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Determination of α-difluoromethylornithine in blood by microdialysis sampling and capillary electrophoresis with UV detection

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Abstract

A procedure is described for the analysis of α-difluoromethylornithine (DFMO), an anti-cancer agent, in plasma microdialysis (MD) samples. DFMO has been shown to be effective alone or in combination with other agents in the treatment of several cancers. Precolumn derivatization of DFMO with naphthalene-2,3-dicarboxaldehyde–cyanide (NDA–CN) in pH 10.0 borate buffer results in the rapid formation of a stable mono-derivatized product (N-substituted 1-cyanobenz[*f*]isoindole, CBI), which is UV active. An analytical method has been developed to separate CBI–DFMO from NDA–CN derivatization products of 20 standard amino acids using capillary electrophoresis (CE). This method is then employed for the determination of DFMO in plasma microdialysis samples. Separation of DFMO from other components in the dialysate was achieved within 20 min. The response for DFMO in Ringer's solution was linear over the range of $1.2 \cdot 10^{-6}$ to $1.6 \cdot 10^{-4}$ *M* after derivatization. The detection limit of DFMO in the plasma dialysate is 5 μ*M* using UV detection at 254 nm. This method has been proven to have adequate sensitivity for quantitation of DFMO in i.v. microdialysate samples and has been successfully applied to monitoring the pharmacokinetics of DFMO by CE–UV.

1. Introduction

α-Difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase, which effectively blocks the biosynthesis of endogenous polyamines (putrescine, spermidine, and spermine) [1]. DFMO inhibits tumor cell growth in vitro and in vivo, and demonstrates chemopreventive activity in a variety of animal tumors [2]. DFMO has been shown to be effective alone or in conjunction with other agents in the treatment of several cancers [3].

DFMO itself has no chromophore; therefore, derivatization is necessary for detection using conventional spectroscopic methods. To date, only a few published reports on the analysis of DFMO in biological fluids have appeared in the literature [4–6]. All of these employed precolumn derivatization with *o*-phthalaldehyde–thiol (OPA–thiol) [4,5] or dansyl chloride [6] and liquid chromatography (LC) with fluorescence or UV detection. The reported methods

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provide adequate sensitivity for plasma analysis, but have certain disadvantages. Derivatization with dansyl chloride requires at least a 4-h reaction time [6]. The reaction with OPA–thiol is rapid, but the instability of the resulting fluorophore is well documented [7,8], making precise timing of the derivatization essential.

In this paper, NDA–CN is evaluated as a reagent for the determination of DFMO by capillary electrophoresis with UV detection (CE–UV). NDA reacts with primary amines in the presence of cyanide to form cyano[*f*]benzoisoindole (CBI) derivatives, which are both spectroscopically and electrochemically active. Both the fluorescence efficiency and stability of the CBI derivatives are better than those of the OPA–thiol derivatives [9]. The determination of DFMO in plasma by LC using NDA–CN has been reported previously [10]. However, difficulties were encountered with this system due to the presence of naturally occurring amines in the sample matrix, which interfered with the separation. Adequate resolution of DFMO from amino acids could not be achieved using a single LC column [10] and, therefore, a multidimensional approach was employed. In addition, CBI–DFMO exhibited problematic chromatography, including peak tailing and splitting.

CE is a powerful analytical technique for the separation of charged substances. Very high separation efficiencies can be obtained using this approach. Amino acids have been determined previously by CE with UV, electrochemical, and fluorescence detection following derivatization with NDA–CN [11–15]. Microdialysis is an established technique for sampling drugs and endogenous compounds in tissues of awake, freely moving animals [16]. It has been used to study the metabolism and pharmacokinetics of a variety of drug substances in blood, liver, bile, and skin [17–21]. One of the advantages of microdialysis over other sampling methods is that dialysates are protein-free and can be directly injected into the analytical system with minimal sample preparation. In addition, since protein-bound molecules cannot pass through the membrane, only the biologically active "free" drug is measured. In this report, microdialysis sampling was employed to investigate the pharmacokinetics of DFMO using CE–UV as the analysis system.

