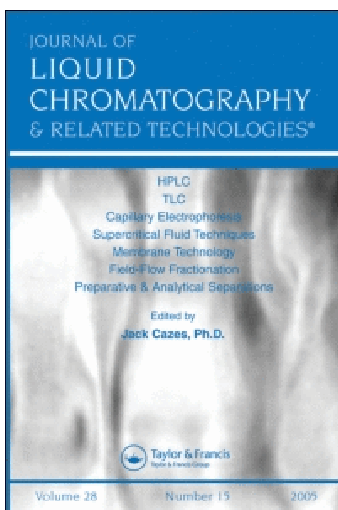
This article was downloaded by:
On: *25 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



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

Determination of U-85575 in RAT Serum by Solid-Phase Extraction and High-Performance Liquid Chromatography

M. G. Williams[a]; W. Z. Zhong[a]
[a] Drug Metabolism Research Upjohn Laboratories, Kalamazoo, Michigan

To cite this Article Williams, M. G. and Zhong, W. Z.(1993) 'Determination of U-85575 in RAT Serum by Solid-Phase Extraction and High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 16: 3, 743 — 753
To link to this Article: DOI: 10.1080/10826079308019561
URL: http://dx.doi.org/10.1080/10826079308019561

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(3), 743-753 (1993)

DETERMINATION OF U-85575 IN RAT SERUM BY SOLID-PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

M. G. WILLIAMS AND W. Z. ZHONG*
Drug Metabolism Research
Upjohn Laboratories
Kalamazoo, Michigan 49001

Downloaded At: 08:52 25 January 2011

ABSTRACT

Solid phase extraction and liquid chromatographic methods are described for the isolation and quantitation of U-85575 from rat serum. The assay utilizes a simple extraction of U-85575 from serum with phenyl solid phase columns. The extracts were chromatographed on a CN reverse-phase column using a mobile phase of acetonitrile-water (32:68, v/v) containing 0.2% triethylamine (pH 6.5 ± 0.05). The method has a limit of quantitation of 50 ng/ml of U-85575 in serum and is linear in the range of 0.05 to 20 μg/ml. The precision and accuracy of this method was proved to be <10% at the three concentrations evaluated. The method was demonstrated to be suitable to determine U-85575 concentrations in rat serum samples collected from a preliminary 15-day toxicity study.

*To whom correspondence should be addressed.

Copyright ©1993 by Marcel Dekker, Inc.

INTRODUCTION

U-85575, 12-chloro-5-(5-cyclopropyl 1,2,4-oxadiazol-3-yl)-2,3-dihydrodiimidazo 1,5-a:1',2'-c quinazoline (Figure 1) is under evaluation as a hypnotic compound. U-85575 has shown clear hypnotic-like effects and good oral potency in mouse, rat, and monkey with limited side effects. To support the preclinical pharmacokinetic and toxicokinetic studies, it is important to have a sensitive and accurate analytical method to determine the concentrations of U-85575 in serum. Because of the unique structure of U-85575, previously developed methods for other quinoxalin analogs (1-4) were not suitable in both chromatographic and extraction characteristics. In this paper, a high performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection using a solid-phase extraction (SPE) procedure for determination of U-85575 in rat serum is described. The applicability of this method to a preliminary toxicology study is also reported.

Downloaded At: 08:52 25 January 2011

MATERIALS

U-85575 and internal standard (IS), U-82217 (Figure 1) were provided by The Upjohn Company (Kalamazoo, MI). HPLC grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI). Acetic acid, ammonium hydroxide, and potassium phosphate (dibasic) were of analytical reagent grade and purchased from Mallinckrodt Inc. (Paris, KY). Purified water was produced by a Milli-Q reagent water system (Millipore Corporation, Bedford, MA).

METHODS

Instrumental Parameters

The chromatographic separation was performed on a cyano reverse-phase column (250 x 4.6 mm ID, 5 μm particle size (Supelco Inc., Bellefonte,

U-85575

U-82217 (IS)

Figure 1: Chemical structures of compounds U-85575 and U-82217 (IS).

Downloaded At: 08:52 25 January 2011

PA) protected by a guard column (Rainen CN Guard Column, 1.5 X 2 mm ID). The mobile phase of acetonitrile-water (32:68) containing 0.2% triethylamine (TEA) with a pH of 6.5 ± 0.05 adjusted by acetic acid was delivered by a Waters M-6000A pump (Milford, MA). The UV absorbance of column effluent was monitored at 325 nm with 0.005 a.u.f.s. sensitivity using a Kratos Spectroflow 783 variable wavelength ultraviolet (UV) detector (Ramsey, NJ). Injections were made by a Perkin-Elmer ISS-100 Autosampler (Norwalk, CT). The chromatographic system was operated at ambient (21-23°C) temperature with an eluent flow rate of 1.0 ml/min. Quantitation was accomplished by peak height ratio of drug to internal standard (IS) using a Harris Computer System. The standard curve, along with a statistical evaluation of standards linear fit, were computed by the linear regression program. The unknown concentration was determined by inverse prediction against the standard curve.

