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DETERMINATION OF CIBENZOLINE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive and selective high-performance liquid chromatographic (HPLC) assay was developed for the determination of cibenzoline (Cipralan™) in human plasma and urine. The assay involves the extraction of the compound into benzene from plasma or urine buffered to pH 11 and HPLC analysis of the residue dissolved in acetonitrile—phosphate buffer (0.015 mol/l, pH 6.0) (80:20). A 10-μm ion-exchange (sulfonate) column was used with acetonitrile—phosphate buffer (0.015 mol/l, pH 6.0) (80:20) as the mobile phase. UV detection at 214 nm was used for quantitation with the di-*p*-methyl analogue of cibenzoline as the internal standard.

The recovery of cibenzoline in the assay ranged from 60 to 70% and was validated in human plasma and urine in the concentration range of 10—1000 ng/ml and 50—5000 ng/ml, respectively. A normal-phase HPLC assay was developed for the determination of the imidazole metabolite of cibenzoline. The assays were applied to the determination of plasma and urine concentrations of cibenzoline and trace amounts of its imidazole metabolite following oral administration of cibenzoline succinate to two human subjects.

INTRODUCTION

Cibenzoline [I] (Cipralan™), 4,5-dihydro-2-(2,2-diphenylcyclopropyl)-1H-imidazole (Fig. 1) is a member of a new class of antiarrhythmics [1].

An electron-capture gas—liquid chromatographic (EC—GLC) procedure for the determination of [I] in plasma and urine was reported [2]. It requires the extraction of the compound into diethyl ether from plasma or urine buffered to pH 10.6, back-extraction into 0.1 *N* hydrochloric acid, readjustment of the sample pH to 10.6 and re-extraction into benzene, the residue of which is reacted with trifluoroacetic anhydride (TFAA) to yield the N-trifluoroacetyl (TFA) derivative for EC—GLC analysis. The internal standard for the assay is

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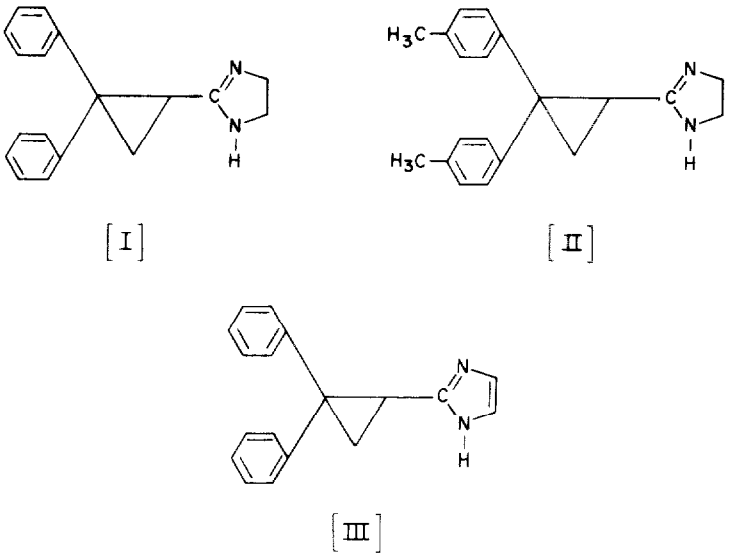

[I] [II] [III]

Fig. 1. Chemical structures of cibenzoline [I], di-*p*-methylcibenzoline (internal standard) [II] and imidazole metabolite [III].

the di-*p*-methyl analogue of cibenzoline 2-[2,2-bis(4-methylphenyl)-1-cyclopropyl]-4,5-dihydro-1H-imidazole [II]. The assay has a sensitivity limit of 10 ng [I] per ml of plasma, and was used in clinical pharmacokinetic studies for the analysis of plasma and urine following single intravenous and oral doses of [I] · succinate (Cipralan) [2].

The present work describes a high-performance liquid chromatographic (HPLC) assay for [I] as the intact compound in plasma and urine utilizing ion-exchange chromatography on a sulfonate column [3] with UV detection at 214 nm. The HPLC procedure is simpler and more expeditious for high sample throughput than the reported EC—GLC assay for [1] due to the elimination of the back-extraction and derivatization steps. The sensitivity limit (10 ng/ml) and precision of the HPLC and EC—GLC assays are equivalent. The HPLC assay also utilizes the di-*p*-methyl analogue of cibenzoline [II] as the internal standard.

