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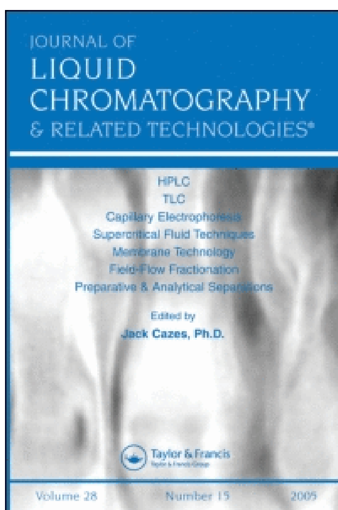

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Florence J. Reavis[a]; John J. Contario[a]
[a] Merrell Dow Research Institute Cincinnati, Ohio

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ALPHA-DIFLUOROMETHYLORNITHINE IN RODENT FEED

Florence J. Reavis and John J. Contario
MERRELL DOW RESEARCH INSTITUTE
Cincinnati, Ohio 45215

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ABSTRACT

A high performance liquid chromatographic method has been developed for the assay of alpha-difluoromethylornithine, an inhibitor of cell growth, in rodent feed at levels from 0.5 to 50 mg/ml. The drug is leached from the feed with water, and a portion of the aqueous solution is passed through a cation exchange resin to remove some feed components which are simultaneously leached. The drug is removed from the resin column and derivatized with 1-fluoro-2,4-dinitrobenzene to enhance its ultraviolet absorptivity. The derivative is isolated from the reaction mixture and chromatographed on a 25-cm LiChrosorb RP-18 column equipped with a guard column, using a mobile phase of pH 4.8 acetate buffer:tetrahydrofuran (80:20) and photometric detection at 360 nm. The method is also applicable for the assay of feed preparations of other amino acids or similar non-UV absorbing materials which react with 1-fluro-2,4-dinitrobenzene to give dinitrophenyl derivatives.

INTRODUCTION

Alpha-difluoromethylornithine (I) is an enzyme activated irreversible inhibitor of ornithine decarboxylase, the first enzyme in the polyamine biosynthetic pathway.(1) Inhibition of this enzyme by (I) has demonstrated significant antiproliferative effects *in vitro* and *in vivo* against a number of tumors and

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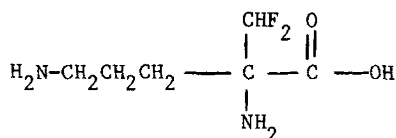
parasitic protozoa. (1,2) The compound is currently undergoing clinical studies in the United States and in Europe.



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The purpose of this work was to provide an analytical procedure to insure adequacy of mixing and stability with time of the drug in feed as required for the toxicological testing. Although an amino acid analytical system had been developed for (I) (3), it was necessary to devise a new analytical procedure for this assay since neither an amino acid analyzer nor a fluorometric detector were available in this laboratory.

Reversed phase liquid chromatography has been used successfully in the past, in this laboratory and by others (4-9), to assay for drugs in feed, and was considered the method of choice for this assay. The following difficulties were encountered in the development of a liquid chromatographic method. Alpha-difluoromethylornithine has the characteristically low UV absorbance of aliphatic amino acids which initially necessitated the detection of the drug at 210 nm. However, at low levels of drug in feed, a large sample was required in order to obtain a measurable amount of drug. Feeds such as Purina Rodent Chow

contain analogous amino acids such as lysine, arginine, etc., at concentrations of over ten times that of the drug in low dosage samples. As a result of this, large feed samples produced high background and interferences from co-leached feed components. These artifacts created difficulties in the ultimate quantitation of the drug despite an additional clean-up procedure.

In order to make UV detection easier, pre-column derivatization with a highly UV absorbant chromophore was explored. Since the detectability of the drug would be increased, a smaller feed sample could be used and lower background absorbance from co-leached feed components would be obtained as a result of detection at a higher wavelength. The reaction of 1-fluoro-2,4-dinitrobenzene with amino acids is known to result in a highly absorbant dinitrophenyl derivative. (10-12) The formation of this derivative of (I) was chosen because the reaction is a fast and simple one, requiring only the mixing of the amino acid and reagent in basic solution to form the derivative, and because it allowed the use of the ubiquitous ultraviolet detector. The reaction of (I) with 1-fluoro-2,4-dinitrobenzene was found to result in the formation of a dinitrophenyl derivative which exhibited the characteristic UV spectrum of dinitrophenyl amino acids, displaying a strong absorption band at 360 nm and a weaker band at 260 nm. A sample preparation procedure was developed employing the dinitrophenyl derivative which would allow the accurate measurement of low levels of (I) in feed using UV detection at 360 nm.

