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Plasma analysis of α-difluoromethylornithine using pre-column derivatization with naphthalene-2,3-dicarboxaldehyde/CN and multidimensional chromatography

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Abstract

A procedure for the plasma analysis of α-difluoromethylornithine (DFMO) has been developed that utilizes pre-column derivatization with naphthalene-2,3-dicarboxaldehyde/cyanide (NDA/CN) in pH 9.2 borate buffer. Selective derivatization of δ-amine of DFMO followed by quenching of the reaction results in the formation of a cyanobenz [f] isoindole (CBI) derivative that is stable for 24 h. Plasma was prepared for derivatization by a single step procedure which resulted in an ultrafiltrate compatible with derivatization and analysis. The DFMO derivative (CBI-DFMO) was separated from plasma interferences by multidimensional chromatography with an analysis time of 28 min. The response for DFMO in plasma was linear over the range of 2.1×10^{-8}–2.1×10^{-6} M after derivatization. This procedure encompasses a useful linear range and offers the advantages of minimal sample preparation and production of a stable fluorophor.

Keywords: Plasma determination; DFMO; Pre-column derivatization; Naphthalene-2,3-dicarboxaldehyde; Fluorescence; Multi-dimensional chromatography

1. Introduction

In recent years, aspects of cancer research have focused on the role of endogenous polyamines (putrescine, spermidine, and spermine) on the growth of rapidly dividing cells. Recognition that depletion of these polyamines could decrease the growth of cancer cells led to a search for inhibitors of this pathway. A compound that has been studied extensively is α-difluoromethylornithine (DFMO). This compound has been shown to be effective alone or in conjunction with other agents in the treatment of several cancers [1–3].

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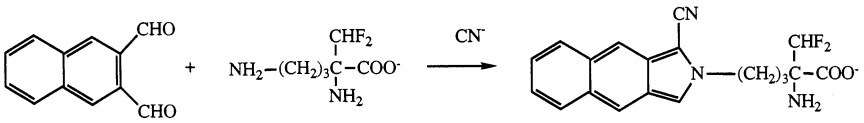

Fig. 1. Reaction pathway for the derivatization of DFMO with NDA/CN showing the CBI-DFMO derivative.

Disease states such as *Pneumocystis carinii pneumonia* and African sleeping sickness caused by *Trypanosoma brucei gambiense* have been successfully treated with DFMO [4,5]. The use of DFMO as a chemoprevention agent for certain disease states has also been investigated [6]. Studies have found plasma levels of DFMO from approximately 4 μg ml^{-1} upwards [3,4,6–8]. Correlation of the therapeutic response with drug levels requires analytical methods that are simple, rapid and reliable. Methods utilizing derivatization with *o*-phthalaldehyde/thiol (OPA/thiol) [6,9,10] or dansyl chloride [11] have disadvantages associated with the derivatizing reagent. Dansyl chloride required a 4 h reaction time [11] and although the derivatization with OPA was rapid, the instability of the resulting fluorophor is well documented [12–14]. The derivatizing reagent NDA/CN reacts rapidly with primary amines to form a strongly fluorescent and stable derivative (Fig. 1). For these reasons, NDA/CN was selected as the derivatization reagent. This reagent system has been used with CE and ultraviolet detection to monitor DFMO kinetic studies in animals [15] with the derivative reported to be stable for up to 5 h. Improvements were sought in the areas of sample preparation, reduce analysis time, and improve derivative stability.

Preparation of biological samples such as plasma for HPLC analysis requires removal of proteins and any particulate matter that might block the system or damage the analytical column. Derivatization requirements added to the challenges already presented by DFMO because of its polar nature and ionic properties. Precipitation of the proteins by the addition of organic solvent or acid can involve multiple steps and results in a media unsuitable for derivatization. Ultrafiltration was identified as a suitable technique for sample preparation because it offered both simplicity and convenience. The structural similarity of DFMO and the amino acids present in plasma, made chromatographic selectivity difficult to achieve without sacrificing analysis time. Multidimensional chromatography was investigated to provide selectivity and minimize analysis time.

