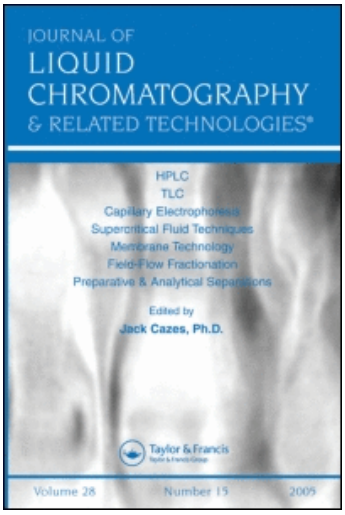

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Welscher, Teresa M. , Armstrong, Karon E. , Rhode, Kathleen A. , Miller, Aldora M. , Sandahl, Jane H. and Chang, Shaw F.(1988) 'Quantitation of R-836, a New Oral Bronchodilator, by High Performance Liquid Chromatography using Manual or Robotic Sample Preparation', Journal of Liquid Chromatography & Related Technologies, 11: 2, 435 — 445
To link to this Article: DOI: 10.1080/01483918809349950
URL: http://dx.doi.org/10.1080/01483918809349950

JOURNAL OF LIQUID CHROMATOGRAPHY, 11(2), 435-445 (1988)

QUANTITATION OF R-836, A NEW ORAL BRONCHODILATOR, BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING MANUAL OR ROBOTIC SAMPLE PREPARATION

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ABSTRACT

A simple and selective high performance liquid chromatography (HPLC) method has been developed for the quantitation of R-836 (an investigational oral bronchodilator) in human plasma and urine, dog plasma and urine, and rat plasma. The method consists of reversed-phase HPLC with ultraviolet detection. Sample preparation involved a one-step protein precipitation procedure and was performed both manually and by a Zymate robotic system. Precision and accuracy results showed excellent reproducibility; results using the robotic procedure were slightly better than the manual procedure. The robotic procedure was capable of preparing the samples with minimal operator handling.

INTRODUCTION

R-836 [2,5-diethyl-7-(4-thiomorpholino)-1,2,4-triazolo-(1,5-c)pyrimidine] is an investigational drug that has been shown to be an effective oral bronchodilator in animal models (1,2). Clinical trials in humans are currently in progress. This paper describes a bioanalytical method for determining levels of R-836 in human plasma and urine, dog plasma and urine, and rat plasma. Sample preparation is accomplished by a protein precipitation method performed either manually or by a Zymate robotic system.

MATERIALS AND METHODS

Reagents

Methanol and acetonitrile were Omnisolv® Grade and were purchased from EM Science (Cherry Hill, NJ). Water was deionized and all other chemicals were analytical reagent grade.

Instrumentation

HPLC analyses were performed using a Laboratory Data Control Constametric III pump (Riviera Beach, FL), a Waters 710B automatic sample delivery system (Milford, MA), and a Shimadzu SPD-2A spectrophotometric detector (Kyoto, Japan). Peak heights were measured by a Hewlett-Packard 3390A integrator (Avondale, PA) or a Spectra-Physics 4100 computing integrator (San Jose, CA). Samples were injected onto an

Applied Science (State College, PA) 5 μm hexyl (C6) reversed-phase column (25 cm x 4.6 mm I.D.) and detected at 280 or 313 nm. Mobile phases were delivered at 1 mL/min and consisted of methanol/0.01 M sodium phosphate buffer (pH=7.0) in the ratios 77/23 (v/v) for System I and 73/27 (v/v) for System II. Mobile phases were filtered through a 0.45 μm Nylon 66 membrane before use. The high speed centrifuge used for manual preparation of samples was an Eppendorf, Model 5414 (rpm=12,000, Brinkman Instruments, Westbury, NY).

The robotic system was purchased from Zymark Corporation (Hopkinton, MA) and consisted of the following components: a robotic arm equipped with a dual purpose hand (gripper and syringe), a single tube vortexer, four racks to hold test tubes and sample vials, two pipet tip racks, a master laboratory station with two solvent reservoirs, a dispensing station, a centrifuge, a capping station, a power and event controller, a system controller, and a printer.

Standard Solutions

All standard solutions of R-836 and the internal standard were prepared in acetonitrile and were made by diluting a 1 mg/mL stock solution of each compound. Concentrations of R-836 used for the animal samples were 0.9, 1.8, 3.6, 9, 18, 36, 54, and 108 μM; concentrations used for the human samples were 1, 2.5, 5, 10, 25, 50, and 100 μM. The internal standard, S-26033 [3,5-di-n-propyl-7-thiomorphilino-1,2,4-triazolo-(1,5-c)pyrimidine bisulfate], was used at a concentration of 18 μg/mL. See Figure 1 for structures of R-836 and the internal standard.

