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Technical Note

A Precolumn Derivatization High-Performance Liquid Chromatographic (HPLC) Procedure for the Quantitation of Difluoromethylornithine in Plasma

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INTRODUCTION

Difluoromethylornithine [2-(difluoromethyl)-DL-ornithine; DFMO] is a specific enzyme-activated, irreversible inhibitor of ornithine decarboxylase (ODC) that has the effect of blocking the synthesis of polyamines. Through this action DFMO has been indicated for the treatment of gambian trypanosomiasis, pneumocystis carinii pneumonia, and human carcinoma (1–4).

Previously, DFMO in plasma has been analyzed by ion-exchange chromatography on an automated amino acid analyzer that utilizes two columns (5). While analysis is in operation on one column, the other is regenerated and equilibrated in preparation for the next sample analysis. There is also a procedure for the quantitation of the (+)- and (−)-enantiomers in plasma and urine by capillary gas chromatography (GC) with electron capture detection (6). The method involves a double derivatization of DFMO and is quite complex, although specific and highly accurate for each enantiomer. This report describes a method that utilizes precolumn derivatization with *o*-phthalaldehyde and separation by reverse-phase high-performance liquid chromatography (HPLC). It is a relatively simple and rapid method that was developed through the application of advances in the analysis of amino acids, where reverse-phase chromatography is replacing the more traditional ion exchange (7–9).

MATERIALS AND METHODS

DFMO was synthesized by Merrell Dow Research Institute (Cincinnati, Ohio). 4-Amino-3-hydroxybutyric acid was from Aldrich Chemical Co., and the *o*-phthalaldehyde and 2-mercaptoethanol were from Sigma Chemical Co. All other chemicals were reagent grade and the solvents used in high-performance liquid chromatography (HPLC) were chromatographic grade. All water was glass distilled.

Instrumentation. Analyses were performed on a Waters HPLC system (Millipore, Waters Chromatography Division) consisting of a Model 720 system controller, two Model 510 pumps, and a WISP, Model 710B autoinjector. The chromatography column was a Waters C18, 5-μm, Radial-Pak cartridge, preceded by a precolumn (Upchurch Scientific, Inc.) packed with Waters Bondapak C18/corasil, 37- to 50-μm particle size. The fluorometric detector was a Kratos Model FS 970 (Schoeffel Instrument Division). The excitation wavelength was 335 nm, and a 418 nm cutoff filter was used on the emission side.

Standard and Reagent Solutions. A stock standard solution for the analysis of DFMO in plasma samples was prepared by dissolving DFMO in water (2 mg/ml). A 5-ml aliquot and a 1-ml aliquot were diluted to 100 ml with human plasma to give standard solutions of 100 and 20 μg/ml. From these two standards, a standard curve of nine samples was prepared by dilution ranging in concentration from 80 to 0.5 μg/ml.

4-Amino-3-hydroxybutyric acid, the internal standard (IS), was prepared in a water solution at 250 μg/ml.

The *o*-phthalaldehyde (OPA) reagent was prepared by dissolving 10 mg of OPA in 1 ml of ethanol. One hundred microliters of 2-mercaptoethanol and 10 ml of 0.1 *M* phosphate buffer, pH 7.5, were added to the ethanol solution. The reagent was freshly prepared every 3 days and stored in the dark at room temperature.

Plasma Analysis. Standard and sample plasma (100 μl) were placed in 100 × 13-mm screw-cap test tubes, to which was added 20 μl of the internal standard solution. To these was added 400 μl of methanol to precipitate the proteins. After centrifugation at approximately 800 *g* for 20 min, the supernatant was removed to a WISP vial. Two hundred microliters of 0.02 *M* phosphate buffer, pH 7.5, was added to it, and the vial was capped. A vial with the OPA reagent was placed in the number 1 position in the carousel. The precolumn derivatization of the sample with OPA is accomplished by programming the WISP to inject alternately reagent and sample under zero-flow conditions (Waters Auto-Tag technique). Chromatographic separation was by gradient elution. Solvent A composition was 92% 0.1 *M* phosphate buffer (pH 7.5), 5% methanol, and 3% isopropanol. Solvent B was 80% methanol, 10% water, 5% aceto-