2. Experimental

2.1. Chemicals

DFMO [2-(difluoromethyl)-DL-ornithine hydrochloride monohydrate] was kindly provided by Dr Milan Slavik (V.A. Medical Research Service, Wichita, KS). Acetonitrile (ACN) and tetrahydrofuran (THF), hexane, ethyl acetate, and chloroform were HPLC grade and were purchased from Fisher Scientific (St. Louis, MO). Sodium phosphate monobasic was also purchased from Fisher Scientific. Sodium citrate was purchased from MCB Manufacturing Chemists (Cincinnati, OH).

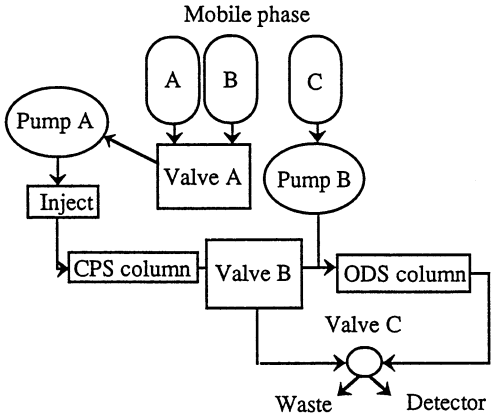

Fig. 2. Illustration of the chromatographic system. Valve A controls delivery of the mobile phase to the CPS column. Valve B directs the eluent from the CPS column to either the ODS column or to valve C. At valve C, the eluent can flow to the detector or be diverted to waste.

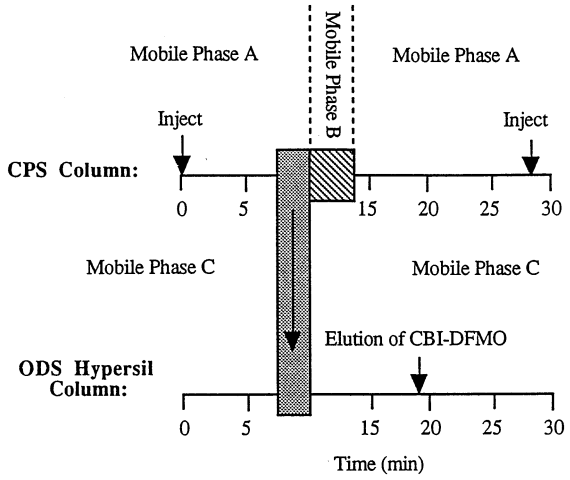

Fig. 3. Solvent and flow programming as a function of time. At time = 0, sample is injected onto the CPS column. Between 8 and 9.5 min the eluent is diverted to the ODS column (continuously eluted with mobile phase C, 1.0 ml min^{-1}), where resolution of the CBI-DFMO derivative from matrix interferences was completed. Concurrently, the CPS column is washed with mobile phase B (3.5 min, 2.0 ml min^{-1}) then re-equilibrated with mobile phase A (15 min, 1.0 ml min^{-1}). Mobile phase A: THF-phosphate buffer (41.0 mM NaH_2PO_4, pH 2.5, 1.35% glacial acetic acid, 0.135% TEA), (26:74, v/v). Mobile phase B: THF-phosphate buffer (50.0 mM NaH_2PO_4, pH 2.5, 1.7% glacial acetic acid, 0.17% TEA), (40:60, v/v). Mobile phase C: THF-citrate buffer (41.0 mM sodium citrate, pH 4.8), (27:73, v/v).

Sodium tetraborate and phosphoric acid were purchased from J.T. Baker (Phillipsburg, NJ). Taurine, triethylamine, carbon tetrachloride, and methyl-*tert*-butyl ether were purchased from Aldrich (Milwaukee, WI). Tetramethylammonium perchlorate was purchased from Eastman Kodak (Rochester, NY). Glacial acetic acid was purchased from Mallinckrodt (Paris, KY). Naphthalene-2,3-dicarboxaldehyde was prepared by Dr Hitesch Chokshi of the Chemistry Department, University of Kansas [16]. Sodium cyanide was purchased from Matheson Coleman and Bell (Cincinnati, OH).

