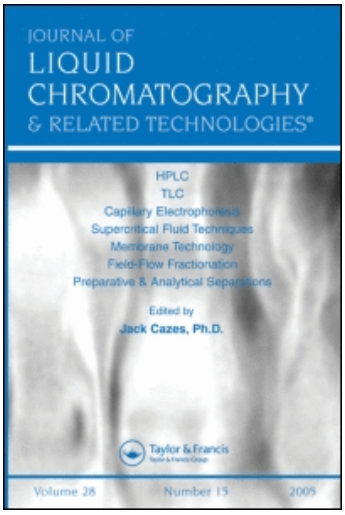

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PURIFICATION OF S-ADENOSYL-1,8,DIAMINO-3-THIOOCTANE (AdoDATO) BY PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Because polyamines are critically involved in the proliferation of neoplastic tissues, enzymes in their synthetic pathway present rational targets for the development of anticancer agents. AdoDATO is a potent and specific transition-state analog inhibitor of the key polyamine biosynthetic enzyme, spermidine synthase, and in in vitro systems leads to the inhibition of cell proliferation via polyamine depletion. In order to evaluate the in vivo effectiveness of AdoDATO, the synthesis of this inhibitor has been scaled up using the reported procedure. This paper describes the development of a preparative HPLC procedure for the purification of AdoDATO by paired-ion chromatography. This methodology could also be used to purify other strongly basic compounds that contain hydrophobic regions.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are essential for normal cell proliferation (1,2). In mammalian cells, these compounds are synthesized from ornithine and S-adenosylmethionine (AdoMet) by four enzymes - ornithine decarboxylase, AdoMet decarboxylase, spermidine synthase, and spermine synthase (3,4). Two compounds that interfere at specific points in the polyamine biosynthetic pathway, difluoromethylornithine (DFMO) and methylglyoxal bisguanylhydrazone (MGBG), have been shown to be effective against rodent tumor systems (5,6) and have been tested in man where they have shown definite but limited activity (7,8). These findings have prompted efforts to identify compounds that inhibit the other enzymes in the polyamine pathway. AdoDATO (Figure 1) was designed by Coward and colleagues (9) as a transitionstate inhibitor of spermidine synthase, the enzyme which catalyzes the conversion of putrescine to spermidine, and has been shown to be a potent specific inhibitor of the enzyme isolated from rat prostate (10). At drug concentrations of 50 - 200 μM, *in vitro* cell growth is inhibited in a dose dependent manner, and this effect can be prevented by the addition of spermidine to the media (10). In order to test the activity of this compound against murine tumor systems and to continue exploring its biological effects, a scale-up of the published procedure for the

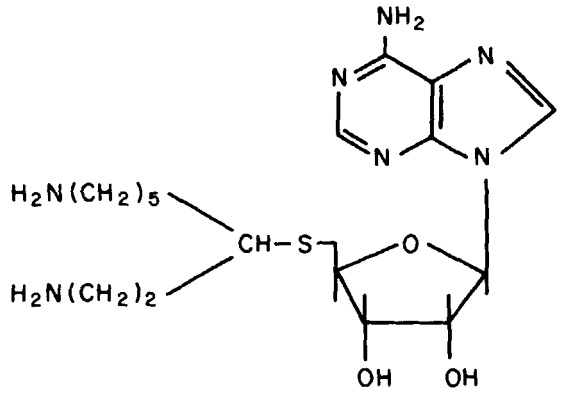

Figure 1: Structure of S-Adenosyl-1,8,Diamino-3-Thiooctane (AdoDATO).

synthesis of AdoDATO (9) was undertaken. In the course of this effort, it became necessary to develop a purification procedure for AdoDATO. This paper describes the paired-ion high performance liquid chromatographic (HPLC) technique which has been successfully applied for this purpose.

MATERIALS AND METHODS

HPLC Instrumentation

The analytical HPLC system (Waters Chromatography Division of Millipore Company, Milford, MA) consisted of a 710 WISP, two M6000A solvent delivery systems, a M720 system controller, a M441 UV absorbance detector, and an RCM100 radial compression module; radial compression cartridges (8 mm X 10 cm) were packed with either μBONDAPAK C18 or with VYDAC C18 (15 - 20 μ) (The

Separations Group, Hesperia, CA). The preparative HPLC system was a PREP 500 (Waters Chromatography Division of Millipore, Milford, MA), a 441 UV absorbance detector, and a PREP-PAK 500 radial compression module containing a 5.7 cm X 30 cm radial compression cartridge packed with VYDAC C18 (15 - 20 μ). Analog data was collected, digitized and stored by a model 4400 Nelson Analytical Chromatography Data System, running XTRACHROM Software (Revision 7.2).

