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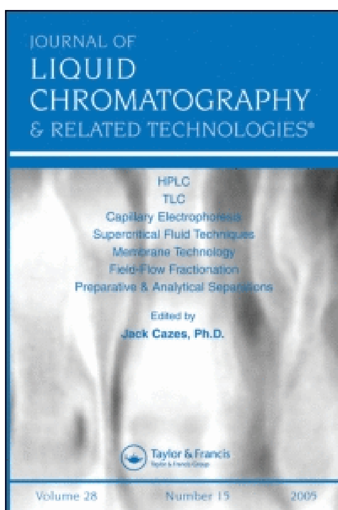

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ABBOTT-53385 IN DOG, RAT, AND HUMAN PLASMA

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ABSTRACT

A simple, sensitive and reproducible high-performance liquid chromatographic (HPLC) method was developed to monitor plasma ABBOTT-53385 (I) levels in dogs, rats and humans. The samples were first supplemented with the internal standard, then extracted on Bond Elut® extraction columns. They were analyzed by reverse-phase HPLC with UV detection at 240 nm. The calibration curve was rectilinear over the range of 0.05-2.0 μg/ml and the interday variance was less than 4%. The limit of detection for this method was about 0.03 μg/ml.

INTRODUCTION

ABBOTT-53385 (I) [8-chloro-3-(2-fluorophenyl)-5,6-dihydrofluoro[3,2-f]-1,2-benzisoxazole-6-carboxylic acid] is a new high-ceiling salidiuretic and uricosuric agent (1-3) currently under investigation at Abbott Laboratories. This drug is a modification of the (aryloxy)-acetic acid pharmacophore series where ethacrynic

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acid serves as the starting point (4). The structure of I and the internal standard used in this assay are shown in Figure 1.

MATERIALS AND METHODS

Chemicals

The acetonitrile and methanol were HPLC grade. The phosphoric acid was reagent grade, 85% from Mallinckrodt. The internal standard and I were obtained from Abbott Laboratories. All chemicals and reagents were used as received.

Chromatography

The following pieces of equipment were used in this study: a Waters Associates M-6000 pump, a WISP® injector, an LDC Spectro Monitor III Ultraviolet Detector and a Spectra Physics 4100 Integrator. The detector was operated at a time constant of 2.0, a range of 0.01 and an excitation wavelength of 240 nm. The chromatography column was a Nucleosil 5 micron C_{18} (15 x 0.46 cm, Alltech Associates). The extraction columns were Bond Elut® Octadecyl (C_{18}) 200 mg/3.0 ml (Analytichem International).

The mobile phase was methanol/acetonitrile/85% phosphoric acid/water (40:18:0.2:41.8, v/v/v/v). Minor manipulations of the acetonitrile content may be required to accommodate column efficiency loss or interferences from atypical plasma samples. The mixture was filtered through a polycarbonate 0.4 micron membrane (Nucleophore or equivalent) and degassed about 5 minutes under house vacuum with stirring. The HPLC system was operated at ambient temperature at a flow rate of about 1.0 ml per minute.

Standard Solutions

Standard curves were prepared by adding 0.2 ml of an acetonitrile solution of I (0.1 mg/ml) to blank plasma. This supplemented sample was then serially diluted with blank plasma to give the working range of 0.05-2.0 μg/ml.

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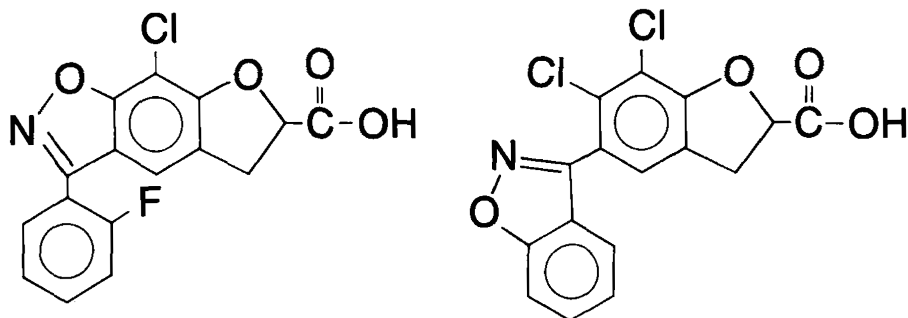

FIGURE 1. Structure of ABBOTT-53385 and the internal standard.