The assay was applied to the determination of plasma and urine concentrations of [I] in two human subjects following oral administration of [I] · succinate.

A normal-phase HPLC assay is also described for the determination of the imidazole metabolite [III] of cibenzoline [4] in plasma and urine.

EXPERIMENTAL

Column

The column used for ion-exchange HPLC analysis was a 25 cm × 4.6 mm I.D. stainless-steel column prepacked with 7—8 μm Zorbax SCX (Dupont Instruments, Wilmington, DE, U.S.A.), which was jacketed in a styrofoam block.

Instrumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model 710B automatic sample injector (WISP) and a Model 441 UV detector equipped with a 214-nm zinc source and wavelength kit (Waters Assoc., Milford, MA, U.S.A.). For the plasma assay the UV detector was operated at 0.05 a.u.f.s. Two low-pass filtered (3 sec time constant) output responses were monitored using a four-channel Model 3314 recorder (Soltec Corporation, Sun Valley, CA, U.S.A.) at 2 and 10 mV/full scale deflection (equivalent to 0.01 and 0.05 a.u.f.s.). For the urine assay the detector was operated at 0.2 a.u.f.s. Three low-pass filtered output responses were monitored at 1, 5 and 10 mV/full scale deflection (equivalent to 0.02, 0.1 and 0.2 a.u.f.s.).

All reservoir-to-pump tubing was changed from Teflon FEP to polyethylene-lined ethylvinyl acetate tubing (Bev-A-Line V-HT, Thermoplastic Scientific, Warren, NJ, U.S.A.) (see Results and discussion).

Analytical standards

Compound [I], cibenzoline; 4,5-dihydro-2-(2,2-diphenylcyclopropyl)-1H-imidazole, $C_{18}H_{18}N_2$, M.W. = 262.33, compound [II], 2-[2,2-bis(4-methylphenyl)-1-cyclopropyl]-4,5-dihydro-1H-imidazole, $C_{20}H_{22}N_2$, M.W. = 290.39 and compound [III], 2,2-diphenylcyclopropyl-1H-imidazole, $C_{18}H_{16}N_2$, MW = 260.32, m.p. = 218—220°C, of pharmaceutical grade purity (>99%) are used as analytical standards (Fig. 1).

Prepare stock solution A, 1.00 mg of [I] per ml by dissolving 50.0 mg of [I] in 50 ml acetonitrile. Dilute stock solution A 1:10 with acetonitrile to prepare intermediate solution B containing 100 μg [I] per ml. Working standards (Numbers 1 to 10) are prepared in 10 ml of acetonitrile by taking aliquots of A or B indicated below:

Solution No.	Amount of Solution A or B (μl)	Final concentration (ng per 50 μl)
1	20 B	10
2	50 B	25
3	100 B	50
4	200 B	100
5	500 B	250
6	1000 B	500
7	150 A	750
8	200 A	1000
9	400 A	2000
10	1000 A	5000

Prepare stock solution C equivalent to 1.00 mg of [II] per ml by dissolving 50.0 mg of [II] in 50 ml acetonitrile. A working standard D of 100 ng per 50 μl [II] is prepared by diluting 100 μl stock solution C to 50 ml with acetonitrile. Working standards are routinely prepared every two weeks from the stock solution, and a fresh stock solution prepared once a month.

Reagents

Reagent grade chemicals are used to prepare: 1 mol/l phosphate buffer, pH 6.0 (prepared by mixing 430 ml of 1 mol/l orthophosphoric acid with 570 ml of 1 mol/l dipotassium dihydrogen orthophosphate and adjusting to pH 6.0), and 1 mol/l phosphate buffer, pH 11.0 [prepared by mixing 530 ml of 1 mol/l dipotassium hydrogen phosphate with 470 ml of saturated (0.7 mol/l) trisodium phosphate and adjusting the pH to 11.0]. Other reagents include acetonitrile (UV grade) and benzene (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

The mobile phase for ion-exchange HPLC is composed of acetonitrile—0.015 mol/l phosphate buffer, pH 6.0 (80:20), prepared by mixing 6.0 ml of a 1 mol/l phosphate buffer, pH 6.0, with 394.0 ml distilled water and diluting to 2 l with acetonitrile. All mobile phases are vacuum degassed prior to use and kept under constant sparging with helium (99.995%). A solution of acetonitrile—water (80:20) is used to flush the phosphate buffer from the ion-exchange column at the completion of a day's analysis to prevent salt build up.