2.2. Instrumentation

The instrumentation (Fig. 2) consisted of the following Shimadzu (Shimadzu, Kyoto, Japan) components: two pumps (model LC-6A), a low pressure switching valve (valve A, model FCV-3AL), a fluorescence detector (model RF-530, ex: 420 nm, em: 470 nm), an autosampler (model SIL-6A) and a model C-R3A integrator. Chromatographic columns (150 × 4.6 mm i.d.) were prepared by the upward slurry technique in these laboratories. The stationary phases were obtained from Shandon (5 μ particle size: ODS Hypersil (ODS), and Cyanopropyl Hypersil (CPS)). A dry packed silica column (50 mm × 4.6 i.d., 0.040–0.063 mm particle size, silica gel 60, E.M. Science, Germany) was placed prior to the CPS column as a saturator column. The analytical columns were thermostated at 40°C in a Shimadzu column oven.

2.3. Mobile phase preparation

The same general procedure was followed for the preparation of HPLC mobile phases. The

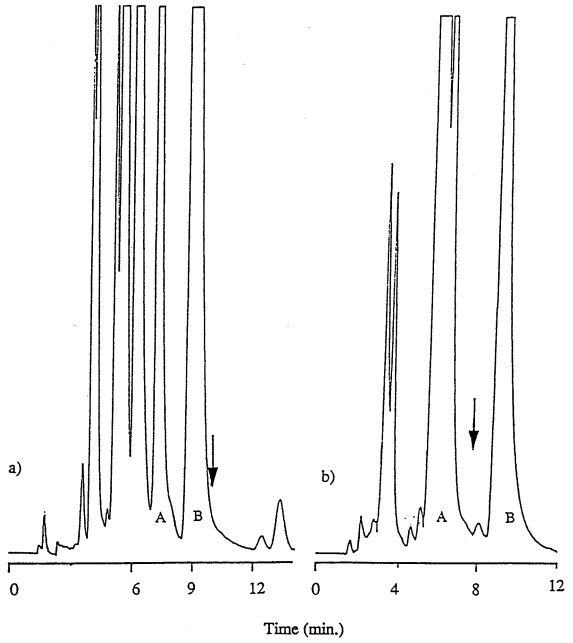

Fig. 4. Chromatograms of derivatized ultfiltrate: water (1:8) with arrows indicating where CBI-DFMO would elute under these conditions. The chromatograms were generated with an ODS column using a mobile phase of organic-phosphate buffer (10 mM NaH_2PO_4, pH 4.0); flow rate: 1.0 ml min^{-1}, 100 μl injection volume. The organic composition was: (a) 30% CAN, (b) 20% THF.

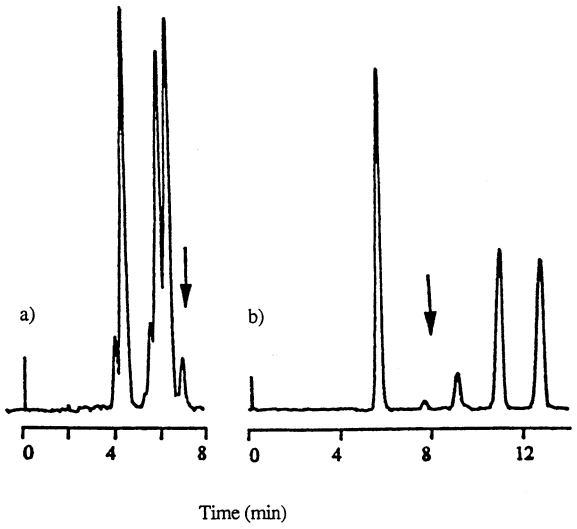

Fig. 5. Chromatograms illustrating the effect of the mobile phase pH on the elution profile of CBI-DFMO and ultrafiltrate derivatized in the absence of DFMO. The study was done using a CPS column with a mobile phase of THF-phosphate buffer (42.3 mM NaH_2PO_4), (29:71, v/v). (a) pH 4, (b) pH 2.5. The respective elution times of CBI-DFMO are 6.8 and 7.8 min (indicated by the arrows).