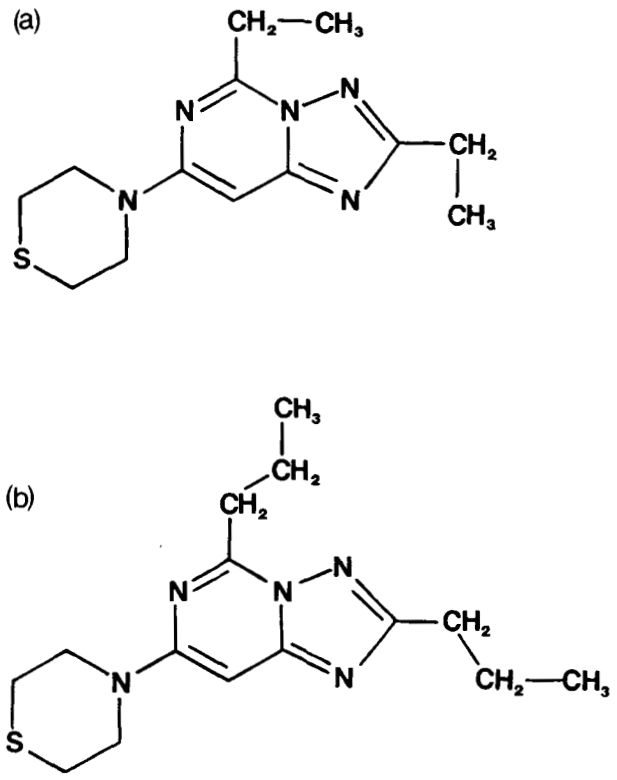

Figure 1. Chemical structures of: (a) R-836, and (b) internal standard.

Sample Preparation

The following reagents were combined in a tube: 250 μl plasma or urine, 250 μl internal standard and 250 μl acetonitrile. For calibration standards, R-836 standard solutions were added in place of acetonitrile. Each sample was vortexed for 15 sec and then centrifuged for 5 min to compact

the pellet. The supernatant was transferred to an autosampler vial and injected into the liquid chromatograph.

When sample preparation was performed manually, the reagents were added to 1.5 mL polypropylene centrifuge tubes; when performed by the robot, the tubes were changed to 15 mL disposable glass centrifuge tubes. The robot initially picked up a centrifuge tube and uncapped it in the capping station. Before returning the tube to the rack, it was brought to the dispensing station where the internal standard and, in the case of the unknown samples, acetonitrile was added. The robot then picked up a pipet tip, uncapped the sample vial and withdrew an aliquot of the sample while the vial was in the capping station. The plasma aliquot was dispensed into the centrifuge tube, the pipet tip was disposed, and the sample vial was capped and returned to the sample rack. When dispensing the R-836 solutions for the calibration standards, the robot performed the same sequence used to aliquot the unknown plasma samples. When all additions had been made, the tube was capped, vortexed and centrifuged (rpm=2000). The robot proceeded to the next sample while the previous sample was in the centrifuge. The supernatant from each sample was manually transferred to an autosampler vial and capped.

Calibration

A calibration curve was constructed by plotting peak height ratios (R-836/internal standard) versus known concentrations of R-836. The slope and intercept obtained from linear regression analysis of the curve were used for the calculation of R-836 concentrations in unknown samples. A calibration curve was run with each set of unknowns.

RESULTS AND DISCUSSION

Human plasma and urine, and rat plasma samples were successfully quantitated using mobile phase System I with detection at 280 nm. Retention times for R-836 and the internal standard were 5.8 and 7.8 min, respectively. Chromatograms of blank human plasma (Fig. 2) and urine (Fig. 3), before and after spiking samples with R-836 are shown.

A peak was detected in blank dog plasma that interfered with the quantitation of R-836 using mobile phase System I. By changing to mobile phase System II with detection at 313 nm, the interfering peak was eliminated; however, detection sensitivity for R-836 was decreased, resulting in the loss of the lowest calibration standard. The retention times of R-836 and the internal standard using System II were 7.0 and 10.2 min, respectively. Chromatograms of blank dog plasma before and after spiking with R-836 are shown in Fig. 4. Mobile phase System II was also successfully used to quantitate R-836 in dog urine.

The linear range for this HPLC method was 0.9 to 108 μM R-836 for rat plasma, 1.8 to 108 μM R-836 for dog plasma and urine, and 1 to 100 μM R-836 for human plasma and urine. In all cases, the lowest quantifiable concentration was the lowest calibration standard used and linearity was not tested beyond the highest calibration standard.