Chromatographic parameters

The flow-rate for the ion-exchange HPLC assay is 1.5 ml/min with a resulting pressure of 4.8 MPa (700 psi), and retention times for [I] and [II] of 5.4 min ($k' = 5.0$) and 4.4 min ($k' = 3.9$), respectively.

Assay sample preparation

Into a 13 × 100 mm disposable borosilicate culture tube (Cat. No. 14-962-10C, Fisher Scientific, Springfield, NJ, U.S.A.) place 50 µl of solution D (100 ng of [II]), 1.0 ml of plasma or urine and 2.0 ml of 1 mol/l phosphate buffer, pH 11.0. (Because of the high concentrations of [I] in urine during the 0—24 h excretion interval, only 0.1 ml is assayed for these samples). Mix well on a Vortex mixer and add 2.5 ml of benzene using a repipet (Model No. 3020-GR, Lab Industries, Berkeley, CA, U.S.A.), stopper using a polyethylene stopper (Cat. No. 127-0019-000, Elkay Products, Shrewsbury, MA, U.S.A.) and then shake on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80 to 100 strokes per min for 10 min, followed by centrifugation at 1460 *g* for 10 min at 10°C (Model PR-J rotor No. 253, Damon/IEC Corp., Needham, MA, U.S.A.). Transfer 2.0 ml of the supernatant to another 13 × 100 mm culture tube and evaporate to dryness in a 65°C water bath under a stream of nitrogen (N-EVAP evaporator, Organomation Assoc., Worchester, MA, U.S.A.). Reconstitute th residue in 400 µl of mobile phase and transfer to a 1.5-ml polypropylene micro test tube (Cat. No. 696, W. Sarstedt, Princeton, NJ, U.S.A.) and cap (Cat. No. A-123, Kew Scientific, Columbus, OH, U.S.A.). Insert the capped micro test-tube into a glass shell vial (Cat. No. 60930-L, Kimble Glass, Toledo, OH, U.S.A.) making sure that the flange of the test-tube is inside the vial not resting on the lip of the vial. Place all vials in the auto-injector carousel.

HPLC analysis of plasma

Inject a 50-µl aliquot of the contents of the auto-sampler vial by automatic injection using pre-programmed parameters. Along with the samples process a

1.0-ml specimen of control plasma and eight 1.0-ml specimens of the same control plasma to which 50 μl of solutions No. 1—8 and 50 μl of solution D (internal standard) have been added (equivalent to 10.0, 25.0, 50.0, 100.0, 250.0, 500.0, 750.0 and 1000.0 ng [I] per ml). These standards are used to establish a least-squares regression calibration curve using a power function for the quantitation of the unknowns using peak height ratio of [I]/[II] vs. concentration of [I] (ng/ml). Appropriate corrections for any changes in sample aliquot or dilutions must be made.

A set of external standards of [I] prepared by adding and mixing 50 μl of solutions No. 1–8, 50 μl of solution D (100 ng of [II]) and 300 μl of mobile phase are also assayed. These samples are used to standardize the chromatographic system and to calculate percent recovery. Typical chromatograms following automatic sample injection are shown in Fig. 2 for (A) control plasma extracts, (B) an extract from a subject following the administration of [I] · succinate, and (C) control plus added standards.

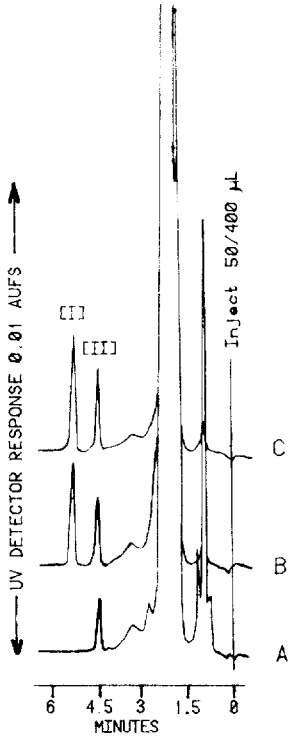

Fig. 2. Ion-exchange chromatograms of human plasma: (A) control containing 100 ng [II] per ml; (B) subject, 18 hours, day 12 following multiple oral dosing of [I] · succinate (see text); and (C) control containing 100 ng [I] per ml and 100 ng [II] per ml (internal standard).