Mobile Phases

All aqueous solutions were made with HPLC-grade water prepared with a Milli-Q Water System (Millipore Company, Bedford, MA). Trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC-grade methanol and acetonitrile were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). NaOH pellets (ACS certified) were purchased from Fisher Scientific Co. (Pittsburg, PA).

Ion Exchange Columns

Dowex 1-X8 (3.2 MEq/mg) was purchased from Bio-Rad Laboratories (Richmond, CA). The resin was converted to the hydroxyl form by running 25 bed volumes of 1 N NaOH over the resin on a fritted glass filter. The resin was then washed with water until the pH was 7.0. The resin was washed with methanol

and dried in a desicator under vacuum for one hour. It was then packed into glass columns where it was held in place by plugs of nylon wool.

Thin Layer Chromatography

Silica gel plates (5 x 10 cm, Merck E60 F_{254}) were developed with a solvent system consisting of n-butanol:glacial acetic acid:5% sodium acetate (60:15:25). The plates were dried and sprayed with a Ninhydrin solution (0.3% Ninhydrin in n-butanol containing 3% acetic acid) to visualize the spots.

AdoDATO

AdoDATO was synthesized by the published procedure (9) in 3 gm batches. A small amount of AdoDATO was kindly supplied by Dr. James Coward (U. Michigan, Ann Arbor) as a reference compound.

RESULTS

Analytical HPLC of AdoDATO Batches

AdoDATO (Figure 1) is a strongly basic compound and contains a hydrophobic heterocyclic ring; a direct approach to developing an HPLC separation of this kind of molecule is the use of paired-ion chromatography on a C18 column. Aliquots (10 μg) of 15 batches of AdoDATO synthesized by The Chemistry Resource Laboratory were injected into an HPLC system consisting of a

radial compression cartridge (8 mm X 10 cm) packed with μBONDAPAK C18 (d_p=10 μm), mobile phase of 0.1% TFA/H_2O, 0.2% TFA/H_2O, 0.1% HFBA/H_2O, 0.2% HFBA/H_2O with methanol or acetonitrile as organic modifier, and a flow rate of 1 ml/min; the components were estimated by making the assumption that the absorbtivity of AdoDATO and its impurities are the same. The best separation was obtained with a mobile phase of 0.2% HFBA/H_2O:acetonitrile (75:25). Under these HPLC conditions, all of the batches synthesized by The Chemistry Resource Laboratory have significant impurities eluting before and after AdoDATO (Figure 2a, 2b). All of the batches of AdoDATO showed at least 4 ninhydrin positive spots on thin layer chromatography (TLC).

Determination of the Parameters for the Preparative Separation

An HPLC separation using acetonitrile as the organic modifier was then developed on a radial compression cartridge (8 mm x 10 cm) containing VYDAC C18 (15-20 μm); with a flow rate of 0.8 ml/min and a mobile phase of .2% HFBA/H_2O:MeCN (80:20), AdoDATO was resolved from the impurities (Figure 3); the α value for AdoDATO and the major impurity was greater than 2.

Aliquots containing increasing amounts of batch B2-97-3 were injected into this HPLC system; the AdoDATO peak was collected and analyzed for purity. Chromatograms in Figure 4 represent the analysis of 10 mg, 20 mg, 30 mg, respectively; the shaded