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MATERIALS

Chemicals

Glacial acetic acid, hydrochloric acid, methanol, sodium acetate, and ethyl ether, all reagent grade, were purchased from J. T. Baker, Inc. (Phillipsburg, N.J.). Ammonium hydroxide and sodium bicarbonate, both reagent grade, were purchased from Matheson, Coleman and Bell (Norwood, OH.). Absolute ethanol, USP grade was purchased from Aaper Alcohol and Chemical Co. (Louisville, KY.). Tetrahydrofuran, HPLC grade, was purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI.). The 1-fluro-2,4-dinitrobenzene, 98%, was purchased from Aldrich Chemical Co. (Milwaukee, WI.). The cation exchange resin was analytical grade, AG 50-W-X2, 100-200 mesh, hydrogen form, purchased from Bio-Rad Laboratories (Richmond, CA.). The feed used was a constant-nutrient rodent feed formulation, Certified Rodent Chow #5002, from Ralston Purina Co. (St. Louis, MO.).

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Apparatus

The high performance liquid chromatographic system consisted of an Altex, model 110 pump, Altex Scientific, Inc. (Berkeley, CA.), a Vari-Chrom variable wavelength detector, Varian Associates, Inc. (Palo Alto, CA.), operated at 360 nm, a Rheodyne Model 70-10 injector, Rheodyne, Inc. (Berkeley, CA.), and a recorder, model 252A, from Linear Instruments Corp. (Irvine, CA.).

METHODS

Chromatographic Conditions

The chromatography was carried out at ambient temperature on a reversed phase C_{18} column, Lichrosorb RP-18, 25 cm x 4.6 mm (ID), E. Merck (Darmstadt, Germany). The analytical column was attached to a guard column containing C_{18} packing, Bondapak C_{18}/Corasil, 37-50 microns, Waters Associates, Inc., (Milford, MA). The mobile phase consisted of pH 4.8 acetate buffer: tetrahydrofuran (80:20). The flow rate was 2 ml per minute.

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Preparation of the Ion-Exchange Column

The cation exchange column was prepared to contain an appropriate volume of resin in the ammonium form corresponding to various levels of drug in feed as listed in Table I. A small pledget of glass wool was inserted in the column. The appropriate volume of resin (hydrogen form) was added as a slurry in water, allowed to settle, then capped with another pledget of glass wool. The resin was treated with 3M methanolic ammonia until the column effluent was basic using Hydrion pH paper, then washed with water until the effluent was neutral.

Preparation of the Sample Solution

A 2-g sample of feed was used for samples having a concentration of 0.5 mg of drug per g of feed. A 1-g sample of feed was used for samples having a concentration greater than 0.5 mg of drug per g of feed. The accurately weighed feed sample was

TABLE 1

Variation of Ion-Exchange Resin Volume and Aliquot Volume With Sample Drug Concentrations

Alpha-difluoro-methylornithine (mg per g)	Resin bed volume (ml)	Aliquot of extract transferred to resin (ml)	Dilution factor (F)
0.5	4	50	0.040
1.0	4	50	0.040
2.0	4	50	0.080
2.5	4	40	0.100
4.0	2	25	0.160
5.0	2	20	0.200
10.0	2	10	0.400
20.0	2	5	0.800
25.0	2	4	1.00
40.0	2	3	1.33
50.0	2	2	2.00

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leached with 250.0 ml of water. The large particulate matter was allowed to settle and a portion of the remaining mixture was centrifuged. An aliquot of the supernatant solution containing 0.4 mg of the drug was transferred to the ion-exchange resin column. Table 1 gives examples of aliquots used for various levels of drug in feed. The sample was drained into the resin bed and the column was washed with 10 ml of water, discarding the eluate. The drug was eluted from the column with 25 ml of 3M methanolic ammonia and the eluate evaporated to dryness. The residue was dissolved in 1 ml of 2% sodium bicarbonate and mixed with 2 ml of 1-fluoro-2,4-dinitrobenzene reagent, prepared by mixing 0.25 ml of 1-fluoro-2,4-dinitrobenzene with 10 ml of absolute ethanol. The reaction mixture was protected from light and shaken for at least 30 minutes.

