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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR DILEVALOL IN HUMAN PLASMA AND URINE USING A PRP-1 COLUMN AND FLUORIMETRIC DETECTION

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SUMMARY

A single high-performance liquid chromatographic (HPLC) assay for the quantitative determination of dilevalol, the *R,R* isomer of labetalol, was developed for both plasma and urine. A significantly improved limit of detection for dilevalol in plasma was accomplished by extensive modification of an HPLC assay originally developed in our laboratory for labetalol. This simplified method is readily adaptable to urine and represents the first reported HPLC assay for the quantitative determination of dilevalol in this biofluid. Drug was recovered from plasma or urine by partition into diethyl ether under mildly alkaline conditions and back-extraction into dilute acid. Reversed-phase separation of dilevalol and the internal standard was accomplished on a 150×4.1 mm column commercially packed with a spherical (5 μm) macroporous copolymer (PRP-1). No interferences were observed in extracts obtained from drug-free plasma or urine. Selectivity for dilevalol in the presence of other β-blockers was established. This method demonstrated a linear detector response to concentrations of unchanged drug typically observed in urine and plasma following once-a-day treatment with dilevalol hydrochloride (100–800 mg). The lowest limit of reliable quantitation was established at 1 ng/ml in plasma. The intra-assay precision (coefficient of variation) remained less than 6% at all concentrations evaluated from 1 to 800 ng/ml. In urine, the lowest limit of quantitation was validated to 20 ng/ml where the intra-assay precision (coefficient of variation) for unchanged drug was less than 4% at all concentrations evaluated up to 4000 ng/ml. This method is suitable for routine quantitation of unchanged drug in human plasma and urine following the administration of therapeutically effective doses of dilevalol hydrochloride.

INTRODUCTION

Dilevalol hydrochloride, the *R,R* isomer of labetalol, 5-{1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl} salicylamide monohydrochloride is a combined direct vasodilator/β-blocker presently under development as an antihypertensive agent. Animal studies [1] indicate that dilevalol has considerably

greater β-blocking and vasodilating potency than labetalol but less α-blocking activity. Dilevalol undergoes extensive first-pass metabolism by glucuronide conjugation and unchanged drug represents less than 10% of the total circulating levels of metabolites [2] in man. Dilevalol presents additional challenges to previously established limits of detection for labetalol, since unlike the latter, dilevalol is intrinsically effective as a once-a-day antihypertensive agent at similar daily doses. Methodology for the determination of labetalol (and by inference dilevalol) in plasma includes high-performance liquid chromatographic (HPLC) assays employing UV [3–5], fluorimetric [6–8] and electrochemical detection [9]. The lowest limits of detection reported for labetalol [3–9] are generally inadequate to reliably quantify dilevalol for three to four half-lives (30–40 h) during the terminal elimination phase [2] in plasma. In addition, since urine can be less intrusively collected to monitor compliance, a method which extends to this biofluid would have added benefit to clinicians. To this end, lower limits of detection and adaptability to urine were accomplished by extensive modification of an HPLC assay for labetalol previously developed in our laboratory [8]. Since the advantages of using a PRP-1 column with fluorimetric detection [8] over other labetalol assays utilizing traditional silica-based columns apply equally to dilevalol, these key analytical components were retained. However, significant changes in the extraction procedure and mobile phase composition were incorporated to facilitate sample processing and improve sensitivity. The development and validation of this improved HPLC method for dilevalol is herein described.

EXPERIMENTAL

Apparatus

Analyses were performed on an HPLC system composed of a WISP (Model 710B) automatic injector (Waters Assoc., Milford, MA, U.S.A.), a Waters pump (6000A or M590) and an LDC/Milton Roy FluoroMonitor III detector (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) equipped with a round 30-μl flow-cell and black fluorescent (360 nm) lamp. Detection of analyte was achieved after excitation at 370 nm (filter No. 801483) and emission at 418 nm (filter No. 801521). A back-pressure coil constructed with 60 cm of 0.23 mm I.D. stainless steel was connected to the outlet side of the detector to prevent bubble formation in the cell. The amplified signal (range = 2×) from the detector (0–10 mV) was connected to a Model 9176 recorder (Varian Assoc., Palo Alto, CA, U.S.A.) in order to generate real-time chromatographic tracings. A computer (HP 3357 laboratory automation system, Hewlett-Packard, Palo Alto, CA, U.S.A.) was interfaced through the 0–12 V signal output for integration of peak areas.