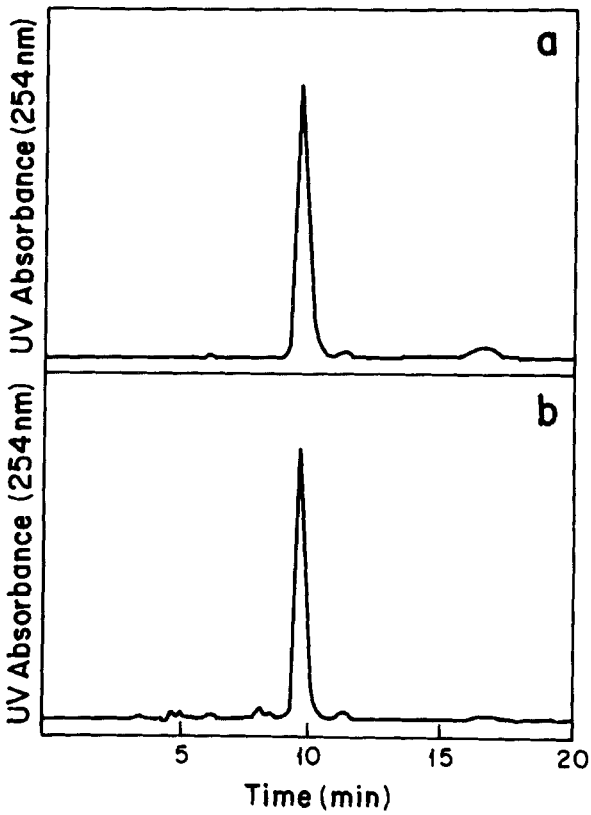

Figure 2: (a) Chromatogram (μBONDAPACK C18) of batch B2-103-1 of AdoDATO prepared by Chemistry Resource Laboratory.
(b) Chromatogram (μBONDAPACK C18) of batch B2-89-1 of AdoDATO prepared by Chemistry Resource Laboratory.

area represents the time of collection for the AdoDATO peak. Chromatograms in Figure 5 represent the analysis of the collected material using the analytical HPLC method described above; the estimated purity of the batches is 99%, 99%, 90%,

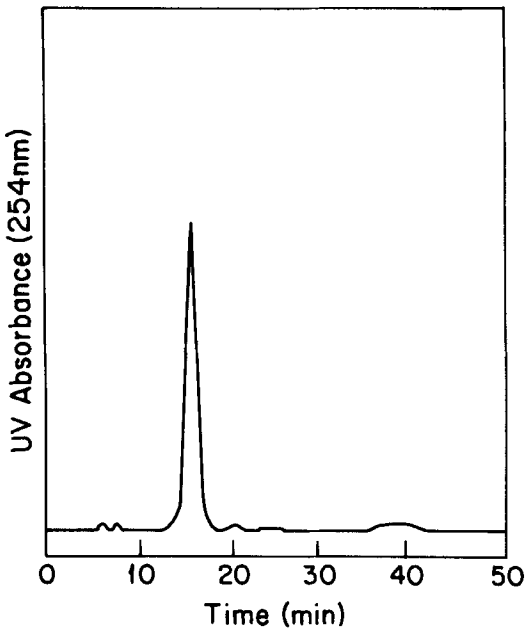

Figure 3: Chromatogram (VYDAC C18) of batch B2-97-3 prepared by Chemistry Resource Laboratory.

respectively. The material collected from the 10 mg and 20 mg separations showed a single ninhydrin positive spot on TLC. These data demonstrate that 20 mg of AdoDATO can be loaded onto the column with little loss of resolution. The preparative radial compression cartridge (RCC) has dimensions 5.7 cm X 30 cm and the volume of its stationary phase is 150 X greater than the analytical RCC; therefore, 3 gms of AdoDATO should be able to be purified in each preparative run. If the mobile phase composition remains the same (0.2% HFBA/H_2O:acetonitrile,

