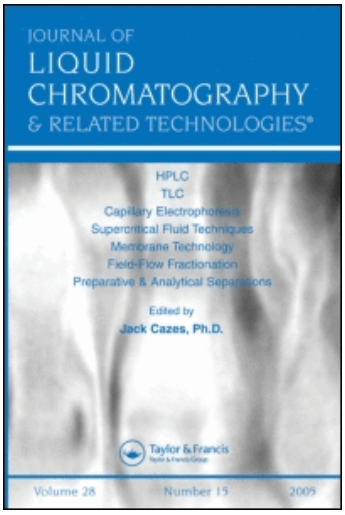

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Ai-Ly Y. Hsieh[a]; Debra M. Bulkowski[a]; William A. Wargin[a]
[a] Wellcome Research Laboratories, Burroughs Wellcome Co. Research, North Carolina

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A NORMAL PHASE HPLC METHOD FOR THE DETERMINATION OF TWO ARYLMETHYLAMINO-PROPANEDIOLS (AMAPs), CRISNATOL AND 773U82 IN PLASMA

AI-LY Y. HSIEH, DEBRA M. BULKOWSKI, AND WILLIAM A. WARGIN
Wellcome Research Laboratories
Burroughs Wellcome Co.
Research Triangle Park, North Carolina 27709

ABSTRACT

A normal phase HPLC method has been developed for the analysis of two potential antitumor agents, crisnatol and 773U82 in plasma samples. The assay procedures for both compounds are essentially the same. The assay uses a silica column and UV detection. The mobile phase consists of dichloromethane: methanol: perchloric acid (95:5:0.02). The plasma sample is extracted with chloroform: methanol (9:1) after potassium hydroxide basification. The reproducibility of the method was demonstrated by the analysis of spiked samples containing 5-1000 ng/ml. Application of the assay to analysis of crisnatol and 773U82 in plasma samples is demonstrated by measurement of the concentrations obtained from patients in Phase I clinical studies.

INTRODUCTION

Crisnatol (2-((6-chrysenylmethyl)amino)-2-methyl-1,3-propanediol methane sulfonate); BWA770U) and 773U82

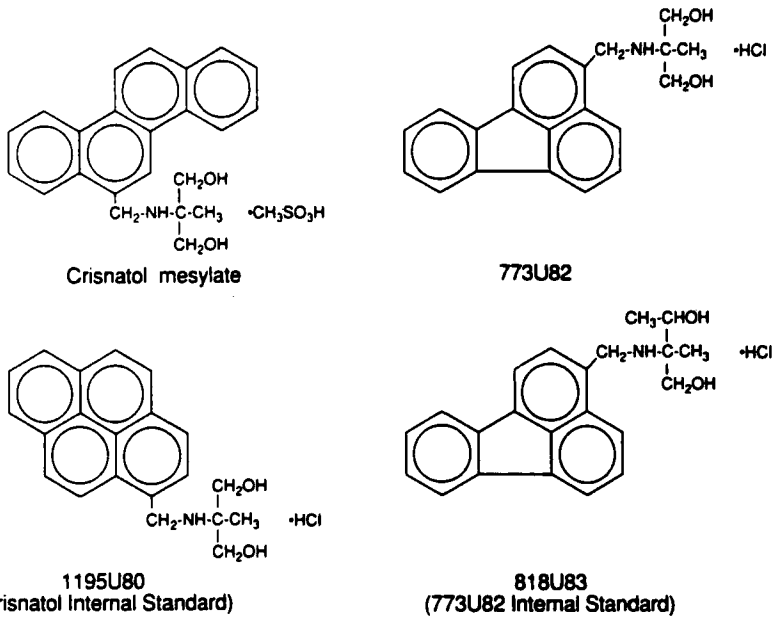

Figure 1. Structures of crisnatol and 773U82 and their internal standards.

(2-((3-fluoranthenyl-methyl)amino)-2-methyl-1,3-propanediol hydrochloride; BWA773U) (Figure 1) are the first two members of a series of arylmethylaminopropanediol (AMAP) DNA intercalators synthesized at Burroughs Wellcome Co. (1), and are currently in clinical trials (2,3). Both compounds have been shown to have marked antitumor activity in murine leukemic and solid tumor models as well as promising tumor sensitivity in the soft agar human tumor stem cell assay (4).

MATERIALS AND METHODS

Instrumentation

The HPLC system consisted of a Waters pump (model 510), a Waters autoinjector (WISP 712), a Kratos Spectroflow 757 variable

wavelength detector (or equivalent), a 5 micron silica column (4.6 mm x 25 cm, ES Industries), a LiChrosorb Si60 guard column (EM Science), and a DS-80Z microcomputer (Digital Specialities) for data acquisitions and analysis. The wavelength of the detector was set at 269 nm for crisnatol and 288 nm for 773U82. The mobile phase consisted of dichloromethane:methanol:perchloric acid (95:5:0.02) and was pumped at a flow rate of 1 ml/min.

