Journal of Chromatography, 224 (1981) 275—288
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 862

DETERMINATION OF THE ANTIHYPERTENSIVE AGENT 1-(2-AMINOETHYL)-3-(2,6-DICHLOROPHENYL)THIOUREA IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received January 28th, 1981)

SUMMARY

A rapid, sensitive and specific normal-phase (adsorption) high-performance liquid chromatographic (HPLC) assay was developed for the determination of 1-(2-aminoethyl)-3-(2,6-dichlorophenyl)thiourea [I] in plasma and urine. The assay involves the extraction of the compound into methylene chloride from plasma or urine buffered to pH 10, and the HPLC analysis of the residue dissolved in methylene chloride—methanol—heptane (85:10:5). A 10-μm silica gel column was used with methylene chloride—methanol—heptane—ammonium hydroxide (85:10:5:0.1) as the eluting solvent. The effluent was monitored at 254 nm and quantitation was based on the peak height vs. concentration technique. The assay has a recovery of 64.5 ± 4.5% (S.D.) from plasma and 96.0 ± 6.3% (S.D.) from urine in the concentration range of 0.1—2 μg per ml and 2—40 μg per 0.1 ml of plasma and urine, respectively, with a limit of detection of 0.05—0.1 μg [I] per ml of plasma using a 1-ml specimen and 0.1 μg per ml urine using a 0.1-ml specimen, respectively. The assay was applied to the determination of plasma levels and urinary excretion of the compound [I] in dog following the oral administration of 28.8 mg of [I] • maleate per kg body weight.

The HPLC assay was also used to determine the stability of [I] and for the measurement of a potential degradation product, clonidine [II] [2-(2,6-dichlorophenylamino)-2-imidazoline] in pooled human plasma stored at —17°C, and pooled human urine stored at —17°C and —90°C, respectively.

INTRODUCTION

The compound 1-(2-aminoethyl)-3-(2,6-dichlorophenyl)thiourea ([I], Fig.1), is one of a series of compounds, structurally related to clonidine [2-(2,6-dichlorophenylamino)-2-imidazoline] ([II], Fig. 1), which is presently under investigation as an antihypertensive agent [1]. Gas—liquid chromatographic analysis of [I] using either electron-capture or mass spectrometric detec-

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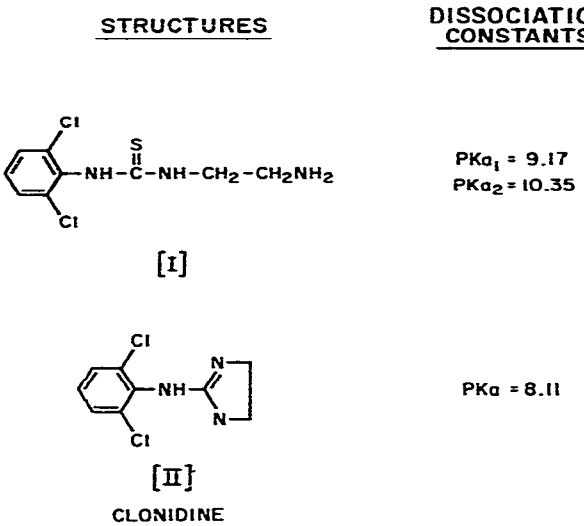


Fig. 1. Chemical structures and dissociation constants of [I] and [II].

tion is not feasible due to thermal rearrangement of [I] in the injection port resulting in the formation of clonidine [II] as a major degradation product [2], necessitating the use of high-performance liquid chromatography (HPLC) analysis, the utility of which was recently described for the determination of [I] in a dietary admix using reversed-phase HPLC [3].

This paper describes a rapid, sensitive, and specific HPLC assay for the determination of [I] in plasma, blood, and urine without chemical derivatization. The assay utilizes normal-phase (adsorption) HPLC with isocratic elution at ambient temperature, monitoring the absorbance of the eluent at 254 nm. The assay was used for preclinical bioavailability studies and for the evaluation of the stability of [I] and its conversion to [II] in pooled human plasma stored at —17°C and urine stored at —17°C and —90°C.

EXPERIMENTAL

Column

The column used was a 30 cm × 4 mm I.D. stainless-steel column containing 10 μm μPorasil silica gel (Waters Assoc., Milford, MA, U.S.A.).

Instumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector, and a Model 440 UV detector with a 254-nm wavelength kit (Waters Assoc.). The mobile phase used for isocratic normal-phase chromatography was a mixture of methylene chloride—methanol—heptane—ammonium hydroxide (85:10:5:0.1). The chromatographic system was operated at ambient temperature, with a flow-rate of 2.2 ml/min, and at pressure of 800 p.s.i. Under the above conditions, the capacity factors k' of compounds [I] and [II] were 4.44 and 1.89, respectively. Concentrations of 40 ng of compound [I] and 15 ng of [II] injected yielded peaks of approximately 50%

of full scale at a detector sensitivity of 5×10^{-3} a.u.f.s. The chart speed on a 10-mV recorder was 30 in./h (0.5 in./min) (Speedomax XL, 625 series, Leeds and Northrup, North Wales, PA, U.S.A.).

