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Analysis of 2-difluoromethyl-DL-ornithine in human plasma, cerebrospinal fluid and urine by cation-exchange high-performance liquid chromatography

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Abstract

An analytical method has been developed based on cation-exchange liquid chromatography for the measurement of 2-difluoromethyl-DL-ornithine (DFMO) in human plasma, cerebrospinal fluid (CSF) and urine. Fluorescence detection at excitation/emission wavelengths of 340/440 nm is followed by postcolumn derivatization with *o*-phthalaldehyde–2-mercaptoethanol. All calibration ranges yielded linear relationships with correlation coefficients better than 0.999. In each case the limit of quantitation was equal to the lowest value of the standard curve. The variability of the assay, expressed as relative standard deviations, was less than 7.1%, 15.3% and 7.1% for plasma, CSF and urine, respectively. The accuracy of the assay (expressed as relative errors) ranged between −4.3% and 2.0% for plasma analysis, between −0.1% and 14.0% for CSF analysis and between −8.0% and 2.0% for urine analysis. Plasma, CSF and urinary DFMO concentrations were measured in samples obtained from patients undergoing treatment for trypanosomiasis. The method was found to be applicable for the measurement of DFMO levels in human body fluids for the determination of pharmacokinetic parameters in clinical studies.

Keywords: 2-Difluoromethyl-DL-ornithine

1. Introduction

DFMO (2-difluoromethyl-DL-ornithine, monohydrochloride, monohydrate, eflornithine, MDL 71 782A) (Fig. 1) is a specific and irreversible inhibitor of ornithine decarboxylase which has been approved for the treatment of the meningoencephalic stage of trypanosomiasis caused by *Trypanosoma brunei gambienze* (also commonly known as African sleeping sickness) [1]. A previous ion chromatographic method based on external standard quantitation [2] has been improved by the use of an internal standard (I.S.) and changes to reduce analysis time. MDL 72 246A (*trans*-2,5-diamino-2-fluoromethyl-3-pentenoic acid, monohydrochloride) (Fig. 1) was chosen as the I.S. due to its structural similarity. This method was applied to the analysis of drug concentrations in the study of the pharmacokinetics of eflornithine in trypanosomiasis patients.

2. Experimental

2.1. Instrumentation

The liquid chromatography apparatus consisted of the following devices: a Waters 590 pump (elution buffer) and a Waters 510 pump [*o*-phthalaldehyde

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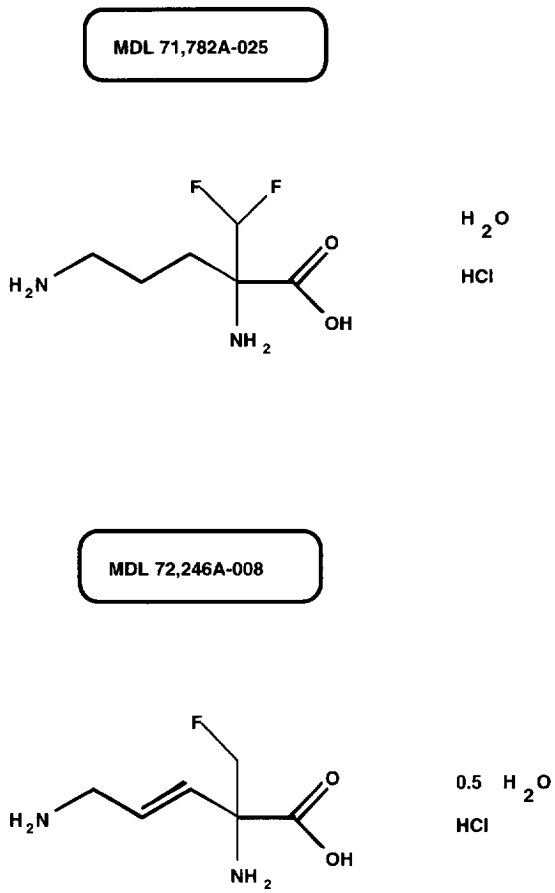

Fig. 1. Chemical structures of DFMO and the internal standard, MDL 72 246A.