Preparation of Standards

A 200 µg/ml stock solution of U-85575 was prepared by dissolving 10 mg accurately weighed compound in acetonitrile and diluting to volume with acetonitrile in a 50 ml volumetric flask. The IS stock solution

(200 µg/ml) was prepared by dissolving 20 mg of U-82217 in 50 ml of acetonitrile and diluting to volume with water in a 100 ml volumetric flask. The stock solutions were diluted with acetonitrile to 20, 2, and 0.2 µg/ml working solutions. Stock and working solutions were stored at 4°C. Serum standards were prepared by aliquoting appropriate volumes of stock and working solutions to 1 ml with control serum (drug free) to produce a concentration series ranging from 5 ng/ml to 20 µg/ml. Reference standards were prepared by aliquoting the same volumes of U-85575 and IS into autosampler vials and bringing the total volume to 1 ml with acetonitrile-water (30:70, v/v) to create a concentration series similar to that of the fortified serum.

Downloaded At: 08:52 25 January 2011

Sample Preparation

Unknown serum samples were prepared by mixing 1 ml of serum sample with 50 µl IS working solution (20 µg/ml) into a disposable culture tube. Twelve phenyl SPE columns (100 mg/1.0 ml, Analytichem International Inc., Harbor City, CA) were placed on the Vacuum Extraction Manifold (Supelco, Inc. Bellefonte, PA) and prewashed with one column volume of acetonitrile followed by one column volume of 0.1 M K_2HPO_4 solution. The prepared serum samples or fortified serum standards were then immediately transferred onto the SPE column from the culture tubes with an approximate vacuum of 86 kPa. After the SPE columns were vacuum-aspirated (approximately 27 kPa) for 5 min, 300 µl of acetonitrile-water (30:70,v/v) followed by 2 ml of 0.1 M K_2HPO_4 solution were applied to rinse each column (86 kPa). The columns were dried with vacuum aspiration (approximately 27 kPa) for 10 min. Compounds of interest were eluted from the column with 300 µl of acetonitrile containing 0.1% TEA by gravity, followed by elution with 350 µl water by applying pressure to the top of the column using nitrogen gas (about 20 kPa). Each eluate was collected in a 2 ml autosampler vial and mixed with 350 µl of purified water, and 100 µl of the mixture was injected onto the HPLC system for analysis.

Method Validation

To determine the linear range of the method, freshly prepared standard curves for U-85575 ranging from 5 ng/ml to 20 μg/ml were analyzed on four different days (5). The limit of quantitation (LOQ) was estimated from the lowest concentration standard which had acceptable precision and accuracy (<15%). The precision and accuracy of the method were evaluated at three concentrations (0.1, 1 and 10 μg/ml). The intra-assay precision was determined by analyzing five fortified serum samples at each concentration on the same day, and the inter-assay precision was obtained by analyzing one fortified serum sample at each concentration on six different days (6). The absolute extraction recovery was studied by spiking U-85575 to control serum in three concentrations and extracting in triplicate based on the procedure previously described. The results were compared with unextracted external reference standards (in acetonitrile-water, 30:70, v/v) containing the corresponding concentrations. In all cases, the means, standard deviations (S.D.), and coefficients of variation (C.V.) were calculated. A p-value of <0.05 was considered as significant in statistical analysis.

Downloaded At: 08:52 25 January 2011

RESULTS AND DISCUSSION

HPLC Characteristics

Initial evaluation of HPLC characteristics of U-85575 was carried out by using the conditions developed for U-82217 (4). However, U-85575 was eluted as a broad peak with obvious tailing. By using a cyano reverse-phase column (250 x 4.6 mm ID, 5 μm particle size, Supelco Inc., Bellefonte, PA) with a mobile phase containing TEA, the peak shape of U-85575 improved dramatically. The optimal mobile phase for separation of U-85575 and IS was established by varying the concentrations of acetonitrile and TEA and was found to be acetonitrile-water (32:68) containing 0.2% TEA (pH of