appropriate quantity of buffer salt was dissolved in water and the pH adjusted with either phosphoric acid (4.0 M) or sodium hydroxide (2.0 M). Additives which would affect the pH were added prior to the pH adjustment. The required volume of buffer was then mixed with the appropriate volume of organic modifier. The mobile phase was filtered through a 0.45 μm nylon membrane filter. The pH of the mobile phase refers to that of the aqueous portion.

2.4. Stock solutions

NDA was prepared in HPLC grade methanol. Separate stock solutions of DFMO, taurine, sodium cyanide, and sodium tetraborate buffer (0.4 M, adjusted to pH 10.0 with 2.0 M NaOH) were prepared in HPLC grade water. NDA and DFMO solutions were protected from light and stored at 5°C.

2.5. Derivatization procedure

Derivatization conditions for the chromatographic studies followed the same general procedure. Ultrafiltrate was derivatized both with and without DFMO. The ultrafiltrate for the chromatography studies was diluted with water for clarity. An aliquot (0.1 ml) of diluted ultrafiltrate or aqueous DFMO (8.5×10^{-8}–4.2×10^{-4} M) was combined with borate buffer (0.1 ml). Volumes of 0.1 ml each of NDA (4.2×10^{-6}–4×10^{-4} M) and NaCN (9×10^{-5}–4.2×10^{-4} M) were added to the reaction mixture. The final volume of the reaction mixture was adjusted to 1.0 ml by the addition of water. After 10 min, the solution was either analyzed or 0.1 ml of taurine (2.5×10^{-2} M) was added to quench the reaction. Data points comprising the calibration curves were prepared individually. On each of three days, plasma containing DFMO was prepared by diluting 0.25 ml of aqueous standards to 5.0 ml with plasma. Spiked plasma was pipetted (1.0 ml) into micropartition devices (MPS-1, YMT membranes, Amicon, Danvers, MA) and centrifuged for 1 h. The derivatizations were carried out in 1.5 ml polypropylene centrifuge tubes and the reactants are listed in the order of addition. Ultrafiltrate or standard (0.1 ml) was pipetted into the tube, followed by an equal volume of borate buffer (0.4 M borate, pH 10.0) and 0.5 ml of water. Aliquots (0.1 ml) of sodium cyanide (4.2×10^{-4} M) and NDA (4×10^{-4} M) were added and tube was vortexed (5 s), protected from light. After 10 min the reaction was quenched by the addition of taurine (2.5×10^{-2} M, 0.1 ml). The samples were placed in the autosampler (thermostated at 20°C).

2.6. Column switching

Column switching was used to achieve separation of the analyte from the derivatized plasma constituents (Fig. 3). The sample was injected (100 μl) onto the CPS column and the elution time frame of the CBI-DFMO was determined. During this time frame or switch window, flow was diverted to the ODS column. Solvent and flow programming were used to wash the CPS column after the switch had occurred. The CPS column