The mixture was treated with 2 ml of water and extracted twice with 5 ml of ether, discarding the extracts. The aqueous phase was acidified with 6M hydrochloric acid and extracted twice with 2 ml of ether, discarding the extracts. The remaining aqueous solution was quantitatively transferred to a 10-ml volumetric flask, and diluted to volume with mobile phase.

Preparation of the Standard Solution

Approximately 25 mg of a standard sample was accurately weighed into a 25-ml volumetric flask. The sample was dissolved in, and diluted to volume with 2% sodium bicarbonate solution. A 1.0-ml aliquot of this solution was reacted with 2 ml of 1-fluoro-2,4-dinitrobenzene in the dark, with shaking for at least 30 minutes. The reaction mixture was extracted as described under "Preparation of the Sample Solution". The remaining aqueous solution was quantitatively transferred to a 25-ml volumetric flask, and diluted to volume with mobile phase. This solution was used for comparison with samples containing more than 1 mg of drug per g of feed. For samples containing 1 mg of drug per g of feed or less, a 5.0 ml aliquot of the solution was diluted to 10 ml before use.

Chromatographic Procedure

A stable baseline was established with the chromatograph operating under the conditions described. The detector was operated at 0.05 AUFS for samples containing 1 mg of drug per g of feed or less, and at 0.1 AUFS for samples containing more than 1

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mg of drug per g of feed. A 20-µl aliquot of the standard solution was injected and its chromatogram recorded. Similarly, a 20-µl aliquot of the sample solution was injected and its chromatogram recorded. Figure 1 illustrates the chromatograms obtained when this method was used to assay a sample prepared to contain 4 mg of drug per g of feed.

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Calculations

For each injection, the area of the peak for the dinitrophenyl derivative of (I) was determined, and the assay was calculated as follows:

$$\frac{Au}{As} \times \frac{Ws}{Wu} \times F = \text{mg of (I) per g of feed}$$

where: Au = area of the dinitrophenyl derivative of (I) in the sample chromatogram.

As = area of the dinitrophenyl derivative of (I) in the standard chromatogram.

Ws = weight of the standard in mg.

Wu = weight of the sample in g.

F = dilution factor (obtained from Table 1).

RESULTS AND DISCUSSION

The major difficulty encountered in the method development was the low UV absorbance of (I). Detection had to be accomplished at 210 nm, which monitored the drug via its end absorption. Because a great number of compounds exhibit some

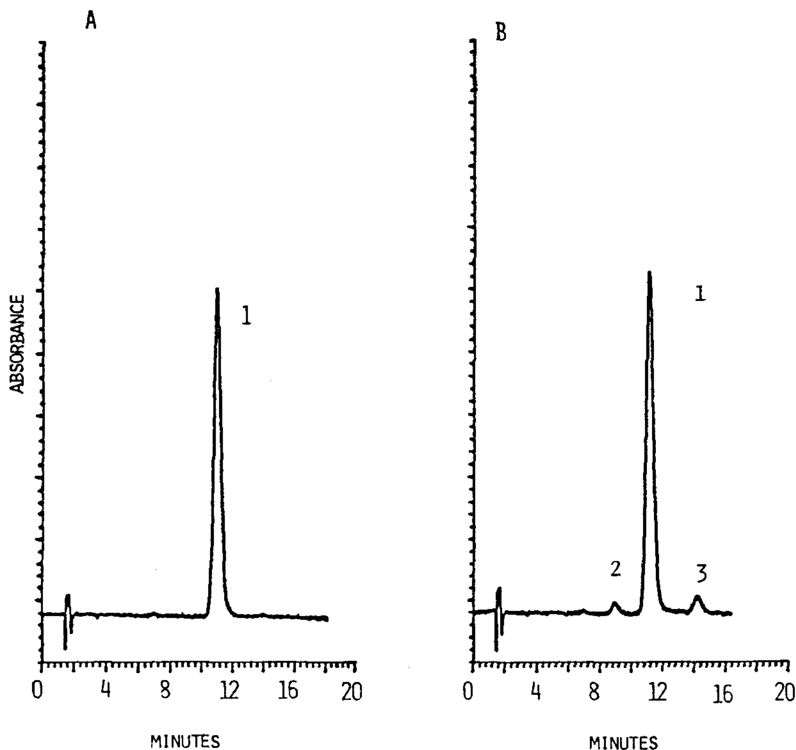

FIGURE 1 - A - Typical chromatogram of the dinitrophenyl derivative of alpha-difluoromethylornithine from a standard solution. B - Typical chromatogram of the dinitrophenyl derivative of alpha-difluoromethylornithine from a feed extract. Key: (1) dinitrophenyl derivative of alpha-difluoromethylornithine leached from feed; (2) dinitrophenyl derivative of arginine leached from feed; (3) dinitrophenyl derivative of unknown leached from feed.