Reagents and solvents

Dilevalol hydrochloride (CAS 75659-08-4) and the internal standard, (*R,R*)-5,2-[4-(4-methylphenyl)-2-butylamino]-1-hydroxyethyl salicylamide hydrochloride, were used as received from the Schering Chemical Distribution Center (Bloomfield, NJ, U.S.A.). All other chemicals except tetrahydrofuran and ammonium carbonate (HPLC grade) were of reagent grade.

Chromatographic conditions

Chromatography was performed on a 150 mm × 4.1 mm I.D. stainless-steel column (Hamilton, Reno, NV, U.S.A.) commercially packed with PRP-1, a spherical (5 μm) macroporous poly(styrene–divinylbenzene) sorbent. Reversed-phase separations were accomplished at ambient temperature using a mobile phase consisting of 0.05 *M* ammonium carbonate–ammonium hydroxide, pH 9.5–tetrahydrofuran (2:1, v/v). The solvent mixture was prepared daily, filtered (0.45 μm) and degassed under reduced pressure before use. The flow-rate (0.4 ml/min) generated a back-pressure of approximately 130 bars.

Standard solution preparation

A solution containing 100 μg/ml free base was prepared by dissolving 55.5 mg of dilevalol hydrochloride in 5 ml of methanol and then diluting to 500.0 ml with distilled water. A solution of the internal standard (55.0 mg) was similarly prepared in a 500-ml volumetric flask. Subsequent dilutions with distilled water were designed so that the desired amount of both drugs could be conveniently delivered (100–200 μl) with automatic pipettes.

Extraction from urine and plasma

Aliquots (0.5 ml) of either biofluid were transferred to 15-ml test tubes (125 mm × 16 mm) fitted with polytef-lined screw caps. After the addition of internal standard, each sample was diluted (1.0 ml) with 0.1 *M* sodium bicarbonate and then extracted with 6.0 ml of anhydrous diethyl ether by agitation on an Eberbach (Ann Arbor, MI, U.S.A.) reciprocal shaker for 10 min. Samples were centrifuged (2°C) for 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 was transferred to a clean 15-ml test tube. An aliquot (0.5 ml) of 0.05 *M* sulfuric acid was added to the diethyl ether and then shaken and centrifuged as above. After freezing the aqueous portion, the organic layer was discarded and the tubes were placed in a water bath (50°C) for 20 min to evaporate the remaining ether. The acidic extract was transferred to a disposable microcentrifuge tube (1.5 ml), and an aliquot (25-150 μl) was injected for analysis by HPLC.

Extraction efficiency

Plasma was purchased from a local blood bank to generate a drug-free plasma pool. The efficiency of extracting drug from both urine and plasma was determined using the following procedure. Dilevalol and the internal standard were added to aliquots (0.5 ml) of plasma ($n \geq 5$ per group) to achieve dilevalol concentrations of 1, 2, 10, 20, 50 and 100 ng/ml with a constant internal standard concentration of 20 ng/ml. Samples were processed as outlined above, and 150 μl of the final extract were injected for analysis by HPLC. Urine from several non-fasted volunteers was collected to generate a drug-free urine pool. Dilevalol and the internal standard were added to aliquots (0.5 ml) of urine ($n = 6$ per group) to achieve dilevalol concentrations of 20, 400, 1000, 2000 and 4000 ng/ml with a constant internal standard concentration of 1000 ng/ml. Samples were extracted as previously described, and 25 μl of the final extract were injected for analysis

by HPLC. Since all phase transfers were essentially quantitative, the recovery of dilevalol 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.

Detector response

Standard curves were generated with two different concentration ranges in an attempt to bracket all anticipated plasma concentrations following the oral administration of 100–800 mg of dilevalol hydrochloride. The response of the detector expected from the lowest doses was investigated after extraction of plasma (0.5 ml) spiked in replicate ($n \geq 5$) to contain 1, 2, 10, 20, 50 and 100 ng/ml of dilevalol and a constant concentration (20 ng/ml) of internal standard. A second set of extracted standards which reflected a wider concentration range was similarly prepared. These included groups of plasma fortified with dilevalol at concentrations of 2, 10, 100, 400 and 800 ng/ml and a constant concentration of internal standard (200 ng/ml). The detector response to anticipated levels of unchanged drug in urine was established using the following procedure. Urine was fortified with dilevalol (20–4000 ng/ml) and the internal standard (1000 ng/ml) as outlined in the previous section. Peak-area ratio (dilevalol/internal standard) versus dilevalol concentration data were evaluated by least-squares fit analysis after the injection of sample extracts from each group.