2. Experimental

2.1. Reagents

All amino acids were purchased from Sigma (St. Louis, MO, USA) and used as received. NDA was supplied by Bioanalytical Systems (West Lafayette, IN, USA). Sodium cyanide, sodium borate, and sodium phosphate (monobasic and dibasic) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). DFMO monohydrate was kindly provided by Dr. Milan Slavik (V.A. Medical Research Service, Wichita, KS, USA).

2.2. Stock solutions

NDA was prepared in HPLC-grade acetonitrile, protected from light and stored at 5°C. All other solutions were prepared in Nanopure water (Sybron-Barnstead, Boston, MA, USA). Sodium borate was dissolved in Nanopure water and titrated to pH 10 with 2 *M* NaOH solution. Phosphate buffers (of different pH values) were prepared as reported in the Handbook of Biochemistry and Molecular Biology [22]. Amino acid mixtures were made by mixing together 1 ml of a 10 m*M* solution of each amino acid to yield a final concentration of 0.5 m*M*. All solutions were passed through a membrane filter (0.2 μm pore size) before use.

2.3. Instrumentation

An ISCO Model 3850 capillary electrophoresis system was driven by a high-voltage d.c. (0–30 kV) power supply connected to a platinum electrode. The fused-silica capillary (50 μm I.D., 360 μm O.D.) was obtained from Polymicro Technologies (Phoenix, AZ, USA). A column length of 75 cm (50 cm to detector) was used. The detection wavelength was set at 254 nm. Sample introduction was accomplished by vac-

uum injection for 4 s, which corresponded to a 3.7 nl injection volume.

2.4. Microdialysis system

Microdialysis sampling was performed using a CMA/100 microinjection pump from Bioanalytical Systems/CMA (West Lafayette, IN, USA) coupled to a microdialysis probe inserted into the jugular vein of the experimental animal. The laboratory-built flexible i.v. microdialysis probe has been described previously [19]. Perfusion was carried out with Ringer's solution (NaCl, 147 m*M*; KCl, 4 m*M*; $CaCl_2$, 2.3 m*M*) at a flow-rate of 0.25 μl/min. Microdialysis samples were collected by a CMA/142 fraction collector.

2.5. Microdialysis probe calibration

In order to ascertain the in vivo concentration of DFMO giving rise to the concentration detected in the perfusion medium, it was necessary to determine the recovery of the dialysis probe. This was accomplished by placing the probe in a standard solution of 0.1 m*M* DFMO prepared in Ringer's solution. The flow-rate was 0.25 μl/min, the same as that used for pharmacokinetic studies. Samples of the perfusate were collected and analyzed. Recovery was then expressed as the ratio of the concentration of DFMO in the perfusate to the concentration of standard. The average recovery for DFMO was $49.6 \pm 3.1\%$ ($n = 3$).

2.6. In vivo pharmacokinetic experiments

Male Sprague–Dawley rats 4–6 months old (450–500 g) were anaesthetized with ketamine and xylazine (80 mg/kg and 4 mg/kg, respectively). The flexible microdialysis probe was implanted into the jugular vein and PE-50 tubing was cannulated into the femoral vein. Ringer's solution was perfused through the microdialysis probes at a flow-rate of 0.25 μl/min, and dialysis samples were collected at 16-min intervals. Blanks were collected immediately following insertion of the microdialysis probe. The pharmacokinetic experiments were begun 24 h after surgery. At that time, the animals were dosed with DFMO (70 mg/kg) in Ringer's solution via the cannula in the femoral vein. Following derivatization with NDA and NaCN using the procedure below, samples were analyzed by CE–UV until DFMO was no longer detected in the dialysate.

2.7. Derivatization procedure

Derivatization conditions varied depending on the requirements of the reaction. In most cases, borate buffer (pH 10) was used as the reaction buffer. For these studies, a 1:1 molar ratio of NDA and NaCN was used. NDA was always added last to avoid the formation of possible side products. The final reaction solution contained a minimum of 20% acetonitrile to prevent precipitation of NDA. For the pharmacokinetic studies, 2 μl of 50 m*M* borate buffer (pH 10) and 2 μl of 15 m*M* NaCN solution were added to 4 μl of microdialysate, followed by 2 μl 15 m*M* NDA solution. The resulting solution was allowed to react for 10 min after the addition of NDA.

