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DETERMINATION OF POLYTHIAZIDE AND PRAZOSIN IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A selective high-performance liquid chromatographic method has been developed for the determination of polythiazide in human plasma down to concentrations of 0.5 ng/ml. Polythiazide and an internal standard (epithiazide) are simultaneously extracted from the sample, the extract is purified on a silica micro-column and analyzed on a μBondapak CN column. Chloroform—methanol (97:3) is the eluent, with spectrophotometric detection at 264 nm.

The extraction methodology developed for the analysis of polythiazide in blood plasma allows the simultaneous quantitative determination of prazosin, which is frequently administered together with thiazide diuretics. The precision and accuracy of both the polythiazide and the prazosin assays are excellent and are not seriously affected by the simultaneous presence of both drugs in the plasma. Therefore, determination of polythiazide and prazosin is possible using a single plasma sample.

INTRODUCTION

Polythiazide (Renese®) (I) is a diuretic used frequently in the management of hypertension either as a sole drug or in combination with antihypertensive drugs [1]. For instance, prazosin (Minipress®) (II) which is a recognized vasodilating agent [2, 3] can be used for this purpose. Since, under certain circumstances, both drugs are administered concurrently, their simultaneous assay in blood plasma is required for evaluation of bioavailability in clinical studies.

A procedure exhibiting sufficient sensitivity for polythiazide analysis has been published recently [1]. In this procedure, polythiazide is extracted from plasma and hydrolyzed to form trifluoroethylthioacetaldehyde which is quantitated using gas—liquid chromatography with electron-capture detection. Since the procedure does not utilize an internal standard and trifluoroethyl-

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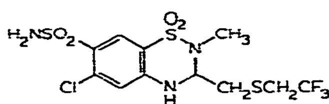
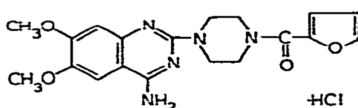
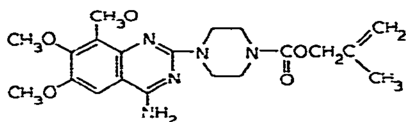


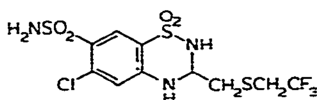
(I) Polythiazide



(II) Prazosin·HCl



(III) ATQ



(IV) Epithiazide

thioacetaldehyde is not available commercially as a primary standard, its practical use requires a high degree of experience.

A relatively simple prazosin determination by high-performance liquid chromatography (HPLC) using fluorescence detection has been described [2, 4]. The procedure involves the 2-methallyl ester of 4-(4-amino-6,7,8-trimethoxy-2-quinazolinyl)-1-piperazine carboxylic acid (ATQ) as an internal standard (III) and provides excellent sensitivity.

Thus, to quantitate both polythiazide and prazosin using a single plasma sample, it was necessary to develop a more practical procedure for polythiazide and couple the method with that for prazosin. The first objective was achieved by development of an HPLC procedure for polythiazide, using epithiazide (IV) as an internal standard, with the eluate monitored by UV detection at 264 nm. Secondly, an extraction procedure was devised which purified and concentrated both drugs and the internal standards. The extract was then chromatographed on two different HPLC systems, one with UV detection for polythiazide, the other with fluorescence detection for prazosin.

EXPERIMENTAL

Materials and equipment

Polythiazide (I), internal standard epithiazide (IV), prazosin (II) in the form of the hydrochloride salt and internal standard 2-methallyl ester of 4-(4-amino-6,7,8-trimethoxy-2-quinazolinyl)-1-piperazine carboxylic acid, ATQ (III) were supplied by Pfizer (Groton, CT, U.S.A.). Reagents included (1) ethyl acetate, glass distilled; (2) chloroform without preservative; (3) *n*-hexane, glass distilled, all, e.g., from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.); (4) methanol, e.g. Matheson, Coleman and Bell (Cincinnati, OH, U.S.A.); (5) acetonitrile (e.g. Burdick and Jackson); (6) acetic acid, Baker (Phillipsburg, NJ, U.S.A.); (7) silica gel 60, particle size 0.063—0.200 mm, Cat. 7734, E. Merck (Darmstadt, G.F.R.), activated by heating in an oven at 100° overnight; (8) ammonium hydroxide (Baker). A vortex evaporator (Buchler) was used at 40°C. In addition, the reagents included methanol treated with *n*-hexane (9) and *n*-hexane treated with methanol (10). (Reagents 9 and 10 were prepared by transferring 100 ml methanol and 500 ml *n*-hexane to a 1-l

