Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 788 (2003) 221–231
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

High-performance liquid chromatographic method for determination of 2-difluoromethyl-DL-ornithine in plasma and cerebrospinal fluid

W. Hanpitakpong, B. Kamanikom, V. Banmairuroi, K. Na-Bangchang*

Pharmacology and Toxicology Unit, Faculty of Allied Health Sciences, Thammasat University (Rangsit Campus), Paholyothin Rd., Pathumthani 12121, Thailand

Received 9 November 2001; received in revised form 28 May 2002; accepted 21 June 2002

Abstract

A simple, sensitive, selective and reproducible method based on anion-exchange liquid chromatography with post-column derivatisation was developed for the determination of eflornithine (2-difluoromethyl-DL-ornithine; DFMO) in human plasma and cerebrospinal fluid. The 1-alkylthio-2-alkyl-isoindoles fluorescent derivative of the drug was separated from the internal standard (MDL 77246A) on an anion-exchange column (PRP-X300, 250×2.1 mm, 7-μm particle size: Hamilton, USA), with retention times of 6.9 and 10.7 min, respectively. Fluorescence detection was set at 430/340 nm (emission/excitation wavelength). The elution solvent consisted of a solution of 30 m*M* potassium dihydrogen phosphate buffer (pH 2.2) and acetonitrile (50:50, v/v), running through the column at a flow-rate of 0.3 ml/min. The chromatographic analysis was operated at 37 °C. Sample preparation for either plasma or CSF (100 μl) was done by single-step protein precipitation with 20% trichloroacetic acid after incubation at 4 °C for 1 h. Calibration curves for plasma (100, 200, 400, 600, 800 and 1200 nmol/100 μl, and 10, 20, 40, 80, 120 and 160 nmol/100 μl for the high and low concentration range curves, respectively) and CSF (1, 2, 4, 8, 16, 32 nmol/100 μl) were all linear with correlation coefficients better than 0.999. The precision of the method based on within-day repeatability and reproducibility (day-to-day variation) at high concentration range was below 15%, whereas at low concentration range was below 20% (% coefficient of variations: %C.V.) Good accuracy was observed for both the intra-day or inter-day assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (below ±15 and ±20% at high and low concentration range, respectively. The limit of quantification was accepted as 0.1 nmol using 100-μl samples. The mean recovery for DFMO and the internal standard were greater than 95%. The method was free from interference from commonly used drugs including antimalarials and antihelminthics. The method appears to be robust and has been applied to a pharmacokinetic study of DFMO in patients with African trypanosomiasis following oral doses of Ornidyl® (Aventis Pharma, Frankfurt, Germany) at 500 mg/kg body weight (125 mg q.i.d.) for 14 days.

Keywords: 2-Difluoromethylornithine; Eflornithine

1. Introduction

Eflornithine (2-fluoromethyl-DL-ornithine; MDL 71782A; DFMO) is a selective, enzyme-activated, irreversible inhibitor of ornithine decarboxylase en-

*Corresponding author. Tel.: +66-2-926-9438; fax: +66-2-516-5379.
E-mail address: nkesara@tu.ac.th (K. Na-Bangchang).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(02)00438-5

zyme, one of the key enzymes in the polyamine biosynthetic pathway [1,2]. It has been used as an antitumor and as antiprotozoan agent in the treatment of the meningoencephalic stage of trypanosomiasis caused by *Trypanosoma brucei gambienze* (African trypanosomiasis) [3–5]. In African trypanosomiasis, DFMO has been approved by the FDA, USA for the treatment of the meningoencephalic stage [6,7]. The drug development process of DFMO in this disease is currently at a relatively early stage, and therefore the full pharmacokinetic characterisation in patients, in conjunction with pharmacodynamics (clinical efficacy/safety) are essential for optimisation of drug therapy. Penetration of the drug into the central nervous system is a pre-requisite for treating patients in late-stage trypanosomiasis with CNS involvement. Concentrations of at least 50 nmol/ml are required for trypanosomidal effect of DFMO [2,8,9]. To support the investigation of its pharmacokinetic profiles or the correlation of drug levels in biological fluids with the therapeutic drug response, an appropriate analytical method (sensitive, selective, reproducible and simple) for quantification of DFMO in biological fluids (plasma and CSF) is essential.

A number of analytical methods have been reported for measuring DFMO in biological fluids and tissue or cell homogenates or organ extracts. These methods involve inhibition immunoassay [10] and high-performance liquid chromatographic techniques (HPLC) [11–14]. An inhibition immunoassay [10] was developed to measure plasma DFMO concentrations in rabbits. Although the method required only a dilution step for sample preparation, the enzyme-linked immunosorbent assay required a laborious antibody producing procedure including immuno-affinity column fractionation.

The HPLC techniques currently available for the quantification of DFMO in biological fluids or tissue/cell homogenates involve either pre- or post-column derivatisation, with ultraviolet or fluorescence detection [11–13]. In a procedure described for the measurement of DFMO in biological specimens (plasma, serum, urine, organ and cell extracts), DFMO was separated from other amino acids using a commercial amino-acid analyser and detected by formation of its alkylthio-isoindole derivative with *o*-phthaldiahyde (OPA) [9]. Fluorescence detection was at excitation and emission wavelengths of 340 and 440 nm. DFMO concentrations of 0.1 nmol could be determined in a sample volume of 100 μl. Sample preparation was done by protein precipitation with 20% trichloroacetic acid or perchloric acid. The assay was used to determine the serum half-life of DFMO, including the relationships between serum and tissue concentrations in several animal species. The time required for each injection was 40 min using a two-column system operated at a temperature of 54 °C.

