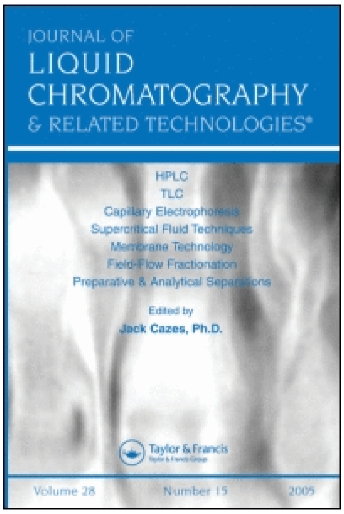

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF R-831, A NEW ANTIALLERGIC AGENT, IN DOGS AND HUMANS

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ABSTRACT

A simple, selective and sensitive HPLC method has been developed to measure R-831 levels in dogs and humans. It is an internal standard technique with a single step extraction and one wash. Samples are chromatographed on a reversed-phase system with ultraviolet detection. The lowest detectable concentration for plasma is 25 ng R-831/ml with a 1 ml sample and the linear range is 25-8000 ng R-831/ml. The lowest detectable concentration for urine is 250 ng R-831/ml with a 0.1 ml sample and the linear range is 250-8000 ng R-831/ml. This method has been used to quantitate levels of R-831 in bioavailability and toxicity studies in dogs, and in pharmacokinetic and efficacy studies in humans.

INTRODUCTION

R-831 [N-(1H-tetrazol-5-yl)quinoline-8-carboxamide, I] is a potential new antiallergic agent. It appears to act similarly to cromolyn sodium through inhibition of mediator release rather than

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through antagonism of mediators at the level of the receptor. A need arose for a sensitive bioanalytical method to measure R-831 levels in dogs and humans during safety, pharmacokinetic, and efficacy studies.

This paper describes a simple and sensitive high performance liquid chromatographic (HPLC) method involving a single step extraction with an internal standard and ultraviolet detection.

MATERIALS AND METHODS

Chemicals and Reagents

The methanol, acetonitrile, and methylene chloride were Omnisolv® (EM Science). The hydrochloric acid, acetic acid, dimethyl sulfoxide and urea were reagent grade. The water was deionized.

Preparation of Standard Solutions

All standards were prepared by diluting a 1 mg/1 ml dimethyl sulfoxide stock solution. The concentrations used for I were 25,50,100,200,400 and 800 ng I/100 µl 10% dimethyl sulfoxide in methanol. Two different internal standards were used for the human and dog samples: 5-nitro-N-(1H-tetrazol-5-yl)-quinoline-8-carboxamide (II) for human, and N-(1H-tetrazol-5-yl)naphthalene-2-carboxamide (III) for dog. A solution of 400 ng II/100 µl 10% dimethyl sulfoxide in methanol was used for the human plasma and urine samples, and a 1 µg III/100µl 10% dimethyl sulfoxide in methanol solution was used for the dog plasma samples. See Figure 1 for structures of I, II and III.

Chromatographic System

The liquid chromatographic system consisted of a Model 6000A pump, Model 710B Sample Processor, and Model 440 absorbance detector (Waters