separatory funnel, shaking the mixture and allowing the phases to separate, the bottom layer being methanol treated with *n*-hexane, the upper layer, *n*-hexane treated with methanol.)

All glassware used during the extraction procedure was cleaned with chromic acid, washed with distilled water, rinsed with ammonium hydroxide and air dried.

Chromatography system

The polythiazide and prazosin assays both utilized the same column system and configuration. A pump (Waters 6000A) and variable volume injection valve (Waters U6K) followed by a guard column (Waters PN 84550) containing Bondapak Phenyl/Corasil packing, particle size 37—50 μm (Waters PN 27282) was used preceding the analytical column μBondapak CN, particle size 10 μm, 30 cm $\times$ 3.9 mm (Waters PN 84042), all from Waters Assoc. (Milford, MA, U.S.A.).

Polythiazide assay. A UV detector (Schoeffel SF 770) set at 264 nm (range 0.04, time constant 5 sec, recorder 1 mV) was used to monitor the column eluate. The mobile phase, consisting of chloroform—methanol (97:3), was degassed for 3—4 min under vacuum and pumped through the column at a flow-rate of 0.7 ml/min. Injection volume was 25 μl for both standards and samples. Before daily analyses, 1 h was allowed for chromatographic equilibration. Under these conditions, polythiazide eluted at approximately 10 min, epithiazide at 15 min.

Prazosin assay. A fluorescence detector (Schoeffel FS 970) set at an excitation wavelength of 248 nm with a 389 emission filter (range 0.1, time constant 6 sec, recorder 1 mV) was used to monitor the column eluate. The mobile phase, consisting of acetonitrile—distilled water—acetic acid (50:47:3), was degassed for 10 min under vacuum and pumped through the column at a flow-rate of 2.2 ml/min. Injection volume was 3 μl for both standards and samples. Before daily analyses, 1 h was allowed for chromatographic equilibration. Under these conditions, prazosin eluted at approximately 6 min, ATQ at 9 min.

Preparation of standard solutions and calibration curves for polythiazide assay

Prepare methanolic solutions containing (a) 10 μg polythiazide per ml and (b) containing 10 μg eipithiazide per ml.

An external calibration curve can be used, since the extraction efficiencies of polythiazide and epithiazide in the procedure were demonstrated to be identical. Typical recoveries are listed in Table I.

For construction of the external calibration curve, take 1.0 ml (2.0 ml or 5.0 ml) of solution (a) and 10 ml of solution (b), combine and dilute to 100 ml with methanol. The calibration solutions will contain 100 ng (200 ng, 500 ng) polythiazide and 1000 ng epithiazide per ml. Transfer 50 μl of each standard solution into separate 10-ml centrifuge tubes. Under the assay conditions, the amounts correspond to 1 ng (2 ng, 5 ng) polythiazide per ml plasma. Evaporate each solution to dryness using a mild stream of nitrogen. Reconstitute each with 50 μl ethyl acetate, vortex 30 sec and inject 25 μl into the HPLC system. An external standard curve is constructed by plotting the polythiazide—internal

TABLE I

POLYTHIAZIDE DETERMINATION IN BLOOD PLASMA

Polythiazide added (ng/ml)	No. of parallel determinations	Recovery (%) (average)	Relative standard deviation (%)
1.0	24	94	8.93
2.0	19	101.5	7.27
5.0	10	93.8	5.69
10.0	10	97.4	4.44
15.0	8	97.9	1.64

standard (epithiazide) peak height ratio vs. ng polythiazide per ml plasma.