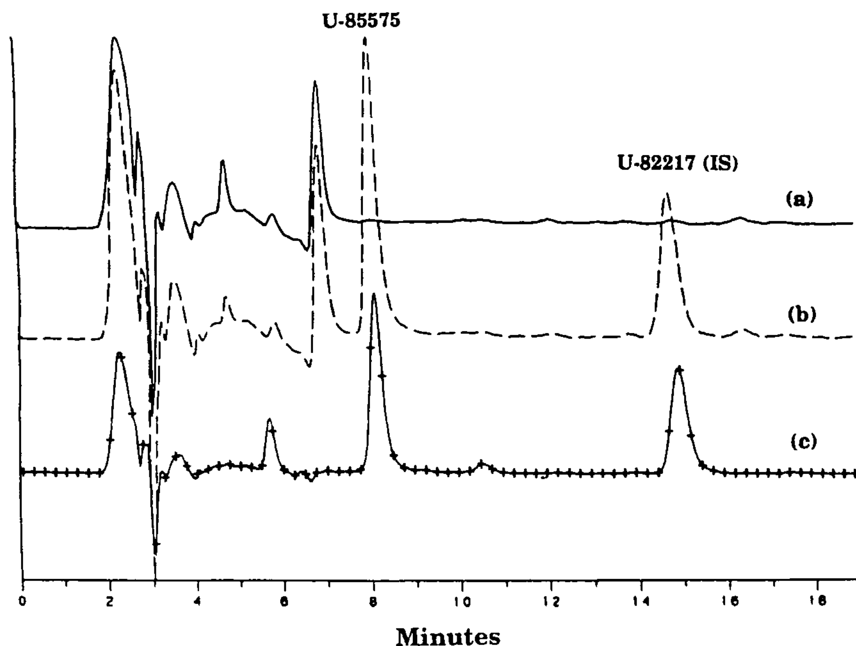


Figure 2: Chromatograms after extraction of (a) a rat serum blank, (b) a serum blank fortified with 0.5 μg/ml each of U-85575 and IS, and (c) a serum sample collected 1 hour after a rat receiving 30 mg/kg oral dose of U-85575 on day 8.

Downloaded At: 08:52 25 January 2011

6.5 ± 0.05 adjusted by acetic acid). Under these conditions, the retention times for U-85575 and IS were 8.2 and 14.9 min, respectively. Figure 2 shows the separation of U-85575 in rat serum using U-82217 as internal standard.

Validation Results

The low limit of quantitation (LOQ) was tested at concentrations of 5, 10 and 50 ng/ml. The LOQ for U-85575 was found to be 50 ng/ml with acceptable precision and accuracy (<15%, n=6). U-85575 was not detectable at concentrations of 5 ng/ml, however, it could be detected following extraction at a concentration of 10 ng/ml, based on a signal-to-noise ratio of 2.

Fortified serum standard curves showed good linear relationship between peak height ratios and concentrations from 50 ng/ml to 20 μg/ml for U-85575 with correlation coefficients greater than 0.998 as shown in Table 1. The intercept was found to be not significant ($p>0.05$) for each of the standard curves by the null hypothesis test. Thus, a forced through-the-origin model can be used for linear regression analysis. The slopes generated from the four calibration curves were not significantly ($p>0.05$) different.

Excellent intra-assay (n=15) and inter-assay (n=18) precision ranging from 2.3 to 5.8% and 6.7 to 9.4%, respectively, was obtained at the three concentrations studied (Table 2). The accuracy ranged from -7.3 to 8.1%.

The mean absolute extraction recoveries (mean ± SD, n=3) at the three concentrations (0.1, 0.5, and 10 μg/ml) for U-85575 and at 1 μg/ml for IS are listed in Table 3. The mean extraction recovery ranged from 91 to 97% for U-85575 and was 93% for IS. Less than 70% extraction recovery was obtained for U-85575 in serum by using the SPE procedure previously developed for the IS (U-82217) (4). When using a phenyl SPE column instead of C_{18} column and eluting the compounds of interest with 300 μl elution solvent of acetonitrile containing 0.1% TEA by gravity, the recovery of U-85575 increased to >90%. The recovery remained similar for IS.

Comparing the extracts of control (drug free) rat serum, control rat serum fortified with U-85575 and IS, and a post-dose serum sample

TABLE 1.

Linear Regression of Fortified Serum Standard Curves

Day	Slope	Intercept ($x10^2$)	Correlation Coefficient
1	0.2457	-0.0116	0.9997
2	0.2593	-0.0231	0.9987
3	0.2312	-0.7694	1.0000
4	0.2398	-0.0216	0.9997

Downloaded At: 08:52 25 January 2011

TABLE 2

Intra- and Inter-Assay Precision and Accuracy

	μg/ml added	Mean ± SD Measured (μg/ml)	Precision (%)	Accuracy (%)
Intra-Assay (n=5)	0.1	0.108 ± 0.003	3.2	8.1
	1	0.940 ± 0.054	5.8	-5.9
	10	9.995 ± 0.227	2.3	-0.05
Inter-Assay (n=6)	0.1	0.101 ± 0.010	9.7	0.9
	1	0.927 ± 0.068	7.4	-7.3
	10	9.721 ± 0.650	6.7	-2.8

Downloaded At: 08:52 25 January 2011

TABLE 3

Absolute Extraction Recovery of U-85575 and IS

Concentration (μg/ml)	Mean ± SD (%)	
	U-85575 (n=3)	U-82217 (IS) (n=9)
0.1	91.5 ± 8.5	
0.5	92.5 ± 5.8	
1		93.4 ± 12.7
10	96.9 ± 6.1	

collected from 14-day toxicity study with U-85575 in rats following the extraction procedures, no additional peaks that could interfere with the determination of U-85575 or IS were present (Figure 2). Several control rat serum pools were tested and showed similar results, indicating the specificity of this method.