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A stock solution of the internal standard was prepared in acetonitrile (1 mg/ml). This solution was serially diluted with acetonitrile/water (1:1, v/v) to give the 5.0 µg/ml working internal standard solution.

Extraction Procedure

Plasma samples (0.5 ml) were accurately pipetted into 12x75 mm tubes. The samples were acidified with phosphoric acid (1M, 0.5 ml) and 0.1 ml of the internal standard solution (5.0 µg/ml) added. The Bond Elut® disposable extraction columns were conditioned by first washing twice with 2 ml washes of methanol then two 1 ml washes of water. The vacuum was released and the above mixed samples were applied to the columns. The samples were drawn through by vacuum and the drug and internal standard absorbed onto the column matrix. The endogenous materials were removed by washing twice with a 2 ml water-methanol mixture (70:30). The drug and internal standard were eluted from the column with 1 ml of methanol. The filtrates were evaporated to dryness in a water bath (50-55°C) under a gentle stream of air.

The residues were dissolved in 0.5 ml of mobile phase and aliquots injected into the HPLC system for drug analysis.

RESULTS AND DISCUSSION

For sample clean-up, the Amicon Ultrafiltration® cones and membranes were first evaluated. This procedure yielded very poor recoveries (less than 20%). The drug is highly protein bound and even with the addition of a detergent displacing agent, the drug could not be filtered free from the biological matrix. An organic solvent extraction scheme was next evaluated. Solvents which extracted the drug quantitively from the acidified plasma yielded high, unacceptable HPLC backgrounds.

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The Bond Elut® extraction system was evaluated and found to be fast, simple and very reproducible. This extraction procedure had been successfully used before (5). Several types of sorbents were screened and the C_{18} 200 mg/3 ml column was found to be the most efficient for the clean-up of I from plasma. The above extraction has been applied to dog, rat and human plasma. Typical chromatograms for extracted blank plasma, the 0.5 µg/ml standard and a dog sample as processed by this method are shown in Figure 2. The retention times were 9.4 minutes for I and 11.3 minutes for the internal standard. As observed, there may be some variation from sample to sample of the compounds eluting at the void volume.

Table 1

Linear Regression Analysis

Species	Slope	Y-Intercept	Corr.
Dog	2.274	0.0259	0.9996
Rat	2.049	0.0195	0.9959
Man	2.059	0.0087	0.9998

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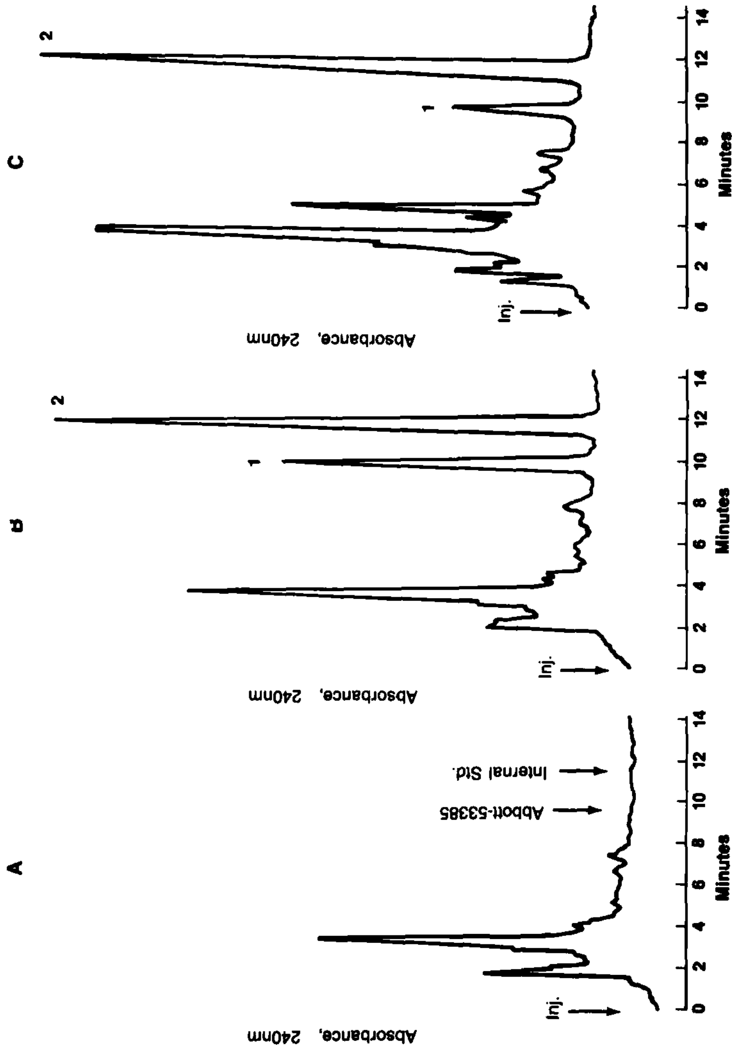