A reversed-phase HPLC method utilising pre-column dansylation was described for the analysis of DFMO in serum [11]. Derivatisation for at least 4 h was necessary for maximum derivative formation. Novaline (2-amino valeric acid) was added as an internal standard and the sample was extracted with three portions of ice-cold 80% ethanol. Chromatography was carried out on a C_8, 15-cm column with a linear gradient from a 95:5 solution of 10 m*M* acetate buffer (pH 4) and tetrahydrofuran, to a 90:10 solution of acetonitrile over a period of 28 min. Detection was by ultraviolet at 330 nm. DMFO and norvaline were eluted at 14.7 and 17.7 min, respectively. The sensitivity of the assay was reported as 42 nmol/ml. The assay was used for the measurement of serial DFMO plasma levels in AIDs patients over a multiple dosing interval (100 mg/kg body weight as an intravenous infusion every 6 h). With this method, the problem of extraneous dansylated amino acid peaks in chromatogram generally confounded pre-column derivatisation procedures, and there was constant interference at the retention time of DFMO, which precluded accurate measurement below 42 nmol/ml plasma.

Another pre-column HPLC method previously reported for the analysis of DFMO from plasma ultrafiltrate was developed by Kilkenny et al. [13]. The use of ultrafiltration was identified as a one-step method of preparing plasma for derivatisation with naphthalene-2,3-dicarboxaldehyde/cyanide (in pH 9.2 borate buffer). The DFMO derivative (cyanobenz(f)isoindole derivative) was separated from plasma interference by multi-dimensional chromatography with ODS Hypersil and cyanopropyl hypersil columns. The analysis time was 28 min. Fluorescence detection was performed at excitation and emission wavelengths of 420 and 470 nm, respectively.

An analytical method based on post-column derivatisation of DMFO with fluorescence detection has recently been developed using cation-exchange liquid chromatography (glass column packed manually with polystyrene–divinylbenzene cross-linked resin beads) for the measurement of DFMO in human plasma, CSF and urine [12]. Fluorescence detection at excitation/emission wavelengths of 340/440 nm was followed by post-column derivatisation with OPA. Sample preparation was done by protein precipitation of biological samples (100 μl) with trichloroacetic acid (20, 40%) or 5-sulfosalicylic acid. Total run time was 53 min. Sensitivity of the method was 5 nmol/ml.

Altogether, the above mentioned methods suffer from one or more of the following shortcomings, namely, relatively large sample volume [11], low sensitivity [11], lack of an internal standard [9,13], long or sophisticated sample preparation or chromatographic procedures [11–13]. In the present report, we describe an alternative method, which is a relatively simple, rapid, sensitive, accurate and reproducible method for determination of DFMO in biological fluids. The total run time was within 15 min. The method was based on anion-exchange chromatography and post-column derivatisation with fluorescence detection. The method has been applied successfully for pharmacokinetic study of DFMO in patients with African trypanosomiasis.

2. Experimental

2.1. Chemicals

All solvents were HPLC grade. Organic solvents were purchased from Fison (Bishop Meadow Road, Loughborough, UK). *O*-Phthaldiadehyde (OPA), Brij 35 solution, 2-mercaptoethanol and trichloroacetic acid (TCA) were obtained from Sigma (St Louis, MO, USA). Ultrapure analytical grade Type I water (r>18 MΩ/cm) was produced by a Milli-Q Plus® water system (Millipore, Bedford, MA, USA). Potassium dihydrogen phosphate and sodium hydroxide were of analytical grade and obtained from APS Ajax Chemicals, Australia. DFMO (MDL 71782A; Fig. 1a) and internal standard (MDL 72246A; Fig. 1b) were obtained from Aventis Pharma (Frankfurt, Germany).

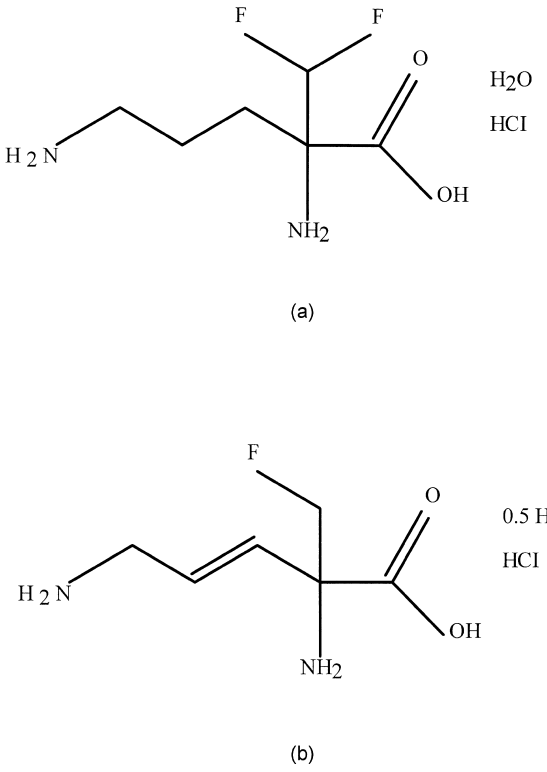


Fig. 1. Chemical structures of (a) DFMO (MDA 71782A) and (b) internal standard (MDA 72246A).