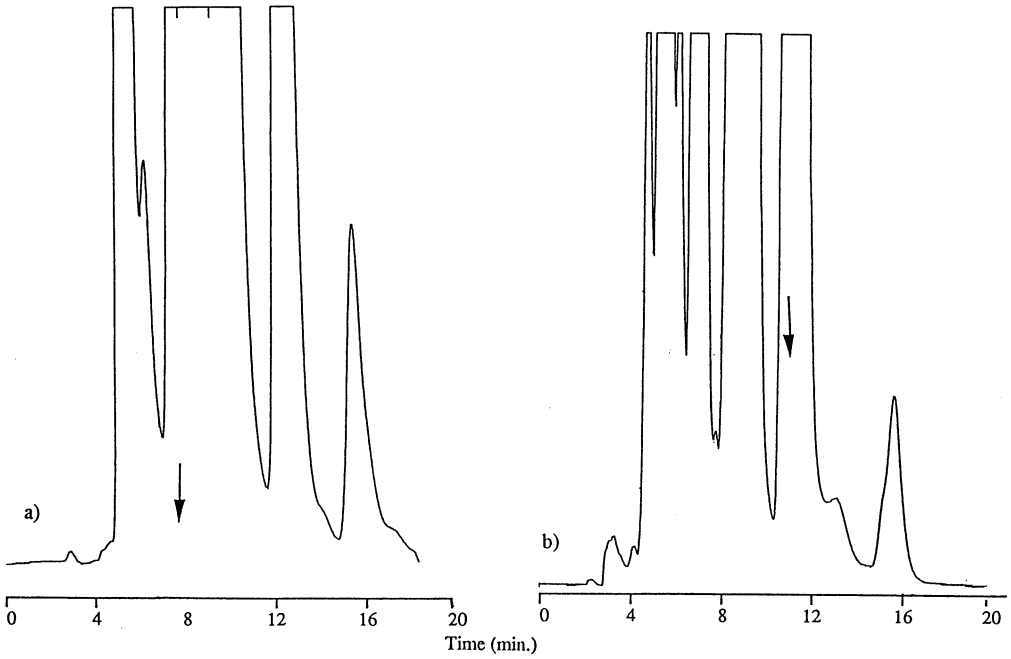

Fig. 6. Chromatography of derivatized ultrafiltrate in the absence of DFMO and without column-switching, the arrow indicates where CBI-DFMO would elute; (a) CPS column, mobile phase: THF-phosphate buffer (42.3 mM, pH 2.5), (26:74, v/v), (b) ODS column, mobile phase: THF-citrate buffer (42.3 mM, pH 4.6), (27:73, v/v).

was re-equilibrated with the initial mobile phase while analysis proceeded on the ODS column. For the CPS column the mobile phases were A and B. Mobile phase A consisted of THF-phosphate buffer (41.0 mM NaH_2PO_4, pH 2.5; 1.35% glacial acetic acid; 0.135% triethylamine), (26:74, v/v). The mobile phase for washing the CPS column (mobile phase B) was THF-phosphate buffer (50 mM NaH_2PO_4 pH 2.5; 1.7% glacial acetic acid; 0.17% triethylamine), (40:60, v/v). Mobile phase C for the ODS column was THF-phosphate buffer (41.0 mM sodium citrate, pH 4.8), (27:73, v/v).

3. Results and discussion

3.1. Derivatization: Reaction conditions and derivative stability

NDA/CN was developed in these laboratories for the determination of primary amines (Fig. 1) [17]. The derivatization has been shown to be sensitive to the reaction media, the nucleophilicity and steric bulk of the amine [17–19]. The unprotonated amine acts as a nucleophile in the reaction, therefore selective derivatization can be achieved by adjusting the pH of the reaction media to be near the pK_a of the target amine. In the case of DFMO, the α-amine and δ-amine pK_a were respectively 6.4 and 10.4. The derivatization of DFMO was conducted at pH 10.0 to ensure that $\sim$50% of the δ amine would be unprotonated [20]. Reactions were allowed to proceed for up to 24 h under identical conditions to generate a kinetic profile for the derivatization of DFMO. The maximal fluorescence was observed at $\sim$10 min and remained constant for 5 h (RSD 1.6%) with a decrease ($\sim$30%) in signal at 24 h. A reaction time of 10 min was selected for further work.

