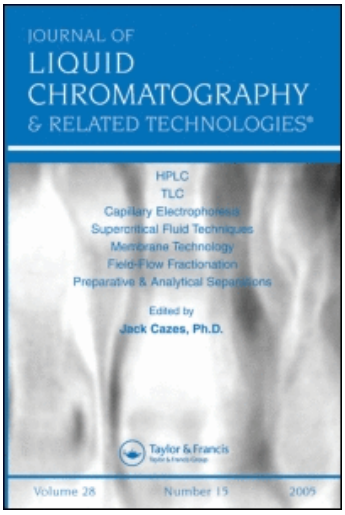

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A Rapid Quantitative Assay of Sparsomycin by High Pressure Liquid Chromatography

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A RAPID QUANTITATIVE ASSAY OF SPARSOMYCIN BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

Fast quantitative analysis of sparsomycin in fermentation broth can be accurately carried out by Amberlite XAD-2 column chromatography followed by normal phase high pressure liquid chromatography using a Porasil T column (2mm ID x 61 cm) and a mobile phase consisting of ethyl acetate-methanol-concentrated ammonium hydroxide (90:10:0.1).

INTRODUCTION

Sparsomycin, a known antitumor and protein synthesis inhibiting agent was isolated by Argoudelis and Herr (1). The structure (Figure 1) was elucidated by Wiley and MacKellar (2). Although a quantitative spectrophotometric papergram assay for sparsomycin was reported (2), there is a need for a much faster quantitative assay of sparsomycin.

EXPERIMENTAL

Apparatus

High pressure liquid chromatography was carried out on a Waters Associates ALC/GPC 200 series instrument, equipped with a

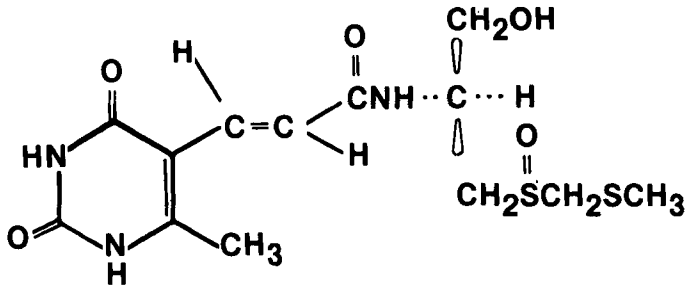

Figure 1

Sparsomycin

U6K septumless injector and a Shoeffel SF770 Spectroflow monitor set at 270 nm (0.04 AUFS) for detection. A Waters Porasil T column (2mm ID x 61 cm, particle size 18-37μ,7450 theoretical plates) was used. Sparsomycin standards were weighed on a Cahn 21 electro-balance.

Materials and Chemicals

Amberlite XAD-2 (20-25 mesh) resin (Mallinckrodt, Inc., St. Louis, MO) was washed with water before using. All solvents used for HPLC were glass-distilled (Burdick and Jackson, Muskegon, MI) and filtered before use. All other solvents were A.C.S. reagent grade. Thin layer chromatography was carried out on prepared plates (E. Merck, silica gel 60, F-254, 250 microns; cellulose F, F-254, 100 microns).

Preparation of Sparsomycin Samples

(a) Preparation of Standard Calibration Curve

Standard solutions were prepared at 0.414, 0.207, 0.106, 0.052 and 0.026 mg/ml by dissolving authentic sparsomycin in water and filtering through a 0.45μ Millipore filter. Ten μl of each standard solution were injected into the HPLC using Porasil T column as stationary phase and ethyl acetate-methanol-concentrated ammonium

hydroxide (90:10:0.1, 2 ml/min) as eluent. Peak heights from triplicate sample injections were plotted against concentration (μg) of sparsomycin to obtain the standard curve (Figure 2).

(b) Isolation and Assay of Sparsomycin from Fermentation Broth

Fermentation broth was centrifuged at 6,000 x g for 20 minutes. An aliquot of 5 ml of the supernatant was adjusted to pH 8, passed at a rate of 1 ml/min through an Amberlite XAD-2 column (1 cm ID x 15 cm), then washed with 20 ml of water. Sparsomycin was then eluted with 20 ml methanol. The methanol solution was concentrated on a rotary evaporator in vacuo, then adjusted to 5 ml with water and filtered through a 0.45 μ Millipore filter. A 10 μl sample was injected using the same HPLC conditions as described above. For a spiked sample, a known amount of sparsomycin was added to the fermentation broth. Quantitative assay and recovery were based on the calibration curve.