Upon completion of a day's analysis, the ammonium hydroxide and any accumulated endogenous material are flushed from the column with methanol, which is then equilibrated in methylene chloride. Although it is reported that high alkalinity reduces the useable life span of silica columns, this one has been in use for ten months without any noticeable deterioration.

Standard solution

Weigh out 10.0 mg of the compound [I] ($C_9H_{11}Cl_2N_3S$, mol. wt. = 264.17, m.p. = 135—137°C) into a 10-ml volumetric flask and dissolve in 10 ml methanol. This stock solution [A] contains 1.0 mg [I] per ml and is used to prepare an intermediate solution in 10 ml containing 100 μg [I] per ml in methanol (solution [B]). In addition, stock solution [A] is used to prepare two working solutions [C] and [D] in 10 ml of 1 *M* phosphate buffer (pH 10.0) containing 100 μg and 10 μg [I] per ml, respectively. Solutions [C] and [D] are used to prepare the internal standards for the quantitation of the unknowns and must be prepared fresh, prior to each day's analysis.

A series of working external standards for the plasma assay is prepared by transferring aliquots of 0.050, 0.125, 0.250, 0.375, and 0.500 ml of solution [B] into 10-ml volumetric flasks and diluting to volume with methylene chloride—methanol—heptane (85:10:5) to yield solutions containing 0.5, 1.25, 2.5, 3.75, and 5 μg/ml. Aliquots (20 μl) of these solutions (equivalent to 0.01, 0.025, 0.050, 0.075, and 0.1 μg) are injected to establish an external standard curve for the calculation of percent recovery from plasma.

A series of working external standards for the urine assay is prepared by transferring aliquots of 0.020 ml from solution [B] and 0.02, 0.10, 0.20, and 0.40 ml of solution [A] into separate 10-ml volumetric flasks and diluting to volume with methylene chloride—methanol—heptane (85:10:5) to yield solutions containing 0.20, 2.0, 10.0, 20.0, and 40.0 μg/ml. Aliquots (10 μl) of these solutions (equivalent to 0.002, 0.020, 0.1, 0.2, and 0.4 μg) are injected to establish an external standard curve for calculation of percent recovery from urine.

Reagents

All reagents are of analytical grade purity and are prepared in deionized, distilled water. Phosphate buffer (1.0 *M*, pH 10) is prepared by mixing 847 ml 1 *M* $K_2HPO_4 \cdot 3H_2O$ (228.23 g/l) and 153 ml saturated solution Na_3PO_4. Mix well and adjust to pH 10.0 with $KH_2PO_4 \cdot 3H_2O$ (1 *M*) or saturated solution Na_3PO_4 as needed. Other reagents include ammonium hydroxide, ACS grade (J.T. Baker, Phillipsburg, NJ, U.S.A.), methylene chloride, methanol, and heptane (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). The mobile phase is deaerated in an ultrasonic bath prior to use.

Assay in plasma or whole blood

Into a 15-ml stoppered centrifuge tube (PTFE No. 13 stopper) add 1.0 ml unknown plasma (or 0.5 ml oxalated whole blood) and 1 *M* phosphate buf-

fer (pH 10) to make up to a volume of 2.5 ml. Mix well and extract the sample with 6 ml of methylene chloride by slowly shaking for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.). Centrifuge the sample in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon/IEC Corporation, Needham, MA, U.S.A.) at 0—5°C for 10 min at approximately 1200 *g*. Immediately aspirate off as much of the aqueous phase as possible and transfer a 5-ml aliquot of the methylene chloride extract into a tapered 15-ml stoppered centrifuge tube (PTFE No. 13 stopper). Evaporate the methylene chloride extract carefully under a stream of clean, dry nitrogen to dryness at 35—40°C over steam vapor using a N-EVAP evaporator (Organomation, Worcester, MA, U.S.A.). Dissolve the residue in 200 μl of methylene chloride—methanol—heptane (85:10:5) and keep the tube in an ice bath. Inject a 20-μl aliquot for HPLC analysis, Fig. 2A.