Difficulties encountered with NDA/CN mainly involve chromatographic interferences associated with the formation of reagent related side-products which appear to be due to reaction of cyanide with NDA [17,18,21]. The use of excess taurine to chemically inhibit derivatization has

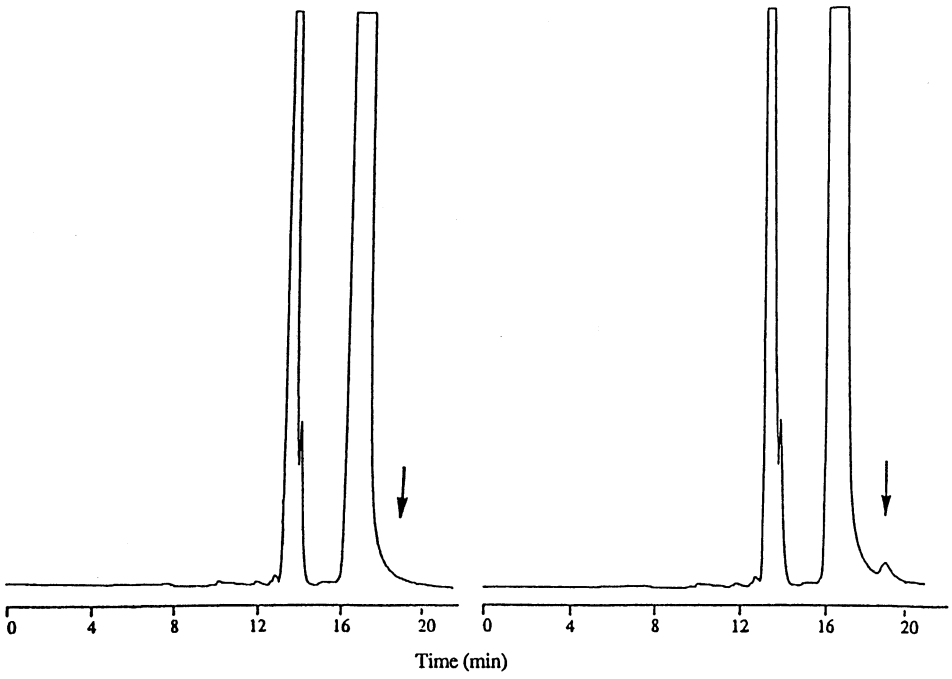

Fig. 7. Typical chromatography of derivatized ultrafiltrate obtained from plasma after column switching; (a) plasma without DFMO, (b) plasma containing DFMO (50 ng ml^{-1}). The time scale shown is the combined retention time of CBI-DFMO (18.7 min) on the CPS and ODS columns. The arrow indicates the elution of the CBI-DFMO peak. (The sample preparation, derivatization, and column switching system with solvent programming procedures are described in Section 2).

been utilized in these laboratories to minimize side reactions. Taurine reacts rapidly with NDA/CN forming CBI-taurine, thus limiting the formation of multiple non-polar side-products. With DFMO, inhibition of the reaction was demonstrated to improve the stability of the derivative. The data generated for the kinetic profile (without taurine) indicated $\sim 30\%$ loss in signal after 24 h. When the reaction was quenched with taurine, recovery at 24 h was 95%. Peak height versus concentration was linear ($R^2 \geq 0.999$) for standards (21 nM–2.1×10^3 nM, after derivatization) assayed over 24 h. No interfering peaks were observed when ultrafiltrate were derivatized in the absence of DFMO and stored for 24 h.

For compounds with multiple reaction sites, selective derivatization of a single site is usually preferred. Compounds with more than one reactive site can potentially undergo multiple derivatizations, which can result in a substantial decrease in the observed fluorescence [17–19,22,23]. Although DFMO has both an α and δ primary amine that could potentially be derivatized, these amines were expected to differ greatly in their reactivity. Repression of the reactivity of the α amine was expected due to steric hindrance. In addition the electronegative fluorine substituents exert an inductive effect on the α-amine reducing the nucleophilicity at the site. As a result the pK_a of the α-amine is ~ 3 pK_a lower than expected. This difference in pK_a of the α- and δ-amine enhanced the selectivity of the reaction. The presumption of mono-derivatization was supported by the data. The DFMO derivative was stable for 5 h when the reaction was not inhibited with taurine. Bis-derivatization would lead to a decrease in fluorescent response. The detected levels of the DFMO derivative were comparable to those reported for CBI derivatives of amino acids capable of only mono-derivatization [17]. Retention time comparison between mono-derivatized taurine (3.5 min) and the DFMO derivative (7.9

min) indicate the derivatives are of similar hydrophobicity. Bis-derivatization of DFMO would result in a molecule considerably more hydrophobic than the mono-derivative. The stability of the derivative, the sensitivity achieved, and the chromatography are evidence consistent with mono-derivatization.