2.2. Standard stock solutions

Stock solutions were made with DFMO and the internal standard. Appropriate amounts of chemicals were dissolved in distilled water in volumetric flasks. Stock solutions for DFMO and internal standard were prepared at a concentration of 1000 nmol/μl. The stock solutions were further diluted to make working solutions at concentrations of 50, 5 and 1 nmol/μl, for DFMO, and 50 and 10 nmol/μl for the internal standard. Standard solutions were stored at −80 °C until use.

2.3. Chromatography

The method was developed on a chromatographic system consisting of a Waters 600 HPLC solvent

Delivery/Controller (elution solvent), Waters 515 HPLC Solvent Delivery (OPA reagent buffer), and Waters 600 Solvent Delivery/Controller (washing buffer), equipped with a Rheodye 7125 injector with a 100-μl loop (Rheodyne, Berkeley, CA, USA) and a fluorescence detector (Jusco FP). The wavelengths were set at 340/440 nm (excitation/emission wavelength). The separation was carried out on an anion-exchange column (PRP-X300, 250×2.1 mm, 7-μm particle size: Hamilton, USA). The elution solvent consisted of a solution of 30 m*M* potassium dihydrogen phosphate buffer (adjusted pH to 2.2 with 85% *o*-phosphoric acid) and acetonitrile (50:50, v/v). The chromatographic analysis was operated at 37 °C. Aliquots of 100-μl samples or standard solutions were injected onto the column with an elution buffer at a flow-rate of 0.3 ml/min. At the column outlet, effluent was mixed with OPA reagent buffer consisting of borate buffer (480 ml), 0.1 N hydrochloric acid (438 ml), 20 mg/ml OPA in methanol (15 ml), and Brij solution (2 ml) in a T-configuration at a flow-rate of 0.2 ml/min in order to form the fluorescent isoindole derivative (1-alkylthio-2-allyl-isoindoles fluorescent derivative). The very rapid complete reaction allowed the use of a short reaction coil (500 mm×0.1 mm I.D.). Sodium hydroxide (0.2 N) containing 500 mg/l ethylenediamine-tetraacetic acid (EDTA) was used as a washing buffer to complex resin-poisoning metallic ions after about 10 chromatographic runs. All buffers were vacuum filtered and degassed through 0.2-μm pore size polymeric PTFE filters.

2.4. Sample preparation

2.4.1. Plasma

This procedure was validated on specimens using 100 μl of spiked human plasma. Outdated human plasma was obtained from the Blood Bank of Bhumipol Adulyadesh Hospital, and stored frozen in aliquots at −20 °C. To 100 μl plasma, 5 or 40 μl internal standard working solutions (250 or 2000 nmol for low or high concentration calibration curve, respectively) was added. After thoroughly mixing, 100 μl of 20% TCA was added in order to precipitate proteins. The sample was mixed thoroughly and allowed to stand for 1 h at 4 °C to complete precipitation of the proteins, and then centrifuged at 10 000 rpm for 5 min. The supernatant (100 μl) was diluted to 500 μl with 400 μl of elution buffer and an aliquot of 100 μl was injected onto the column.

2.4.2. Cerebrospinal fluid (CSF)

Distilled water was used as a substitute for CSF in all calibration curves. To 100 μl distilled water, 5 μl internal standard working solution (50 nmol) was added. After thoroughly mixing, 100 μl of 20% TCA was added in order to precipitate proteins. The sample was mixed thoroughly and allowed to stand for 1 h at 4 °C to complete precipitation of the proteins, and then centrifuged at 10 000 rpm for 5 min. The supernatant (100 μl) was diluted to 500 μl with 400 μl of elution buffer and an aliquot of 100 μl was injected onto the column.

2.5. Calibration curves

2.5.1. Detector linearity

Solutions of DFMO in distilled water at concentrations ranging from 1 to 2000 nmol/100 μl were injected into the HPLC system in order to assess detector linearity. Peak height was plotted against the quantity of DFMO injected. DFMO was linear ($r>0.999$) over the concentration range observed.

2.5.2. Plasma

Calibration curves were prepared by replicate analysis of six plasma samples (100 μl each) spiked with varying concentrations of DFMO and a fixed concentration of the internal standard as follows:

High concentration range: DFMO at concentrations of 100, 200, 400, 600, 800 and 1200 nmol/100 μl, with a fixed concentration of internal standard (2000 nmol/100 μl).

Low concentration range: DFMO at concentrations of 10, 20, 40, 80, 120 and 160 nmol/100 μl, with a fixed concentration of internal standard (250 nmol/100 μl).

Samples were analysed as described in Section 2.4.1.

2.5.3. CSF

Calibration curves were prepared by replicate analysis of six sets of samples of distilled water (100 μl each) spiked with a fixed concentration of the

internal standard (50 nmol), and with varying concentrations of DFMO (0, 2, 4, 8, 16 and 32 nmol/100 μl). Samples were analysed as described in Section 2.4.2.

2.6. Data analysis

Peak height ratios of DFMO/internal standard were calculated. Concentrations of DFMO were determined by matching peak height responses against a calibration curve of response ratio (height of DFMO/height of internal standard) versus concentration, obtained from standard sample injection. The internal standard corrected for variation in the sample preparation (protein precipitation/dilution) steps used. Peak detection, peak height integration, peak height ratio calculation, calibration curve fitting (least-squares regression without weighting) and calculation of sample concentrations were carried out with Millenium 2000 Chromatograph® software.