3. Results and discussion

3.1. Derivatization reaction conditions

DFMO has both alpha and delta primary amino groups, which can potentially be derivatized. The alpha amino group is attached to the tertiary carbon and is more sterically crowded than the delta amino site. Therefore, for DFMO, the mono-derivatized product was expected to be dominant over the bis-derivatized product. Previously, experiments regarding the reaction of NDA–CN with primary amino groups have shown that the reaction rate is maximal when the pH of the reaction solution is equal to the pK_a of the primary amine [9]. Since the delta amine has a pK_a of 10.4 [10], a pH 10.0 borate buffer was used as the reaction buffer.

Fig. 1 shows the electropherograms of solutions containing (A) NDA and (B) DFMO reacted with NDA–CN in pH 10.0 borate buffer. NDA itself is UV active and can therefore serve

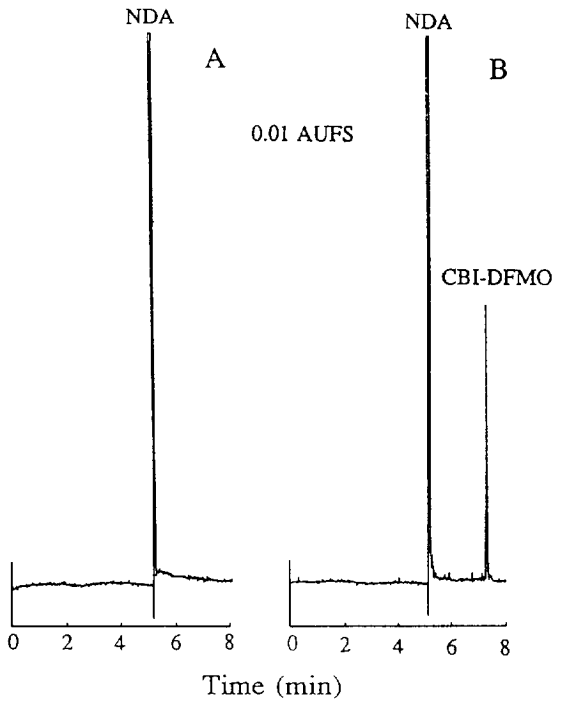

Fig. 1. Electropherograms of (A) 1.0 m*M* NDA and (B) 0.1 m*M* DFMO reacted with NDA–NaCN (1.0 m*M* each) for 10 min in pH 10 borate buffer. Separation conditions: 75 cm (50 cm to detector) × 50 μm I.D. capillary in 20 m*M* sodium borate (pH 9.0); voltage, 20 kV (18 μA).

as neutral marker in these studies (Fig. 1A). The derivatization of DFMO with NDA–CN gives rise to a new peak that corresponds to the mono-CBI–DFMO derivative (Fig. 1B). The reaction of DFMO with NDA–CN at pH 10.0 is very fast (ca. 3 min), and can be monitored by a change from colorless to bright yellow. While several different wavelengths (210, 254, and 420 nm) can be employed for the detection of the CBI derivative, 254 nm was found to provide the best sensitivity and was used for all subsequent studies.

In order to optimize the derivatization reaction, the effect of borate concentration and reaction time on product yield were evaluated. To maintain pseudo-first-order reaction in DFMO, excess NDA–NaCN should be used. However, side products may be formed at very high concentrations of NDA and NaCN. For these studies, a 1:1 molar ratio of NDA and CN was used. Using a constant concentration of DFMO (0.05 m*M*), the mono-CBI-derivative peak increased with increasing concentrations of NDA and NaCN. The peak height achieved its maximum value (Fig. 2) after the concentration of NDA–CN reached ten times that of DFMO.