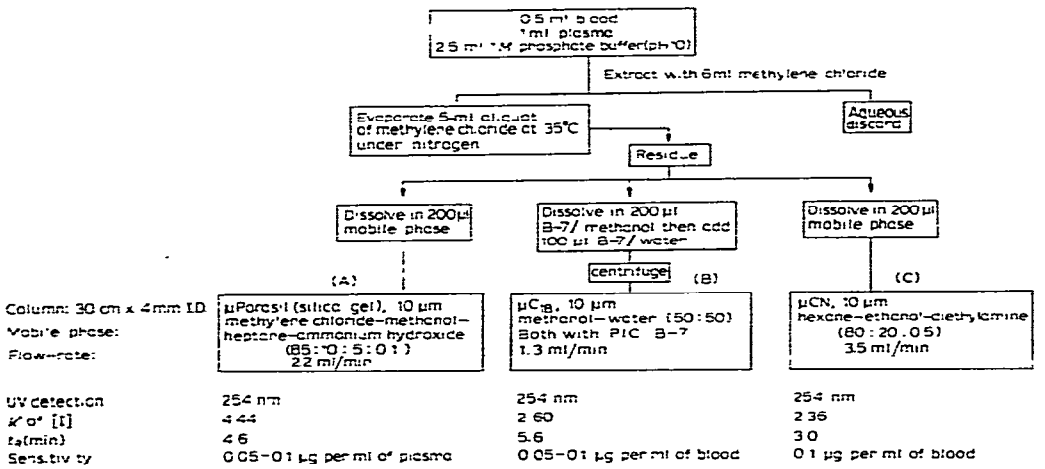


Fig. 2. Flow diagram of HPLC assays for compound [I] in biological fluids. PIC B-7 = 1-heptanesulfonic acid, 0.005 *M*, pH 3.5.

A specimen of 1.0 ml of control plasma and five 1.0-ml aliquots of control plasma containing 0.010, 0.025, 0.050, 0.075, and 0.100 ml of solution [D] (equivalent to 0.1, 0.25, 0.5, 0.75, and 1.0 μg of the compound [I] per ml plasma), respectively, are processed with the samples as recovered standards (Fig. 3). These standards are used to establish a calibration curve for the direct quantitation of the unknowns.

Assay in urine

Into a 15-ml stoppered centrifuge tube, add 0.1 ml urine (0.1 ml of 1:10 dilution of 0—24 h specimen) and sufficient of 1 *M* phosphate buffer (pH 10) to bring the buffer to a volume of 0.5 ml, mix well, and extract the sample with 1 ml of methylene chloride—methanol—heptane (85:10:5) by mixing for 1 min on a Vortex-Genie (Ace Scientific Supply Co., Linden, NJ, U.S.A.). Centrifuge the samples immediately in a refrigerated centrifuge at 0—5°C for 10 min at approximately 1200 *g*. Aspirate off all the aqueous phase, transfer 0.5—0.6 ml of the organic phase in a clean tube and place the tube in an ice bath. Inject a 10-μl aliquot for HPLC analysis.

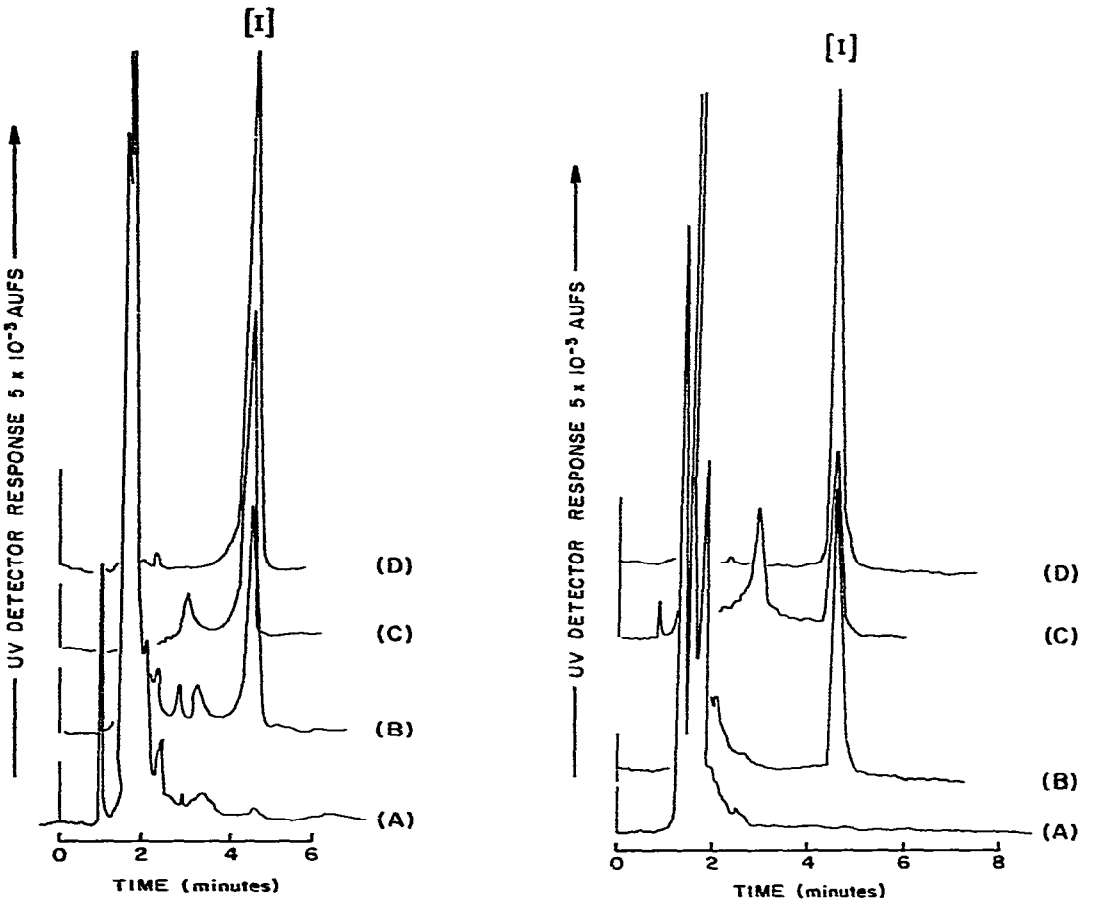


Fig. 3. Chromatograms of HPLC analysis of plasma extracts on a μPorasil silica gel column of (A) control plasma; (B) 6 h dog plasma post dose; (C) control plasma containing added [I] authentic standard, and (D) authentic standard [I].