2.7. Method validation

2.7.1. Precision

The precision of the method based on *within-day repeatability* was determined by replicate analysis of six sets of samples spiked with five to eight different concentrations of DFMO. For plasma samples, validation was performed at the concentrations of 100, 200, 400, 600, 800 and 1200, and 1, 5, 10, 20, 40, 80, 120, and 160 nmol/100 μl for the high and the low concentration range curves, respectively. For CSF samples (distilled water was used as a substitute medium), validation was performed at concentrations of 1, 2, 4, 8, and 16 nmol/100 μl. The *reproducibility* (*day-to-day variation*) of the method was validated using the same concentration ranges of plasma and CSF as described above, but only a single determination of each concentration was made on six different days. Coefficients of variation (C.V.) were calculated from the ratios of standard deviation (SD) to the mean and expressed as percentage.

2.7.2. Accuracy

The accuracy of the method was determined by replicate analysis of six sets of samples at five to eight different levels of DFMO and comparing the difference between the spiked value and that actually found (theoretical value). For plasma samples, validation was performed at concentrations of 100, 200, 400, 600, 800, and 1200, and at 1, 5, 10, 20, 40, 80, 120, and 160 nmol/100 μl, for the high and low concentration range curves, respectively. For CSF samples (distilled water was used as a substitute), validation was carried out at DFMO concentrations of 1, 2, 4, 8, 16 and 32 nmol/100 μl.

2.7.3. Recovery

The analytical recovery of the sample preparation procedure for DFMO was estimated by comparing the peak heights obtained from samples (plasma, CSF) prepared as described in Section 2.4, with those measured with equivalent amounts of DFMO in distilled water. For plasma samples, triplicate analysis was carried out at concentrations of 100, 600, and 1200, and 10, 80 and 160 nmol/100 μl for high and low concentration range curves, respectively.

2.7.4. Selectivity

The selectivity of the method was verified by checking for interference by commonly used drugs, i.e. antimalarials (chloroquine, quinine, mefloquine), anthelminthics (albendazole, praziquantel), paracetamol and dimenhydrinate after subjecting them to sample preparation procedures.

2.7.5. Limit of quantification

The limit of quantification (LOQ) of the assay procedure was determined from the lowest concentration of DFMO (in spiked plasma sample) that produced a peak height three times the baseline noise at a sensitivity of −0.2 μA in a 100-μl sample.

2.7.6. Stability

The stability of DFMO was determined by storing spiked plasma samples (concentrations 10, 100, 600, 1200 nmol/100 μl; triplicate analysis for each concentration) in a −80 °C freezer (Sanyo, Japan) for 6 months. Concentrations were measured periodically (1, 2, 3 and 6 months). For freeze and thaw stability, samples were frozen at −80 °C for at least 24 h and thawed unassisted at room temperature (25 °C). When completely thawed, the samples were transferred back to the original freezer and refrozen

for at least 24 h. The process was repeated for three cycles.

2.8. Quality control

Quality control (QC) samples for DFMO were made up in plasma using a stock solution separate from that used to prepare the calibration curve, at the concentrations 10, 100, 600, 1200 nmol/100 μl. Samples were aliquoted into cryovials, and stored frozen at −80 °C for use with each analytical run. The results of the QC samples provided the basis of accepting or rejecting the run. At least four of the six QC samples had to be within ±20% of their respective nominal value. Two of the six QC samples could be outside ±20% of their respective nominal value, but not at the same concentration.

2.9. Application of the method to biological samples

The method was applied to the investigation of the pharmacokinetics of DFMO in plasma and for determination of drug levels in CSF in an African patient (from Cote d'Ivoire) with African trypanosomiasis (aged 28 years, weighing 50 kg) who received treatment with DFMO (Ornidyl®, Aventis Pharma, Frankfurt, Germany) at the dose of 500 mg/kg body weight (125 mg q.i.d.) for 14 days. This was part of a large clinical trial to compare clinical efficacy, tolerability and pharmacokinetics of DFMO when given at 400 and 500 mg/kg body weight. The study was approved by the Ethics Committee, Ministry of Public Health, Cote d'Ivoire. Written informed consents for study participation were obtained from all patients. Venous blood samples (8 ml) were collected into EDTA-coated plastic tubes at the following time points: day-1 (5 min before first dose), day-10 (15 min before the second dose), day-15 (15 min before the last dose, and 10, 20, 30 and 45 min, and 1, 2, 3, 6, 9, 14 and 24 h after the last dose). Plasma was separated by centrifugation at 2000 *g* for 10 min, immediately after collection and frozen at −80 °C until analysis. CSF samples (5 ml) was taken at diagnosis, 5 min before the second dose on day-10, and 5 min before the last dose of treatment on day-15.

