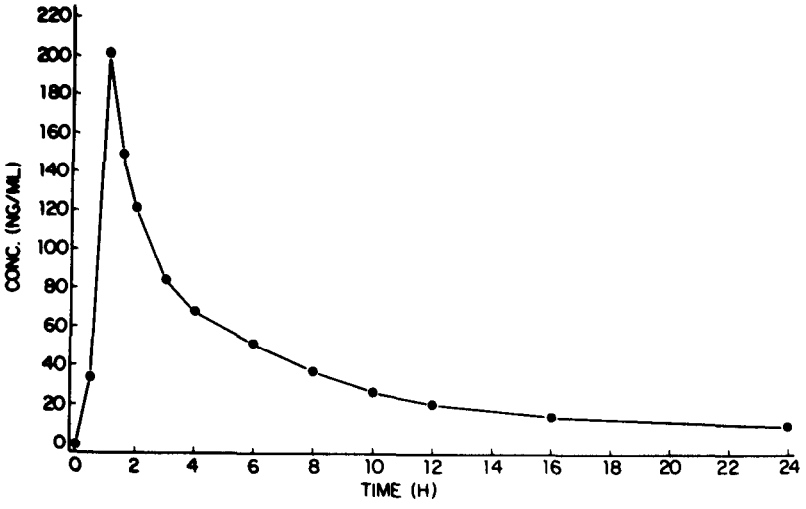

Fig. 4. Plasma concentration—time curve for a subject who received (per os) 300 mg of labetalol.

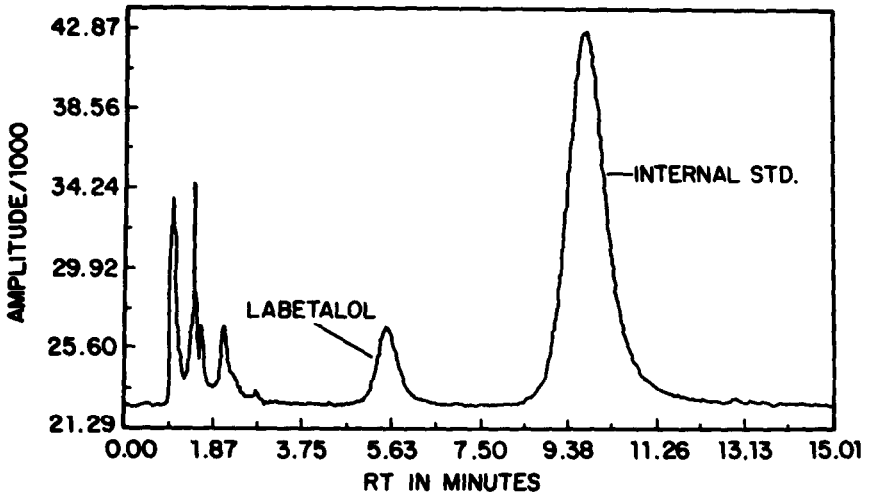

Fig. 5. Computer-reconstructed chromatogram of a 12-h plasma extract which was determined to contain 20.7 ng/ml labetalol.

Assay feasibility

A plasma concentration—time curve is shown in Fig. 4 for a subject who received (per os) 300 mg of labetalol. Duplicate aliquots (0.5 ml) were extracted and analyzed by HPLC as described herein. A chromatogram generated by a 12-h plasma extract (20.7 ng/ml) from this subject is illustrated in Fig. 5. Concentrations of unchanged labetalol remained in excess of the validated lower limit of quantitation (4 ng/ml) throughout the first 24 h of plasma collection. The relative standard deviation of the duplicate analyses ranged from ± 0.04 to ± 5.43%.

CONCLUSIONS

In summary, an HPLC method for the quantitative determination of labetalol in human plasma has been validated for concentrations ranging from at least 4 to 300 ng/ml. This assay takes advantage of a novel separation of labetalol and the internal standard on an analytical column commercially packed with a macroporous co-polymer (PRP-1). Since the support of this column is stable with a moderately basic (pH 9.5) mobile phase, optimized response of labetalol to fluorometric detection can be achieved without post-column alkalination. The increased sensitivity and simplicity of this procedure represents a significant advantage over previously published methods.

Drug agents such as hydrochlorothiazide, trichlorothiazide and propranolol, which may be administered concomitantly with labetalol, demonstrated no potential for interference. This method has been shown to provide quantitatively accurate and precise determinations for labetalol in human plasma and can be routinely employed following the administration of therapeutically effective doses to man. This method is also suitable for quantitating the plasma levels of dilevalol (Sch 19927), the *RR* isomer of labetalol.

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