For spiking plasma samples with internal standard, accurately prepare a standard solution of epithiazide in methanol containing 1 ng/μl (solution *X*).

Preparation of standard solutions and calibration curves for prazosin assay

Prepare methanolic solutions containing (a′) 10 μg prazosin · HCl per ml and (b′) 1 mg ATQ per ml.

To construct a calibration curve, take 1.0 ml (5.0 ml or 15.0 ml) of solution (a′) and 0.25 ml of solution (b′), combine and dilute to 100 ml with methanol. Transfer 50 μl of each calibration standard solution into separate 50-ml centrifuge tubes. Under the assay conditions, the amounts correspond to 1 ng (5 ng, 15 ng) prazosin·HCl per ml plasma. Evaporate the calibration solutions to dryness using a mild stream of nitrogen, add 5.0 ml human control plasma, mix and proceed as described under Sample preparation. A standard curve is constructed by plotting the prazosin—internal standard peak height ratio vs. ng prazosin·HCl per ml plasma.

For spiking plasma samples with internal standard, accurately prepare a standard solution of ATQ in methanol containing 2.5 ng/μl (solution *Y*).

Since the extraction efficiencies of prazosin and ATQ internal standard are not identical in the procedure, both substances have to be added to blank plasma and carried through the procedure in order to obtain a calibration curve. Typical recoveries are listed in Table II.

TABLE II

PRAZOSIN DETERMINATION IN BLOOD PLASMA

Prazosin added (ng/ml)	No. of parallel determinations	Recovery (%) (average)	Relative standard deviation (%)
1.0	12	103	7.24
2.0	14	100	6.62
5.0	14	97	4.97
15.0	10	99	4.59

Extraction of plasma samples

A flow di°gram is presented in Fig. 1. Transfer 5.0 ml of a plasma sample to a 50-ml centrifuge tube, add 50 ng epithiazide internal standard (50 μl of solution X) and 125 ng ATQ internal standard (50 μl of solution Y) and mix well. Extract the plasma sample with 2 × 20 ml n-hexane, inverting the centrifuge tube 30 times for each extraction. Centrifuge and remove the n-hexane by