Chemicals and Reagents

Methanol, dichloromethane and chloroform were HPLC grade (EM Science). Potassium hydroxide was reagent grade (Mallinckrodt). Perchloric acid (70%) was high purity reagent grade (GFS Chemicals). Water was purified by Milli-RQ system (Millipore).

Preparation of Plasma Standard Curve

Stock solutions of crisnatol and 773U82 were prepared at 0.5 mg/ml in methanol and working solutions of 0.05, 0.5 and 5 µg/ml were prepared from this stock solution. Compounds 1195U80, 2-methyl-2((1-pyrenylmethyl)amino)-1,3-propanediol hydrochloride, and 818U83, (+-)-(2R*, 2S*)-2-(((3-fluoroanthenyl)-methyl)amino)-2-methyl-1,3-butanediol hydrochloride (Figure 1), were used as internal standards for crisnatol and 773U82 assay, respectively. Stock solutions for internal standards were prepared at 0.5 mg/ml in methanol and 100-fold dilutions were made to obtain the 5 µg/ml working solutions. A standard calibration curve (5-1000 ng/ml) was generated by adding the desired amount of crisnatol or 773U82 solution to 16 x 100 mm test tubes which contained 40 µl of internal standard (5 µg/ml). These spiked amounts were evaporated to dryness and 0.1 ml blank human plasma and 0.4 ml water were added. These standard samples were then basified with 50 µl of 0.8 N KOH solution and extracted with 2.5 ml

chloroform:methanol (9:1). The tubes were gently rotated on a multi-purpose rotator for 10 min and then centrifuged for 10 min at 1000 x g. The organic layer was then transferred to 12 x 75 mm disposable glass culture tubes and evaporated to dryness in a 40°C water bath under a gentle stream of nitrogen. The residue was reconstituted with 200 µl dichloromethane and 20-100 µl was injected onto the column. The standard calibration curve was constructed by plotting the peak area ratio of crisnatol or 773U82 to their internal standards versus concentration.

Recovery and Assay Validation

Recovery was determined by comparing the peak area ratio of drug to internal standard from the extracted samples to those from direct injections of the equivalent amounts. A six-point calibration curve, four replicates at each concentration, was prepared for the recovery study for both crisnatol and 773U82. Equivalent amounts of crisnatol and 773U82 from the working standard solutions were evaporated to dryness and reconstituted in dichloromethane for direct injections onto the column. The peak area ratios of the direct injection standards and the extracted samples were measured.

To study the inter-assay precision and accuracy, a 5-point crisnatol and 773U82 calibration curves, 3-5 replicates at each concentration, were prepared on six and seven different days, respectively, for crisnatol and 773U82 over a period of 3 and 8 weeks. Another three spiked samples, represented the low, medium and high concentration of the expected ranges in the patients, were designated as quality control samples and analyzed each day in 2-3 replicates.

Analysis of Patient Plasma Samples

Portions (50-500 µL) of plasma samples were added to the tubes which contained previously dried internal standard and were