It has been reported that the concentration of borate has a catalytic effect on the derivatization reaction of NDA–CN with primary amines [9]. Therefore, the effect of borate concentration on reaction rate was also investigated over the range of 10–50 m*M* (final concentration). Fortunately, the concentration of borate was found not to have a substantial effect on the rate of this particular derivatization reaction. Using 10 m*M* borate buffer, the reaction of DFMO with NDA–CN was complete within 3 min and the derivative was stable for at least 5 h. Higher concentrations of borate buffer did not substantially increase the reaction rate. However, it was found that column efficiency decreased with increasing concentration of borate buffer, as shown in Fig. 3. This phenomenon has been

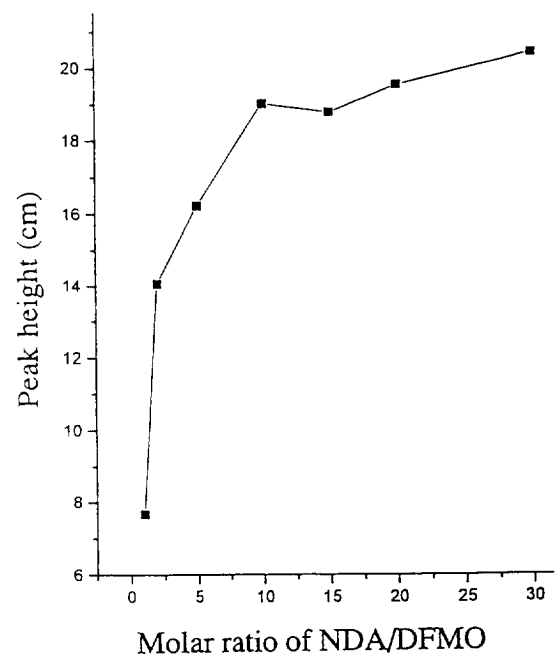

Fig. 2. Effect of molar ratio of NDA–DFMO on peak height of CBI–DFMO derivative. Concentration of DFMO, 0.05 m*M*; reaction time, 10 min.

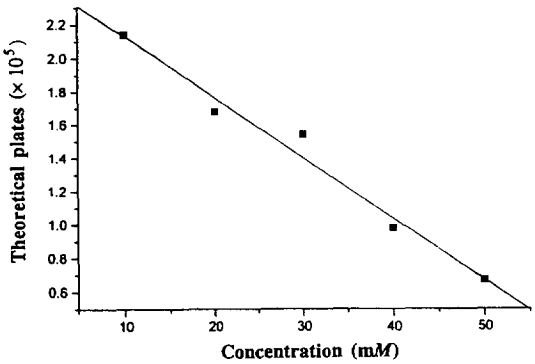

Fig. 3. Efficiency of CBI–DFMO peak as a function of borate buffer concentration in the derivatization reaction. Separation conditions: 75 cm (50 cm to detector) × 50 μm I.D. capillary; 100 m*M* phosphate (pH 6.5); voltage, 15 kV (49 μA).

described previously for the determination of CBI–amino acids by CE–UV [12]. Therefore, 10 m*M* pH 10.0 borate buffer was chosen to be reaction buffer throughout the studies.

3.2. Capillary electrophoresis method development

When performing i.v. microdialysis, there are a number of naturally occurring amino acids and small peptides that are present in the dialysate. Therefore, separation conditions that will isolate the CBI–DFMO from the rest of the amino acids must be developed. In order to accomplish this, we investigated the migration behavior of the 20 standard amino acids found in proteins relative to CBI–DFMO. CBI–amino acids with a neutral or negatively charged R-group are negatively charged above pH 3 and thus are easily resolved from CBI–DFMO. CBI–arginine possesses a positively charged R-group with a high pK_a and migrates early in the electropherogram. In the case of CBI–DFMO, the major interference was expected to be histidine since the pK_a of the free amino group (6.0) is close to that of DFMO (6.4).