FIGURE 2. (A) Chromatogram of extracted blank dog plasma as processed by this method. (B) Chromatogram of extracted dog plasma supplemented with I, 0.5 μg/mL (1) and internal standard (2). (C) Chromatogram of processed dog plasma follow p.o. dosing of 0.3 mg/kg, 0.5 hr post dosing.

Method Validation

The linear response of the UV detector was established by constructing calibration curves from plasma supplemented with I. The parameters for typical standard curves (0.05 - 2.0 μg/ml) are shown below in Table 1.

To assess the precision of the method over the standard curve range, standard curves were analyzed over five separate days by the procedure described above. The results of this inter-assay variance study are summarized in Table 2. The mean assay coefficient of variations (CV) for these species were: rat 3.7%, dog 2.0% and humans 3.0%.

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The recovery of I from the plasma assay procedure was determined by comparing the peak height of the drug from processed samples to the peak height of prepared reference samples. The recovery was 99.9% at 1.0 μg/ml. The recovery of the internal standard in the extraction procedure was greater than 96%. The detection limit of I was estimated to be about 0.03 μg/ml.

Stability

The stability of I in dog plasma was assessed at refrigeration (4°C), room temperature (25°C) and freezer (-20°C) storage.

TABLE 2

Precision of the Analytical Procedure

Theory	Found - Mean ± SD					
μg/ml	Rat		Dog		Human	
	μg/ml	C.V.%	μg/ml	C.V.%	μg/ml	C.V.%
0.05	0.05±0.002	4.0	0.05±0.001	2.0	0.05±0.001	2.0
0.13	0.13±0.006	4.6	0.13±0.003	2.3	0.13±0.005	3.8
0.25	0.25±0.009	3.6	0.26±0.005	1.9	0.25±0.010	4.0
0.50	0.49±0.008	1.6	0.50±0.005	1.0	0.50±0.019	3.8
1.00	0.99±0.057	5.7	0.99±0.025	2.5	1.02±0.023	2.3
2.00	1.98±0.051	2.6	1.98±0.041	2.1	1.97±0.035	1.8
	Mean	3.7		2.0		3.0

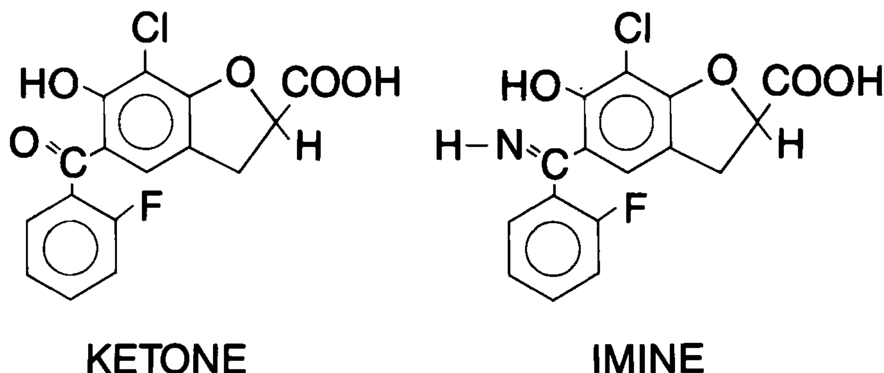

FIGURE 3. Structures of the metabolites of I.

