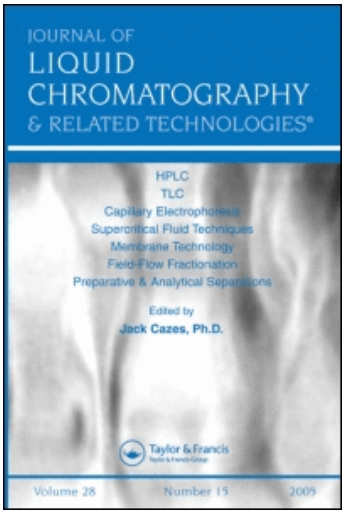

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Sensitive High Pressure Liquid Chromatographic Technique by Which to Measure Didemnin B in Biological Fluids

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To cite this Article Hartshorn, Jeanette N. , Tong, William P. , Stewart, James A. and McCormack, John J.(1986) 'A Sensitive High Pressure Liquid Chromatographic Technique by Which to Measure Didemnin B in Biological Fluids', Journal of Liquid Chromatography & Related Technologies, 9: 7, 1489 — 1501
To link to this Article: DOI: 10.1080/01483918608076698
URL: http://dx.doi.org/10.1080/01483918608076698

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(7), 1489-1501 (1986)

A SENSITIVE HIGH PRESSURE LIQUID CHROMATOGRAPHIC TECHNIQUE BY WHICH TO MEASURE DIDEMNIN B IN BIOLOGICAL FLUIDS

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INTRODUCTION

Didemnin B, (NSC-325319) (Fig. 1), a cyclic depsipeptide isolated from a Caribbean tunicate, has been recently determined to be active against B16 melanoma, both in vitro and in vivo, P388 leukemia in vivo (1,2,3,4), and L1210 in vitro (1,3). This agent also inhibits DNA, RNA and protein synthesis (1,2,5,6,) and possesses antiviral activity (3). Using an in vitro stem

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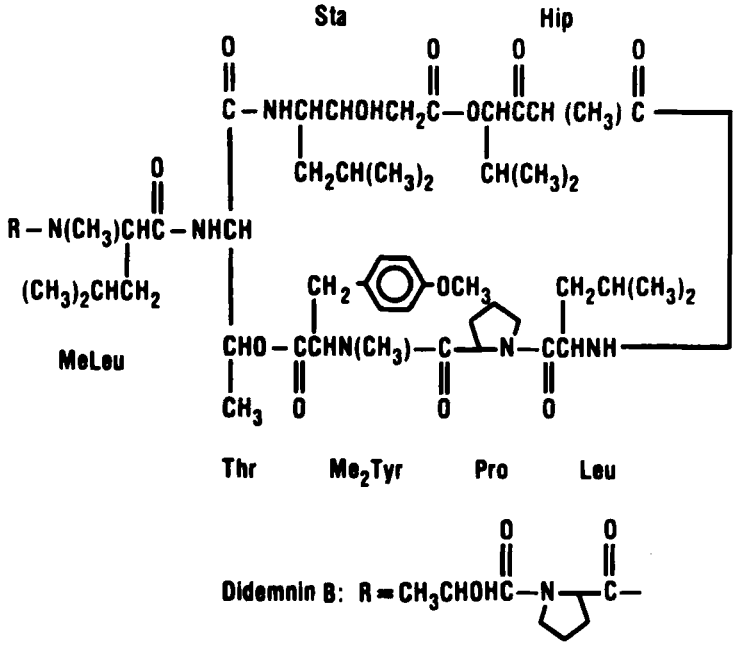

Figure 1: Structure of Didemnin B

cell assay, both Jiang, et al (5), and Rossof, et al (7), have demonstrated Didemnin B to have significant antitumor activity against several human tumor types, including carcinoma of the kidney, lung, breast, and ovary, mesothelioma, sarcoma, and hairy cell leukemia.