HPLC analysis of urine

The procedure for the HPLC analysis in urine is the same as for the plasma assay except for the concentration range of the standards used to establish a least-squares regression calibration curve. These are prepared by adding 50 μl

of solutions No. 3—10 (equivalent to 50.0, 100.0, 250.0, 500.0, 750.0, 1000.0, 2000.0, and 5000,0 ng [II] per ml) and 50 μl of solution D to 1.0 ml of control urine. Solutions No. 3—9 are also used in a similar manner for the preparation of the external standards.

RESULTS AND DISCUSSION

Analytical parameters

Due to the absorbance of oxygen at 214 nm it was necessary to include precautions against oxygen diffusing into the aqueous mobile phase. The mobile phase was rendered free of oxygen by constant sparging with helium and the replacement of the standard Teflon FEP tubing with non-oxygen permeable polyethylene-lined ethylvinyl acetate tubing.

The efficiency of the ion-exchange HPLC column decreased with continuous use resulting in a diminution of the peak height response and resolution of compounds [I] and [II]. This loss in sensitivity and resolution limited the useful lifespan of the column to approximately 2—3 months. It is therefore essential that column performance be monitored routinely using external calibration standards. The column was jacketed in a styrofoam block to maintain better column temperature (25°C) regulation, which provided a relatively noise-free baseline.

The precision and accuracy of the plasma and urine assays were determined by the construction of least-squares regression calibration curves (power function) from the responses of the recovered standards. Mean intra- and inter-assay precision for the plasma assay and intra-assay precision for the urine assay are typically 4—5% (Table I). Definitive inter-assay data are presently unavailable for the urine assay; however, preliminary results indicate comparable precision and accuracy. The validated sensitivity limits for [I] were 10.0 ng/ml and 50.0 ng/ml of plasma or urine, respectively, using a 1.0-ml sample for analysis.

TABLE I

STATISTICAL VALIDATION OF THE ION-EXCHANGE HPLC ASSAY FOR [I] IN PLASMA AND URINE

Intra-assay — plasma				Inter-assay — plasma			Intra-assay — urine		
Amount added (ng/ml)	Amount found ± S.D. (ng/ml)	C.V. (%)	*n*	Amount found ± S.D. (ng/ml)	C.V. (%)	*n*	Amount found ± S.D. (ng/ml)	C.V. (%)	*n*
10.0	11.6 ± 1.0	8.5	3	10.1 ± 0.4	4.1	4	—		
25.0	24.0 ± 1.9	7.9	3	25.7 ± 0.8	2.9	4	—		
50.0	44.2 ± 0.7	1.5	3	47.7 ± 1.9	3.9	4	51.1 ± 0.4	8.5	3
100.0	93.3 ± 0.8	0.9	3	98.9 ± 1.0	1.1	4	103.3 ± 6.1	5.9	3
250.0	251.8 ± 5.2	2.1	2	252.8 ± 20.5	8.1	4	—		
500.0	513.9 ± 35.3	6.9	3	496.8 ± 23.3	4.7	4	472.1 ± 5.5	1.2	3
750.0	774.7 ± 19.9	2.7	3	747.2 ± 25.2	3.4	4	—		
1000.0	1032 ± 60	5.8	3	1022 ± 37	3.6	4	1024 ± 27	2.6	2
5000.0	—			—			5295 ± 350	6.6	3
	Average =	4.5%		Average =	4.0%		Average =	5.0%	
	$y = 0.06899\ x^{0.9474}$						$y = 1.315\ x^{0.997}$		
	$r = 0.999$						$r = 0.999$		

The recovery of [I] from human plasma and urine using the described assay was determined in the concentration range of 10 to 1000 ng/ml of plasma and 50 to 5000 ng/ml urine by substitution of the peak height obtained for these samples into the equation describing the calibration curve of the external (non-recovered) standards (see above); with the appropriate correction for the sample aliquot injected (50/400 μl) and for the extraction aliquot factor (2.0/2.5). The recovery of compound [I] in plasma and urine ranged from 60 to 70%.