3.2. Sample preparation

Separation of polar analytes from biological fluids is difficult and extraction conditions often involve pH manipulation. The process is further hindered by the presence of comparatively high levels of molecules with similar physical and chemical properties. As a small, polar compound DFMO is charged over the pH range 1–14, presenting a particular challenge in terms of sample preparation. Techniques relying on differences in polarity e.g.—extraction by organic solvents, differences in molecular weight—size exclusion, gel filtration, or ultrafiltration could be applied for gross cleanup to remove large or hydrophobic constituents. Consideration of the subsequent derivatization introduced sample dilution, addition dilution was undesirable. The use of acid as a precipitant was impractical since the derivatization required basic media. Ultrafiltration avoided sample dilution and the pH of the ultrafiltrate was ~ 8 which was compatible with the derivatization conditions and analysis.

3.3. Chromatographic studies

Derivatization of the ultrafiltrate resulted in the labeling of naturally occurring amines present in the sample matrix, and thus chromatographic separation of the DFMO derivative from matrix interferences was necessary. The chromatography of CBI-DFMO was studied to establish the retention profiles of the analyte and determine approaches for manipulating retention.

Retention maps were generated for the DFMO derivative in the absence of ultrafiltrate as a function of stationary phase (CPS and ODS) and organic modifier. Although tetrahydrofuran and acetonitrile resulted in similar capacity factors with the ODS column, comparison of asymmetry values revealed that THF improved peak shape. As expected, shorter retention times were observed with the CPS column.

When elution profiles of diluted ultrafiltrate and CBI-DFMO were compared on the ODS column with acetonitrile, it was observed that CBI-DFMO eluted between two peaks of such magnitude that resolution of the analyte was compromised (Fig. 4a). These peaks were arbitrarily identified as peaks A and B since their retention was only of interest in relation to that of the analyte. The organic modifier was changed to THF and the result was a change in the elution order (Fig. 4b). CBI-DFMO now eluted after both peaks A and B, indicating the sensitivity of the relative retention order to the organic modifier.

The retention of the analyte and diluted ultrafiltrate was established as a function of pH on the ODS column with THF as the organic modifier. It was observed that between pH 4 and 5, CBI-DFMO eluted after both peaks A and B. At either lower or higher pH, the DFMO derivative eluted between peak A and peak B. The effect of pH on the retention of CBI-DFMO was also observed with the CPS column at pH 2.5 and 4 (Fig. 5). A significant change in the elution profile of the ultrafiltrate was observed at the lower pH in that CBI-DFMO eluted ahead of the large interferences instead of among them. This pH dependent selectivity can perhaps be explained by consideration of the matrix composition. After ultrafiltration, the majority of matrix interferences were expected to be amino acids. Typical pK_a values of the α-amine and the carboxylic acid groups of common amino acids ($pK_{a_{\alpha\ COOH}}$, 2.1, $pK_{a_{\alpha\ amine}}$, 9.5) [24] are roughly 2 pK_a units higher than those of DFMO ($pK_{a_{\alpha\ COOH}}$, 0.08, $pK_{a_{\alpha\ amine}}$, 6.4, $pK_{a_{\delta\ amine10.4}}$) [10,20]. Assuming that derivatization did not affect pK_a's, the DFMO derivative and matrix derivatives might exhibit different ionic profiles based on pH. Over a limited pH range (3–5), the CBI derivatives of most amino acids would exist as mono- or dianions with the exception of those with a free α-

amine, such as lysine. Both lysine and DFMO would exist as zwitterions in this pH range. At lower pH, because of the unusually low $pK_{a_{\alpha\ COOH}}$ of DFMO, the derivative would be predominately in the zwitterionic form whereas the majority of the lysine derivative would have a positive charge. These differences could result in subtle selectively patterns and permit resolution of the DFMO derivative from matrix intereferences.