Accuracy and precision

Drug levels in plasma and urine were routinely determined by the integrating computer using a single-point calibration procedure. Extracts from three samples prepared at a single concentration of dilevalol were injected to determine an average response factor. In plasma, calibration for the low concentration group (1–100 ng/ml) was performed using samples fortified to contain 20 ng/ml of both dilevalol and the internal standard. A calibration point containing 100 ng/ml dilevalol and 200 ng/ml internal standard was selected for the second group (2–800 ng/ml) of plasma samples. The calibration factor for urine was determined by injection of samples prepared to contain 400 ng/ml dilevalol and 1000 ng/ml internal standard. All remaining samples (see previous section) were treated as unknowns. Dilevalol concentrations were then automatically calculated by the integrating computer using the method of internal standardization. Computer-generated concentration estimates were evaluated to establish intra-assay precision and accuracy.

Selectivity

Drug-free human plasma and urine were routinely analyzed as described above and the resultant chromatograms examined for the presence of endogenous co-extractants which could interfere with the measurement of either dilevalol or the internal standard. The chromatographic behavior of the antihypertensive agents labetalol, metoprolol, nadolol, prazosin, timolol, propranolol and verapamil were also evaluated to determine their potential for assay interference.

RESULTS AND DISCUSSION

Chromatography

Baseline separation was achieved between dilevalol (ca. 9 min) and the internal standard (ca. 12.5 min) under the chromatographic conditions described. Optimization of chromatography to accommodate column-to-column variation in performance was accomplished by modest changes in the ratio of tetrahydrofuran to buffer. Labetalol, which has two chiral centers, is partially resolved into its diastereomers (*RR,SS* and *SR,RS*) under the chromatographic conditions described; therefore, this assay may not be appropriate for trace determinations of labetalol. Verapamil, nadolol and metoprolol showed no fluorescent emission (420 nm) throughout a 45-min analysis time. Prazosin, timolol and propranolol showed peaks which eluted at 6.0, 12.2 and 22.4 min, respectively. Based upon the poor sensitivity observed from on-column injection (3 μg) of each compound, an insignificant response to therapeutic levels of these agents in plasma would be expected. However, their presence in urine at higher concentrations could interfere with the determination of dilevalol. The potential for interference by metabolites of this group of antihypertensive agents was not investigated. Extracts from drug-free plasma and urine were found to be free of interfering peaks (Fig. 1). Representative chromatograms of extracts from plasma and urine collected during the conduct of a clinical study are shown in Fig. 2.

Detector linearity

Multi-point standard curves were generated at two different concentration ranges following the extraction of plasma samples prepared to contain dilevalol varying from 1 to 100 and 2 to 800 ng/ml. The integrated peak-area ratio of dilevalol to the internal standard was chosen as the quantitative measure of detector response. Data from both curves were then subjected to weighted (1/variance) least-squares analysis to determine the best-fit straight-line relationship between detector response and dilevalol concentration. Peak-area ratio data from extracted urine samples (20–4000 ng/ml) were similarly evaluated. Slope, intercept and coefficient of determination (r^2) values are summarized in Table I. Since all curves effectively degenerate to a simple linear ($y=mx$) relationship, the single-point ratio calibration procedure used by the integrating computer to routinely calculate drug concentration was validated.

Extraction efficiency

The average efficiency of extracting dilevalol from plasma samples ranging in concentration from 1 to 100 ng/ml was determined to be $74.36 \pm 2.68\%$ (mean $\pm$ S.D.). The internal standard (20 ng/ml) was extracted with an efficiency equal to $78.07 \pm 2.24\%$ (mean $\pm$ S.D.).

Dilevalol was extracted from fortified urine samples ranging in concentration from 20 to 4000 ng/ml with an efficiency equal to $69.96 \pm 3.74\%$ (mean $\pm$ S.D.) while $79.3 \pm 4.1\%$ (mean $\pm$ S.D.) of the internal standard (1000 ng/ml) was similarly extracted. Statistical analysis revealed that the efficiency of extracting both