3. Results and discussion

3.1. Chromatographic separation

A number of HPLC chromatographic systems were investigated to optimise the separation of the derivatised product of DFMO and the internal standard (MDL 72246A). Retention maps were generated for both compounds as a function of stationary phase (anion-exchange or cation-exchange column) and elution solvent (mobile phase). For, the elution solvent, a composition of 0.1 *M* citric acid and distilled water or phosphate buffer (pH) resulted in similar capacity factors with either the cation- (PPR-X300, 10 μm, 250×2.1 mm) or anion-exchange (PPR-X300, 7 μm, 250×2.1 mm) columns. The retention times (capacity factor) of DFMO fluorescent derivative and the internal standard were approximately 2.0 and 2.5 min, and therefore the peaks of both compounds were not resolved. The anion-exchange column, with elution solvent consisting of potassium dihydrogen phosphate (pH 2.2), acetonitrile and distilled water was shown to give optimal separation with a 15-min run time. Adjusting the composition of these three components, by increasing the organic phase—acetonitrile and decreasing the ratio of distilled water improved the shape of the peak. Finally, the elution solvent consisting of 30 m*M* potassium dihydrogen phosphate buffer (pH

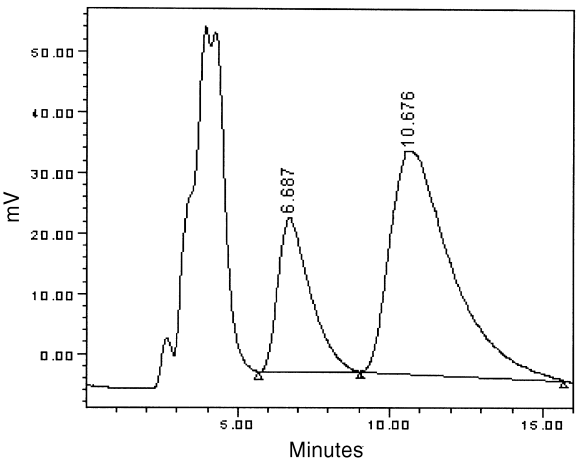
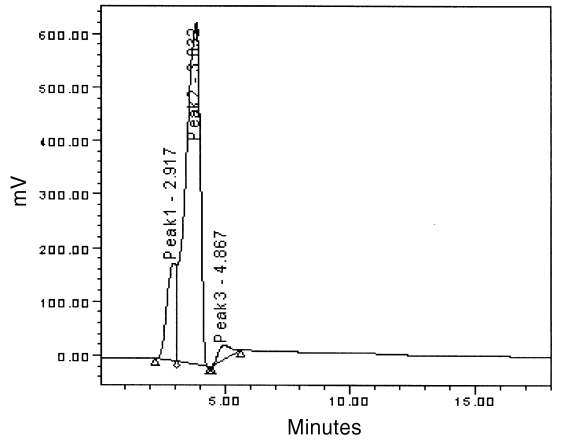


Fig. 2. Chromatogram of standard solution of DFMO (20 nmol) and internal standard (250 nmol), with retention times of 6.9 and 10.7 min, respectively.

2.2) and acetonitrile at the ratio of 50:50 (v/v) was chosen as an appropriate elution solvent as it resulted in optimal separation. The retention times of DFMO, and the internal standard were approximately 6.9, and 10.7 min, respectively. The chromatograms showed a good baseline separation. A chromatogram of standard solution of DFMO (20 nmol/100 μl) and internal standard (250 nmol/100 μl) is shown in Fig. 2.



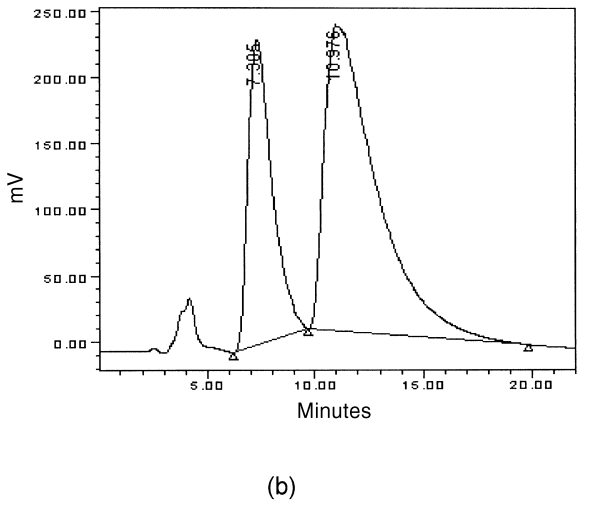
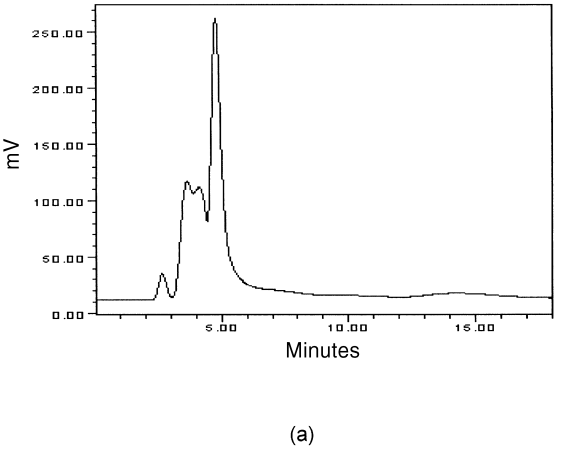


Fig. 3. Chromatogram of (a) blank plasma, (b) plasma spiked with 600 nmol/100 μl DFMO and 2000 nmol/100 μl internal standard (retention times of 7.3 and 10.9 min, respectively).

3.2. Sample preparation

The sample preparation step used in this study was very simple as it involved only one-step protein precipitation. Precipitation of plasma or CSF with 20% TCA at 4 °C for a minimum of 1 h was found to be the optimal condition for sample preparation as it resulted in a clean chromatogram. The internal standard corrected for variation in the sample preparation (protein precipitation/dilution) steps used. Protein precipitation and further dilution with elution solvent introduced a 10-fold dilution for both plasma and CSF.