Fig. 4. Chromatograms of HPLC analysis of urine extracts on a μPorasil silica gel column of (A) control urine; (B) control urine containing added authentic standard [I]; (C) 24—48-h dog urine post dose; and (D) authentic standard [I].

A specimen of 0.1 ml of control urine and five 0.1-ml aliquots of control urine containing 0.200 ml of solution [D] and 0.020, 0.100, 0.200, and 0.400 ml of solution [C] (equivalent to 0.2, 2, 10, 20, and 40 μg of the compound [I] per 0.1 ml urine), respectively, are processed along with the samples as recovered standards (Fig. 4). These standards are used to establish a calibration curve for the direct quantitation of unknowns.

Calculations

The concentration of [I] in μg per ml of plasma or μg per 0.1 ml of urine are determined from their respective standard curves using the peak height vs. concentration technique. For the urine assay, the results are corrected for the appropriate dilutions and calculated as [I] μg per ml of urine.

RESULTS

Statistical validation of the method in plasma and urine

Plasma. The intra-assay linearity and precision of the method was evaluated in plasma over a concentration range of 0.1—2 μg [I] per ml (Table I). Duplicate samples at each concentration of compound [I] were added to 1 ml of plasma, and taken through the analytical procedure. The data shown in Table I are best described by a linear function equation of the form: $y = Bx + A$ ($y = 16.134x + 0.02$) with a correlation coefficient (r) of 0.997, indicating the high degree of linearity of the method. The method showed good precision over the concentration range investigated with an average coefficient of variation of 4.1%. The recovery of compound [I] from plasma was 64.5 ± 4.5% (S.D.) and the sensitivity limit was 0.05—0.1 μg of [I] per ml.

Urine. The intra-assay linearity and precision of the method was evaluated in urine over a concentration range of 2—40 μg per 0.1 ml. Duplicate samples at each of the above concentrations of compound [I] were added to 0.1 ml urine, and then taken through the analytical procedure. The recovery data, shown in Table I, were described by a linear equation at the form: $y = Bx + A$ ($y = 0.443x$—0.45) with a correlation coefficient (r) of 0.998 and with an average coefficient of variation of 3.7%. The recovery of compound [I] from the urine samples was 96.0 ± 6.3% (S.D.) and the sensitivity limit was 1 μg/ml.

Inter-assay linearity and precision data for the method were generated during the stability studies with [I] (Table II). The data showed correlation coefficients (r) of 0.992 and 0.990 with average coefficients of variation

TABLE I

LINEARITY AND INTRA-ASSAY PRECISION OF THE HPLC ASSAY FOR COMPOUND [I] IN PLASMA AND URINE
$N = 2$ in all cases.

Concentration range	Concn. added	Mean concn. found ± S.D.	Coefficient of variation (%)	Mean recovery (%)
Plasma* 0.1—2 μg/ml	0.10	0.107±0.003	2.8	58.4
	0.20	0.237±0.003	1.3	71.3
	0.50	0.473±0.021	4.4	67.5
	1.00	0.960±0.074	7.7	62.5
	2.00	2.015±0.082	4.1	63.1
Mean ± S.D.			4.1	64.5±4.5
Urine** 2—40 μg/0.1 ml	2.00	2.36±0.15	6.5	83.3
	4.00	4.07±0.35	8.7	100.0
	10.00	10.3 ±0.35	3.5	100.0
	20.00	19.1 ±0.14	0.7	100.0
	30.00	29.4 ±0.52	1.8	92.9
	40.00	40.8 ±0.50	1.2	100.0
Mean ± S.D.			3.7	96.3±6.3

*$y = 16.13x + 0.02$ ($r = 0.997$).
**$y = 0.443x - 0.45$ ($r = 0.998$).