In developing the separation of CBI–DFMO from the CBI–amino acids, several variables were investigated, including the pH and composition of the run buffer, the length of the capillary, and the applied voltage. Of these, pH and composition of run buffer were found to have the greatest effects on the separation. At pH values above 9, both CBI–DFMO and the CBI–amino acid derivatives are anionic and the electroosmotic flow is very fast, resulting in poor resolution. Zwitterionic buffers were investigated at lower pH values, but no good separation was obtained over the range pH 7–9. Phosphate buffers in the range 8.5–5.5 were also investigated. With a pH 8.0 phosphate buffer, lysine was not resolved from CBI–DFMO and at pH 6 histidine comigrated with CBI–DFMO. However, all three compounds could be resolved between pH 6.5 and 7.5. A 50 m*M* phosphate (pH 6.7) buffer provided the best resolution of CBI–DFMO from all of the amino acids studied. Fig. 4 shows the separation of CBI–DFMO from the CBI–amino acid mixture. DFMO migrated between NDA and histidine.

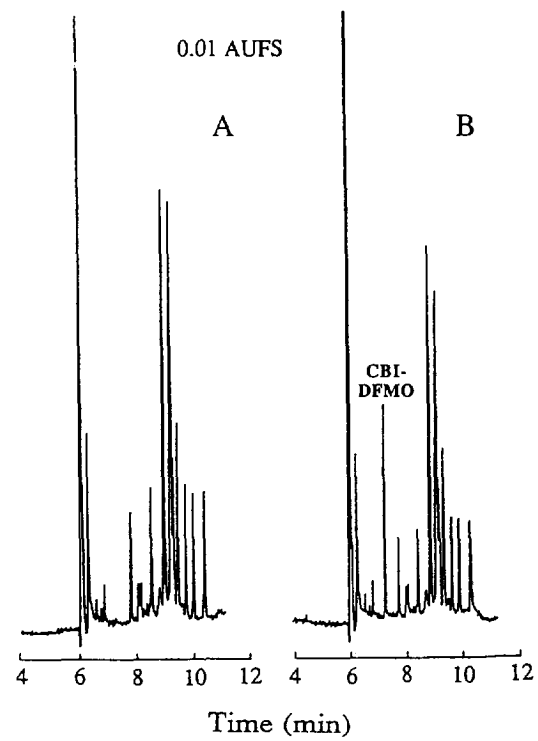

Fig. 4. Electropherograms of (A) 20 amino acids (0.02 m*M* each) and (B) DFMO (0.05 m*M*) and 20 amino acids (0.02 m*M* each) derivatized with NDA–CN. Separation conditions: 75 cm (50 cm to detector) × 50 μm I.D. capillary; 50 m*M* phosphate (pH 6.7); voltage, 20 kV (33 μA); λ = 254 nm.

A blank plasma microdialysis sample produced an electropherogram very similar to that of standard amino acids. However, a small unidentified peak appeared whose migration time was very close to that of CBI–DFMO (Fig. 5). Thus, the separation conditions required further optimization to resolve CBI–DFMO from the unknown peak. It was found that the use of higher concentrations of run buffer, lower applied voltages, and a longer capillary improved resolution. However, these conditions resulted in longer analysis times. In addition, the use of high concentrations of borate buffer resulted in high separation currents and increased Joule heating. Considering all of these factors, the best separation was obtained under the following conditions: 100 m*M*, pH 6.5, phosphate buffer, 75 cm (50 cm to detector) × 50 μm capillary, 15 kV applied voltage. Fig. 5 shows the electropherograms of (A) a blank plasma microdialysis sample and (B) a sample spiked with DFMO. It can be seen that under these conditions, a baseline separation between DFMO and the unknown small peak was obtained ($R = 1.7$). In this case, the migration time for CBI–DFMO was about 11 min, and the overall analysis time was less than 20 min.