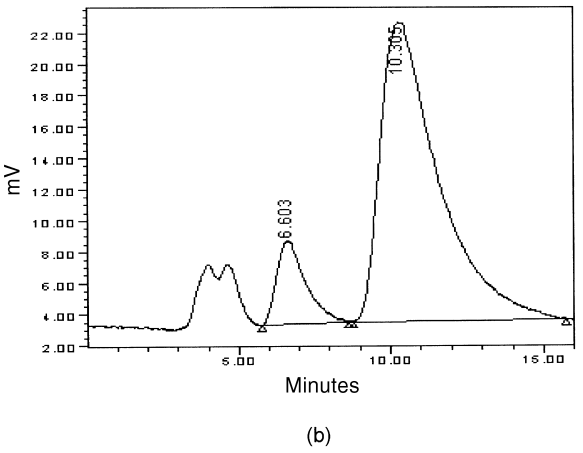


Fig. 4. Chromatogram of (a) blank distilled water, (b) distilled water spiked with 2 nmol/100 μl DFMO and 50 nmol/100 μl internal standard (retention times of 6.6 and 10.3 min, respectively).

Chromatograms of blank plasma and plasma spiked with DFMO at a concentration of 600 nmol/100 μl DFMO (with a fixed concentration of internal standard of 2000 nmol/100 μl), are shown in Fig. 3a and 3b, respectively. Fig. 4a,b represents chromatograms of distilled water (a substitute for CSF) and distilled water spiked with DFMO at a concentration of 2 nmol/100 μl DFMO (with a fixed concentration of internal standard of 50 nmol/100 μl).

3.3. Calibration curves

Plasma analysis was calibrated using low (10–160 nmol/100 μl) and high (100–1200 nmol/100 μl) concentration range curves due to the possibility of large differences in plasma concentrations expected. CSF analysis was calibrated over the range 2–32 nmol/100 μl. All calibration ranges yielded linear relationships with correlation coefficients of 0.999 or better.

3.4. Method validation

3.4.1. Precision

Little variation of DFMO assays was observed; coefficients of variation (C.V.) for six analysis at high concentration range were all below 15%, whereas C.V. at very low concentrations, i.e. at 1 and 2 nmol/100 μl were below 20%. The intra-assay (within-day) and inter-assay (day-to-day) variation for DFMO assay at high (100–1200 nmol/100 μl) and low (10–160 nmol/100 μl) plasma concentration ranges and CSF (2–32 nmol/100 μl) are summarised in Table 1a–c. For intra-day assay validation in plasma, the coefficients of variation varied between 0.8 and 1.8%, and between 3.9 and 16.7%, for the high and low concentration ranges,

Table 1
Summary of assay precision and accuracy (intra-assay and inter-assay) for DFMO assay in plasma and CSF

Concentration added (nmol/100 μl)	Precision (%C.V.)		Accuracy (%DMV)[a]	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
(a) Plasma: high concentration range (100–1200 nmol/100 μl)				
100	2.0	6.1	−0.8 (2.0)	0.3 (6.3)
200	1.8	6.7	−2.2 (1.8)	4.2 (4.5)
400	1.5	3.9	−2.8 (1.4)	0.2 (3.8)
600	0.8	4.1	−2.7 (0.8)	−2.2 (3.9)
800	1.8	2.7	−1.5 (1.8)	−0.6 (2.9)
1200	1.1	4.5	−1.5 (1.1)	2.5 (4.0)
(b) Plasma: low concentration range (10–160 nmol/100 μl)				
1	16.7	17.8	−11.7 (14.7)	5.0 (18.7)
5	11.6	16.8	5.0 (9.2)	1.0 (17.0)
10	14.1	7.9	0.2 (14.1)	14.3 (4.0)
20	5.0	13.5	−1.3 (4.2)	9.1 (14.3)
40	4.5	8.0	2.2 (4.4)	2.8 (14.3)
80	3.9	6.3	0.02 (3.9)	−1.7 (6.2)
120	4.0	4.8	0.7 (3.9)	−2.2 (4.7)
160	4.6	5.4	2.3 (4.7)	1.3 (5.5)
(c) Distilled water (substitute to CSF): concentration range 2–32 nmol/100 μl				
1	17.5	19.0	6.6 (18.6)	0.0 (19.0)
2	18.2	19.7	−6.7 (17.8)	1.2 (19.5)
4	6.3	15.4	−2.5 (6.1)	3.7 (16.0)
8	12.7	9.9	5.0 (13.4)	10.0 (10.9)
16	8.3	7.1	−3.1 (8.0)	3.3 (7.3)
32	8.1	11.5	−1.1 (8.0)	4.4 (12.0)

[a] %DMV, deviation of mean value from theoretical value (%).

respectively. The corresponding values for inter-day assay validation in plasma were 2.7–6.7 and 4.8–17.8%, respectively. For intra- and inter-day assay variation in CSF, the coefficients of variation of DFMO varied between 6.3 and 18.2%, and between 7.1 and 19.7%, respectively.