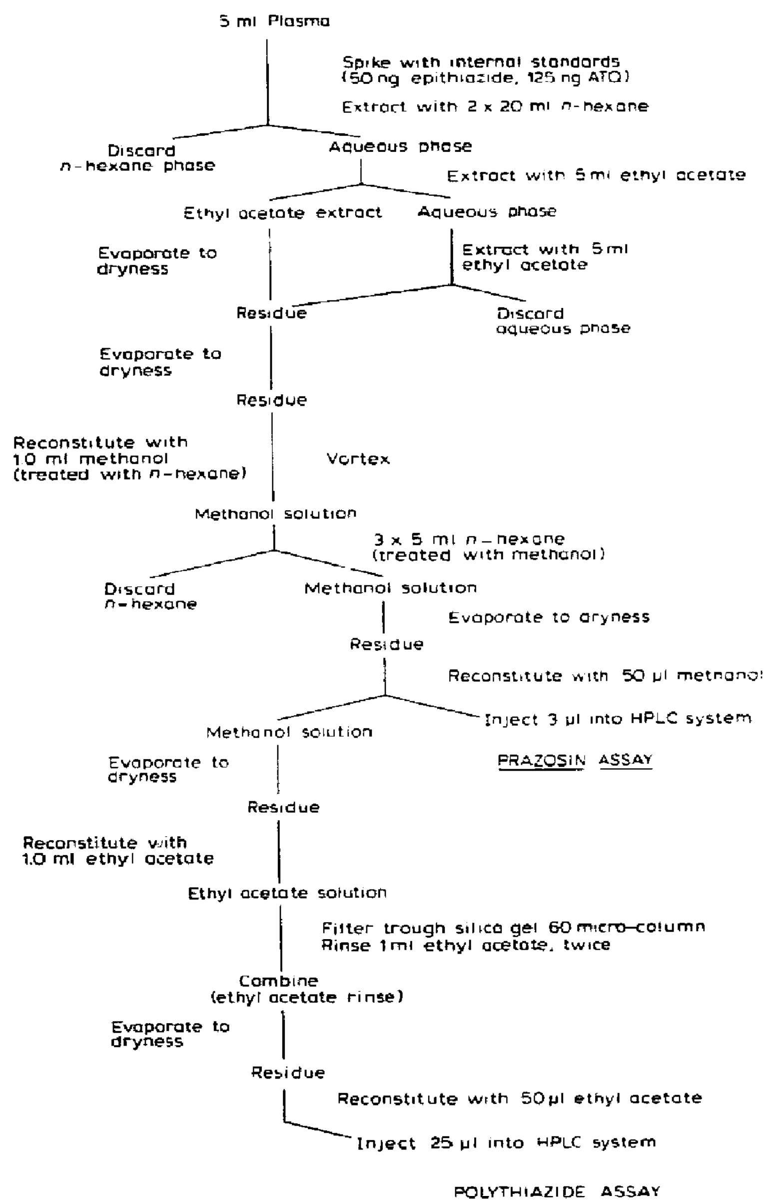


Fig. 1. Schematic diagram for the extraction of polythiazide and prazosin from human plasma.

aspiration. Continue by extracting the plasma sample with 5 ml of ethyl acetate, vortexing 30 sec. Centrifuge the mixture, transfer the ethyl acetate to a 15-ml centrifuge tube, and evaporate the solvent on a vortex evaporator. Repeat the ethyl acetate extraction, combining the solvent with the residue from the first extract. Repeat the evaporation. Reconstitute the residue with 1.0 ml methanol (treated with *n*-hexane). Add 5 ml *n*-hexane (treated with methanol), mix 30 sec, centrifuge. Discard the *n*-hexane layer using a disposable Pasteur pipet. Repeat the extraction with 2 × 5 ml *n*-hexane (treated with methanol). Transfer the methanol to a clean 15-ml centrifuge tube and evaporate the methanol to dryness on a vortex evaporator. The residue (*r*) is used for determining the content of prazosin and polythiazide.

Sample assay for prazosin

Reconstitute the sample residue (*r*) with 50 μl of methanol, mixing 30 sec. Inject 3 μl of the sample solution(s) into the HPLC system for prazosin analysis. Determine the peak heights of prazosin and its internal standard ATQ. Calculate the ratio by dividing peak height of prazosin by peak height of internal standard. Using a calibration curve prepared concurrently with each series of plasma samples, report the content of prazosin in plasma.

Sample assay for polythiazide

Using a vortex evaporator, evaporate to dryness the sample solution(s) remaining after the prazosin assay. Reconstitute the sample residue with 1 ml ethyl acetate, mixing 30 sec. Prepare a micro-column by plugging the tip of a disposable Pasteur pipet (approximately 16 cm long) with glass wool. Transfer 0.5 g of activated silica gel 60 to the column and wet with ethyl acetate. Transfer the ethyl acetate sample solution onto the wet silical gel column with a disposable Pasteur pipet. Collect the eluate in a 15-ml centrifuge tube by applying pressure with a rubber bulb on top of the column. Add an additional 1.0 ml ethyl acetate to the sample tube, mixing 30 sec. Again transfer the ethyl acetate to the silica gel column, collecting the eluate in the 15- ml centrifuge tube. Repeat with an additional 1.0 ml ethyl acetate, the final volume being ca. 3 ml. Rinse the walls of the centrifuge tube with ca. 0.2 ml ethyl acetate. Evaporate the ethyl acetate to dryness on the vortex evaporator and reconstitute the sample residue with 50 μl ethyl acetate, mixing 30 sec. Inject 25 μl of the prepared sample onto the HPLC system for polythiazide analysis. Measure peak heights of polythiazide and its internal standard epithiazide. Calculate ratio by dividing peak height of polythiazide by peak height of internal standard (epithiazide). Using the calibration curve which is constructed concurrently with each series of samples, report the amount of polythiazide present in plasma samples.