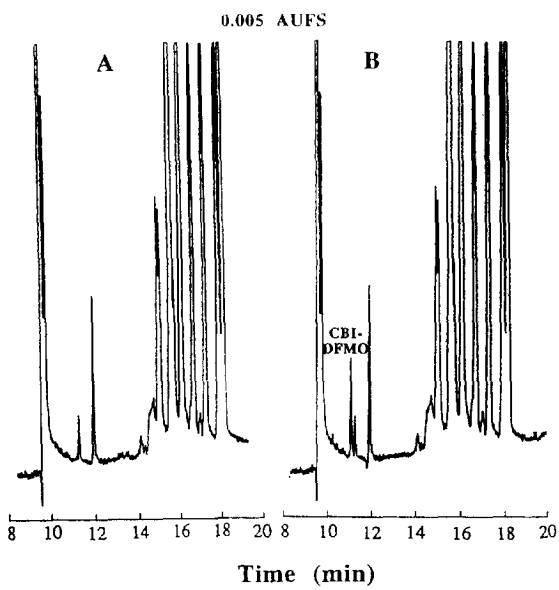

Fig. 5. Electropherograms of (A) blank plasma microdialysate sample and (B) plasma microdialysate spiked with 10 n*M* DFMO. Separation conditions: 75 cm (50 cm to detector) × 50 μm I.D. capillary; 100 m*M* phosphate (pH 6.5); voltage, 15 kV (49 μA).

3.3. Calibration and detection limits

The system was calibrated over the concentration range expected in the microdialysis samples. The relationship between peak height and concentration of DFMO was linear over the range 3–400 μ*M* (original concentration of DFMO before derivatization) with a correlation coefficient of 0.9995. Since 4 μl of DFMO was derivatized to give 10 μl of final solution, the actual concentration range in the final solution after derivatization was between 1.2 and 160 μ*M*. In these studies, DFMO was dissolved in Ringer's solution in order to yield a matrix similar to that of microdialysis sample, and the NDA and NaCN concentrations used were at least ten times the concentration of DFMO.

The effect of NDA–CN concentration on the yield of CBI–DFMO in dialysates was also examined. Because the exact total concentration of amino acids in the microdialysis samples is unknown, it is important to add enough NDA to react with all of the amines in the sample. Therefore, the effect of NDA concentration on the yield of CBI–DFMO in a spiked microdialysis sample (10 μ*M*) was determined. It was found that using 15 m*M* NDA and NaCN ensured almost 100% yield of DFMO. Thus, the calibration curve of DFMO obtained in Ringer's solution under these conditions was used for the calculation of concentration values for pharmacokinetic studies.

The limit of detection (signal-to-noise ratio = 3) for CBI–DFMO in Ringer's solution was 3 μ*M*. It was slightly higher in microdialysate samples (5 μ*M* before derivatization, 2 μ*M* after derivatization). Using 3.7 nl as the injection volume, the corresponding mass detection limit was 7.4 fmol in the microdialysis sample. Based on a 50% recovery of DFMO with the flexible dialysis probe, the limit of detection for DFMO was approximately 10 μ*M* in vivo.

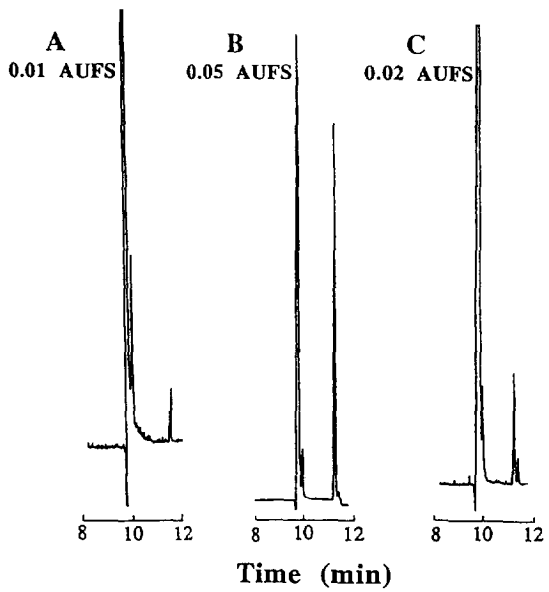

Fig. 6. Electropherograms obtained in the pharmacokinetic study. (A) Blank plasma dialysate, (B) 16 min after a 70 mg/kg i.v. dose of DFMO, (C) 64 min after same dose. Separation conditions: 75 cm (50 cm to detector) × 50 μm I.D. capillary; 100 m*M* phosphate (pH 6.5); voltage, 15 kV (49 μA).