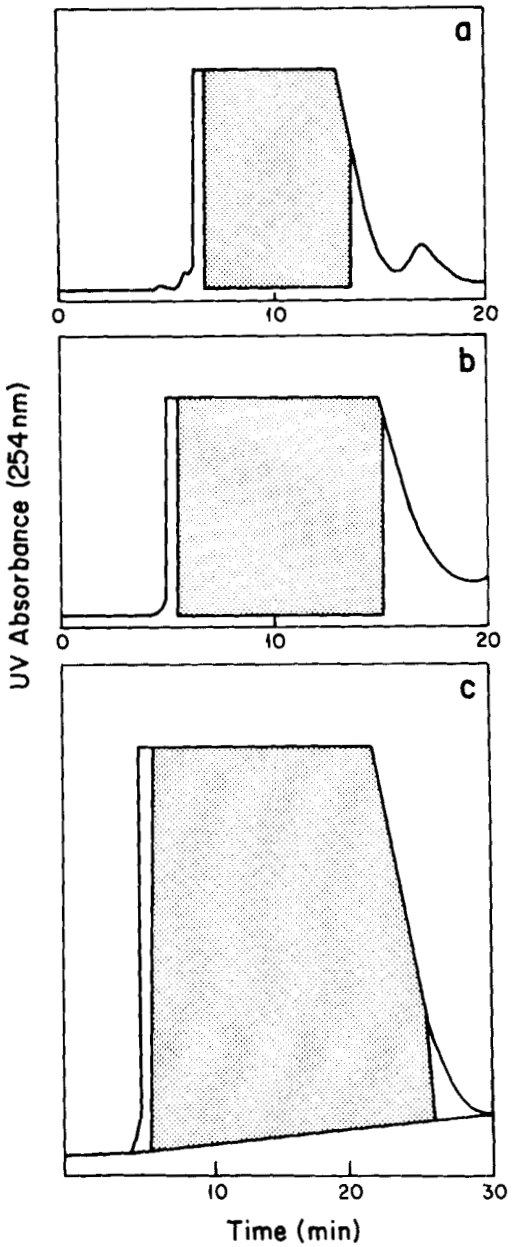

Figure 4: Chromatogram of 10 mg (a), 20 mg (b) and 30 mg (c) of batch B2-97-3 of AdoDATO loaded onto a VYDAC C18 RCC.

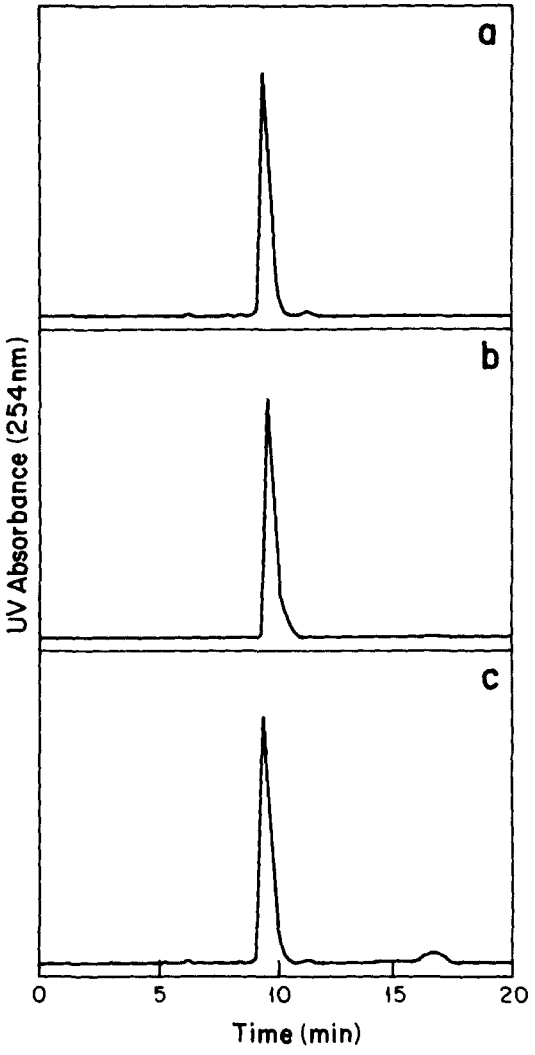

Figure 5: Chromatogram (μBONDAPACK C18) of material eluted from a VYDAC C18 RCC loaded with 10 mg (a), 20 mg (b) and 30 mg (c) of batch B2-97-3.

80:20) and the flow rate is increased to 120 ml/min (150 x 0.8 mL/min), a separation similar to that seen in these loading experiments would be expected on a theoretical basis.

Preparative HPLC

Because of the pressure limitations of the PREP 500, the maximum flow rate that could be attained was 34 ml/min. In order to test the preparative procedure at this flow rate, 3 gms of AdoDATO (Batch B2-103-1) was loaded onto a Vydac C18 preparative RCC of dimension 5.7 x 30 cm and eluted with a mobile phase of 0.2% HFBA/H_2O:acetonitrile (80:20). Figure 6 shows the chromatogram of the preparative separation; the shaded area represents the time of collection of the eluant from the column. After the collected material was lyophilized, it was pure by analytical HPLC and TLC and had an elemental analysis consistent with the HFBA salt of AdoDATO (AdoDATO · $2H_2O$· 2 HFBA). Because of the potential toxicity of HFBA to the in vitro and in vivo test systems, a procedure to remove the HFBA was developed.