TABLE II

LINEARITY AND INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR COMPOUND [I] IN PLASMA AND URINE

Concentration range	*N*	Concn. added	Mean concn. found ± S.D.	Coefficient of variation (%)	Mean recovery (%)
Plasma* 2—6 μg/ml	15	2.00	1.98±0.09	4.4	60.3±7.2
	3	3.00	3.03±0.07	2.4	58.6±4.2
	17	4.00	4.02±0.21	5.2	70.1±6.8
	15	5.00	5.01±0.15	3.0	74.6±12.5
	9	6.00	5.96±0.32	5.4	69.3±7.5
Mean ± S.D.				4.1	66.6±7.6
Urine** 0.1—0.5 μg/0.1 ml	20	0.100	0.105±0.015	14.2	59.5±14.1
	20	0.200	0.192±0.017	8.6	60.5±10.4
	19	0.300	0.299±0.020	6.6	61.5±9.7
	21	0.400	0.405±0.027	6.6	60.3±18.3
	19	0.500	0.498±0.018	3.6	60.0±8.8
Mean ± S.D.				7.9	60.4±12.3

*$y = 4.91x - 2.75$ ($r = 0.992$). Each determination was combined from eight different dates of assay.
**$y = 24.74x - 0.07$ ($r = 0.990$). Each determination was combined from ten different dates of assay.

of 4.1 and 7.9% for plasma and urine, respectively. The overall recoveries were 66.6 ± 7.6% and 60.4 ± 12.3% in the plasma and urine, respectively. Lower recoveries of [I] were measured in the urine in the inter-assay precision study at concentrations of 0.1—0.5 μg per 0.1 ml as compared to the intra-assay precision study at concentrations of 2—40 μg per 0.1 ml. This lower range of concentrations, near the sensitivity limit of the assay, was selected to measure [I] in the urine at the concentrations anticipated in man.

Stability evaluation of [I] in biological samples

The ionization constants of [I] and [II] (pK_a values, see Fig. 1) are approximately equal which precludes differential solvent extraction of the compounds from each other. The determination of the stability of [I] in biological samples as indicated by its recoverability by HPLC analysis and the rate of formation of [II] measured by radioimmunoassay (RIA) [4] is described below:

Preparation of pooled biological samples. Four 50-μl aliquots of stock solution [A] were added to four separate 10-ml aliquots of control human plasma and combined (total 40 ml) to yield a pooled plasma sample containing 5 μg [I] per ml. The pooled plasma sample was then subdivided into 1-ml aliquots in 15-ml glass-stoppered tubes and stored frozen at —17°C.

Aliquots of 350 μl and 300 μl of standard solution [B] were added to 10 ml of control human urine to yield two pools containing 0.35 μg [I] per 0.1 ml and 0.30 μg [I] per 0.1 ml for stability studies at —17°C and —90°C, respectively. The pooled urine sample was subdivided into 0.1-ml aliquots

in 15-ml polypropylene centrifuge tubes and stored at —17°C and —90°C (Revco Ultra-low temperature freezer, Rheem Refrigeration Products Division, Columbia, SC, U.S.A.).

Assay procedure. Normal-phase HPLC as described in the experimental section was used for analysis which was carried at fixed time interval using five individual plasma or urine test samples which had been stored at —17°C or —90°C.

A stock solution containing 100 μg of the free base of [II] per ml in methanol was prepared by weighing 11.588 mg of [II]·HCl, ($C_9H_9Cl_2N_3$·HCl, mol. wt. = 266.54) into a 100-ml volumetric flask and dissolving with methanol. Four individual series of freshly spiked plasma and urine standards prepared from solutions of [I] and [II] individually in 1 *M* phosphate buffer (pH 10.0) containing 2—6 μg [I] and 0.2—2 μg [II] per ml of plasma, respectively, and 0.1—0.5 μg [I] and 0.05—0.2 μg [II] per 0.1 ml of urine, respectively, were processed with each set of pooled samples for measurement of [I] and to determine the rate of formation of [II].

Inter-assay linearity and precision data for [II] using the assay yielded correlation coefficients (r) of 0.998 and 0.998, and coefficients of variation of 4.2% and 2.5%, respectively, for plasma and urine. The recovery of [II] from the plasma and urine was 86.1 ± 9.9% (S.D.) and 76.1 ± 0.8% (S.D.), respectively (Table III). Typical chromatograms of [II] from the stability evaluation in plasma and urine are shown in Figs. 5 and 6, respectively.

TABLE III

LINEARITY AND INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR COMPOUND [II] IN PLASMA AND URINE

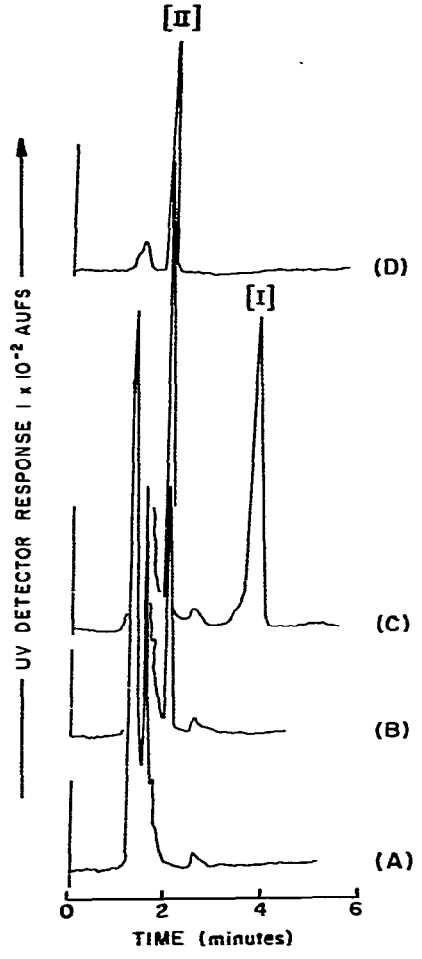
Concentration range	*N*	Concn. added	Mean concn. found ± S.D.	Coefficient of variation (%)	Mean recovery (%)
Plasma* 0.2—2 μg/ml	2	0.200	0.193±0.004	2.1	73.5
	2	0.500	0.504±0.043	8.5	74.5
	2	1.00	1.00 ±0.039	3.9	94.4
	2	1.50	1.50 ±0.039	2.6	94.4
	2	2.00	1.99 ±0.074	3.7	93.8
Mean ± S.D.				4.2	86.1±9.9
Urine** 0.05—0.2 μg/0.1 ml	4	0.050	0.050±0.002	4.0	75.8
	4	0.100	0.100±0.002	2.0	75.4
	4	0.150	0.148±0.003	2.0	75.8
	4	0.200	0.201±0.004	2.0	77.5
Mean ± S.D.				2.5	76.1±0.8