3.4. Multidimensional chromatography

As discussed in the Section 2, diluted ultrafiltrate was used for the chromatographic studies. The successful separation of the CBI-DFMO derivative from plasma interferences was not realized when undiluted ultrafiltrate was derivatized. Individually neither the ODS or CPS column provided sufficient selectively to resolve the DFMO derivative from the matrix interferences (Fig. 6). Derivatization of ultrafiltrate resulted in late eluting peaks under the conditions studies. Therefore, a two column-switching system was assembled and solvent flow programming was implemented to wash highly retained peaks from the CPS column (Fig. 3). The system was programmed such that while analysis was occurring on the ODS column, the CPS column could be eluted with a stronger mobile phase at a higher flow rate in order to elute these compounds. Then the CPS column could be re-equilibrated with the first mobile phase and returned to starting conditions. The mobile phases for the CPS column were THF-phosphate buffer (41.0 mM NaH_2PO_4, pH 2.5; 1.35% glacial acetic acid; 0.135% triethylamine), (26:74, v/v). The mobile phase for washing the CPS column was THF-phosphate buffer (50 mM NaH_2PO_4 pH 2.5; 1.7% glacial acetic acid; 0.17% triethylamine), (40:60, v/v). The mobile phase for the ODS column was THF-phosphate buffer (41.0 mM sodium citrate, pH 4.8), (27:73, v/v). Under these conditions, CBI-DFMO eluted at 19 min (Fig. 7). It was critical to the success of the method that the switch window remain constant, therefore an ample re-equilibration time was allowed and injections were made every 28 min.

3.5. Linearity and recovery

Sequential injections were made and the data points represent the mean value ($n = 3$) or separately prepared derivatizations both from aqueous standards and spiked plasma. Regression analysis indicated a linear response between peak height and concentration over the range of 0.21–21 μM (concentration of DFMO before derivatization) with coefficients of determination of at least 0.999. The derivatization procedure introduced a 10-fold dilution resulting in a final concentration of 21 nM–2.1×10^3 nM DFMO. The recovery of the analyte from spiked plasma was calculated by comparing the slope of the aqueous calibration curve and the spiked plasma calibration curve for each day of the study (Mean recovery, 99%; SD, 066; and % RSD, 6.6). The data reflect essentially 100% recovery from the spiked plasma and indicate that the micropartition units did not affect the concentration of the DFMO in the ultrafiltrate.

4. Conclusion

An HPLC method for the analysis of DFMO from plasma ultrafiltrate has been developed. The use of ultrafiltration was identified as a one-step method of preparing plasma for derivatization and HPLC analysis. This procedure produced a matrix suitable for derivatization and direct injection. Derivatization of DFMO using the NDA/CN reagent system produced the mono-derivative of DFMO. When the reaction was chemically inhibited with taurine, the CBI-DFMO was found to be stable for 24 h. Retention maps provide information from which conditions were identified for a column switching system that resulted in resolution of the DFMO derivative and efficient use of analysis time. Peak height versus concentration was linear from 21 nM–2.1×10^3 nM (based on the final concentration after derivatization) with a coefficient of determination of at least 0.999. Multidimensional chromatography combined with pre-column derivatization with NDA/CN and chemical inhibition of the reaction has been demonstrated to be a useful method for the

analysis of DFMO from plasma. This methodology offers the following advantages: minimal sample preparation, rapid derivatization, increased derivative stability, and column switching with solvent programming minimizes the analysis time.

The greatest potential for variation involved the purging the re-equilibration of the CPS column because reproducibility is dependent on the consistency of conditions on this column. Incomplete re-equilibration would cause variation in the switch window. Although not addressed here, optimization of the solvent flow programming might permit a decrease the analysis time.

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