3.4. Pharmacokinetics studies

Pharmacokinetic studies were performed on awake, freely moving rats. Typical electropherograms of blood dialysates by in vivo sampling are shown in Fig. 6. The blank sample was run at the high-sensitivity detector gain setting; no peak co-migrating with the CBI–DFMO derivative was present in the blank at this high gain (Fig. 6A). Fig. 6B shows an electropherogram of blood dialysate 16 min after an i.v. dose of 70 mg/kg DFMO. In the initial samples, the CBI–DFMO peak overwhelmed the interference peak. The concentration of DFMO in the microdialysate rapidly decreased, as shown in Fig. 6C.

Since microdialysis is a continuous sampling technique, each sample represents the average concentration of analyte in the blood during the sampling interval. A representative concentration versus time profile (plotted as a bar graph) of the microdialysis samples is shown in Fig. 7. The semi-log plot (log concentration DFMO versus time) gave a straight line (Fig. 7, inset),

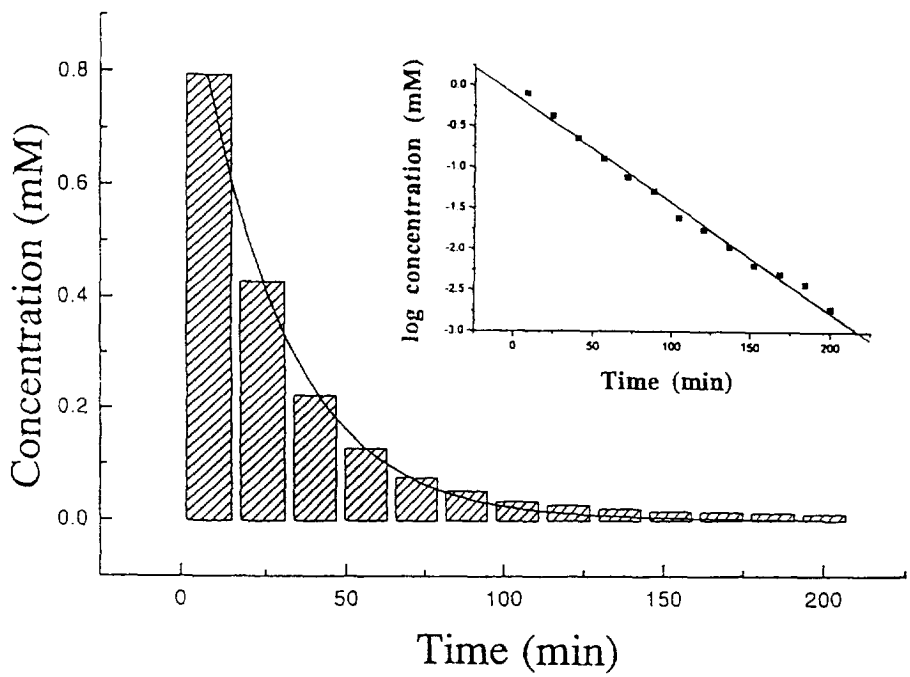

Fig. 7. Concentration versus time profile for a 70 mg/kg i.v. dose of DFMO in an awake, freely moving rat. Inset: plot of log concentration versus time.

indicating that the rapid i.v. dose of DFMO was distributed in the body according to a one-compartment model and eliminated by apparent first-order kinetics. The average half-life for an i.v. dose of DFMO ($n = 4$) was 21.0 ± 4.8 min.

4. Conclusions

A capillary electrophoresis method for the analysis of CBI–DFMO derivatives in blood dialysates has been developed. The derivatization of DFMO using NDA and NaCN in pH 10 borate buffer produced the mono-CBI–DFMO derivative, which is UV active. For the CE separation, a phosphate run buffer (100 mM, pH 6.5) was found to provide the best separation of CBI–DFMO from the endogenous CBI-derivatized amino acids native to the dialysate samples. Peak height versus concentration was linear from 1.2 to 160 μM with a correlation coefficient of 0.9995 (based on the final concentration after derivatization). The detection limit for DFMO in an i.v. dialysate was 5 μM. The use of UV detection at 254 nm provided adequate sensitivity for the detection of the drug in blood microdialysates. The separation was faster and more efficient than those previously reported using LC with fluorescence detection. This method was used to monitor the pharmacokinetics of DFMO following an i.v. dose of 70 mg/kg of DFMO to awake, freely moving rats.

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