RESULTS AND DISCUSSION

An HPLC procedure for the determination of polythiazide in human plasma has been developed. The extraction methodology allows simultaneous determination of prazosin, which is, under certain circumstances, administered together with polythiazide. The procedure was validated in three steps. First,

reference plasma (plasma blank) was spiked with internal standard (epithiazide) and known amounts of polythiazide (corresponding to 1—15 ng polythiazide per ml plasma). The mixture was analyzed according to the procedure described above. A linear calibration curve was obtained. A typical chromatogram is depicted in Fig. 2A. The results summarized in Table I clearly demonstrate the reproducibility and accuracy of the procedure. Second, known amounts of prazosin (corresponding to 1—15 ng prazosin per ml plasma) and internal standard ATQ were added to blank plasma and prazosin content was determined as described. A linear calibration curve was obtained. Fig. 3A shows the separation of prazosin and its internal standard ATQ, while Table II illustrates the accuracy and precision obtained. Amounts as low as 1 ng prazosin per ml plasma and 1 ng polythiazide per ml plasma can be reliably quantified. Finally, polythiazide was determined in blood plasma containing prazosin and ATQ (Table III), and prazosin was determined in blood plasma containing polythiazide and epithiazide (Table IV). No interferences were observed in the chromatographic backgrounds in either procedure. Consequently, polythiazide and prazosin can be analyzed with adequate precision and sensitivity using a single blood plasma sample.

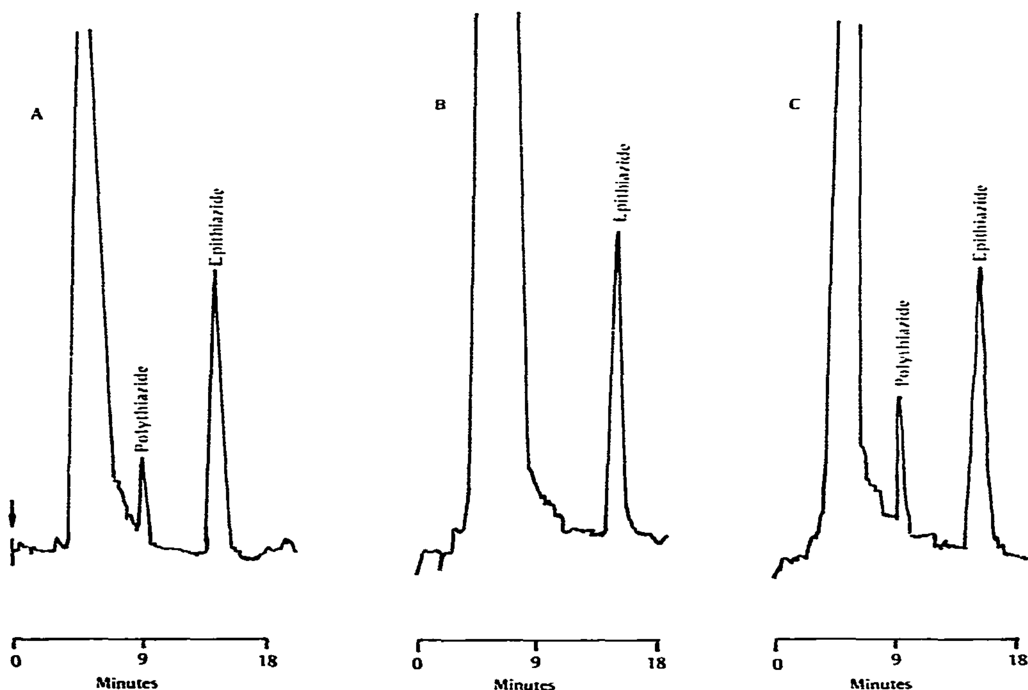


Fig. 2. HPLC chromatograms of (A) the extract from blank plasma spiked with 2 ng polythiazide and 10 ng epithiazide per ml plasma; (B) the extract of plasma sample obtained from a test subject prior to administration of Minizide® spiked with 10 ng epithiazide per ml plasma; (C) the extract of a plasma sample obtained from a test subject 8 h after oral administration of Minizide®.