*$y = 12.9x - 0.038$ ($r = 0.998$).

**$y = 90.5x - 0.24$ ($r = 0.998$). Each determination was combined from two different dates of assays.

Stability results

The plasma samples stored at —17°C showed no significant degradation for seven days (Table IV). Decomposition of 4.4—19.2%, measured as loss



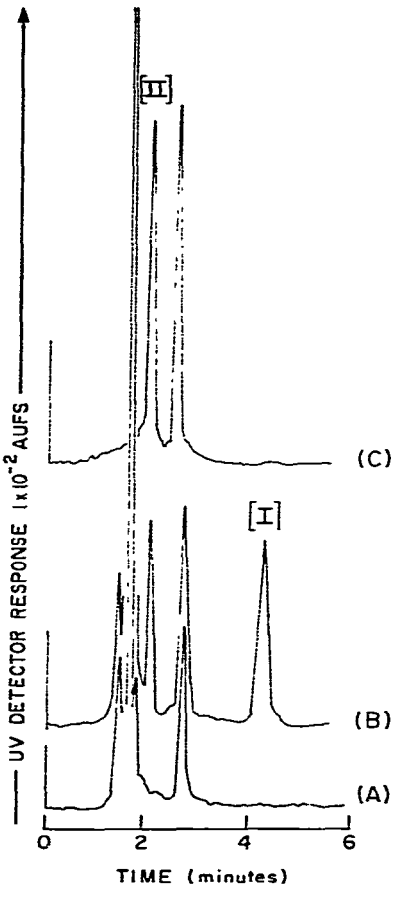


Fig. 5. Chromatograms of HPLC assay of plasma containing added [I], stored at —17°C for stability evaluation: (A) control plasma, (B) control plasma containing added [II] authentic standard, (C) plasma stability sample on day 49, and (D) authentic standard [II].

Fig. 6. Chromatograms of HPLC assay of urine containing added [I], stored at —17°C for stability evaluation: (A) control urine, (B) urine stability sample on day 43, and (C) control urine containing added [II] authentic standard.

of [I], was found from the 24th to 70th day. The degradation product [II] had the same retention volume as authentic clonidine measured by HPLC. Aliquots of this plasma pool assayed by RIA showed an increase in "clonidine like" material of 9 to 16% on the 35th to 45th day [4]. The good agreement between the non-specific RIA and the specific HPLC assay indicated that the product measured by RIA is predominantly clonidine [II].

The urine samples stored at —17°C showed as much as 52% breakdown over a 43-day period (Table V). Only a small portion (11—17%) could be accounted for as [II] by HPLC which suggests other routes of degradation. The urine samples stored at —90°C showed complete stability over a 68-day period (Table V). A third study demonstrated complete stability of [I] in

TABLE IV

STABILITY OF [I] IN PLASMA, STORED AT −17°C DETERMINED BY HPLC ANALYSIS

The pooled plasma concentration was 5.00 μg/ml, for each determination.

Day of study	N	Amount [I] found ± S.D.	[I] lost (%)
0	5	4.90±0.19	n.m.*
1	4	5.00±0.12	n.m.
7	5	5.10±0.14	n.m.
24	5	4.78±0.09	4.4
49	5	4.07±0.13	18.6
70	5	4.04±0.06	19.2

*Non-measurable.

TABLE V

STABILITY OF [I] AND ESTIMATION OF [II] IN URINE STORED AT −17°C AND −90°C

Storage temperature	Duration of storage (days)	Pooled urine conc. (μg/0.1 ml)	N	Amount [I] found ± S.D.	[I] lost (%)	[II] found (%)
−17°C	0	0.35	5	0.35±0.016	—	—
	1	0.35	4	0.33±0.007	(5.7)	n.m.*
	7	0.35	5	0.25±0.012	28.6	n.m.
	14	0.35	5	0.21±0.030	40.0	10.9
	35	0.35	5	0.17±0.020	51.4	15.1
	43	0.35	5	0.17±0.017	52.0	16.9
−90°C	0	0.30	5	0.30±0.021	—	—
	1	0.30	5	0.32±0.014	—	—
	13	0.30	5	0.30±0.013	—	—
	27	0.30	5	0.34±0.032	—	—
	48	0.30	4	0.32±0.030	—	—
	68	0.30	5	0.29±0.020	—	—

*Non-measurable.

urine for a period of 2 h at 37°C and approximately 20% degradation during the third hour. This would indicate that urine specimens should be frozen immediately on collection.