3.4.2. Accuracy

Good accuracy was observed from both the intra-day or inter-day assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (actual amount added). The intra-assay (within-day) and inter-assay (day-to-day) accuracy for DFMO assay at high (100–1200 nmol/100 μl) and low (10–160 nmol/100 μl) plasma concentration ranges and CSF (2–32 nmol/100 μl) are summarised in Table 1a–c. For intra-day assay accuracy in plasma, the mean deviation from the theoretical values varied between −0.8 and −2.8%, and between −11.7 and +5.0%, for the high and low concentration ranges, respectively. The corresponding values for inter-day assay validation in plasma were −2.2 to +4.2, and −2.2 to +14.3%, respectively. For intra- and inter-day assay accuracy for distilled water (used as substitute for CSF), the mean deviation from theoretical values varied between −6.7 and +6.6, and between 0 and +10.0%, respectively.

3.4.3. Recovery

The mean recoveries for DFMO in plasma at high (100, 600, 1200 nmol/100 μl) and low (10, 80, 160 nmol/100 μl) concentration range curves including the internal standard in all cases were greater than 95%. The results reflect essentially 100% recovery from the spiked plasma and indicate lack of interference from the sample preparation procedure.

3.4.4. Selectivity

Selectivity of the chromatographic separation was demonstrated by the absence of interferences from endogenous peaks in plasma and CSF (Figs. 3a, 4a and 5a,b).

3.4.5. Limit of quantification

The limits of quantification (LOQ) in human plasma and CSF for DFMO were accepted as 0.1 nmol using 100 μl plasma and CSF.

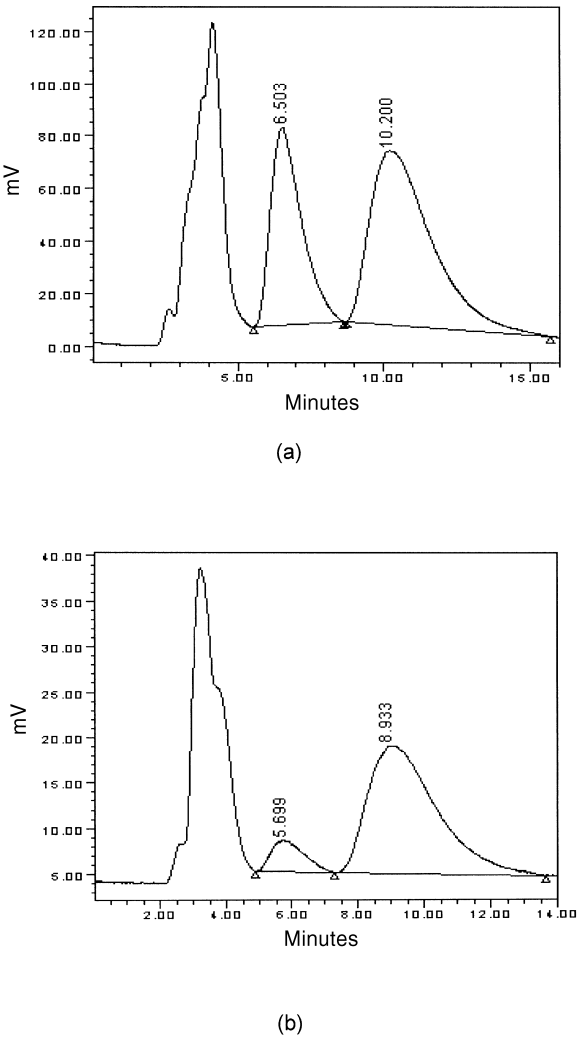


Fig. 5. Chromatogram of (a) plasma sample collected 3 h after the last dose of DFMO on day-15 (spiked with 250 nmol/100 μl internal standard), (b) CSF sample collected 5 min before the last dose of DFMO on day-15 (spiked with 50 nmol/100 μl internal standard).

3.4.6. Stability

Plasma samples containing DFMO at concentrations of 10, 100, 600 and 1200 nmol/100 μl were found to be stable when stored in a −80 °C freezer for a minimum of 6 months without significant decomposition of the drug. Freezing and thawing of the spiked samples did not appear to affect the quantification of the analytes (Table 2a). Mean deviation (%) of measured concentrations after stor-

Table 2
Storage stability data for DFMO in plasma at concentrations of 10, 100, 600 and 1200 nmol/100 μl

Time period (months)	Concentration (nmol/100 μl)	Concentration measured (nmol/100 μl)				
		Assay 1	Assay 2	Assay 3	Mean (SD)	% DEV[a]
(a) Long-term stability at 1, 2, 3 and 6 months						
1	10	11.1	12.1	9.7	11.0	9.67
	100	105.0	108.8	97.5	103.8	3.77
	600	615.0	589.1	591.5	598.5	−0.24
	1200	1212.0	1220.9	1189.7	1207.5	0.63
2	10	9.1	11.5	10.2	10.3	2.67
	100	98.7	102.5	97.3	99.5	−0.8
	600	612.3	580.4	580.1	591.0	−1.5
	1200	1176.0	1245.2	1240.0	1220.4	1.7
3	10	8.9	10.5	9.8	9.7	−2.67
	100	95.7	109.1	95.4	100.0	−0.07
	600	615.8	612.2	597.2	608.4	1.4
	1200	1189.0	1223.6	1205.0	1205.9	0.49
6	10	12.0	9.8	10.5	10.8	7.67
	100	95.4	92.4	107.8	98.5	−1.47
	600	623.1	589.2	598.2	603.5	0.58
	1200	1213.0	1245.0	1223.1	1227.0	2.25
(b) Freeze and thaw stability						
	10	10.5	11.4	9.5	10.5	4.67
	100	95.7	108.2	93.2	99.0	−0.97
	600	612.2	589.3	580.2	593.9	−1.02
	1200	1200.7	1212.5	1244.0	1219.1	1.59

[a] %DMV, deviation of mean value from theoretical value (%).

age at the observed periods (1, 2, 3 and 6 months) varied between −2.7 and +9.7%. Freezing and thawing for three successive cycles did not affect the measured concentrations. Mean deviation from the theoretical values varied between −1.0 and +4.7% (Table 2b).