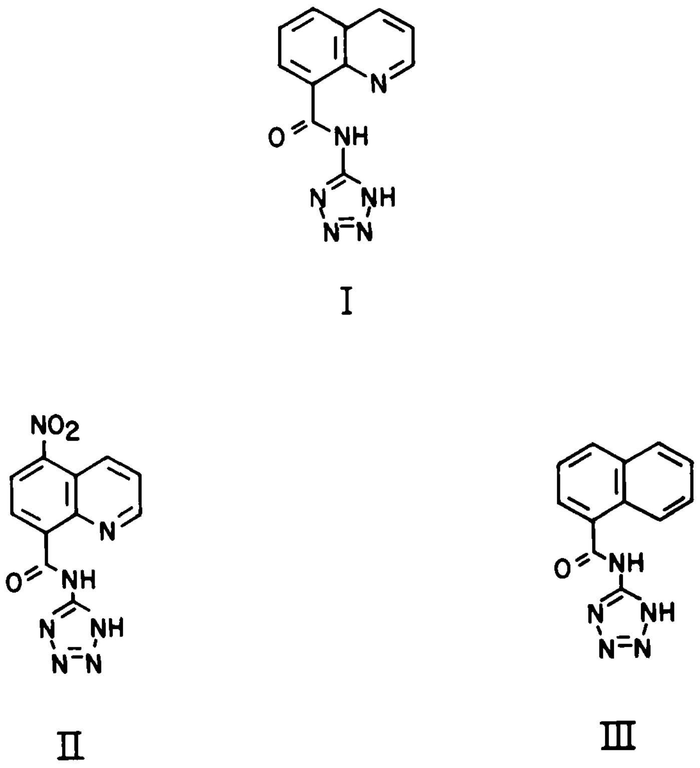

FIGURE 1. Chemical structures of I, II and III

Associates, Inc.). Peak heights were measured by a Model 4100 computing integrator (Spectra-Physics). The column used was a 5 μm Supelcosil® LC-8, 4.6 mm I.D. X 15 cm (Supelco, Inc.). The mobile phase was prepared by mixing acetonitrile and 1% acetic acid (30/70, v/v), and filtering through a 0.45 μm filter prior to use. The flow rate was 1 ml/min and the effluent was monitored at 313 nm.

Extraction Procedure

The extraction is carried out in 12 ml glass round bottom culture tubes with polyethylene lined screw caps. The tubes are prerinsed with approximately 1 ml of methylene chloride before use. The following is added to the tube: 100 µl 10% dimethyl sulfoxide in methanol (replaced with the standard solutions for the calibration curve), 100 µl internal standard solution, 1.0 ml plasma or 0.1 ml urine plus 0.9 ml water, 1 ml 10M urea, 0.2 ml 2M hydrochloric acid, and 5 ml methylene chloride. The tubes are capped, shaken on a reciprocal shaker for 15 minutes, and centrifuged at 1600xg for 10 minutes. The aqueous phase is removed, and 2 ml water and 0.2 ml 2M hydrochloric acid are added to the methylene chloride phase. The tubes are capped, shaken for 5 minutes, and centrifuged for 5 minutes. The aqueous phase is removed and at least 4.0 ml of the methylene chloride phase is transferred to a 15 ml conical tube. This is evaporated to dryness under a stream of N_2 at 40-45°C. The residue is reconstituted in 100 µl of 10% dimethyl sulfoxide in methanol, vortexed for 1 minute, and 20 µl is injected into the liquid chromatograph.

Calibration and Quantitation

A least squares linear regression line of the peak height ratio of I/internal standard vs. I concentration was calculated. The slope and intercept of the line were used to calculate the concentration of I in unknown samples. A calibration curve was run with each set of unknowns.

RESULTS AND DISCUSSION

Sample Preparation

Sample preparation is relatively simple involving only a single extraction with one wash; forty samples plus the calibration curve can be prepared in approximately four hours of analyst time. Prerinsing the tubes with methylene chloride and centrifuging at a high speed decreased the thickness of the pellet that formed between the aqueous and organic phases during the extraction. By decreasing this layer, it became possible to transfer a larger volume of the organic phase and thereby increase the percent of I recovered. Urea was added to denature the serum proteins so the binding of I to the proteins was reduced to a minimum. In this particular case, reduction of the protein binding likely facilitates the extraction of the drug and internal standard from the aqueous media to the organic phase. The one minute vortex time prior to injection into the HPLC was necessary to ensure the complete dissolution of the drug.

For urine, the volume analyzed had to be decreased from the 1.0 ml used for plasma to 0.1 ml to reduce the amount of interfering substances to a negligible level and thus make quantitation of the drug possible.

Chromatography

In dog plasma, a large interference peak eluted at the same retention time as II, the internal standard used for human samples. Thus, another analog of I was used as an internal standard (III) for the dog samples. The retention times of I and III were 5.0 min and 7.0 min, respectively. Sample chromatograms for blank dog plasma and plasma from a dog dosed with I are shown in Figure 2.