(OPA) buffer], both fitted with microflow pump heads, a Waters solvent select valve for buffer switching, and a WISP 710 autosampler. The inert glass column (400 mm×2.5 mm I.D.), fitted with a circulating water jacket from Kontron (Zürich, Switzerland), was thermostatically controlled at 60°C by a Polystat 5 circulating water bath (Bioblock, France). Detection was achieved by a Waters 420-AC fluorimetric detector connected to a WW 600 Tarkan analogic recorder (Kontron) and a Waters SIM (system interface module). The entire system was controlled through the SIM by a Waters Millennium Chromatography Manager installed on a Compaq Deskpro 486 computer.

2.2. Chemicals and solvents

All aqueous solutions were prepared using MilliQ water (R>10 MΩ/cm). Citric acid (trisodium salt, dihydrate, amino acid analysis grade), 5-Sulfosalicylic acid (SSA), ethylenediaminetetraacetic acid (EDTA), 2-mercaptoethanol and Brij 35 were purchased from Sigma (St. Louis, MO, USA). Trichloroacetic acid 40% (w/v) (TCA), boric acid, potassium hydroxide, sodium hydroxide and methanol were of reagent grade (Merck, Darmstadt, Germany). Hydrochloric acid, 6 M, constant boiling, twice distilled was prepared from 37% reagent grade hydrochloric acid (Merck). Liquified phenol was amino acid analysis grade from Baker (Deventer, Netherlands). OPA was purchased from Karl Roth KG (Karlsruhe, Germany). DFMO and MDL 72 246A were synthesized at the Marion Merrell Research Institute in Strasbourg.

Solvent A: The elution buffer was sodium citrate buffer (0.2 M Na^+) adjusted to a pH of 6.25 by dropwise addition of 6 M hydrochloric acid. To avoid bacterial growth, 1 ml of liquified phenol was added as a preservative to one liter buffer. Solvent B: Sodium hydroxide (0.2 M) containing 500 mg/l EDTA was used as a washing buffer between samples in order to complex resin-poisoning metallic ions. Solvent C: The OPA reagent (300 mg), dissolved in 15 ml methanol, 2 ml Brij 35 solution, and 2 ml 2-mercaptoethanol, was added to one liter of borate buffer (pH 10.5). All buffers were vacuum filtered and degassed through 0.2 mm pore size polymeric PTFE filters.

2.3. Analytical procedure

The glass column (400 mm×2.5 mm I.D.) was packed manually to a final resin bed height of 280 mm by the slurry method with cation-exchange polystyrene–divinylbenzene cross-linked resin beads (6–12 μm size) purchased from Labotron (Münich, Germany). The resin bed was protected by a low dead volume column inlet filter fitted with a 2 μm frit (Rheodyne).

Aliquots of 50 or 100 μl were injected onto the column with an elution buffer (A) flow-rate of 300 μl/min. After 21 min, the solvent select valve was switched to the wash buffer (B) for 5 min. The column was then re-equilibrated with elution buffer (A) for 27 min giving a total run time of 53 min. Flow-rate was kept constant at 300 μl/min for both wash and re-equilibration steps.

At the column outlet, effluent was mixed with OPA reagent buffer (C) in a T-configuration at a flow-rate of 200 μl/min in order to form the fluorescent isoindole derivatives. The very quick complete reaction time allowed the use of a short reaction coil (500 mm×0.1 mm I.D.). Fluorimetric detection was performed with an excitation wavelength of 340 nm and emission wavelength of 440 nm during 35 min.

2.4. Sample preparation

To 100 μl plasma, 50 μl I.S. solution (0.2 m*M* and 2 m*M* for low and high range level determination, respectively) in TCA 20% was added in order to precipitate proteins. The sample was mixed thoroughly and then centrifuged at 10 000 rpm for 5 min. The supernatant (50 μl) was diluted to 500 μl with 450 μl of elution buffer to obtain dilution SD1. For low range level determination, 100 μl of SD1 dilution were injected directly. For high range level determination, the SD1 dilution (50 μl) was diluted further to 500 μl with 450 μl of elution buffer to obtain the final dilution, SD2. Aliquots of 100 μl of SD2 dilution were injected onto the column.

