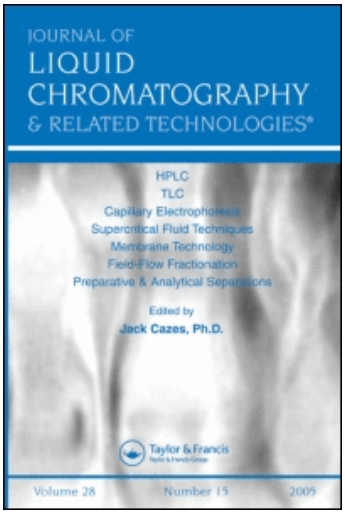

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A High Performance Liquid Chromato-Graphic Method for a Novel Serotonin Reuptake Inhibitor 5-Chloro-2-[[2-(dimethylamino)methyl]phenyl]benzyl Alcohol (403U76)

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A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR A NOVEL SEROTONIN REUPTAKE INHIBITOR 5-CHLORO-2-[[2-(DIMETHYLAMINO)METHYL]PHENYL]BENZYL ALCOHOL (403U76)

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ABSTRACT

A sensitive and selective high performance liquid chromatographic assay using UV-detection was developed for the analysis of 403U76, a structurally novel serotonin reuptake inhibitor, and two demethylated metabolites, 515U88 and 336U76 in rat plasma. The compounds were isolated from plasma by liquid extraction followed by back extraction into 0.1N HCl. Extracts were chromatographed on an Apex I phenyl HPLC column cartridge (4.6 mm x 12.5 cm) using a mobile phase of 74% buffer (0.005M PIC-B5 low UV adjusted to pH 2.5 with 85% phosphoric acid) and 26% acetonitrile. The compounds of interest were detected at 210 nm with a lower limit of detection of 5 ng/mL. Overall precision of the assay for 403U76, 515U88, and 336U76, expressed as percent deviation of measured values from true values and percent coefficient of

variation, respectively, were ≤ 9.7 % at plasma concentrations between 5 and 500 ng/mL. This assay has been successfully used to analyze plasma samples from preclinical pharmacokinetic and disposition studies in rats.

INTRODUCTION

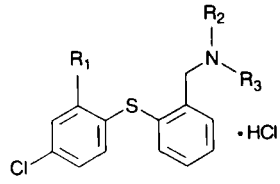
403U76, 5-chloro-2- [[2-[(dimethylamino) methyl]phenyl] thio] benzyl alcohol hydrochloride (Fig. 1), is a structurally novel serotonin reuptake inhibitor which is being studied as a potential antidepressant. Preclinical pharmacology indicated that 403U76 inhibits the neuronal uptake of serotonin (5-HT) and to a lesser extent, inhibits the neuronal uptake of norepinephrine (NE).[1] Consequently, 403U76 may be useful for the treatment of major depressive illness.[2-4]

403U76 is extensively metabolized and two metabolites have been identified in plasma as 5-chloro-2-[[2-[(methylamino) methyl] phenyl] thio]benzyl alcohol (515U88) and 5-chloro-2-[[2-[(amino) methyl] phenyl] thio]benzyl alcohol (336U76).[5] 515U88 is also a potent serotonin reuptake inhibitor while 336U76 has weak activity.[1] To provide analytical support for preclinical development of 403U76, a high performance liquid chromatographic (HPLC) assay was developed and validated for quantitation of 403U76 and its two metabolites in rat plasma. This assay has been used to monitor plasma concentrations during pharmacokinetic and disposition studies of 403U76 in rats.

MATERIALS AND METHODS

Materials

403U76, 515U88, 336U76, and the internal standard 516U88 (5-chloro-2-carbomethoxy-2-[(amino)methyl]diphenyl sulfide) were synthesized at Burroughs Wellcome Co., Research Triangle Park, NC. The chemical structures of 403U76, its metabolites, and internal standard are shown in Fig 1. Octane sulfonic acid, (PIC-B5, low UV) was purchased from Waters, Millipore Division, Milford, MA. Phosphoric acid (85%) was A.R. grade (Mallinckrodt, Paris, KY.) Hydrochloric acid (0.1N) was purchased from Fisher Scientific, Raleigh, NC. Acetonitrile, methyl-t-butyl ether and water were HPLC grade (Omnisolv, EM Science, Cherry Hill, NJ).