A different column of the same type and manufacturer was used for the analysis of the human samples. The resolution achieved by the second column was not as good as that achieved by the original column; therefore, the mobile phase ratio was adjusted to 25.5:74.5, (acetonitrile:1% acetic acid) so that baseline separation between I and II could be achieved. The retention times were 7.0 min for I and 7.6 min for II. Sample chromatograms for blank human plasma and a subject dosed with I are shown in Figure 3. There were no endogenous peaks in any of the blank samples which interfered with the integration of the drug or internal standard.

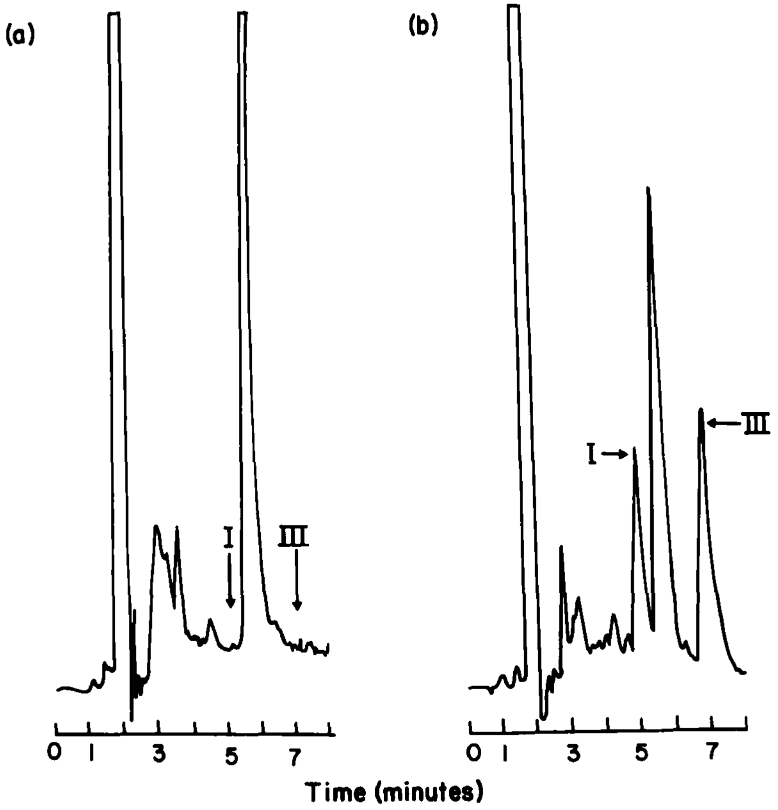

FIGURE 2. Typical chromatograms for dog plasma samples: (a) blank plasma, (b) plasma from a dog dosed with I, concentration level is 116 ng I/

Precision and Accuracy

The intra-day precision and accuracy of this method was determined by analyzing quadruplicate samples at various concentration levels in dog and human plasma. The precision, expressed as the coefficient of variation, ranged from 2.1 to 3.5% in dog plasma (Table 1) and from 1.5 to 2.8% in human plasma (Table 2). The accuracy, expressed as the relative error, ranged from -5.8 to +3.3% and from +7.5 to +9.2% in dog and human plasma, respectively.