Intra-day precision and accuracy were determined for the manual sample preparation method (Table 1) and the robotic method (Table 2) by analyzing five replicate samples at each of seven concentrations. The precision for the manual method, expressed as the coefficient of variation, ranged from 0.7% to 3.7%. The accuracy, expressed as the relative error, ranged from -12.7% to +1.8%. The coefficient of variation for the

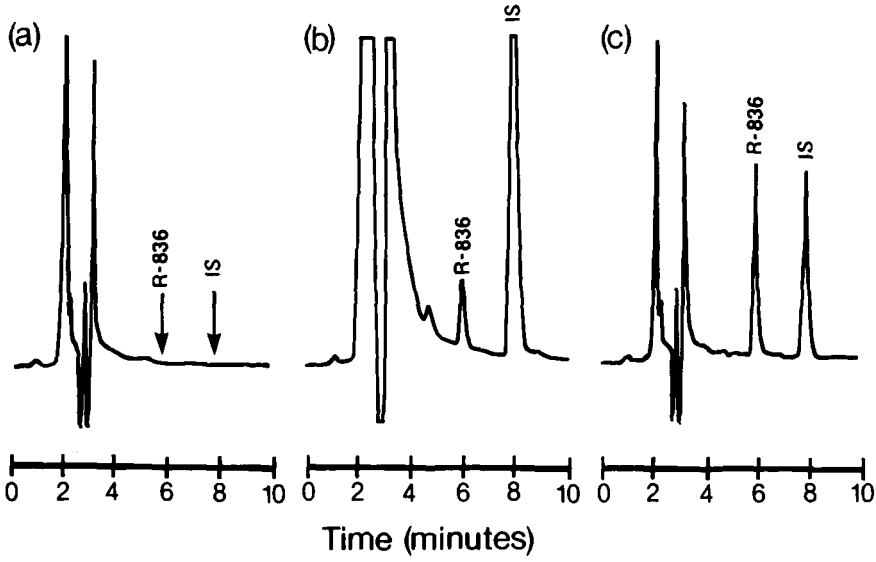

Figure 2. Typical chromatograms of human plasma: (a) blank plasma, injection volume is 2 μl, (b) spiked plasma, concentration is 0.9 μM R-836, injection volume is 20 μl, (c) spiked plasma, concentration is 36 μM R-836, injection volume is 2 μl.

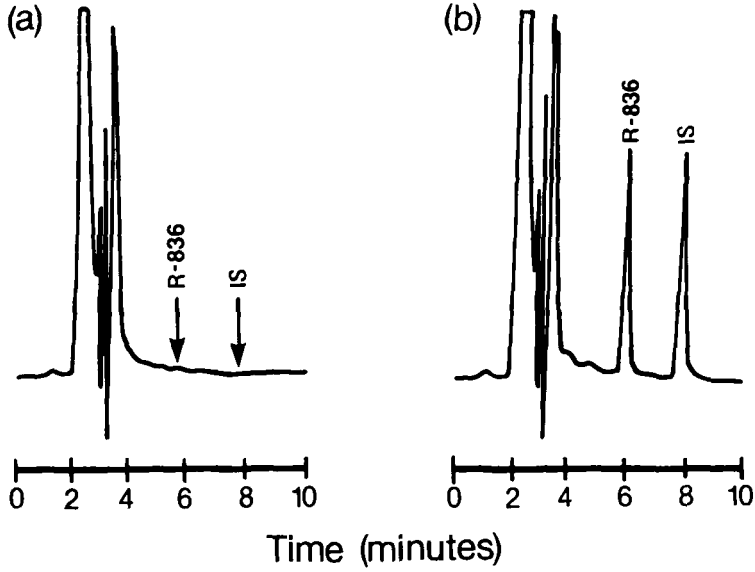

Figure 3. Typical chromatograms of human urine: (a) blank urine, (b) spiked urine, concentration is 36 μM R-836, injection volume is 2 μl.