Selectivity

Metabolite investigation has shown that [I] is biotransformed to [III] [4]. A preliminary method for cibenzoline utilized a reversed-phase HPLC assay using a 10-μm C-18 column with phosphate buffer, pH 3.5—methanol as the mobile phase. This assay showed that compounds [I] and [III] could not be chromatographically resolved. The reversed-phase assay was replaced by the ion-exchange assay to permit the specific quantitation of [I], in which metabolite [III] elutes in the solvent front. Attempts to quantitate [I] and [III] simultaneously by either reversed-phase, normal-phase or ion-exchange HPLC were not successful, hence a separate normal-phase HPLC assay was used for the quantitation of [III] in the benzene extract (see below).

The normal-phase assay for [III] differs from the ion-exchange assay for [I] mainly by the use of a 30 cm × 3.9 mm I.D. stainless-steel column prepacked with 10 μm Porasil (Waters Assoc.) [mobile phase: heptane—ethanol—concentrated ammonium hydroxide (90:10:1)], a SpectroMonitor III variable-wavelength UV detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.) operated at 229 nm at 0.01 a.u.f.s. and a Hewlett-Packard (Paramus, NJ, U.S.A.) Model 7132 dual-channel recorder with 10 mV output. The residue of the benzene extract is reconstituted in 200 μl of heptane—ethanol (90:10), 20 μl of which are injected for analysis. Using these chromatographic parameters, the retention time for [III] was 6.02 min (k' = 2.1). The precision and accuracy of the normal-phase methods for plasma and urine were determined in a similar manner to that described for the ion-exchange methods except for the use of linear (instead of power function) least squares regression calibration curves:

$y = 29.16x + 0.046$, $r = 0.999$ (plasma)

$y = 62.52x + 0.45$, $r = 0.999$ (urine)

where y = peak height and x = concentration of [III] per ml of plasma or urine.

The recovery of [III] from human plasma and urine using the normal-phase HPLC assay was 88.5 ± 8.7% (S.D.) (n = 11) in plasma and 82.1 ± 7.3% (S.D.) (n = 5) in urine.

The sensitivity limit was 10.0 ng/ml of [III] using a 1.0-ml sample of human plasma and 50.0 ng/ml of [III] using a 1.0-ml sample of human urine. The intra-assay coefficient of variation (C.V.) for the analysis of [III] over the concentration ranges 0.01—0.5 and 0.05—2.5 μg/ml for plasma and urine, respectively, was approximately 5%. Typical chromatograms following automatic sample injection are shown in Fig. 3 for (A) control plasma extracts, (B)

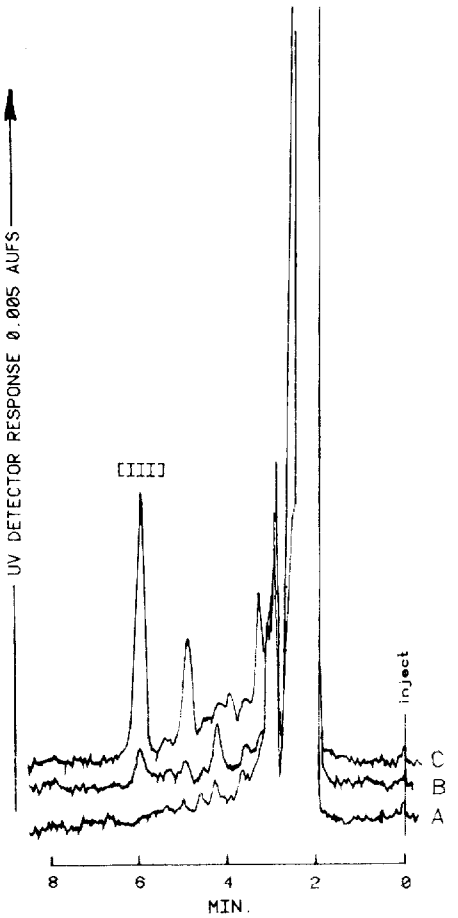

Fig. 3. Normal-phase chromatograms of human plasma: (A) control (20/200 μl injection aliquot); (B) subject, 2-hours, day 12, following multiple oral dosing of [I] · succinate (20/200 μl injection aliquot) (see text); and (C) control containing 250 ng [III] per ml (10/200 μl injection aliquot).

an extract from a subject following the administration of [I] · succinate, and (C) control plus added standards.