Application of the method to biological specimens

The assay was applied to the quantitation of [I] in plasma and urine samples (Figs. 3 and 4) in a dog following the oral administration of 28.8 mg of [I] · maleate ($C_9H_{11}Cl_2N_3S \cdot C_4H_6O_4$, mol. wt. = 382.17) per kg of body weight. The samples were frozen (−17°C) upon collection and assayed within one week. Following this dose, a peak plasma concentration of 1.9 μg [I] per ml was measured at 1 h and declined to nonmeasurable amounts

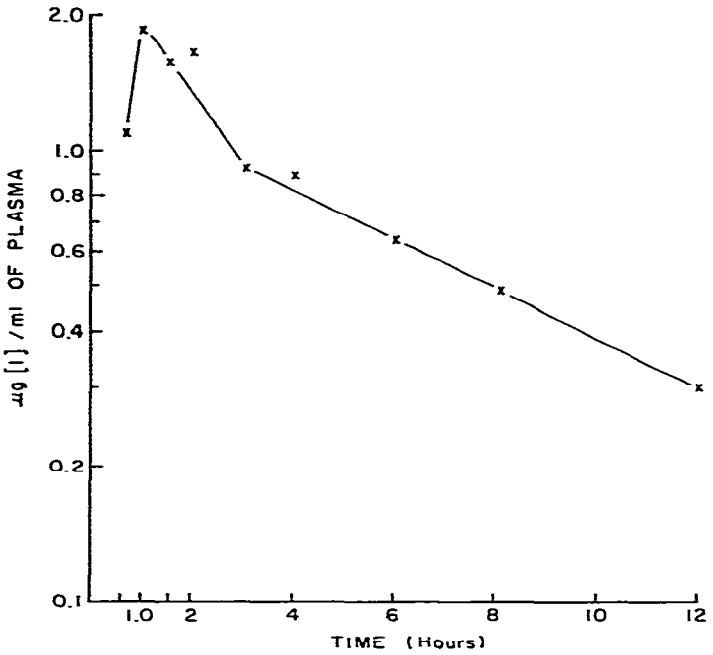


Fig. 7. Plasma level fall-off curve in a dog following oral administration of 28.8 mg [I]· maleate per kg dose, equivalent to 20 mg of free base per kg.

(⩽0.05 μg/ml) after 12 h (Fig. 7). Concentrations of [II] were non-measurable (⩽0.2 μg/ml) in the plasma throughout the experimental period.

The urinary excretion of [I] following this dose was 52.3 and 0.4% of the dose recovered as [I] in the 0—24 and 24—48 h periods, respectively. Non-measurable amounts (⩽2 μg/ml) were observed in the 48—72 h excretion period.

DISCUSSION

Thermal rearrangement of [I] to clonidine [II] in the injection port precludes GLC analysis of the parent drug since it is also metabolized to [II] in vivo, and therefore would result in a non specific assay. Clonidine [II] per se can, however, be analyzed by electron-capture GLC utilizing the pentafluorobenzyl [5] and diheptafluorobutyryl [6] derivatives of the compound. Gas chromatographic—mass spectrometric assays based on selective ion monitoring of clonidine [II] and its deuterated internal standard by electron-impact ionization as either the intact compound [7] or as the dimethyl derivative produced by on-column methylation with trimethylanilinium hydroxide [8] have also been used to determine [II] in plasma. These assays [5, 8] are capable of measuring as little as 25 pg/ml using a 4-ml plasma sample. Plasma concentrations of [II] have been measured in man following oral doses of 50—300 μg of [II] · hydrochloride [6, 9—11]. Due to the aforementioned problems, compound [I] had to be determined by a specific HPLC

assay capable of resolving [I] from [II] and other potential metabolites and/or breakdown products.

Development of normal-phase HPLC analysis

The choice of 254 nm for measurement of [I] and [II] is based on their UV spectra in methylene chloride—methanol—heptane—ammonium hydroxide (85:10:5:0.1) which showed maxima at 247 and 250 nm, respectively.

The strongly basic nature of [I] and [II] required extraction at alkaline pH and the use of either a basic mobile phase for normal-phase (adsorption) chromatography or "paired ion" chromatography for reversed-phase HPLC analysis. Both techniques were investigated and normal-phase HPLC was selected eventually as the method of choice since it yielded the best resolution and chromatographic performance in biological fluids.