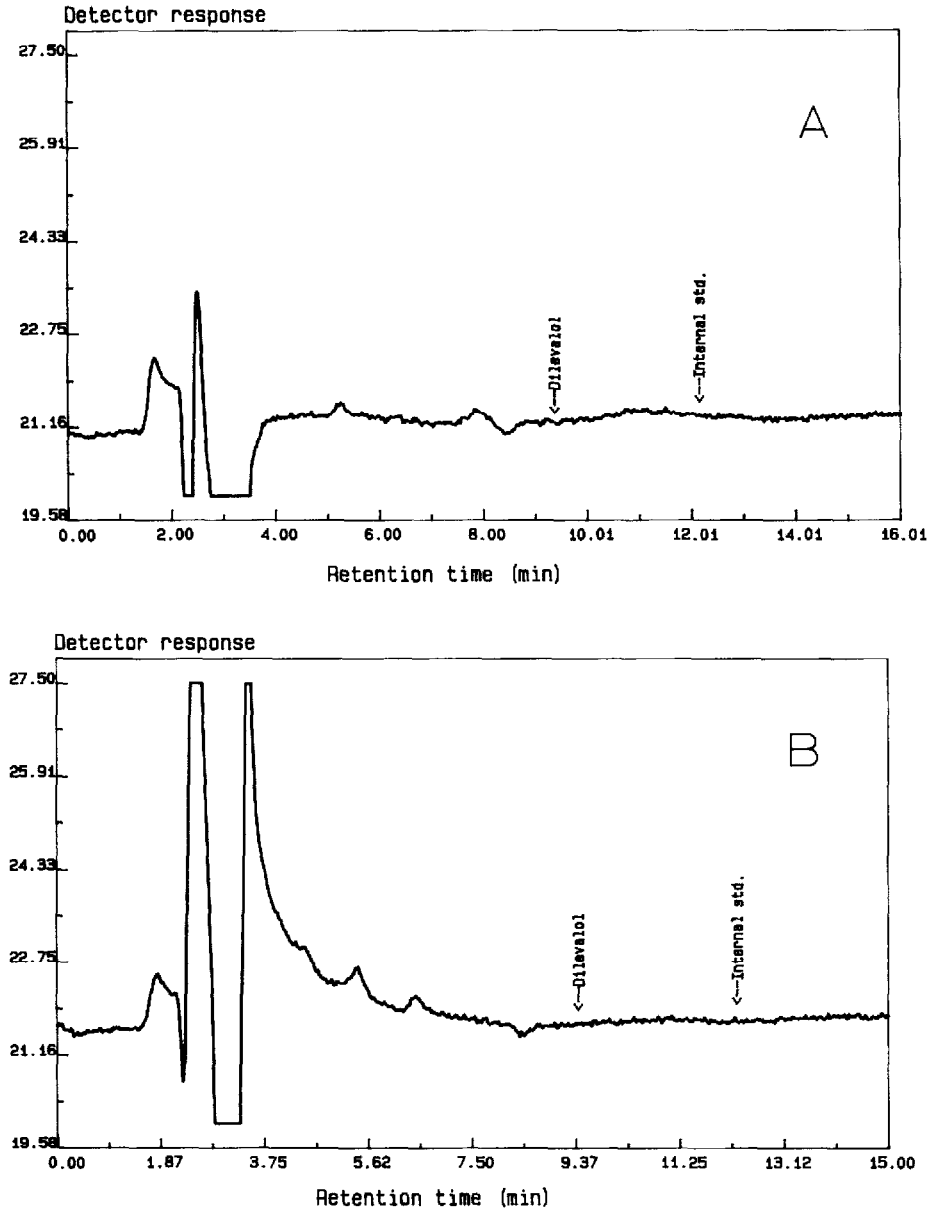

Fig. 1. Typical chromatograms of extracts from drug-free plasma (A) and urine (B).

compounds was not dependent on the concentration of dilevalol over the ranges which were investigated.

Accuracy and precision

Concentration estimates from plasma spiked to contain 1, 2, 10, 20, 50 and 100 ng/ml dilevalol and 20 ng/ml internal standard are shown in Table II. These levels correspond to the range of dilevalol concentrations that would typically be