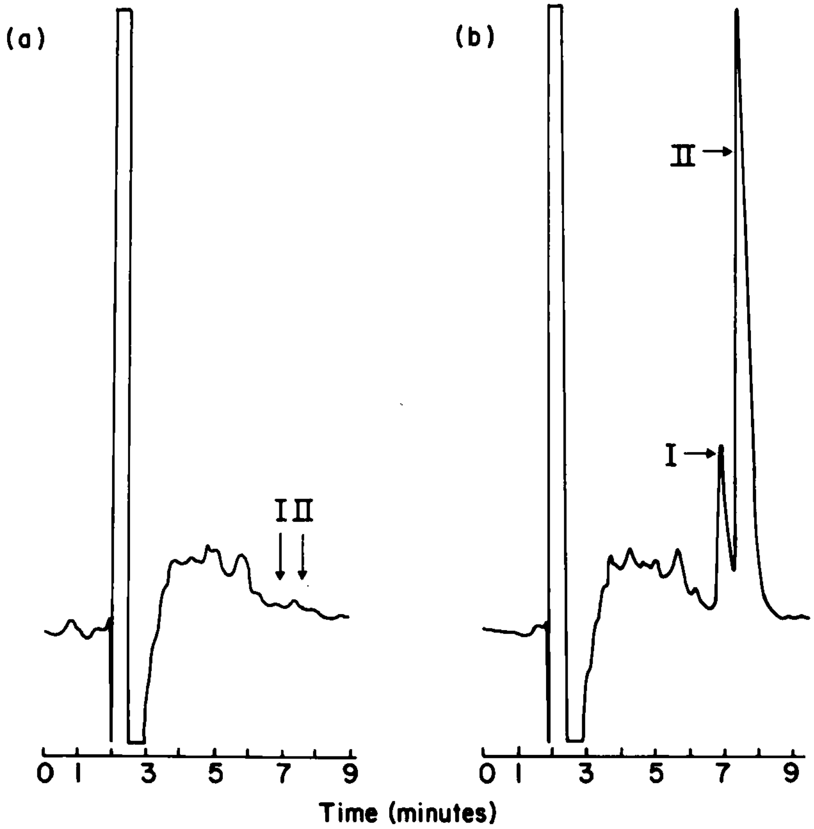

FIGURE 3. Typical chromatograms for human plasma samples: (a) blank plasma, (b) plasma from a subject dosed with I, concentration level is 83 ng I/ml.

TABLE 1

Intra-Day Precision and Accuracy for Dog Plasma

Sample Concentration (ng/ml)	Number of Samples	$\bar{x} \pm$SD (ng/ml)	Coefficient of Variation (%)	Relative Error (%)
75	4	77.5±1.7	2.2	+3.3
150	4	144.8±3.0	2.1	-3.5
300	4	285.8±10.0	3.5	-4.8
600	4	565.0±14.3	2.5	-5.8

TABLE 2

Intra-Day Precision and Accuracy for Human Plasma

Sample Concentration (ng/ml)	Number of Samples	$\bar{x}$±SD (ng/ml)	Coefficient of Variation (%)	Relative Error (%)
75	4	80.6±1.3	1.6	+7.5
150	4	163.5±4.6	2.8	+9.0
300	4	327.7±4.9	1.5	+9.2

The inter-day precision and accuracy was determined over a six week period for dog plasma and a two week period for human plasma. The coefficient of variation ranged from 1.2 to 8.7% in dog plasma (Table 3) and 0.5 to 5.5% in human plasma (Table 4). The relative error ranged from -2.9 to +1.7% and from -8.0 to +2.5% in dog and human plasma, respectively.

Extraction Recovery

The standard extraction procedure was modified to assess the recovery of I from human plasma. In the modified procedure, the internal standard was added after the extraction was completed. Recovery was calculated by comparing these ratios with ratios obtained by direct injection of a series of I and internal standard mixtures. Three concentration levels were analyzed in triplicate. The percent recovery was 79.2±1.8, 82.2±1.5, and 80.5±0.7 for 75, 150, and 300 ng I/ml respectively. This data indicates consistent extraction recovery between samples over the concentrations analyzed.

Linearity and Sensitivity

The calibration curve used for dog and human plasma was 25-800 ng I/ml with a 1.0 ml sample. The calibration curve used for human urine was

TABLE 3

Inter-Day Precision and Accuracy for Dog Plasma

Sample Concentration (ng/ml)	Number of Samples	$\bar{x}\pm SD$ (ng/ml)	Coefficient of Variation (%)	Relative Error (%)
50	11	48.6+4.2	8.7	-2.9
100	11	97.3±3.1	3.2	-2.7
200	11	199.0±5.4	2.7	-0.5
400	11	407.0±19.0	4.7	+1.7
800	11	797.2±9.7	1.2	-0.4

TABLE 4

Inter-Day Precision and Accuracy for Human Plasma

Sample Concentration (ng/ml)	Number of Samples	$\bar{x}\pm SD$ (ng/ml)	Coefficient of Variation (%)	Relative Error (%)
25	6	23.0±1.3	5.5	-8.0
50	6	47.5±1.9	3.9	-5.0
100	6	99.8±2.5	2.5	-0.2
200	6	205.0±2.7	1.3	+2.5
400	6	406.5±8.0	2.0	+1.6
800	6	795.8±3.9	0.5	-0.5