Studies were initially conducted on the use of normal-phase (adsorption) chromatography utilizing a 30 cm × 4 mm I.D. stainless-steel column containing 10 μm μBondapak CN (chemically bonding a cyano group to μPorasil, 9% by weight, Waters Assoc.) and a mobile phase of hexane—ethanol—diethylamine (80:20:0.5) (Fig. 2C). This procedure provided a solvent system for the assay of [I] in blood, in which the lipid materials in the sample residue are totally soluble in the mobile phase system. The assay is straightforward and obviates the need for clarification of the reconstituted sample extract usually required for reversed-phase HPLC. Normal-phase HPLC analysis at a flow-rate of 3.5 ml/min with the above mobile phase and a head pressure of 950 p.s.i., resulted in a capacity factor (k') for compound [I] of 2.36. The overall recovery of [I] is 77.6 ± 7.4% (S.D.) in the range of 0.05—5 μg per 0.5 ml blood, with a limit of detection of 0.1 μg/ml of blood using 0.5 ml per assay.

Unfortunately, the chromatographic response became nonreproducible following continuous use with this mobile phase, possibly due to the hydrolysis of the cyano groups at the high alkalinity of 0.5% diethylamine in the solvent system and further work using the μBondapak CN column was discontinued.

The successful use of small amounts of concentrated ammonium hydroxide (< 0.5%) as a modifier in the mobile phase for use with silica columns in normal-phase HPLC analysis is well-documented [12—15]. The substitution of concentrated ammonium hydroxide for diethylamine in the mobile phase led to the successful use of methylene chloride—methanol—heptane—ammonium hydroxide (85:10:5:0.1) as the mobile phase for HPLC and resulted in the assay previously described for the preclinical evaluation of [I] in plasma and urine in dog specimens.

Preliminary investigation of reversed-phase HPLC analysis

This mode of analysis is generally used for the determination of polar, ionizable (cationic, anionic or zwitterionic) compounds, which usually chromatograph poorly (as broad tailing peaks) by normal-phase chromatography.

The use of an appropriately buffered mobile phase renders the molecule in a non-ionized "lipophilic" state enabling retention on chemically bonded octadecylsilane (ODS-C_{18}) columns.

Preliminary investigations for the analysis of [I] in body fluids involved reversed-phase chromatography, with water—methanol—acetic acid (80:20:0.5) as the mobile phase, and a 30 cm × 4 mm I.D. stainless-steel column containing 10 μm μBondapak C_{18} (Waters Assoc.). The parent drug [I] was extracted into methylene chloride from blood or plasma buffered to pH 10.0 with 1 *M* phosphate buffer, the residue of which was reconstituted in water—methanol of varying composition. It was noted that coextracted lipids were insoluble in the water—methanol mixtures, resulting in a colloidal suspension which interfered with HPLC analysis.

The reversed-phase chromatographic system was then modified in an attempt to increase the solubility of the residue by increasing the methanol concentration and by the introduction of an ion-pair reagent as the counter-ion. A mobile phase of water—methanol (50:50) containing heptanesulfonic acid (PIC Reagent B-7, Waters Assoc.) with an ionic strength of 0.005 *M* (pH 3.5) was found to be suitable. The assay required reconstitution of the methylene chloride extract in 200 μl of 0.005 *M* heptanesulfonic acid in methanol by vortex mixing, followed by the addition of 100 μl of 0.005 *M* heptanesulfonic acid in water. The lipids which were coextracted from the sample still precipitated out upon addition of the aqueous solution. The sample was clarified by centrifuging at approximately 1200 *g* to obtain a clear supernatant, 30 μl of which were assayed by HPLC (Fig. 2B). The mobile phase and μBondapak C_{18} column previously described were used at a flow-rate of 1.3 ml/min, and a head pressure of 2.1 × 10^3 p.s.i., and gave a capacity factor (k') for compound [I] of 2.60. The overall recovery of [I] was 83.0 ± 5.7% (S.D.) with a limit of detection of 0.05 μg [I] per ml of plasma or 0.1 μg [I] per ml of blood. However, this procedure was very time-consuming since the mobile phase required filtration through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) prior to use and the reconstituted sample extracts required clarification by centrifuging prior to analysis.

Attempts to increase the sensitivity of measurement by post-column derivatization of [I] with fluorescamine (Fluram, Hoffmann-La Roche, Nutley, NJ, U.S.A.) following the reversed-phase HPLC using mixtures of water, methanol, and acetic acid were unsuccessful. Losses in sensitivity were observed due to the need to dilute the acidic HPLC effluent with an alkaline reagent prior to derivatization. Compound [II] does not react with fluorescamine.

CONCLUSIONS

A normal-phase HPLC assay is described for the measurement of [I] in plasma and urine with sufficient sensitivity and specificity to measure the drug in preclinical bioavailability and toxicology studies. Based on the stability of the drug in biological fluids, it is recommended that the samples be analyzed within one week if stored at −17°C and that storage for extended periods of time should be at −90°C. All samples should be analyzed expeditiously after thawing.

ACKNOWLEDGEMENT

The authors wish to thank Mr. T. Daniels for the drawings of the figures presented, and Mesdames W. Morley and V. Waddell for the preparation of this manuscript.

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