Application in biological samples

Plasma samples were collected immediately prior to and at 2, 6, 12, 18, 24, 48, and 72 h after the last 81.25-mg oral dose from two patients receiving cibenzoline · succinate for three days at each dose of 32.5 mg, 65.0 mg, and 81.25 mg q.i.d. The concentrations of [I] were measurable up to 72 h in subject 1 and up to 48 h in subject 2, with maximum observed plasma concentrations of 741 and 650 ng/ml (Fig. 4).

The ratio of metabolite [III]/cibenzoline [I], determined in subject 2 using the normal-phase HPLC assay showed only trace concentrations, i.e., 2—4% of [I] after chronic oral administration.

Concentrations of [I] and [III] in urine were also determined in a subject

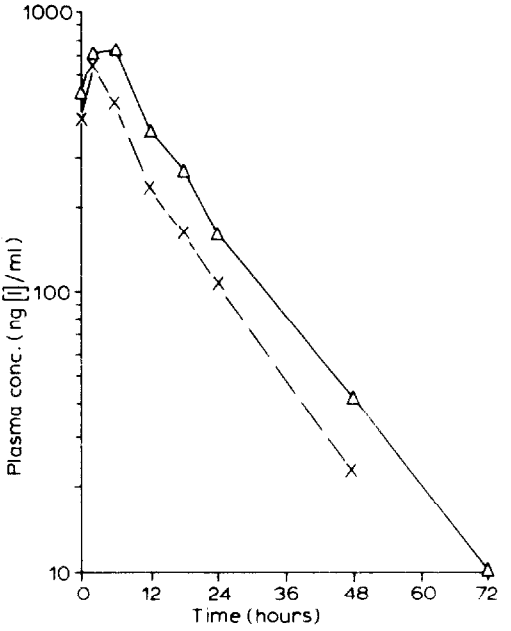

Fig. 4. Plasma concentration—time curves from two subjects following multiple oral dosing of [I] · succinate (see text).

TABLE II

URINARY EXCRETION PROFILE OF [I] AND [III] IN A SUBJECT FOLLOWING A SINGLE 65-mg ORAL DOSE OF [I]·SUCCINATE

Time interval (h)	Conc. [I] (μg/ml)	Conc. [III] (μg/ml)	Total mg [I]	Total mg [III]	Percent of dose as	
					[I]	[III]
0—2	35.2	1.11	5.28	0.169	11.3	0.38
2—4	17.0	0.52	1.70	0.052	3.80	0.12
4—8	19.6	0.52	4.40	0.117	9.83	0.26
8—24	6.11	0.30	6.05	0.297	13.5	0.67
24—48	0.44	N.M.*	0.41	—	0.91	—
				Total	39.34	1.43

*N.M. = ⩽ 0.05 μg/ml.

following a single 65-mg dose. The urine samples were collected immediately before and at 0—2, 2—4, 4—8, 8—24, and 24—48 h periods after the 65-mg dose. These samples were assayed for [I] and [III] using the ion-exchange and normal-phase HPLC methods, respectively. The results of the metabolic profile (Table II) showed approximately 40% of the dose excreted as [I] and approximately 1% of the dose as [III] over the excretion interval. Other subjects showed similar excretion profiles.

CONCLUSIONS

A sensitive and selective ion-exchange HPLC assay was developed for the determination of cibenzoline [I] in plasma and urine and was validated in the concentration range of 10 to 1000 ng/ml using UV detection at 214 nm. Removal of oxygen from the mobile phase through constant helium sparging and the use of special solvent lines is a requirement for the assay.

The assay was used to determine plasma and urine concentrations of [I] in human subjects following multiple oral dosing regimens of cibenzoline · succinate.

A normal-phase HPLC assay was also developed for the determination of the imidazole metabolite [III] and was applied to its quantitation in plasma and urine samples.

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