To 150 μl cerebrospinal fluid (CSF), 50 μl I.S. solution (0.2 m*M*) in 5% SSA was added in order to precipitate proteins. The sample was mixed thoroughly and then centrifuged at 10 000 rpm for 5 min. The supernatant (50 μl) was diluted to 500 μl with 450 μl elution buffer of which 50 to 100 μl were injected onto the column.

To 100 μl urine, 250 μl of I.S. solution (10 m*M*) was added. The sample volume was adjusted to 10 ml with MilliQ water and mixed thoroughly. The above dilution (50 μl) was added to 450 μl of elution buffer and mixed to obtain a 500 μl final dilution of which 50 μl were injected onto the column.

All calibration curves were prepared in duplicate and injected before and after unknown and quality control samples which were prepared in an identical fashion. Five replicates of each control sample were analysed on the same day to obtain within-day accuracy and precision. The same procedure was repeated over two additional days yielding fifteen replicates in order to establish the day-to-day accuracy and precision.

2.5. Data analysis

Concentrations of DFMO were determined by matching peak height responses against a calibration curve of response ratio (height DFMO/height I.S.) vs. concentration, obtained from standard sample injection. The I.S. corrects for variation in the numerous dilution steps used.

Peak detection, peak height integration, peak height ratio calculation, calibration curve fitting (least squares regression without weighting) and calculations of sample concentrations were performed by the Millenium Chromatography software.

3. Results

3.1. Chromatographic separation

Selectivity of the chromatographic separation was assessed by the absence of interferences from endogenous peaks in each matrix studied (i.e. plasma, CSF, urine). Representative chromatograms obtained from blank and spiked plasma, CSF and urine are shown in Fig. 2. The retention times (capacity factors) for DFMO and the I.S. were 13.3±0.1 min (5.65) and 31.1±0.3 min (14.55), respectively.

3.2. Linearity and limit of quantitation

Plasma analysis was calibrated using low (5–50 nmol/ml) range ($y=-0.01291+0.0311x$) and high (50–2000 nmol/ml) range ($y=0.2413+0.00309x$) curves due to the large differences in plasma concentrations expected. CSF analysis was calibrated over the range 5 to 200 nmol/ml ($y=-0.01322+0.0453x$). A Ringer's solution was used as a substitute for CSF in calibration standards. Urine analysis was calibrated over the range 5 to 50 mmol/ml ($y=-0.01232+0.11534x$). All calibration ranges yielded linear relationships with correlation coefficients better than 0.999. In each case the limit of quantitation was equal to the lowest value of the standard curve. As the plasma, CSF and urine concentrations expected in the biological samples was within this range of calibration concentrations, no attempt was made to increase assay sensitivity and determine a true limit of detection. Recovery of