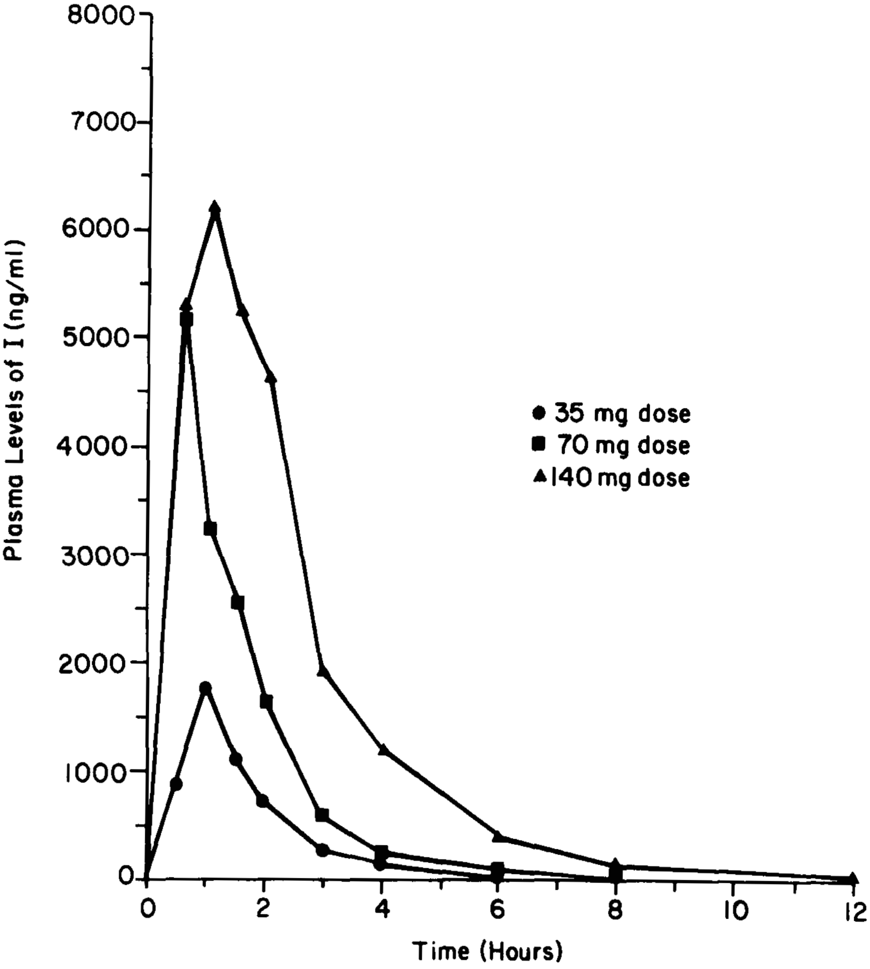

FIGURE 4. Plasma concentrations of unchanged I in a human subject following oral administration of single 35, 70, and 140 mg doses of I.

250-8000 ng I/ml with a 0.1 ml sample. In each case, the response for the lowest standard was 8-10 times the noise level of the blank samples. The correlation coefficient (r) for the calibration curve was generally 0.999 or better.

Application

The procedure for I in plasma has been used successfully for both dog and human studies. In a safety/pharmacokinetic study in healthy, human subjects with single, escalating oral doses of I, plasma concentrations of I were found to be reasonably proportional to dose (Figure 4); measured plasma concentrations of I ranged from 25 ng/ml to 7230 ng/ml. In this same study, human urine levels of unchanged I were generally too low (<250 ng/ml) to be quantitated.

CONCLUSION

A simple and selective method has been developed to quantitate I at nanogram levels in dog plasma and human plasma and urine. Forty samples can be analyzed in approximately four hours of analyst time. The intra and inter-day precision and accuracy results have shown excellent within run and day-to-day reproducibility. The extraction recovery was consistent over the entire concentration range. The method has been used to measure levels of I in bioavailability and toxicity studies in dogs, and safety, pharmacokinetic, and efficacy studies in humans.

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