Varying amounts of AdoDATO salt prepared by the preparative HPLC procedure was dissolved in water, filtered through a large fused glass filter, applied to freshly prepared Dowex 1-X8 mini columns for a final AdoDATO:Resin ratio of 1:1, 1:1.5 or 1:2; the AdoDATO was then eluted with H_2O and lyophilized. This material was pure by analytical HPLC and TLC and had an

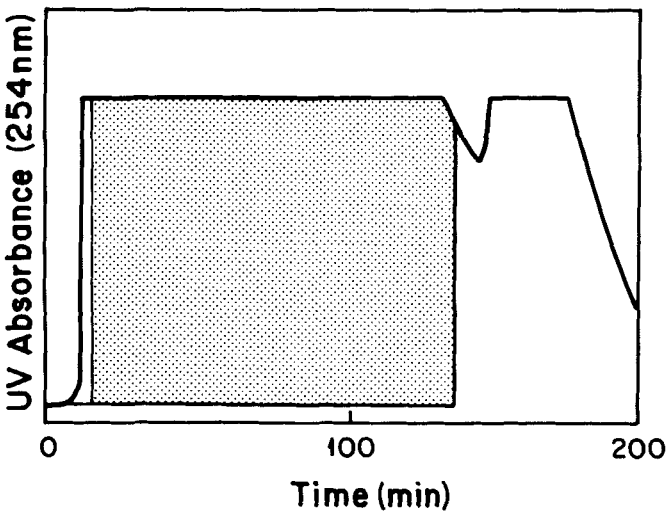

Figure 6: Chromatogram of 3 gm of batch B2-103-1 loaded onto a VYDAC C18 preparative RCC of dimensions 5.7 X 30 cm.

elemental analysis consistent with the carbonate salt of AdoDATO (AdoDATO $\cdot 2H_2O \cdot H_2CO_3$).

Application of Preparative Procedure

Twenty grams of AdoDATO was purified by the procedure outlined above. The lyophilized material obtained is >99% pure by analytical HPLC and shows a single spot on TLC; its elemental analysis is consistent with the structure AdoDATO $\cdot 2H_2O \cdot H_2CO_3$. The overall yield of pure material was 13 g.

Nuclear Magnetic Resonance (NMR) Spectroscopy of Purified AdoDATO

A 400 MHz Proton NMR spectrum (Figure 7) was obtained on approximately 200 mg of AdoDATO dissolved in 0.5 ml of D_2O

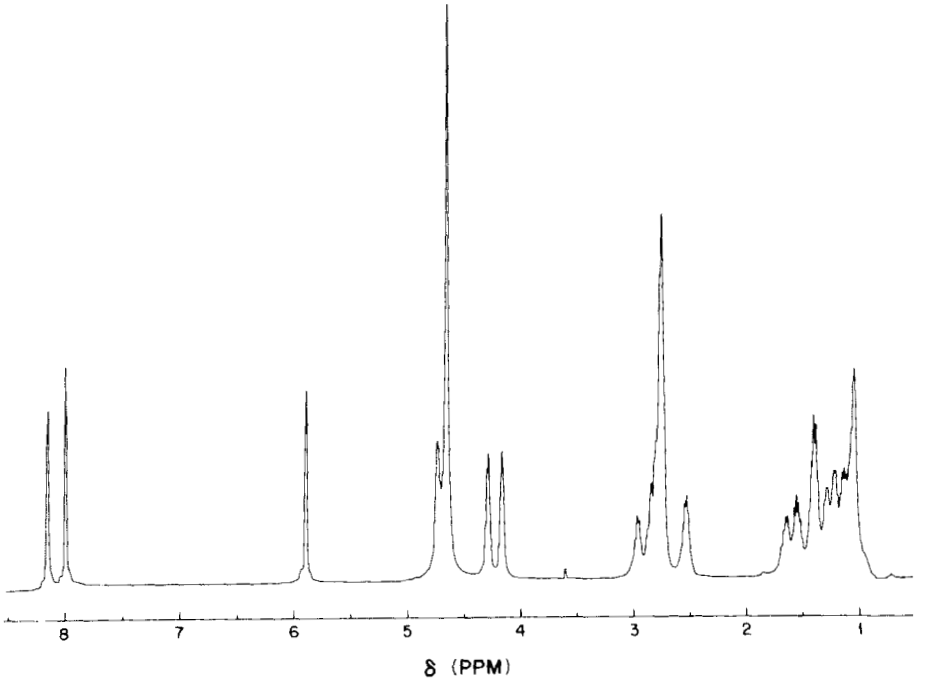

Figure 7: 400 MHz Proton NMR spectrum (40°C) of AdoDATO, obtained by the preparative HPLC procedure, in D_2O (pH 9.5).