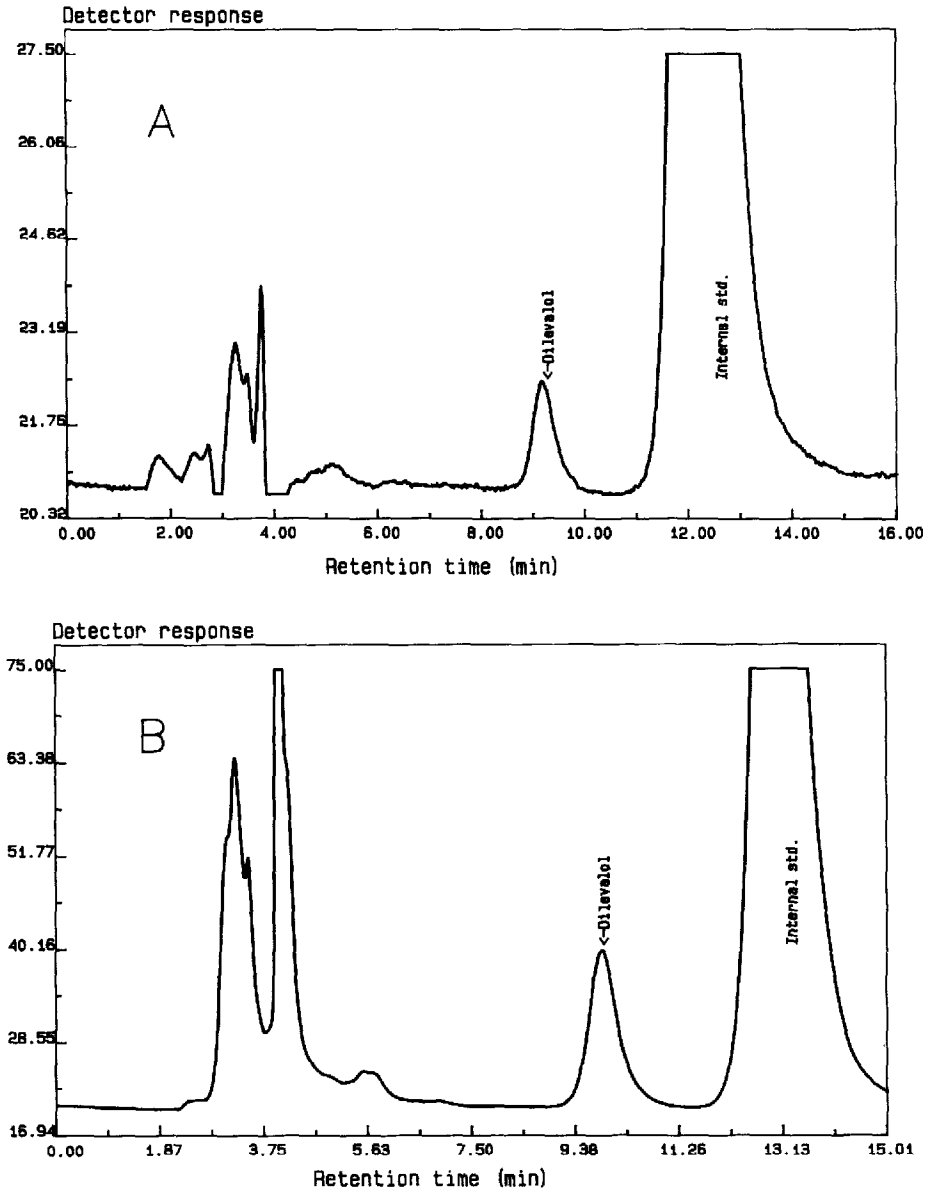

Fig. 2. Representative chromatogram of a plasma extract determined to contain 4.5 ng/ml dilevalol (A) and a chromatogram from urine which was determined to contain 56.0 ng/ml dilevalol (B). Internal standard was added as previously described for each biofluid.

found following the administration of doses approximating 100 mg dilevalol hydrochloride. Accuracy and precision data from plasma fortified with dilevalol ranging from 2 to 800 ng/ml are displayed in Table III. These correspond to the range of dilevalol concentrations that would typically be observed with larger doses (200–800 mg) of dilevalol hydrochloride. Mean data from similarly prepared samples which were used as daily quality controls over a two-month period

TABLE I

WEIGHTED (1/VARIANCE) REGRESSION OF PEAK-AREA RATIOS (DILEVALOL/INTERNAL STANDARD) ON THREE DIFFERENT CONCENTRATION RANGES OF DILEVALOL IN PLASMA AND URINE

Sample	Concentration (ng/ml)		Slope (ng^{-1})	Intercept* (ng)	r^2
	Dilevalol	Internal standard			
Plasma	1- 100	20	$5.42 \cdot 10^{-1}$	$1.13 \cdot 10^{-3}$	0.998
	2- 800	200	$5.36 \cdot 10^{-2}$	$7.8 \cdot 10^{-4}$	0.999
Urine	20-4000	1000	$1.01 \cdot 10^{-3}$	$-1.03 \cdot 10^{-3}$	0.999

*Not significantly different from zero at the 95% confidence interval.