Compound	R_1	R_2	R_3
403U76	CH_2OH	CH_3	CH_3
515U88	CH_2OH	CH_3	H
336U76	CH_2OH	H	H
516U88	$COOCH_3$	H	H

Figure 1. Structure of 403U76, its metabolites, and internal standard 516U88.

Instrumentation

A Hewlett Packard HP-1090 HPLC system (Hewlett Packard, Avondale, PA) was used in conjunction with an Apex I phenyl analytical cartridge (4.6 mm x 12.5 cm) (Jones Chromatography, Litton, CO) for the separation of 403U76 and its metabolites. A precolumn cartridge containing the same bonded phase was coupled directly to the analytical cartridge. The analytical cartridge was maintained at 45°C within the heated column compartment of the HPLC. Samples were injected onto the column with a HP-1090 autoinjector and the analytes were quantitated with a Waters 486 variable wavelength detector set at 210 nm. The HPLC mobile phase was 26% acetonitrile and 74% buffer (0.005M PIC-B5 adjusted to pH 2.5 with 85% phosphoric acid). With a flow rate of 1 mL/min, the retention times of 403U76, 515U88, 336U76 , and the internal standard were approximately 12.8, 9.9, 8.3, and 14.9 min, respectively. Chromatographic data acquisition, peak-area analysis and data reduction were performed on a HP-Chemstation (Hewlett-Packard, Avondale, PA).

Preparation Of Standards And Solutions

Analytical standard solutions of 403U76, 515U88 and 336U76, (100 μg/mL) and the internal standard (50 μg/mL) were prepared in methanol. The hydrochloride salts of each compound were used and weights were corrected such that concentrations represented the free base. Analytical standards were diluted with methanol to prepare working stock solutions containing 5-500 ng/mL of 403U76, 515U88 and 336U76. Calibration standards were prepared by evaporating aliquots of the methanol solutions under nitrogen in a Zymark Turbo Vap LV (Zymark, Hopkinton, MA) followed by the addition of 1 mL of rat plasma. Quality control (QC) samples were prepared in a similar manner at concentrations of 5, 50, and 500 ng/mL. These samples were frozen at -80°C and replicate samples (n=2) were assayed with each analysis of experimental samples.

Extraction Procedures

Plasma samples (1 mL) and calibration standards (5-500 ng/mL) were spiked with internal standard (50 μg/mL of 516U88) and extracted with 4 mL of methyl-t-butyl ether. Samples were shaken for 15 min and then centrifuged at 1000 x g for 5 min. The tubes were immersed in a dry-ice acetone bath to freeze the aqueous phase, and the organic phase was transferred to a graduated tapered test tube containing 150 μL of 0.1N HCl. The tubes were shaken for 15 min, and centrifuged at 1000 x g for 5 min. The acid fraction was pipetted into autoinjector vials and a 50 μL aliquot was injected onto the HPLC column.

Assay Validation

To determine the intra-day accuracy and precision of the assay, replicate (n=3) plasma samples at seven different concentrations (5-500 ng/mL) were analyzed using SAS JMP® statistical software (SAS Institute, Cary, NC). The peak area ratios of each compound, and the internal standard, were fitted to different regression models; namely, an unweighted least-squares regression, a weighted least-squares regression (1/c, $1/c^2$ or 1/variance) and a least-squares regression of the logarithmically-transformed data. Residual plots were generated for each regression model and the best fit determined, based on the variance observed over the concentration range. For each compound, the residual plot from least-squares regression of the logarithmically-transformed calibration curve data showed equal variances over the concentration range. Therefore, this regression model was used to calculate the concentrations of