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absorbance at 210 nm, the background absorbance from other co-leached feed components increased as the size of the feed sample increased, making quantitation impossible for low levels of drug in feed.

A clean-up procedure was developed in which an ion-exchange resin was used to separate other feed components from (I). As

indicated in Table I, the resin bed volumes were increased when the concentration of drug in feed was low enough to require the treatment of more than 25-ml of feed extract. This was necessary to avoid loss of (I) from an overloaded resin column. Although this procedure improved the quality of the chromatograms, the background absorbance remained high especially at low levels of drug in feed.

In order to overcome this obstacle, a sample preparation procedure was developed which would allow the accurate measurement of the derivatized drug in feed at a detection wavelength of 360 nm. By preparing the dinitrophenyl derivative of (I) leached from feed, a small amount of this normally low UV absorber could be easily detected. In addition to allowing the use of a smaller sample size, less background was obtained as a result of detection at 360 nm and the detector response was increased 140 times when compared to that which was previously obtained for underivatized (I) at 210 nm. The feed itself contains several other amino acids which could also react with the derivatizing reagent. Because of their structural composition, the derivatives of most of these are soluble in ether after acidification of the aqueous reaction mixture and are therefore eliminated with the ether extraction. Those which may remain in the aqueous phase are arginine, lysine, histidine, and threonine. Arginine has been identified as one of these amino acids which is co-leached from the feed, derivatized, and may subsequently appear in the sample chromatogram at a retention time of about 8 minutes. Another peak in the sample chromatogram at about 14.5 minutes is unidentified, however it has

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been shown not to be any of the other amino acids listed as present in the feed.

The structure proof of the derivative was accomplished through elemental analysis and proton magnetic resonance spectroscopy. The elemental analysis was in agreement with the formula for a mono-derivative. The spectroscopy confirmed this, and indicated that the dinitrophenyl chromophore was attached to the terminal amino group. The lack of derivatization at the alpha amino group, under the conditions of the reaction, may be due to steric hindrance.

The optimum reaction conditions were determined by varying the reaction time and temperature. A reaction time of thirty minutes at room temperature was found to be optimum, although reaction times up through 2 hours gave similar results. At times of 5 hours and longer, the derivative level began to drop off.

The linearity of detector response was determined for peak areas *vs* column loading from 0.2 to 1.0 µg. The detector response was found to be linear over the range. The 20-µl injection used in the method contains 0.8 µg or 0.4 µg of the dinitrophenyl derivative depending on the level of drug in feed of the sample being assayed.

To determine the accuracy and precision of the method, five synthetic samples were prepared at the 0.5, 5, and 50 mg/g levels by adding known amounts of drug to blank feed samples and assaying by the method. Table 2 lists the recoveries obtained from synthetic samples. As can be noted from the table, recovery

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TABLE 2

Recoveries of Alpha-difluoromethylornithine from Synthetic Samples at Three Levels (% Recovery)

0.5 mg/g	5 mg/g	50 mg/g
93.5	95.9	100.0
93.8	93.5	98.4
97.5	94.4	96.1
90.9	96.7	98.3
85.6	98.0	96.2
x = 92.1%	x = 95.7%	x = 97.8%
s = ± 4.3%	s = ± 1.7%	s = ± 1.6%

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decreases as the amount of drug in the sample decreases. This phenomenon is not observed when blank feed is leached, and the resulting solution spiked with drug. The decrease in recovery is believed to be caused by some type of irreversible binding of a small amount of (I) to the feed matrix itself.

CONCLUSIONS

A pre-column derivatizaion procedure has been developed for the precise assay of (I), a low UV absorbing aliphatic amino acid. By reacting with 1-fluoro-2,4-dinitrobenzene, a highly UV absorbing dinitrophenyl chromophore was attached, allowing detection and accurate measurement of low levels of the drug in feed. The reaction is simple and fast, and can easily be adapted to the assay of other non-UV absorbing compounds which may be formulated in feed at low levels.

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