TABLE II

INTRA-ASSAY PRECISION AND ACCURACY OF DILEVALOL QUANTITATION FROM HUMAN PLASMA USING A LOW-DOSE CALIBRATION

Theoretical concentration (ng/ml)	n	Observed concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Bias (%)
1	5	1.05 ± 0.03	2.85	+5.00
2	6	2.07 ± 0.07	3.30	+3.50
10	6	10.7 ± 0.24	2.22	+6.70
20	6	19.5 ± 0.68	3.50	−2.30
50	6	51.1 ± 1.52	1.52	+2.28
100	6	102.9 ± 2.82	2.82	+2.92

TABLE III

INTRA-ASSAY PRECISION AND ACCURACY OF DILEVALOL QUANTITATION FROM HUMAN PLASMA USING A HIGH-DOSE CALIBRATION

Theoretical concentration (ng/ml)	n	Observed concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Bias (%)
2	5	2.18 ± 0.13	5.82	+9.00
10	5	9.75 ± 0.27	2.81	−2.50
100	8	100.3 ± 1.30	1.29	+0.31
400	5	396.7 ± 2.28	0.58	−0.83
800	5	792.1 ± 15.49	1.96	−0.99

are presented in Table IV. Concentration estimates from urine spiked to contain dilevalol (20–4000 ng/ml) are shown in Table V. Mean quality control data accumulated daily over a two-week period during the analysis of urine samples from a clinical study are presented in Table VI. In all instances, the relative accuracy (bias, %) and precision (coefficient of variation, C.V., %) remained within acceptable limits for reliable quantitation.

TABLE IV

INTER-ASSAY PRECISION AND ACCURACY OF DILEVALOL QUANTITATION FROM HUMAN PLASMA

Theoretical concentration (ng/ml)	n	Mean observed concentration (ng/ml)	Coefficient of variation (%)	Bias (%)
2	102	1.98	12.09	−0.81
4	31	4.25	8.65	6.18
10	100	9.49	5.28	−5.10
20	32	19.5	3.26	−2.56
100	149	99.8	2.13	−0.22
400	102	404.3	4.74	1.07
800	32	792.9	2.39	−0.89

TABLE V

INTRA-ASSAY PRECISION AND ACCURACY OF DILEVALOL QUANTITATION FROM HUMAN URINE

Theoretical concentration (ng/ml)	n	Observed concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Bias (%)
20	6	19.3 ± 0.20	1.03	−3.65
400	6	400.0 ± 3.73	0.93	0.00
1000	6	1012 ± 6.79	0.67	+1.20
2000	6	1977 ± 61.25	3.10	−1.16
4000	6	4062 ± 9.90	0.24	+1.55

TABLE VI

INTER-ASSAY PRECISION AND ACCURACY OF DILEVALOL QUANTITATION FROM HUMAN URINE

Theoretical concentration (ng/ml)	n	Mean observed concentration (ng/ml)	Coefficient of variation (%)	Bias (%)
20	20	17.7	10.77	−11.55
500	17	503.1	1.52	0.60
2000	20	1965	2.19	− 1.78
4000	18	3888	1.71	− 2.80

Stability

Since dilevalol undergoes extensive first-pass metabolism by glucuronide conjugation [2], unchanged dilevalol represents a fractional part ($<10\%$) of the total drug-related species found in plasma and urine. Glucuronide conjugates of dilevalol can be readily hydrolyzed in the presence of β-glucuronidase from *Helix pomatia*. Studies were designed to evaluate the stability of dilevalol and its con-

jugates under a variety of storage conditions. Selected plasma samples from a clinical study were stored at room temperature for three days and for at least eight months at $-20°C$. The results from these stability studies revealed that no significant hydrolysis of conjugates or dilevalol degradation had occurred.

CONCLUSIONS

An HPLC assay has been validated for plasma concentrations of dilevalol ranging from 1 to 800 ng/ml and urine concentrations of unchanged drug ranging from 20 to 4000 ng/ml. Selectivity for dilevalol in the presence of other antihypertensive agents was established. The limit of reliable quantitation using 0.5 ml of plasma was determined to be 1 ng/ml. This assay is readily adaptable to the determination of trace levels of drug in human urine without any changes in the extraction procedure or chromatography. The limit of quantitation for dilevalol in urine (0.5 ml) has been validated to 20 ng/ml. This method is routinely employed for the quantitation of unchanged drug in human plasma and urine following the administration of therapeutically effective doses of dilevalol hydrochloride.

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