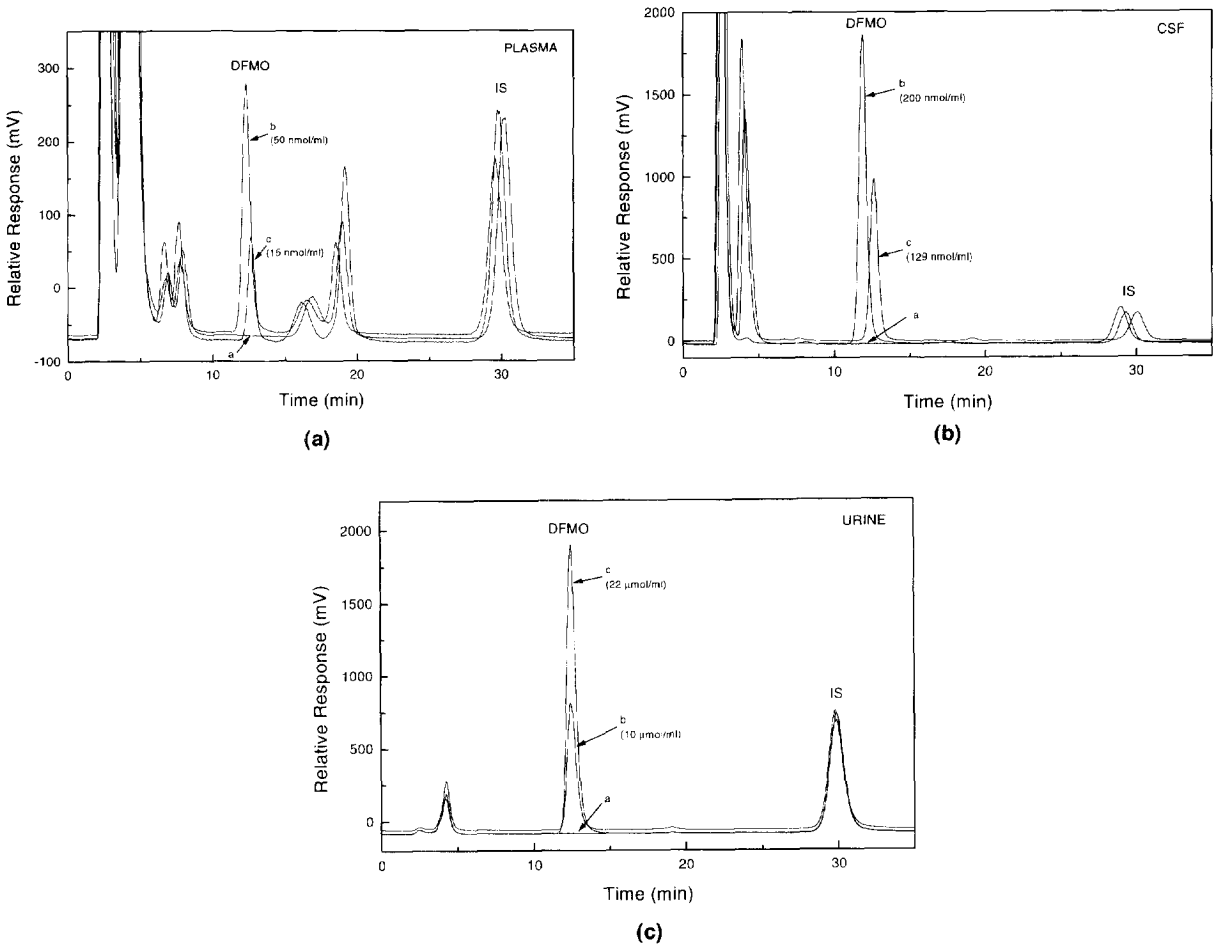

Fig. 2. Chromatograms of DFMO and I.S. separations in plasma, CSF and urine. In each case chromatogram "*a*" refers to blank, "*b*" refers to a calibration standard and "*c*" refers to an unknown. Chromatograms: (a) plasma; (b) CSF; (c) urine.

DFMO and its I.S. after the protein precipitation step was quantitative (data not shown).

3.3. Accuracy

The accuracy was determined by calculating the difference between the mean observed value and the theoretical value as a function of the theoretical value. Relative errors (R.E.) expressed as a percentage were between −8.0 and 14.0% for the different biological fluids (Tables 1 and 2).

3.4. Precision

Precision of the assay was determined by examination of the within-day and day-to-day reproducibilities. The within-day coefficients of variation (C.V.) (Table 1) determined by dividing the standard deviation by the mean and expressing as a percentage were less than 4.9% for all concentrations and in all fluids. The day-to-day C.V.'s (Table 2) were less than 15.3%.

3.5. Application

This method has been used to measure DFMO concentrations in the plasma, CSF and urine obtained from ten patients undergoing treatment of trypanosomiasis by venous DFMO infusions over a fourteen day period. Plasma concentrations ranged

Table 1
Within-day accuracy and precision for the analysis of spiked plasma, CSF and urine samples.