TABLE 4

U-85575 Serum Concentrations for a 15-day Toxicity Study

Treatment (mg/kg)	Mean ± SD (n=5) (ng/ml)		
	Day 1	Day 8	Day 15
0	<LOD*	<LOD	<LOD
3	68.0 ± 15.6	42.2 ± 17.2	93.1 ± 44.1
10	236.4 ± 49.4	219.4 ± 52.9	303.8 ± 54.4
30	458.4 ± 123.6	634.2 ± 176.7	1288.5 ± 416.8

*:LOD=limit of detection (10 ng/ml)

Downloaded At: 08:52 25 January 2011

Applicability

The analytical method described above has been applied for the determination of U-85575 concentrations in the serum for a preliminary 15-day toxicity study. Twenty rats in four dose groups (vehicle, 3, 10, and 30 mg/kg/day) received daily single oral administration of the vehicle or U-85575 solution. Five rats were sacrificed for blood samples 1 hr after dosing on days 1, 8, and 15. Serum was obtained by centrifugation and stored at -20°C until analysis.

Table 4 lists the mean serum concentrations for this study. Serum samples collected from rats in the vehicle control group contained no detectable U-85575. At dose levels of 3 and 10 mg/kg, there are no significant differences in the serum concentration among days 1, 8, and 15 ($p>0.05$). However, at 30 mg/kg dose, U-85575 serum concentration significantly increased from day 1 to day 15, suggesting potential bioaccumulation of U-85575 in systemic circulation after 15 days daily dosing with a dose level of 30 mg/kg.

CONCLUSION

An HPLC method for determination of U-85575 in rat serum has been developed. U-85575 in serum can be rapidly and reproducibly extracted by

using phenyl solid phase extraction columns with a minimum volume of organic solvent. Twelve serum samples can be extracted within 30 min. This method has proved to be suitable for use in toxicokinetic studies and appears to have excellent potential for other bioavailability and pharmacokinetic studies during preclinical development of this drug.

ACKNOWLEDGEMENTS

The authors wish to thank T.A. Jackson, J.J. Cypher, D.M. Brussee, B.W. Jones, and T.L. VandeGiessen for their collaboration in conducting the toxicity study with U-85575 in rats, and acknowledge the CNS chemists at Novo Nordisk A/S, Denmark for supplying U-85575. The authors also thank J.E. Katz for assisting in the preparation of this report.

Downloaded At: 08:52 25 January 2011

REFERENCES

1. Puglisi, C.V., Pao, J., Ferrara, F.J. and de Silva, J.A.F., Determination of Midazolam (versed) and Its Metabolites in Plasma by High Performance Liquid Chromatography, *J. Chromatogr.*, 344, 199, 1985.

2. Puglisi, C.V., Pao, J., Ferrara, F.J., and Bekersky, I., Determination of the Agonist-Antagonist Anxiolytic Agent 6-(2-chlorophenyl)-4-Hydroxy-4H-imidazo (1,5 a)-(1,4) Benzodiazepine-3-carboxamide and Its Benzophenone Carboxylic Acid Metabolite in Plasma by High Performance Liquid Chromatography, *J. Chromatogr.*, 417, 347, 1987.

3. Zhong, W.Z., Determination of 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-(1-methylethyl)-imidazo-<1,5-a>- quinoxalin-4(5h)-one in serum by high-performance liquid chromatography, *Journal of Chromatogr.*, 563, 428, 1991.

4. Zhong, W.Z., Determination of 6-chloro-3-(3-cyclopropyl 1,2,4,-oxadiazol-5-yl)-5-methyl-imidazo<1,5-a-quinoxalin-4(5h)-one in rat serum, urine, and brain by solid-phase extraction and liquid chromatography, *Journal of Pharmaceutical & Biomedical Analysis*, 9, 573, 1991.

5. Connors, K.A., A Textbook of Pharmaceutical Analysis, 3rd ed., Wiley, NY, 1982, pp. 595.

6. Peng, G.W., and Chiou, W.L., Analysis of drugs and other toxic substances in biological samples for pharmacokinetic studies, *J. Chromatogra.*, 531, 3, 1990.

7. Thompson, M., Variation of Precision with Concentration in an Analytical System, *Analyst*, 113, 1579, 1988.

Received: April 10, 1992
Accepted: July 1, 1992

Downloaded At: 08:52 25 January 2011