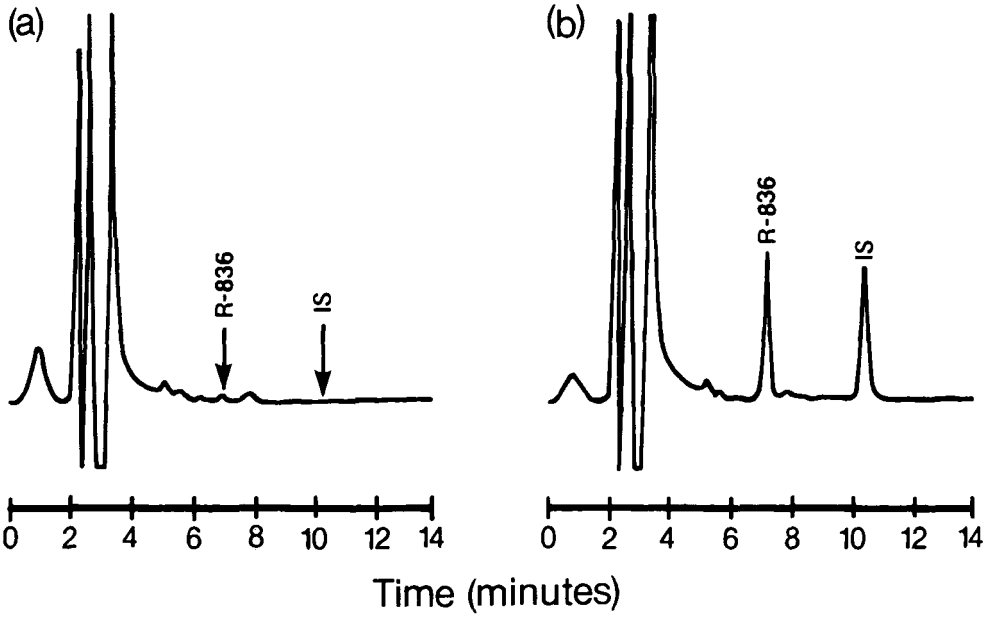

Figure 4. Typical chromatograms of dog plasma: (a) blank plasma, (b) spiked plasma, concentration is 36 μM R-836, injection volume is 10 μl.

TABLE 1

Intra-day Precision and Accuracy Using the Manual Method[a]

Concentration (μM)	Mean ± SD (μM)	Coefficient of Variation (%)	Relative Error (%)
0.9	0.79 ± 0.029	3.7	-12.7
1.8	0.71 ± 0.036	2.1	-4.8
3.6	3.49 ± 0.048	1.4	-3.1
9	8.84 ± 0.058	0.7	-1.8
18	18.33 ± 0.192	1.1	+1.8
36	36.06 ± 0.261	0.7	+0.2
54	54.21 ± 0.365	0.7	+0.4
108	108.49 ± 1.313	1.2	+0.5

[a]Values represent five replicate samples

TABLE 2

Intra-day Precision and Accuracy Using the Robotic Method[a]

Concentration (μM)	Mean ± SD (μM)	Coefficient of Variation (%)	Relative Error (%)
1	1.10 ± 0.000	0.0	+10.0
2.5	2.60 ± 0.000	0.0	+4.0
5	4.96 ± 0.152	3.1	-0.8
10	9.94 ± 0.055	0.6	-0.6
25	25.10 ± 0.212	0.8	+0.4
50	50.12 ± 0.705	1.4	+0.2
100	100.08 ± 0.887	0.9	+0.1

[a]Values represent five replicate samples

robotic method ranged from 0% to 3.1%, and the relative error ranged from -0.8% to +10.0%. Inter-day precision was determined over a 15 day period by analyzing samples using the robotic method (Table 3). The coefficient of variation ranged from 0.4% to 13.8%

The sample preparation procedure was modified to determine the recovery of R-836 from human plasma. In the modified procedure, the internal standard was added after the protein precipitation step was completed. Recovery was calculated by comparing the peak height ratios of these samples with ratios obtained by direct injection of a series of solutions containing both R-836 and internal standard. Five replicate samples at each of seven concentrations were analyzed (0.9, 1.8, 3.6, 9, 18, 36, 54, and 108 μM R-836). The recovery ranged from 90.8% to 95.4%.

TABLE 3
Inter-day Precision[a]

Concentration (μM)	Mean ± SD (μM)	Coefficient of Variation (%)
1	1.21 ± 0.168	13.8
2.5	2.57 ± 0.076	2.9
5	4.97 ± 0.111	2.2
10	9.97 ± 0.198	2.0
25	24.97 ± 0.206	0.8
50	49.26 ± 0.725	1.5
100	100.37 ± 0.355	0.4

[a]Values represent seven replicate samples

TABLE 4
Compounds Tested for Interference

acetaminophen	isoproterenol
ampicillin	1-methyluric acid
caffeine	3-methyluric acid
cephaloridine	7-methyluric acid
cephalothin	1-methylxanthine
corticosterone	3-methylxanthine
dilantin	7-methylxanthine
1,3-dimethyluric acid	phenobarbital
1,7-dimethyluric acid	theobromine
1,7-dimethylxanthine	theophylline
ephedrine	uric acid
hypoxanthine	xanthine

A series of drugs which might be taken concurrently with R-836 in the clinical environment and various dietary xanthines were tested by direct injection of a standard solution into the HPLC for possible interference with quantitation of R-836. None of the compounds tested (Table 4) were found to interfere.

This procedure has been successfully used for determination of plasma levels during safety studies in humans, and pharmacokinetic and bioavailability studies in dogs and rats.

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