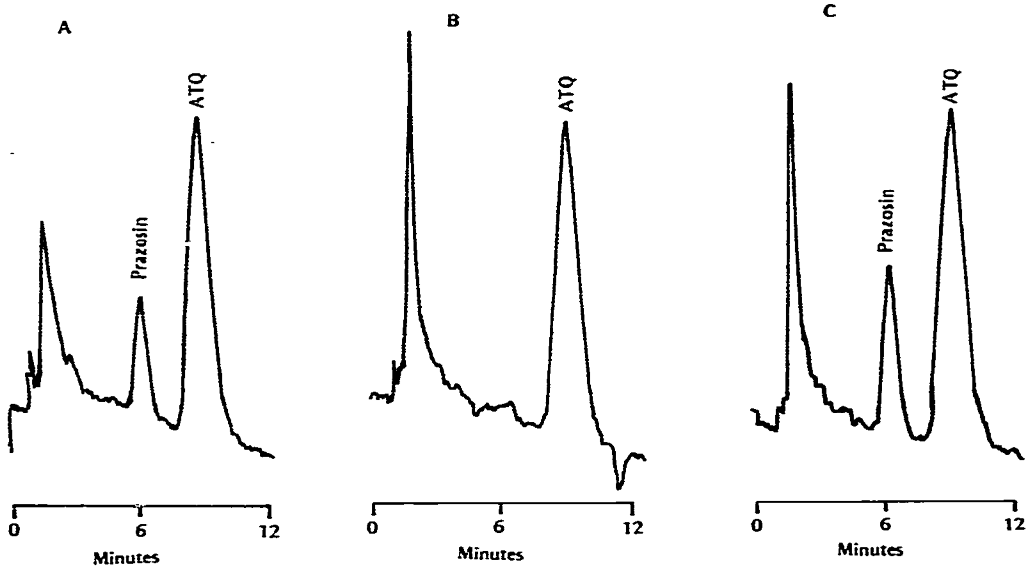


Fig. 3. HPLC chromatograms of (A) the extract from blank plasma spiked with 5 ng prazosin · HCl and 25 ng ATQ per ml plasma; (B) the extract of plasma sample obtained from a test subject prior to administration of Minizide® spiked with 25 ng ATQ per ml plasma; (C) the extract of a plasma sample obtained from a test subject 0.5 h after administration of Minizide®.

TABLE III

POLYTHIAZIDE DETERMINATION IN BLOOD PLASMA IN THE PRESENCE OF PRAZOSIN

Amounts of prazosin added varied from 1 ng per ml plasma to 15 ng per ml plasma at all polythiazide concentration levels studied.

Polythiazide added (ng/ml)	No. of parallel determinations	Recovery (%) (average)	Relative standard deviation (%)
1.0	9	94.4	9.32
2.0	9	102.2	6.95
5.0	10	95.8	6.90

TABLE IV

PRAZOSIN DETERMINATION IN BLOOD PLASMA IN THE PRESENCE OF POLYTHIAZIDE

Amounts of polythiazide added varied from 1 ng per ml plasma to 15 ng per ml plasma at all polythiazide concentration levels studied.

Prazosin added (ng/ml)	No. of parallel determinations	Recovery (%) (average)	Relative standard deviation (%)
1.0	12	112	6.67
2.0	12	105	4.27
5.0	12	98	3.66

The described methodology was applied to large numbers of blood plasma specimens from a clinical study in which prazosin and polythiazide were administered in the form of the combination drug, Minizide®. Plasma levels for both drugs were determined, with representative chromatograms illustrated in Figs. 2C and 3C.

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