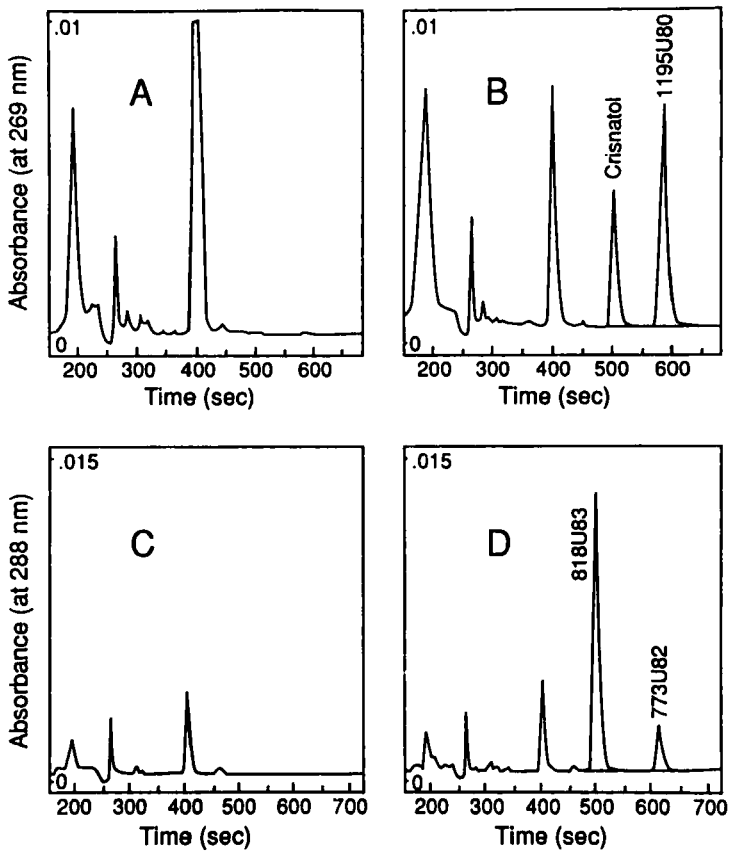

Figure 2. Typical chromatograms of extracted plasma samples. (A) Blank plasma, (B) extracted crisnatol standard of 50.2 ng/ml, (C) blank plasma, (D) extracted 773U82 standard of 50 ng/ml.

extracted using the procedure described above for plasma standards. If less than 500 µL samples were used, the volume was made up to 500 µL with water. A standard curve was prepared daily to calculate the unknown concentrations using linear least-squares regression analysis. Also, low, medium and high quality control samples were processed along with the regular

TABLE 1 Recovery and Intra-Assay Precision of Crisnatol and 773U82 Plasma Calibration Curve

Spiked Conc. (ng/ml)	Peak Area Ratio		Intra-Assay Precision (% C.V.)	Recovery (%)
	Neat Standard	Extracted Standard		
Crisnatol	(n=2)	(n=4)		
5.02	0.077 ± 0.003	0.060 ± 0.002	4.1	77.9
50.2	0.674 ± 0.014	0.519 ± 0.006	1.2	77.0
100	1.30 ± 0.020	1.07 ± 0.007	0.7	82.1
251	3.44 ± 0.069	2.61 ± 0.024	0.9	75.9
502	6.48 ± 0.334	5.08 ± 0.076	1.5	78.4
1004	12.1 ± 0.085	9.67 ± 0.019	0.2	80.0
Regression Equation:	y=0.0120x+0.1708 r^2 = 0.999	y=0.0096x+0.1000 r^2 = 0.999		
773U82				
	(n=3)	(n=4)		
4.99	0.085 ± 0.004	0.085 ± 0.007	7.8	100
49.9	0.208 ± 0.003	0.189 ± 0.006	3.0	90.9
99.9	0.372 ± 0.008	0.354 ± 0.006	1.6	95.2
250	0.772 ± 0.006	0.741 ± 0.020	2.6	96.0
499	1.44 ± 0.018	1.43 ± 0.008	0.6	99.0
999	2.80 ± 0.216	2.79 ± 0.038	1.3	99.6
Regression Equation:	y=0.0027x+0.083 r^2 = 0.999	y=0.0027x+0.067 r^2 = 0.999		

plasma samples to monitor the long-term reproducibility of the assay.

RESULTS AND DISCUSSION

The use of a 5 micron particle size silica column in a normal phase mode with a mobile phase of dichloromethane: methanol:perchloric acid (95:5:0.02) produced excellent separation of crisnatol or 773U82 and their internal standards from endogenous peaks in the plasma (Figure 2). Crisnatol and

TABLE 2 Inter-Assay Accuracy/Precision of Crisnatol and 773U82

Crisnatol	Target Conc. (ng/ml)			Slope ($x10^{-3}$)	Intercept
	10.0	100	502		
day 1	10.3	102	502	10.1	0.001
day 2	9.32	100	501	10.1	0.004
day 3	9.29	102	498	10.3	0.004
day 4	11.3	102	503	10.3	-0.009
day 5	9.50	100	502	10.4	0.006
day 6	9.45	102	509	9.98	0.010
Mean	9.85	101	502		
SD	0.80	0.80	3.8		
Precision (% C.V.)	8.1	0.79	0.76		
Accuracy[1] (%)	98.1	101	100		

773U82	Target Conc. (ng/ml)			Slope ($x10^{-4}$)	Intercept
	10.0	100	500		
day 1	12.4	101	491	27.4	0.063
day 2	9.86	99.9	497	27.0	0.064
day 3	12.4	101	489	26.6	0.057
day 4	11.6	104	498	26.5	0.064
day 5	1.0	103	491	27.5	0.060
day 6	10.8	103	497	27.5	0.060
day 7	11.5	101	499	28.5	0.058
Mean	11.4	102	494		
SD	0.85	1.37	3.67		
Precision (% C.V.)	7.5	1.3	0.74		
Accuracy (%)	114	102	98.9		

