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Determination of eflornithine enantiomers in plasma, by solid-phase extraction and liquid chromatography with evaporative light-scattering detection

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Abstract

A bioanalytical method for determination of eflornithine (DFMO) in 1000 μL human plasma has been developed and validated. DFMO and the internal standard (IS) were analysed by liquid chromatography with evaporative light-scattering detection (ELSD). Separation was performed on a Chirobiotic TAG (250 mm × 4.6 mm) column with ethanol (99.5%):0.01 mol/L acetic acid-triethylamine buffer at the rate of 25:75% (v/v) with flow rate of 1.0 mL/min. For D-DFMO in plasma the inter-assay precision was 6.5% at 75 μmol/L, 6.6% at 375 μmol/L and 5.8% at 750 μmol/L. For L-DFMO in plasma the inter-assay precision was 10.4% at 75 μmol/L, 6.5% at 375 μmol/L and 5.0% at 750 μmol/L. The lower limit of quantification (LLOQ) was determined to 25 μmol/L where the precision was 4.3% and 5.7%, respectively.

Keywords: Eflornithine; DFMO; 2-Fluoromethyl-DL-ornithine; Chiral chromatography; Chirobiotic TAG; Human African Trypanosomiasis; Evaporative light-scattering detection; ELSD; HPLC; Chirality

1. Introduction

Human African Trypanosomiasis (HAT), sleeping sickness, is a parasitic disease transmitted to man through the bite of the tsetse fly. In 1960, HAT was nearly extinguished but during the last 15 years the disease has re-emerged [1]. WHO estimates that 300,000–500,000 people are now infected and up to 60 million people in 36 countries are at risk of contracting the disease [2]. There are two forms of African sleeping sickness, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodiense* [3]. There are drugs to treat sleeping sickness, but serious side effects are a problem with all of them, and also resistance is increasing. Before the parasites pass the blood–brain barrier and enter the central nervous system (CNS), suramin and pentamidine are the drugs used against *rhodiense* and *gambiense*, respectively. The later stage is treated with the arsenical drug melarsoprol. For *gambiense*, eflornithine (DFMO) is also used [4]. DFMO was synthesized in the 1970s as a potential anticancer drug. It works by inhibiting ornithine decarboxylase (ODC), a key enzyme involved in polyamine biosynthesis, and the inhibition leads to impairment of cellular division [3]. DFMO is the only registered drug available when patients do not respond to melarsoprol and this drug is becoming less and less effective [1]. In some areas, the melarsoprol fails to cure up to 30% of the patients [5]. For successful treatment it is of major importance that the DFMO concentration in CSF is above 50 μmol/L, to attain consistent clearance of parasites [6]. In plasma, DFMO concentration is roughly 7.5 times CSF concentration [7]. DFMO is a chiral drug and is administered as a racemic mixture of enantiomers (D/L-DFMO). Enantiomers often differ in biological systems and may have different pharmacodynamic, pharmacokinetic and toxicological activities [8,9]. There is an indication that both L- and D-DFMO are potent and irreversible inhibitors of ODC, but that L-DFMO is a more effective inhibitor compared to the other enantiomer or the racemic mixture [10]. To investigate the behaviour of DFMO enantiomers, a chiral method of determination for DFMO enantiomers in biologi-

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cal matrices is of value. There are chromatographic methods described for determination of racemic DFMO concentrations in biological matrices such as human plasma, cerebrospinal fluid (CSF) and urine. All uses pre- or post-column derivatization with either dansyl chloride, *O*-phthaldialdehyde (OPA) or naphthalene-2,3-dicarboxaldehyde/cyanide (NDA/CN) and detection with fluorescence or ultraviolet absorption [11–16]. A chiral method for determination of DFMO enantiomers has been described, by derivatization, with liquid and gas chromatography, but not for determination of D-DFMO and L-DFMO in biological matrices [17]. DFMO lack chromophores or fluorophores and therefore, derivatization are necessary for detection using conventional spectroscopic methods [15]. This is also the situation when detecting underivatized amino acids and therefore, it is common practice to create amino acid derivatives with strong chromophore/fluorophore groups. However, derivatization is associated with a variety of problems, for instance derivative instability, reagent interferences, and increased void-volume for post-column derivatization assays [18]. Methods have been described for determination of different underivatized amino acids by evaporative light-scattering detection (ELSD) [18,19]. The calibration curve is approximately linear in a certain region, but below and above this region, sensitivity decreases [20]. Linear regression can be applied after logarithmic transformations of responses and concentrations [18,20,21]. Chirobiotic TAG is a macrocyclic antibiotic type of chiral stationary phase (CSP), based on native teicoplanin aglycone. Antibiotic CSPs can be used in a wider range of mobile phases (normal-, reversed- and organic-phase) and they show greater stability with higher capacities compared to protein-based stationary phases [22]. Chirobiotic TAG has been used for the enantiomeric separation of amino acids [23,24]. In the presented report, a direct chiral chromatographic method is described, for the determination of enantiomers of DFMO in human plasma. The major advantage of the described method is determination of DFMO enantiomers, and an additional advantage is to be able to do so without the need of derivatization. Regression analysis has been performed in order to select the optimal regression model for D-DFMO and L-DFMO in plasma. The method has been validated with respect to accuracy, precision, linearity, selectivity, stability and extraction recovery, according to published guidelines [25].