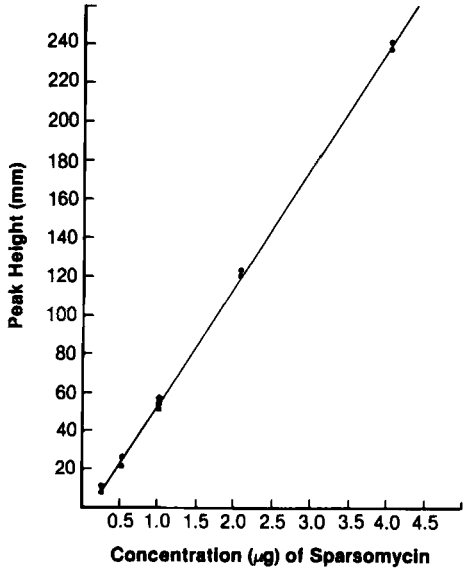

Figure 2

Standard calibration curve for sparsomycin

RESULTS AND DISCUSSION

Sparsomycin was originally isolated from fermentation broths using carbon adsorption as the first step in the purification (1). In our attempt to develop a rapid quantitative assay for sparsomycin, we initially used carbon adsorption to purify the fermentation broth. Using a spiked fermentation broth, it was found that recovery from the carbon column was less than 30%. Substituting Amberlite XAD-2 resin for the carbon column gave a better recovery of sparsomycin (75-80%). Additionally, the use of methanol rather than aqueous solvent to desorb the compounds from the column allowed for rapid concentration of the solvent, thus minimizing heat and light decomposition (2).

The major co-product in the fermentation broth with sparsomycin is tubercidin. These two compounds can be separated with our HPLC system as shown in Figure 3. Sparsomycin can be

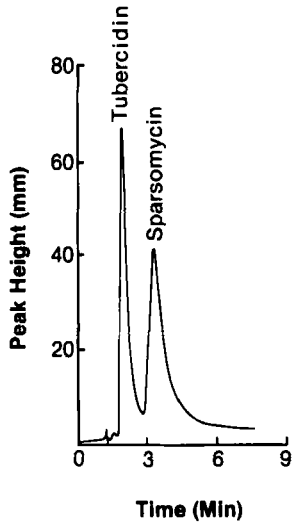

Figure 3

HPLC chromatogram of standard sparsomycin and tubercidin. Conditions: as described in experimental section

quantitatively assayed in the fermentation broth in the presence of up to 50% tubercidin by weight.

A standard calibration curve of sparsomycin showed linearity in the range of 4.14 µg to 0.26 µg as seen in Figure 1. In order to determine the titer of a fermentation broth, an aliquot of 5 ml of centrifuged broth was worked up as described in the experimental section and subjected to HPLC; another aliquot of 5 ml centrifuged broth was spiked with a known amount of sparsomycin and worked up accordingly. The HPLC chromatograms of both the unspiked and spiked samples are shown in Figure 4. Using the standard calibration curve (Figure 2), it was found that the titer of one of the fermentation broths

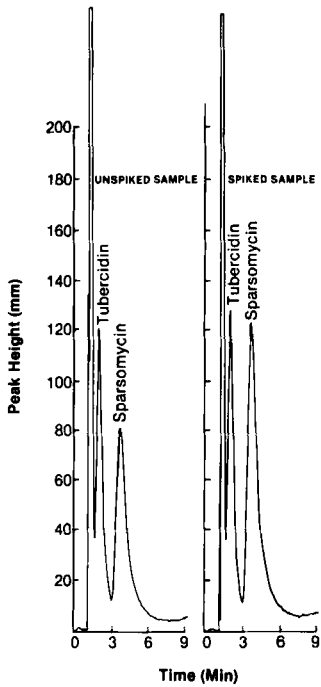

Figure 4

HPLC chromatogram of fermentation broth, both unspiked and spiked samples. Conditions: as described in experimental section

was 146 μg/ml with recovery of 80%. Additional assays of other fermentation broths gave recoveries of 75-80%.

To confirm the presence of sparsomycin in the fermentation broths, the analytical procedure was used preparatively. The materials collected from the HPLC column (both sparsomycin and tubercidin) were spotted along with standard sparsomycin and tubercidin on silica gel plates with ethyl acetate-methanol-concentrated ammonium hydroxide (70:30:1) and chloroform-methanol-concentrated ammonium hydroxide (80:20:1) elution and on a cellulose plate with n-butanol-water-acetic acid (2:1:1) elution. Visualization of the plates under UV light showed that the sparsomycin and tubercidin isolated from the HPLC had the same R_f as that of the standard compounds.

ACKNOWLEDGEMENTS

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