Biological fluid	Theoretical concentration (nnmol/ml)	Observed concentration (mean±S.D.; $n=5$) (nmol/ml)	R.S.D. (%)	R.E. (%)
Plasma (low level)	5	5.1± 0.1	2.2	2.0
	50	49.9± 1.0	2.0	−0.2
Plasma (high level)	100	96.9± 2.5	2.6	−3.1
	2000	1976.6±23.8	1.2	−1.2
CSF	5	5.4± 0.1	1.2	8.0
	200	202.1± 9.9	4.9	1.1
Urine*	5	5.1± 0.0	0.9	2.0
	50	49.1± 0.5	1.1	−1.8

* Theoretical and observed concentrations are in μmol/ml. R.S.D.=relative standard deviation; R.E.=relative error.

from 6.6 to 2446.6 nmol/ml. CSF concentrations ranged from 4.9 to 227.3 nmol/ml and 24 h urinary DFMO excretion ranged from 3.43 to 21.34 g.

4. Discussion

The present method was developed as a modification of a previously described method [2], which used the external standard method for correcting for losses during the analytical procedures, in order to enhance the reproducibility, accuracy and precision of the measurements. MDL 72 246A was chosen as the I.S. due to its close structural similarity to DFMO (Fig. 1) and due to the fact that it eluted without interferences from endogenous amino acids in any of the biological fluids. Commercial alternatives such as homoarginine, norleucine or norvaline could also be used as I.S.. The present method was also developed to validate the method for CSF analysis since penetration of the drug into the central nervous system (CNS) is a prerequisite for treating patients in late stage trypanosomiasis with CNS involvement. The new method resulted in a significant shortening of the total analysis time from 80 min to 53 min largely due to the fact that the decreased variability due to inclusion of the I.S. permitted shorter washing and equilibration steps. The specificity of fluorescent detection results in minimal sample preparation which involves only protein precipitation and sample dilution. Due to the relatively large doses used in trypanosomiasis treatment and the relative ease with which the drug passes the blood–brain barrier, the present method was sufficiently sensitive to measure the drug even in CSF; however, given the lack of interferences in all biological fluids it is theoretically possible to enhance the sensitivity further by decreasing the volumes of or totally omitting the dilution steps. At an early stage in the investigation of DFMO concentrations, assays were performed

Table 2
Day-to-day accuracy and precision for the analysis of spiked plasma, CSF and urine samples.

Biological fluid	Theoretical concentration (nnmol/ml)	Observed concentration (mean±S.D.; $n=15$) (nmol/ml)	R.S.D. (%)	R.E. (%)
Plasma (low level)	5	5.0± 0.2	4.0	0.0
	50	50.8± 1.0	2.0	1.6
Plasma (high level)	100	95.7± 6.8	7.1	−4.3
	2000	2017.0±105.0	1.2	0.9
CSF	5	5.7± 0.9	15.3	14
	200	199.9± 7.9	4.0	−0.1
Urine*	5	4.6± 0.3	7.1	−8.0
	50	46.8± 2.4	5.2	−6.4

* Theoretical and observations concentrations are in μmol/ml. R.S.D.=relative standard deviation; R.E.=relative error

only to determine DFMO concentrations in CSF. Due to the small amounts of protein found in CSF, even in those coming from patients, the weak protein precipitation agent, SSA, was used. The extension of the analysis to plasma samples required larger amounts of SSA to achieve complete protein precipitation, leading to a very broad injection peak as a result of the strong fluorescent signal of SSA. Therefore, it was replaced by the stronger protein precipitation agent, TCA, which did not exhibit this strong fluorescent interference.

A recently developed inhibition immunoassay [3] was used to measure plasma DFMO concentrations in rabbits but has not been validated for urine or CSF analysis. Both methods require only a dilution step for sample preparation although the enzyme-linked immunosorbent assay requires a laborious antibody producing procedure including immunoaffinity column fractionation.

The present method has been applied to the measurement of DFMO concentrations in plasma, CSF and urine samples obtained for the determination of pharmacokinetic parameters from patients undergoing treatment for trypanosomiasis. Plasma concentrations ranged from 6.6 to 2446.6 nmol/ml. CSF concentrations varied between 4.9 to 227.3 nmol/ml and 24 h urinary excretion varied between 3.43 and 21.34 g. We believe that the method has the sensitivity, precision and accuracy required for the unambiguous measurement of DFMO levels in human body fluids for the determination of pharmacokinetic parameters in clinical studies.

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