Phase I clinical trials of Didemnin B have recently been initiated at the Vermont Regional Cancer Center. We, therefore, have developed a high pressure liquid chromatographic (HPLC) technique by which to measure drug levels in the patients receiving this compound. There is no published method suitable to measure concentrations of

Didemnin B in biological samples, and which is applicable to clinical pharmacokinetic studies. The HPLC method described here affords both sensitivity and accuracy, while also being relatively simple and rapid.

MATERIALS AND METHODS

Standards and Reagents

Didemnin B in a powder form was kindly provided by Dr. Matthew Suffness (Division of Cancer Treatment, National Cancer Institute, Bethesda, MD). Stock solutions (1.0 mg/ml) were dissolved in methanol/water (50/50), and serial dilutions were made in the mobile phase. Stock solutions remained stable for at least two weeks when maintained at -20°C.

Reagents and solvents included Baker HPLC grade water, methanol, and acetonitrile (J.T. Baker Chemical Company, Brick Town, NJ), Mallinckrodt acetic acid (Mallinckrodt, St. Louis, MO), and Fisher potassium hydroxide, triethylamine, and HPLC grade monobasic potassium phosphate (Fisher Scientific, Pittsburgh, PA). Control plasma was donated by the American Red Cross (Burlington, VT), and control urine was obtained from normal healthy volunteers. The vacuum manifold and cyano Bond Elut sample extraction columns with 500 mg column beds were purchased from Analytichem International (Harbor City, CA).

Chromatographic Apparatus and Conditions

A Spectra Physics Model 8700 liquid chromatograph (Spectra Physics, Piscataway, NJ) was used to perform the separations, along with a Bio-Rad model AS-48 autosampler (Rockville Centre, NY), equipped with a refrigerated sample compartment, and fit with a Bio-Rad Model 70-10 injection valve and a 20 ul sample loop.

The separations were effected on an Alltech C3 RSiL column (250 x 4.5 mm ID) connected to a trimethylsilane adsorbosphere guard column cartridge (10 mm x 4.6 mm) (Alltech Associates, Deerfield, IL), both 5um particle size, placed within a circulating water jacket (Alltech Associates, Deerfield, IL), at 58°C. The mobile phase consisted of acetonitrile/50 mM KH_2PO_4 (57/43), with 0.01% triethylamine, and was run at a flow rate of 1.0 ml/min. UV detection was carried out with a Kratos model 757 variable-wavelength spectrophotometer set at 220nm and 0.005 AUFS.

Sample Preparation

Lensmeyer, et al (8), have described a procedure for the extraction of another cyclic polypeptide, cyclosporine-A, from whole blood, serum, and plasma. A modification of this method was used for the extraction of both plasma and urine samples, employing cyano Bond Elut solid phase extraction columns.

Extraction columns were initially pre-wet with three column reservoirs each of methanol followed by

water. Then, for the preparation of plasma samples, one ml aliquots of spiked and blank control or patient samples were diluted 1:3 in water/acetonitrile (80/20) and then loaded onto the cyano columns. After the samples were passed (with suction) through the columns, one ml of water was added to each column followed by two ml of 0.001 N KOH as a wash. Didemnin B was eluted using 1.75 ml of methanol. The samples were then partially evaporated under a stream of nitrogen, after which they were diluted with mobile phase, and their volumes were measured. Samples were then vortexed and chromatographed.

Urine samples were also diluted before loading onto pre-wet cyano columns; a 1:2 dilution in water/acetonitrile (60/40) was found to yield optimal recovery. After the samples (spiked and blank controls and patient urines) have been passed through the columns, the columns were washed sequentially with 3 ml of a 0.5N acetic acid/acetonitrile (80/20) solution, 1 ml of a 0.5N acetic acid/acetonitrile (60/40) solution and finally 0.1 ml of 0.001 N KOH. Didemnin B was eluted with 1.75 ml of methanol after which these samples were then treated in the same manner as the processed plasma samples.