with a Bruker AM-400 NMR Spectrometer. The integration of the spectrum confirms the presence of the expected 23 non-exchangeable protons that are assigned as follows: H-1 NMR (D_2O, pH 9.5) 40°C δ8.15 (1H, Ado-H8), 7.95 (1H, Ado-H2), 5.88 (1H, furanose H1'), 4.76 (1H, furanose H2'), 4.26 (1H, furanose H3'), 4.12 (1H, furanose H4'), 2.4-3.0 (7H, furanose H5' and H5", two CH_2 adjacent to each NH_2, methine hydrogen on carbon bonded to sulfur), 0.8 - 1.7 (10H, five CH_2 in aliphatic amine side

chains that are not adjacent to the NH_2). These assignments have been confirmed by one-dimensional spectral studies at pH 8.5 and pH 9.5 and by a 2-dimensional COSY (scalar correlated) NMR spectrum. The assignment regions for the chemical shift positions of the aliphatic amine methylene groups are consistent with those reported for other amines (11).

DISCUSSION

This study demonstrates that it is possible to develop preparative separation in a straightforward way from data obtained on analytical columns and outlines the preparation of 13 g of pure AdoDATO by preparative HPLC. The material obtained was >99% pure by analytical HPLC and TLC; the structure of the purified product was consistent with that of AdoDATO by elemental analysis and high resolution NMR.

The approach used in this study to develop a preparative separation for AdoDATO follows that outlined by Snyder and Kirkland (12) for the isolation of the major component from a mixture and adapted to radial compression technology by the Waters Chromatography Division of Millipore Company (13): 1) develop an analytical procedure that resolves the component of interest from the other components of the mixture; 2) adapt this separation to a small diameter (8 mm) radial compression cartridge (RCC) containing the same packing to be used in the large scale purification with special attention to maximizing α;

3) determine the maximum amount of the mixture that can be loaded onto the RCC before resolution decreases to such an extent that pure compound cannot be isolated without an excessively narrow "heart cut"; 4) compute the preparative LC conditions from that of the scale-up operation; 5) perform a trial preparative separation using these parameters; and 6) test the collected material for purity.

Although this concept of developing the preparative separation is straight-forward, the particular physical properties of AdoDATO make the implementation of this approach difficult. Since AdoDATO is a strongly basic compound, it was not possible to elute AdoDATO from an analytical column packed with silica even though mixtures of organic solvents were doped with acids to decrease the interaction of the primary amine groups of AdoDATO with the silanol groups of the silica. Therefore, it was necessary to use reverse phase chromatography for this separation. In general, the bulk of reported preparative separations have been carried out with normal phase chromatography; the only class of compounds that have been extensively prepared with the reverse phase technique are the polypeptides and proteins (14). The peptides are prepared by ion-pair chromatography on a C-4 or C-18 stationary phase with TFA or HFBA as a counter ion; since AdoDATO is charged at low pH, this HPLC system would also be appropriate for resolving it from a complex mixture. Our results demonstrate that it is possible to

develop a preparative procedure for AdoDATO using pair-ion chromatography with HFBA as a counter-ion and with a VYDAC C-18 stationary phase by following the approach outlined above. The analysis time for the preparative separation was longer than computed because the pumping system used could not generate the required flow rates secondary to back pressure limitations but the resolution obtained was similar.

Since polyamines are essential components for cell growth, compounds that interfere with the activity and/or regulation of the enzymes required for their synthesis present rational targets for antitumor agents. Given the information available on the structural requirements for transition-state analog inhibitors of those enzyme systems involved in spermidine and spermine synthesis (i.e., the synthases), it would be expected that such compounds would contain polar regions that could carry a positive charge (e.g., primary or secondary amines) and non-polar hydrophobic regions. When large amounts of these compounds are synthesized, the procedure outlined in this paper should be applicable in their purification.

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