To the best of our knowledge there are no published methods for the chiral quantification of DFMO in human plasma.

2. Experimental

2.1. Chemicals

D/L-DFMO (2-fluoromethyl-DL-ornithine) was provided by UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR, Geneva, Switzerland). The internal standard (IS), DL-Norvaline was obtained from Sigma–Aldrich (St. Louis, MO, USA). The molecular structures are shown in Fig. 1. D-DFMO and L-DFMO were synthesized by Lipitek International Inc. (San Antonio, USA). Deionized water was prepared by a Milli-Q deionised water system (Millipore, Bedford, USA). Drug free human plasma was obtained from the Department of Blood Transfusion, Falun Central Hospital, Sweden.

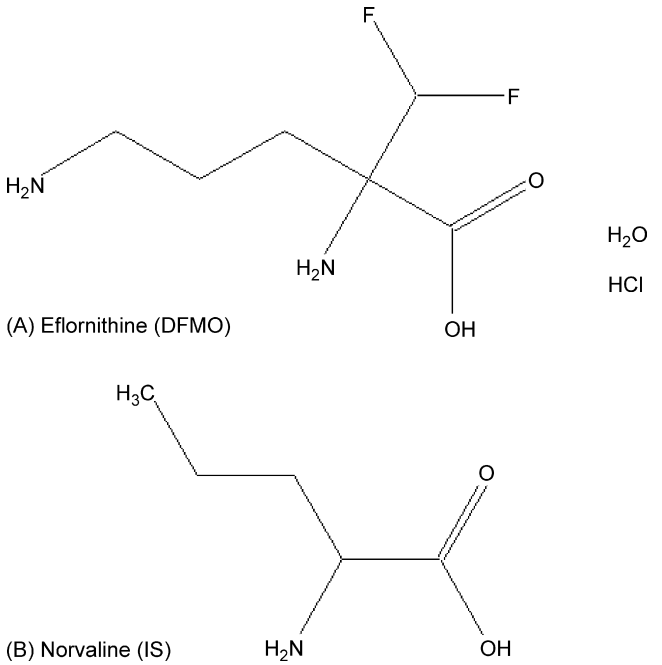

Fig. 1. Structures of (A) eflornithine (DFMO) and (B) the norvaline internal standard (IS).

2.2. Instrumentation and chromatographic conditions

Solid-phase extraction was performed using strong cation exchange (IST SCX-2) columns (100 mg, 1 mL, IST, Hengoed, Glamorgan, UK) on an automated SPE system, ASPEC XL 4 with Gilson 735 Sampler software v4.04 (Gilson, Middleton, WI, USA). The ASPEC uses a positive air pressure in order to push fluids through the column. The LC system consisted of a Degasys DG-1310 degasser (Uniflows, Tokyo, Japan), a WATERS 600E Multisolvent delivery system (Waters, Milford, MA, USA), a Gilson 234 autoinjector (Gilson, Middleton, WI, USA) and a WATERS 3420 Evaporative light scattering detector (Waters, Milford, MA, USA). The nebulizer was off (i.e., room temperature), the drift tube was at 50 °C and nitrogen gas pressure was 10 psi. A Parker Balston nitrogen generator N2-4000 (Parker Haniffin Coorperation, Haverhill, MA, USA) was used to deliver nitrogen gas to the detector. Data acquisition was performed using Data apex Clarity Chromatography station for windows v2.3.0.188 (DataApex Ltd., Prague, The Czech Republic). The mobile phase was ethanol (99.5%):0.01 mol/L acetic acid–triethyl amine buffer at the ratio of 25:75% (v/v) with flow rate 1.0 mL/min. The acetic acid-triethyl amine buffer solution was prepared by diluting 1000 μL concentrated acetic acid and 270 μL concentrated triethyl amine to 1 L with Milli-Q deionized water. The chromatographic separation was achieved on a Chirobiotic TAG (250 mm × 4.6 mm) column (ASTEC, New Jersey, USA). Statgraphics Plus for Windows 4.0 (Herndon, Virginia, USA) was used for statistical calculations.