[1]Accuracy = $\frac{\text{assayed conc.}}{\text{target conc.}}$ x 100

773U82 are readily extracted from plasma in a single step using chloroform:methanol (9:1) after potassium hydroxide basification. The recovery was found to be 75-80% for crisnatol and 90-100% for 773U82 by comparing the peak area ratio of the extracted standards with those of neat standards (Table 1). There was no trend of increase or decrease in recovery as a function of concentration. The intra-assay precision of an extracted plasma

TABLE 3 Long-Term Reproducibility of Crisnatol and 773U82 Plasma Assay

Sample	Target Conc. (ng/ml)	Average Conc. (ng/ml)	S.D.	Precision (% C.V.)	Accuracy (%)
Crisnatol					
QC low	25.0	23.4	1.3	5.6	93.4
QC medium	250	240	10.1	4.2	96.0
QC high	700	666	27.3	4.1	95.1
773U82					
QC low	12.0	13.7	1.2	8.8	114
QC medium	100	89.2	2.9	3.2	89.2
QC high	450	435	12.6	2.9	96.7

n=10

standard curve (5-1000 ng/ml), 4 replicates for each concentration, are shown in Table 1. The coefficient of variation (C.V.) for the replicates is less than 10% over a concentration range of 5-1000 ng/ml and the correlation coefficient (r^2) is greater than 0.999 after least-squares linear regression analysis for both crisnatol and 773U82. The lower limit of quantification was established as 5 ng/ml using 0.5 ml plasma for analysis of crisnatol and also 5 ng/ml using 0.25 ml of plasma for 773U82 by the criteria of 10% C.V. for precision and 100 ± 20% for accuracy.

The inter-assay accuracy and precision results are shown in Table 2. The accuracy was expressed as % of the amount assayed by HPLC divided by the nominal amount spiked in the plasma. The accuracy was within 100 ± 2% for crisnatol and 773U82 at all

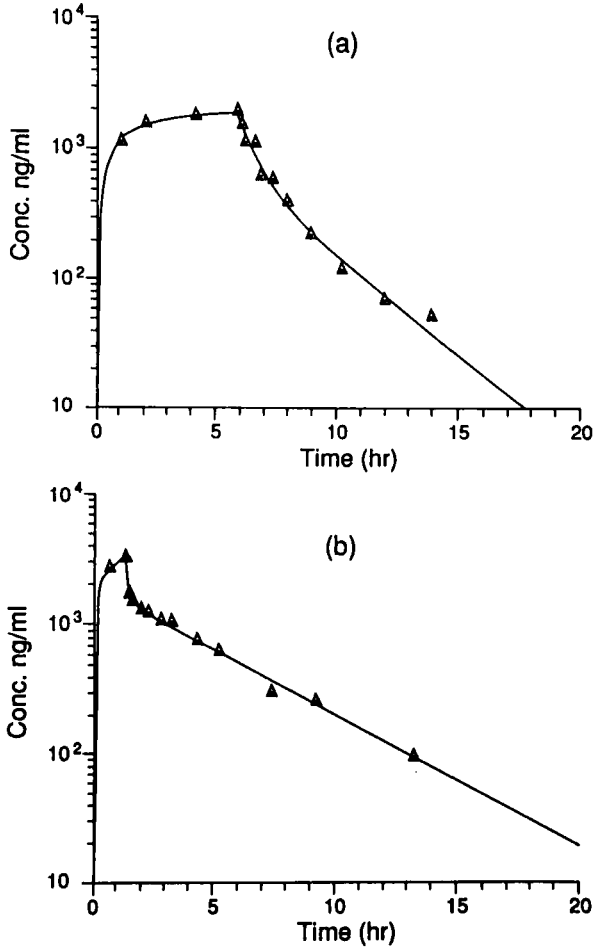

Figure 3. Plasma profile of (a) crisnatol following a six hour infusion at 30 mg/m^2, (b) 773U82 following a one hour infusion at 480 mg/m^2.

concentrations except at 10 ng/ml for 773U82, where it was 114%. Inter-assay precision was acceptable since the C.V. was less than 10% for both crisnatol and 773U82 over the concentration range studied. Low, medium and high quality control samples were analyzed each day and the method showed good long-term reproducibility over five months (Table 3).

This plasma assay method has been applied to the analysis of patient samples obtained from clinical studies of crisnatol and 773U82. A crisnatol Phase I trial utilizing a six hour infusion repeated every 28 days was initiated at a dose of 7.5 mg·m^2. A 773U82 Phase I trial utilizing a one hour infusion was initiated at a dose of 15 mg/m^2. Blood samples were taken during infusion and at frequent times after cessation of infusion. Typical plasma profiles for crisnatol and 773U82 observed in patient from each of these trials are shown in Figure 3. These drugs are currently being studied in Phase II trials for evaluation of their efficacy in the treatment of selected solid tumors.

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