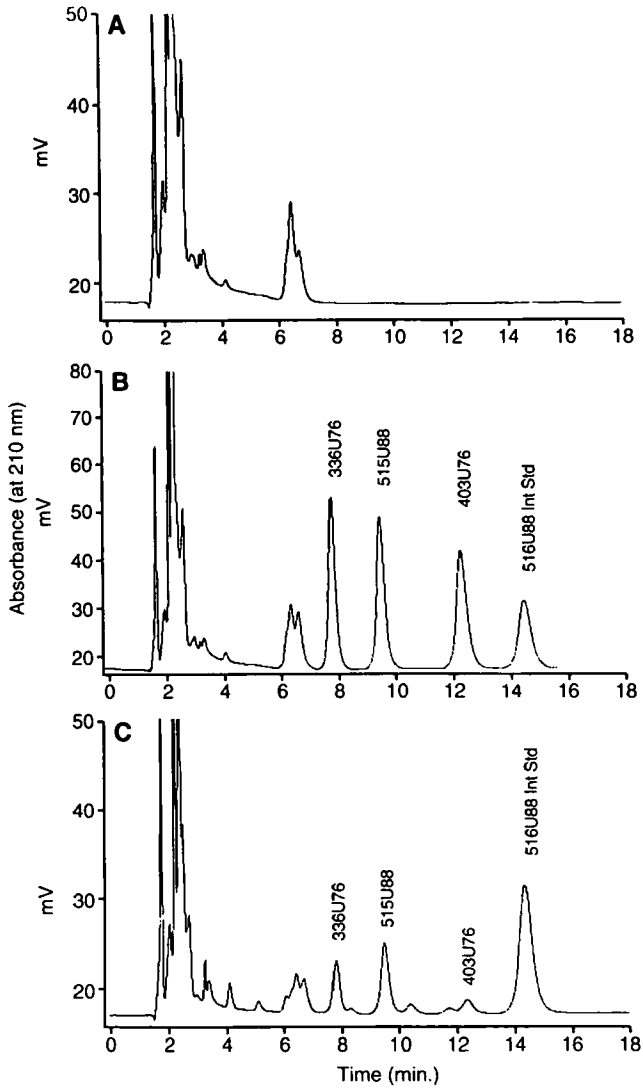


Figure 2. HPLC chromatograms of extracted rat plasma samples. A: blank plasma. B: plasma containing 500 ng/mL of 403U76 (peak 1), 515U88 (peak 2), 336U76 (peak 3) and50 μg/mL internal standard 516U88 (peak 4). C: actual plasma sample collected from a rat dosed with 403U76.

each compound in unknown samples. Precision was estimated from the standard deviation expressed as the percentage of the mean (% coefficient of variation, CV). Accuracy was calculated as the percentage difference between the mean calculated concentration and the amount added (% bias). To determine the inter-day accuracy and precision of the assay, spiked plasma pools were prepared at three concentrations (5, 50 and 500 ng/mL) of each compound. The pools were separated into 1 mL aliquots and frozen at -80°C. Duplicate samples from each pool were analyzed with each calibration curve over a 7-day period to assess the inter-day accuracy and precision of the assay. An analysis of variance (ANOVA) was performed using SAS JMP® statistical software (SAS Institute, Cary, NC) to determine assay precision.

The stability of 403U76, 515U88, 336U76, and internal standard 516U88 in rat plasma stored at -80°C was determined. Plasma was spiked with 25 and 500 ng/mL of each compound. Replicate samples (n=2) were analyzed immediately and at various times after storage at -80°C. The stability of each compound was also determined in standard solutions stored at -20°C and in plasma samples after five freeze-thaw cycles.

The extraction efficiency of the assay was determined by comparing the peak areas for each compound in extracted standards with those obtained by injection of unextracted standards.

The pharmacokinetics and disposition of 403U76 and its metabolites were studied in male and female rats after an oral (15 mg/kg) and intravenous (5 mg/kg) dose of ^{14}C-403U76.[5] Venous whole blood samples were collected at various times after dosing and plasma was separated by centrifugation. Plasma samples were frozen at -80°C until analyzed.

RESULTS AND DISCUSSION

A sensitive and selective HPLC assay using UV-detection was developed for the analysis of 403U76 and its two demethylated metabolites in rat plasma. The assay is specific for 403U76 and its metabolites with no interfering peaks in chromatograms of control plasma (Fig. 2). Fig. 2 also shows a chromatogram of a calibration standard (50 ng/mL) and a chromatogram of extracted plasma from a rat dosed with 15 mg/kg 403U76. The lower limit of quantitation for each compound was 5 ng/mL plasma.

Table 1

Intra-day Precision and Accuracy for the Analysis of 403U76, 515U88 and 336U76 in Rat Plasma

Compound	Mean Conc. Added (ng/mL)	Mean Conc. Found (ng/mL)	CV (%)	Bias (%)
403U76	5	4.7	8.5	-6.4
	10	10.5	4.4	4.8
	20	22.1	9.3	9.5
	50	50.5	0.5	1.0
	100	101.2	1.2	1.2
	250	247.7	2.5	-0.9
	500	481.1	0.7	-3.9
515U88	5	4.8	4.8	-4.2
	10	10.4	4.9	3.8
	20	20.3	4.9	1.5
	50	50.8	0.5	1.6
	100	102.0	1.7	2.0
	250	242.8	1.1	-3.0
	500	497.2	0.2	-0.6
336U76	5	4.8	9.7	-4.2
	10	10.2	6.1	2.0
	20	20.3	1.8	1.5
	50	51.7	1.1	3.4
	100	103.1	1.0	3.0
	250	246.9	1.6	-1.3
	500	483.4	1.2	-3.4

Replicate analyses (n=3). Precision was derived from the standard deviation and is expressed as the percent coefficient of variation. Accuracy is expressed as bias of the theoretical concentration.