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Blank plasma was supplemented with I at 1.0 µg/ml. Aliquots were set at 4° and 25°C and analyzed four days later for drug content. No degradation was observed in the plasma samples stored at 4°C. However, at room temperature, the plasma samples showed about a 10% loss of drug and a large increase in HPLC background. Thus, extended room temperature storage of plasma samples containing I is not recommended. After six months storage at -20°C, plasma samples of I showed no degradation or changes in the HPLC background.

Pharmacokinetic Study

This method has been used to analyze over 2000 plasma samples. The main metabolites, following oral or intravenous administration of I to animals, are shown in Figure 3. Under the chromatographic conditions described here, they elute at a retention time of 7.9 minutes for the ketone and 2.9 minutes for the imine (I was at 9.4 minutes). The drug was mainly excreted in feces as a metabolite so urinary levels of I were not routinely determined. A typical plot of plasma concentration versus time post dosing (P.O.) for dogs is shown in Figure 4.

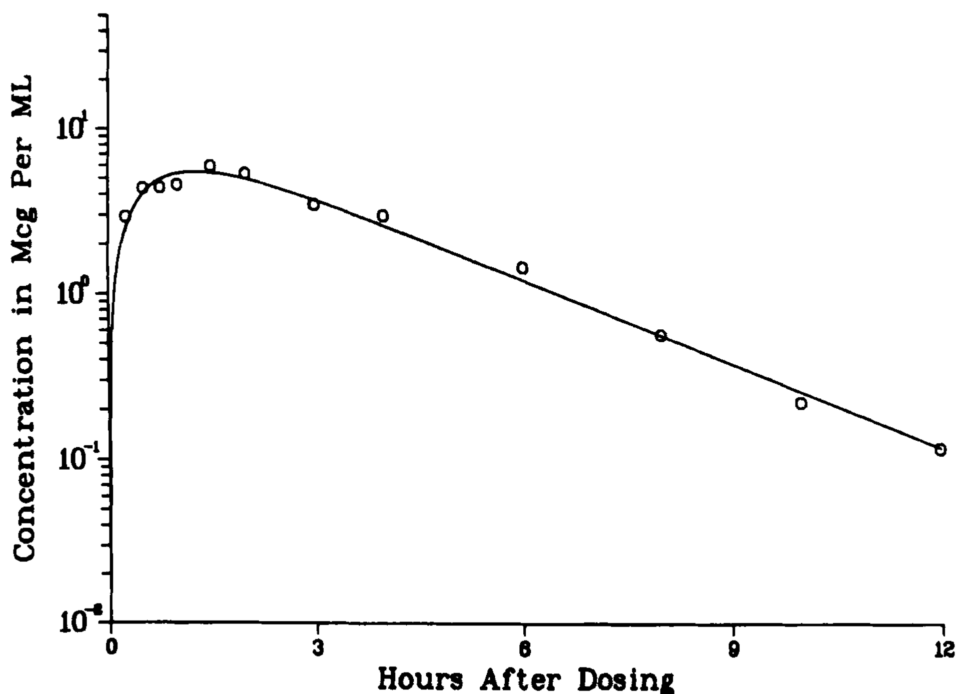

FIGURE 4. Average plasma levels of I following p.o. dosing of 3.0 mg/kg to nine dogs.

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Conclusions

This method has been validated for dog, rat and human plasma. Generate of acceptable chromatograms and standard curves would serve to validate the procedure for other types of biological fluids.

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