3.5. Application of assay and analysis of specimens

To demonstrate the clinical applicability of the method, plasma and CSF concentrations levels of DFMO were determined in a patient following an oral dose administration of 500 mg/kg body weight (125 mg q.i.d.) for 14 days. Fig. 5a represents a chromatogram of a plasma sample collected 3 h after the last dose of DFMO on day-15, and Fig. 5b represents a chromatogram of a CSF sample collected 5 min before the last dose of DFMO on day-15. Plasma concentration–time profiles of DFMO are shown in Fig. 6. Plasma concentrations of DFMO at three time points (at diagnosis, 5 min before the second dose on day-10, and 5 min before the last dose on day-15) were 0, 36.1 and 28.3 nmol/ml, respectively; these concentrations were below the minimum level required to kill the parasite (50 nmol/ml) [14]. This patient however, had no relapse during the 24-month follow-up. Maximum plasma concentration of 731 nmol/ml was achieved 3 h after the last dose of DFMO on day-15.

4. Conclusions

We describe a HPLC assay procedure based on anion-exchange chromatography and post-column

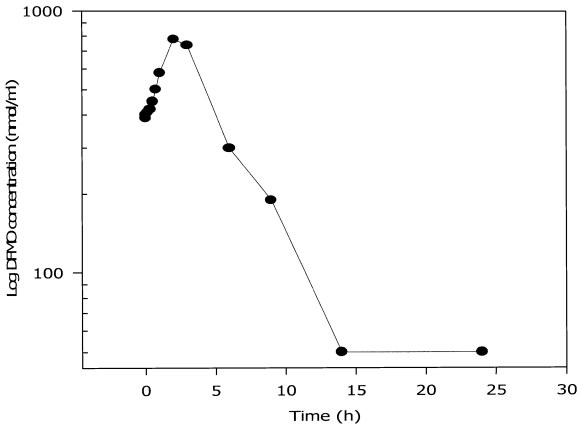


Fig. 6. Plasma concentration–time profile of DFMO in a patient following the last dose on day-15 (125 mg/kg body weight).

derivatisation with fluorescence detection, for the specific, sensitive, accurate and reproducible quantitative analysis of DFMO in human plasma and CSF samples. Total run time was within 15 min. The analytical method for the determination of DMFO in biological fluids established in this study meets the criteria for application to routine clinical drug level monitoring or pharmacokinetic study. The advantage of the method over previously reported ones are basically its rapidity, simplicity and high sensitivity. In addition, the sample preparation procedure is simple, faster and less expensive compared to the method that used the ultrafiltration technique.

Acknowledgements

This investigation received financial support from the UNDP/World/Bank/WHO Special Programme for Research and Training in Tropical Diseases. We thank Felix Kuzoe, PDT on African Trypnosomiasis Chemotherapy, WHO/TDR for his support during the study.

References

[1] C.J. Bacchi, B. Goldberg, J. Carofalo-Hannan, D. Rattendi, P. Lyte, N. Yarlett, Biochem. J. 309 (1995) 737.
[2] A.J. Bitonti, C.J. Bacchi, P.P. McCann, A. Sipertsma, Biochem. Pharmacol. 34 (1985) 1773.
[3] P.P. McCann, C.J. Bbacchi, A.B. Clarkson, Med. Biol. 59 (1981) 434.
[4] A. Sjoerdsma, Clin. Pharmacol. Ther. 30 (1981) 3.
[5] A. Sjoerdsma, P. Schechter, Clin. Pharmacol. Ther. 35 (1984) 287.
[6] P.P. McCann, A.E. Pegg, Pharmacol. Ther. 54 (1992) 195.
[7] J. Pepin, C. Guerin, F. Milord, P.J. Schecter, Lancet (1987) 1431.
[8] C.J. Racchi, H.C. Nathan, S.H. Hutner, P.P. McCann, A. Siperdsma, Science 210 (1980) 332.
[9] J. Grove, J.R. Fozard, P.S. Mamont, J. Chromatogr. 223 (1981) 409.
[10] P.C. Gunaratna, G.S. Wilson, M. Slavik, J. Pharm. Biomed. Anal. 12 (1994) 1249.
[11] J.L. Cohen, R.J. Ko, A.T.L. Lo, M.D. Shields, T.M. Gilman, J. Pharm. Sci. 78 (1989) 114.
[12] H.D. Huebert, J.J. Schwartz, K.D. Haegele, J. Chromatogr. 762 (1997) 293.
[13] M.L. Kilkenny, M. Slavik, C.M. Riley, J.F. Stobaugh, J. Pharm. Biomed. Anal. 17 (1998) 1205.
[14] F. Milford, L. Loko, L. Ethier, B. Mpia, J. Pepin, Trans. R. Soc. Trop. Med. Hyg. 87 (1993) 473.