RESULTS AND DISCUSSION

This method employing Bond Elut cyano columns demonstrates reproducible recoveries while eliminating

all compounds co-eluting with Didemnin B in plasma and urine under the chromatographic conditions described here. Chromatograms of blank and spiked plasma samples are depicted in Figure 2, while Figure 3 shows those of blank and spiked urine samples. The KOH wash described in the extraction of both plasma and urine samples was required in order to achieve good baseline resolution between the Didemnin B peak and the solvent front.

Standard curves, consisting of concentrations of Didemnin B both below and above the sample concentrations, were run daily to ascertain consistency in detector response as measured both by the integrations of peak areas and the measurements of peak heights of the drug. These curves have been demonstrated to be linear over the range of 4 ng to 356 ng of Didemnin B injected (187.5nM to 16 uM) using a 20 ul sample loop. Using least squares regression analysis, standard curves of both concentration versus peak area and concentration versus peak height had a linear regression coefficient of 0.9999 (n=8), however, the average interday coefficient of variation at each concentration, with the number of samples per concentration varying between 14 and 22, was 9.1% for peak area, and 23.7% for peak height. We therefore chose to use peak area integrations rather than peak height measurements to determine drug recoveries from biological samples and drug levels in unknown samples. Degradation of the guard column, due to its

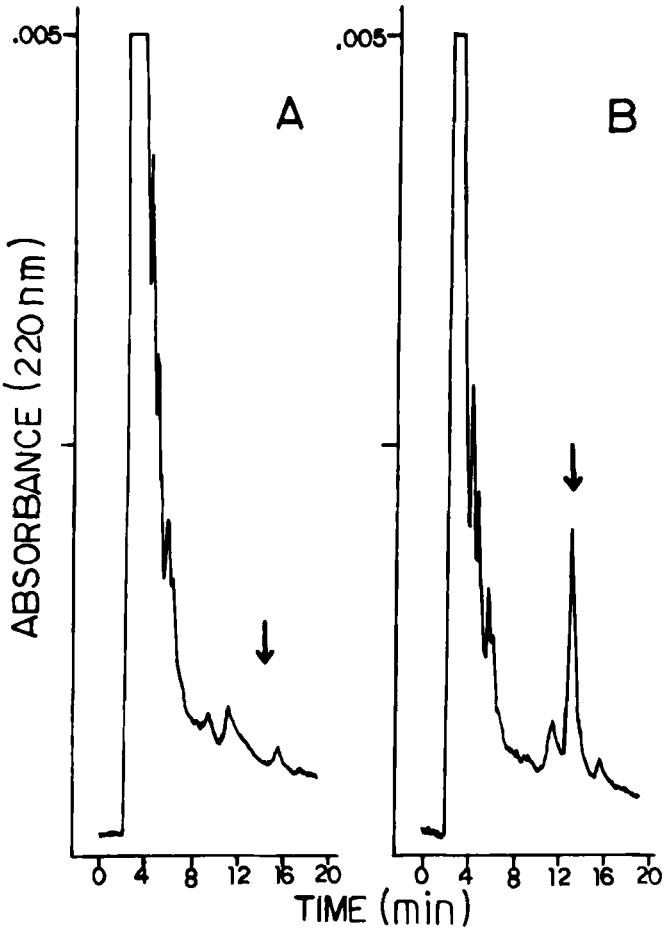

Figure 2: a) Chromatogram of 1 ml blank control plasma
b) Chromatogram of 1 ml 1 uM spiked control plasma
Arrows indicate where Didemnin B elutes.