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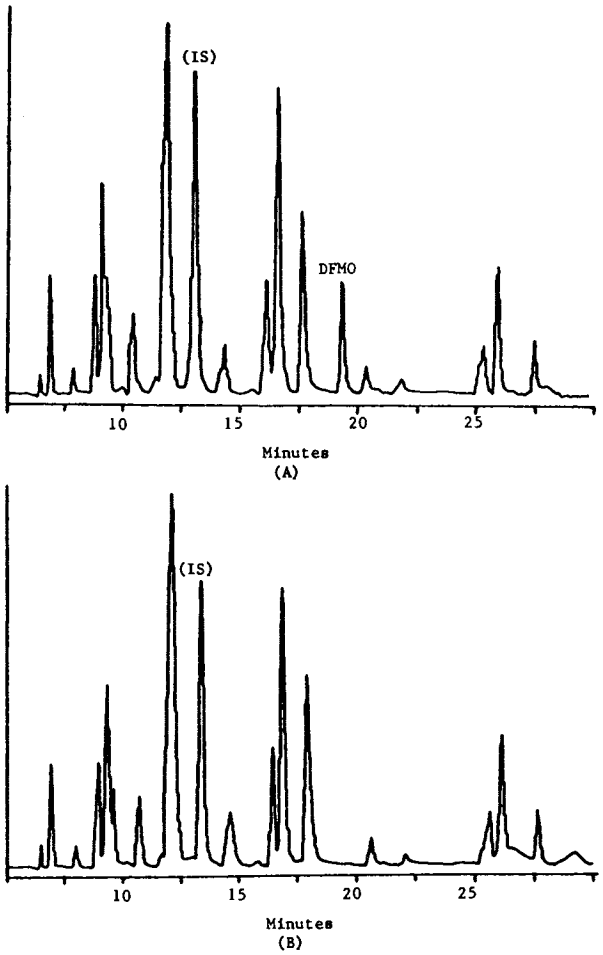

Fig. 1. Chromatograms of a plasma sample of 20 μg/ml of DFMO (A) and a plasma blank (B). The retention times of the IS and DFMO are 13 and 19 min, respectively.

nitrile, and 5% isopropanol. There was an initial 3-min isocratic flow of 80% A, 20% B. The flow rate the first minute was 0.2 ml/min, changing to 1.5 ml/min after that and remaining as such for the rest of the program. The linear gradient, beginning at 3 min, was to 50% A, 50% B over 15 min, where it remained isocratically for 5 min before returning to the initial conditions. The column equilibrated for 7 min before the flow was stopped and the next injection began. There was, therefore, approximately 35 min between sample injections since the reagent and the sample each take approximately 1.5 min for the sampling sequence. Detection was by fluorescence. Data were analyzed to give the peak area ratio of DFMO to the IS. Values for the samples were determined from the daily standard calibration curve, which was calculated by linear regression.

RESULTS AND DISCUSSION

The OPA/2-mercaptoethanol reagent reacts with the α-amino group of DFMO to form a fluorescent 1-alkyl-thio-2-alkyl-substituted isoindole. The pK_a of the acid function of DFMO is 0.08 and that of the α-amino group is 6.4. Even after derivatization this increased polarization from the difluoromethyl group has the effect of decreasing the retention time on reverse-phase chromatography, placing the DFMO in the middle of the pack of the endogenous α-amino acids. Normally, the OPA-derivatized ornithine and lysine elute late, after the neutral amino acids in plasma (9). Thus, gradient elution was necessary to achieve complete separation.

As stated previously, the maximum fluorescence for the amino acid/OPA moiety is obtained when the pH of the OPA reagent is in the range of 9.5 to 10 (10). However, there was noticeable column degradation after approximately 40 sample injections when the derivatizing reagent was at a pH of 9.5. This did not occur when the reagent was at a pH of 7.5. Although the fluorescence of DFMO derivatized with a reagent of pH 7.5 was approximately 15–20% less than at a pH of 9.5, there was adequate sensitivity and the need for frequent column changing was eliminated.

This method is linear over a concentration range of 0.5 to 80 μg/ml, with a minimum quantifiable limit of ca. 0.5 μg/ml. The limit of detection is ca. 0.25 μg/ml. Comparison of a sample chromatogram with that of a plasma blank indicates that the region of DFMO is free from interfering substances (Fig. 1). The method was validated in the range of 0.5 to 80 μg/ml by the analysis of eight unknown samples in duplicate on 3 different days. The unknowns were compared each day to an eight-point standard curve plus a blank in duplicate. Table I presents the values for each assay as well as the mean, standard deviation (SD), and coefficient of variation (CV) for each unknown. The mean values of the seven unknowns range from 94.2 to 106.9% of the theoretical values. All blanks were zero. Overall, the results indicate very good accuracy and precision.

A Phase I clinical pharmacokinetic study of DFMO in cancer patients reported plasma concentrations from a single oral dose of 1.5 g/m² (approximately 40 mg/kg) in the range of 0.6 to 21.3 μg/ml (11). These values fall within the validated range and confirm the adequacy of this method,

Table I. Results of 3-Day Validation Study

Theoretical value (μg/ml)	71.4	57.1	25.0	16.6	6.66	2.86	0.86
Assay value (μg/ml)	68.5	60.2	25.1	19.2	7.28	2.91	0.90
	69.6	58.8	24.4	16.7	6.82	2.94	0.70
	73.8	53.4	26.1	17.1	7.23	2.94	0.84
	63.6	—	23.5	17.0	6.38	2.96	0.90
	78.4	65.4	26.4	18.0	6.52	2.48	0.85
	67.3	58.9	25.5	18.4	7.21	2.70	0.69
Mean	70.2	59.3	25.2	17.7	6.91	2.82	0.81
SD	5.20	4.28	1.08	0.97	0.39	0.19	0.09
CV (%)	7.54	7.25	4.25	5.41	5.64	6.73	11.1

which is a relatively simple and rapid method. It is, therefore, ideal for bioavailability and pharmacokinetic studies that entail the analysis of large numbers of samples.

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