Table 2

Inter-day Precision and Accuracy for the Analysis of 403U76, 515U88 and 336U76 in Rat Plasma

Compound	Mean Conc. Added (ng/mL)	Mean Conc. Found (ng/mL)	CV[a] (%)	Bias (%)
403U76	5	4.9	3.7	-1.2
	50	53.0	1.5	5.7
	500	477.1	2.3	-4.8
515U88	5	5.3	5.9	4.9
	50	50.9	2.4	1.7
	500	499.9	2.9	0.0
336U76	5	5.1	7.3	2.3
	50	48.6	4.4	-2.8
	500	539.9	3.5	7.4

Replicate analyses (n=14) for low, medium and high concentrations.
Precision was derived from the standard deviation and is expressed as the percent coefficient of variation. Accuracy is expressed as bias of the theoretical concentration.
[a] Inter-day precision was derived using an Analysis of Variance (ANOVA).

The intra-day precision and accuracy of the assay for 403U76, 515U88, and 336U76 in rat plasma are shown in Table 1. The intra-day %CV was 9.7 or less for 403U76 and its metabolites over the concentration range of 5-500 ng/mL. The % bias was less than 9.5% for all compounds. The inter-day accuracy and precision of the assay, measured daily over a 14 day period, is shown in Table 2. The inter-day % CV was 7.3 % or less and the % bias was 7.4% or less for all three compounds.

The extraction efficiencies for 403U76, 515U88, and 336U76 over the concentration range of 5-500 ng/mL are shown in Table 3. The mean extraction efficiencies were 99.5, 102.1 and 102.9% for 403U76, 515U88 and 336U76, respectively.

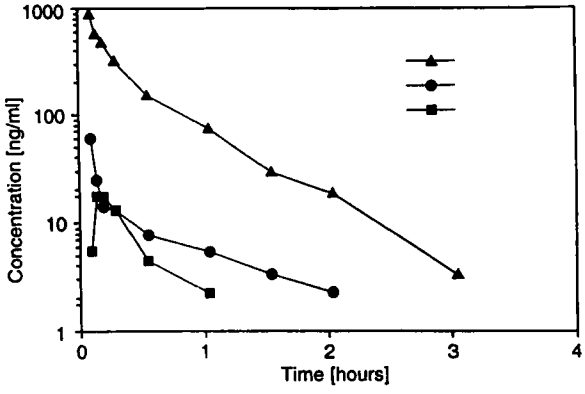


Figure 3. Plasma concentration profile for 403U76 (▲)515U88 (●) and 336U76(■) in rats following administration of a 5 mg/kg single intravenous dose of 403U76.

Table 3

Extraction Efficiencies for the Analysis of 403U76, 515U88 and 336U76 in Rat Plasma

Compound	Conc. (ng/mL)	Recovery (Mean ± S.D.) (%)
403U76	5	973 ± 12.0
	50	105.9 ± 4.8
	500	95.4 ± 3.1
515U88	5	105.2 ± 9.7
	50	101.3 ± 4.1
	500	99.9 ± 3.1
336U76	5	102.3 ± 14.9
	50	98.6 ± 5.1
	500	107.9 ± 3.8

Replicate analyses (n = 14) for low, medium and high concentrations.

The stability of 403U76 and its metabolites in plasma stored at -80°C was evaluated. No degradation of either 403U76, 515U88 or 336U76 was detected in plasma samples stored for at least 56 weeks at this temperature. In addition, no degradation of either compound was detected in plasma samples after five freeze-thaw cycles.

This assay was used to quantitate 403U76 and its metabolites from a rat pharmacokinetic and disposition study.[5] As an example of the utility of the assay, a plasma concentration versus time curve for each compound following an intravenous dose of 5 mg/kg of ^{14}C-403U76 to rats is shown in Fig. 3.

In summary, this assay allowed for the quantitation of 403U76, and its metabolites 515U88, and 336U76 in rat plasma and provides evidence that the method can be used to support preclinical pharmacokinetic and disposition studies.

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