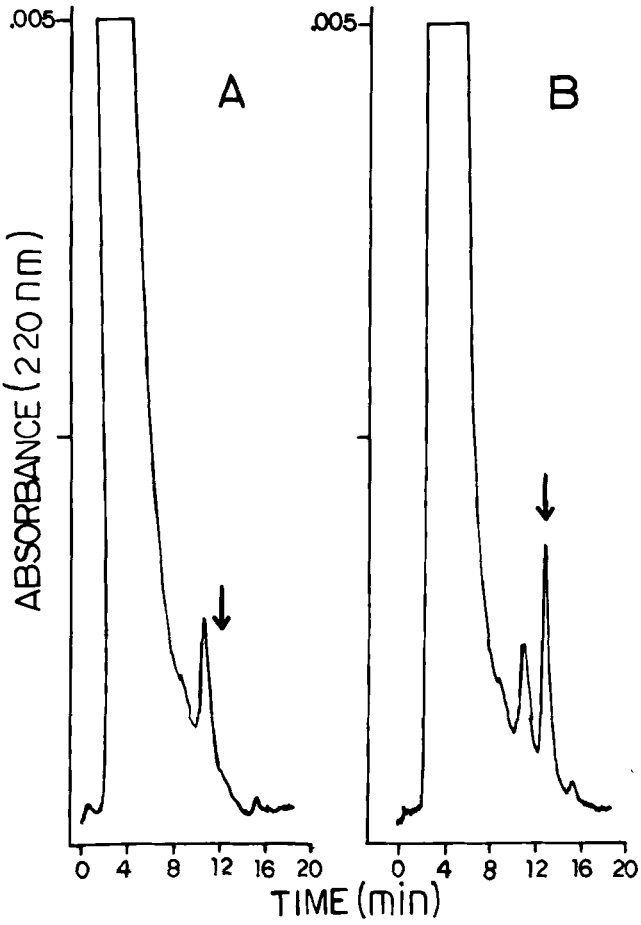

Figure 3: a) Chromatogram of 1 ml blank control urine
b) Chromatogram of 1 ml 1 uM spiked control urine
Arrows indicate where Didemnin B elutes.

temperature being maintained at 58°C appears to be the principal reason for the high coefficient of variation in peak heights, since after changing the guard column, the peak heights at each concentration re-established interday consistency. The average lifetime of a guard column in this assay was approximately two to three weeks, or 250 runs, after which high back pressure and broadened peaks resulted.

Using 1 ml sample aliquots, spiked control plasma and urine concentrations ranging from 0.125 uM to 4.5 uM in plasma and 0.25 uM to 8.0 uM in urine were tested, with the minimum detection limits being 5 ng for plasma and 11 ng for urine. Intraday and interday recoveries from processed spiked plasma were 78.2% (CV = 2.2%) and 77.9% (CV = 7.5%), respectively, while those for urine were 77.4% (CV = 9.5%) and 77.3% (CV = 11.2%), respectively. To determine the loading capacity of the extraction columns, up to 4 ml of spiked plasma and up to 10 ml of spiked urine were tested, with no significant difference in recovery demonstrated. Plasma samples up to 4 ml yielded an average recovery of 74.3% (CV = 3.9%) and urine samples up to 10 ml yielded an average recovery of 74.6% (CV = 4.1%). Figure 4 compares the concentration versus peak area curve of Didemnin B standards with those of processed spiked plasma and urine, demonstrating the accuracy with which Didemnin B levels can be measured using this assay.

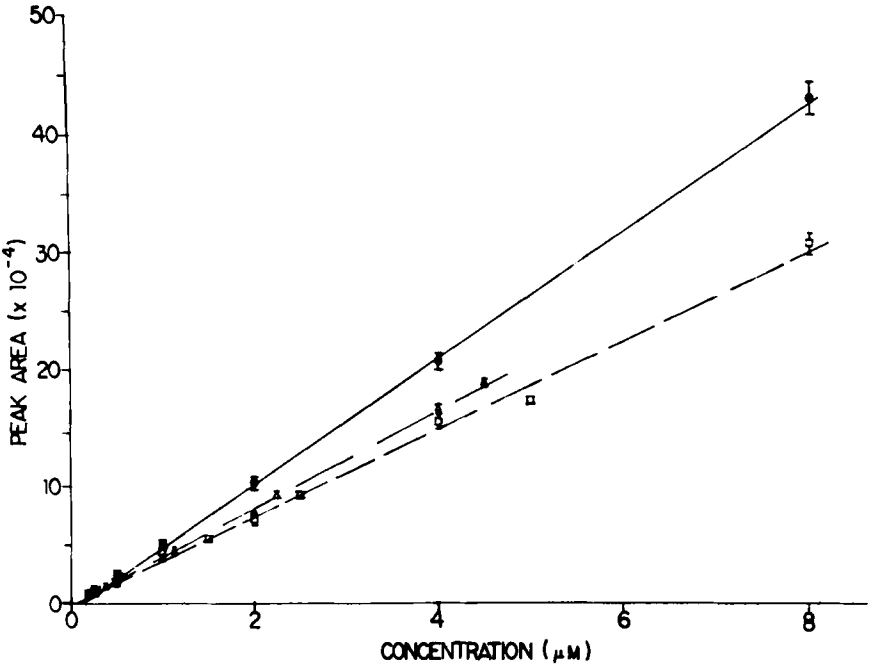

Figure 4: Standard curve for Didemnin B as represented by average interday peak area per concention ± standard error of the mean. The dots represent standards (r=0.9999), the triangles represent extracted spiked control plasmas (r=0.9994), and the squares represent extracted spiked control urines (r=0.9978).

Doses received by patients in the Phase I study recently have been elevated to a level whereby Didemnin B is detectable in both plasma and urine samples. These samples, obtained from a patient receiving 0.67 mg/m^2/d qd x 5, have been analyzed using this assay. Representative chromatograms appear in Figure 5. Spiked controls were prepared simultaneously with the patient samples and

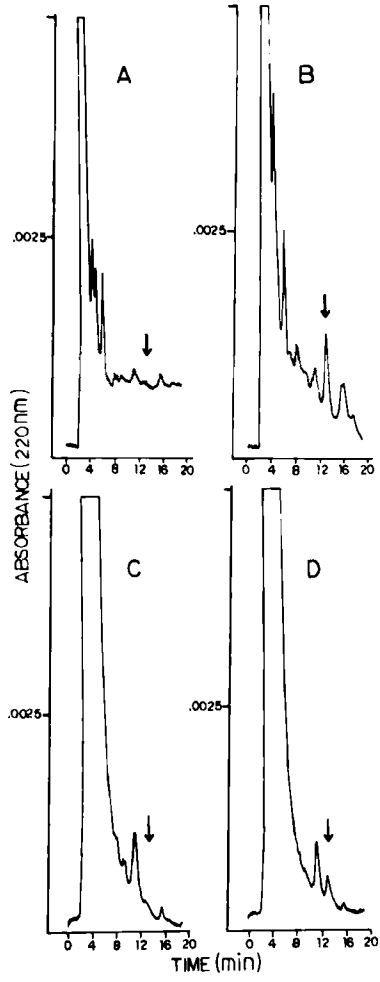

Figure 5: Chromatograms of extracted plasma and urine samples obtained from a patient receiving 0.67 mg/m^2
a) extraction of 2.5 ml baseline plasma
b) extraction of 2.5 ml plasma obtained ten minutes after drug administration
c) extraction of 10 ml baseline urine
d) extraction of 10 ml urine collected for the first four hours following drug administration

chromatographed in order to confirm recoveries of the drug.

As has been observed by other investigators (9,10,11) for another cyclic polypeptide cyclosporine-A, very little of the parent drug, Didemnin B, was found to be excreted in the first four hour urine collection following drug administration, suggesting that rapid metabolism may be involved in elimination. We will be using this assay in future studies to investigate the pharmacokinetics and metabolism of this compound during the course of the Phase I study at the Vermont Regional Cancer Center.

ACKNOWLEDGEMENTS

The authors wish to thank the nursing and laboratory personnel of the University of Vermont's General Clinic Research Center for their technical assistance.

This work was supported by grants/contracts PHS PO1 CA 24543-05/8, PHS P30 CA 22435/3-06, and NO1 CM 27547, awarded by the National Cancer Institute.

James A. Stewart is a recipient of an American Cancer Society Junior Faculty Award.

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