Table 1
ASPEC SPE procedure for the extraction of DFMO in 1000 μL human plasma

SPE-step	Liquid dispensed	Dispensing volume (μL)	Dispensing flow rate (μL/min)	Pressuring air volume (μL)
Conditioning	Methanol	2000	1000	200
	Phosphoric acid $C = 0.05$ M	2000	1000	500
Sample loading		8000	500	800
Washing	Methanol:phosphoric acid $C = 0.05$ (75:25, v/v)	2000	1000	700
Elution	Methanol:triethylamine (90:10, v/v)	1000	500	0
Elution	Methanol:triethylamine (90:10, v/v)	1000	500	700

2.3. Preparation of calibration standards and quality control samples

Stock solutions of D/L-DFMO (50 mmol/L) and IS (500 mmol/L) were prepared in Milli-Q deionized water. D/L-DFMO stock solution was used to prepare D/L-DFMO working solutions (20.0, 5.0 and 2.0 mmol/L) in Milli-Q deionized water. All solutions were stored dark at +4 °C. D/L-DFMO working solutions were added to blank human plasma to obtain calibration standards of D-DFMO and L-DFMO ranging between 25 μmol/L and 1000 μmol/L; all together six different concentrations and a blank, and the QC (quality control) samples used for the determination of accuracy, precision and recovery (LQC: 75; MQC: 375 and HQC: 750 μmol/L). All spiked samples contained less than 4% working solution (v/v). Thousand of microliters human plasma was transferred into cryo tubes and stored at −86 °C until analysis.

2.4. Sample preparation

To 1000 μL human plasma, 60 μL of IS 500 mmol/L was added and the tubes were mixed for 10 s. Protein precipitation was performed by adding 500 μL trichloro acetic acid (20%, m/v) and the samples were mixed again for 10 s. Thereafter, the samples were placed at +4 °C for one hour for a more efficient protein precipitation [11]. Samples were centrifuged at 23,900 × g for 10 min. The liquid phase was transferred to polypropylene tubes, diluted with 6500 μL Milli-Q deionized water, and loaded onto SPE columns. The extraction procedure for the ASPEC XL is shown in Table 1. Elutes were evaporated at 70 °C under a stream of air and then reconstituted in 200 μL Milli-Q deionized water each. Hundred micro liters of the reconstituted sample was injected into the LC system.

2.5. Method development

2.5.1. Regression analysis

The selection of an inadequate calibration model can lead to an increase of the experimental error, which could significantly affect the results and conclusions of the study [26]. Regression analysis can be performed by evaluating effects of weighting or transformation of data in order to select optimal regression model [27,28]. The response function can be determined by applying different regression models and selecting the model with the most suitable percentage relative error to concentration plot (%RE plot) [29]. Regression analysis was performed, on human plasma samples, by calibration curves with six different concentrations ranging between 25 μmol/L and 1000 μmol/L over 5 days ($n = 5$), and quality control samples at three different concentrations (LQC, MQC and HQC) ($n = 15$). Data was evaluated for normality and an F-test was performed to examine whether data was homoscedastic or heteroscedastic. Models evaluated were linear regression (non-weighted, $1/x$ and $1/x^2$), log–log transformation with linear regression (non-weighted), quadratic regression (non-weighted, $1/x$ and $1/x^2$) and Box–Cox transformation of responses with linear regression (non-weighted). Selection of suitable model was based on percentage RE plots and accuracy of QC samples. This was performed for both D-DFMO and L-DFMO in human plasma.

2.6. Method validation

2.6.1. Enantioseparation

Enantioseparation of D-DFMO and L-DFMO was verified by injecting pure enantiomers in Milli-Q deionized water.

2.6.2. Accuracy, precision, lower limit of quantification (LLOQ)

The accuracy and precision of the method were estimated by analysis of 1000 μL human plasma at the three QC-levels in triplicate for 5 days. Concentrations were determined by calibration curves prepared on the day of analysis and the intra- and inter-precisions were calculated, as well as accuracy at each concentration level. The LLOQ was set as the concentration where the analyte response of a spiked sample was five times the response of a blank sample and where the analyte response had precision and accuracy within ±20%, respectively.

2.6.3. Linearity

Calibration graphs were constructed using six different concentrations ranging between 25 μmol/L and 1000 μmol/L. The ratio of DFMO enantiomer peak area and IS peak area at each concentration was plotted as a function of DFMO enantiomer concentration using the regression model determined optimal for the data. Deviations of back-calculated concentrations of calibration standards were evaluated.

2.6.4. Extraction recovery

Three concentrations of DFMO enantiomers (LQC, MQC, HQC) in triplicate were analysed for 5 days. DFMO enantiomer

peak area was compared to direct injections at the same concentrations as after reconstitution. IS extraction recovery was determined in the same manor. The direct injections were prepared in Milli-Q deionized water and stored at −86 °C until the day of analysis. Hundred microliters was injected into the LC system.

2.6.5. Selectivity

Blank human plasma from six healthy volunteers were analysed in order to investigate endogenous compounds that could interfere with quantification of D-DFMO and L-DFMO and the IS. A number of pharmaceuticals used for treatment of tropical diseases and side effects of DFMO treatment were analysed (i.e., albendazole, acetamoniphen, chloroquine, pyrimethamine, amodiaquine, loperamide, diazepam, amoxicillin, metronidazole, dextroproxyphene, fluphenazine, cetirizine, nifurtimox, melarsoprol, suramin, trimethoprim, sulfadoxine and sulfamethoxazole). Direct injections, ranging between 500 μmol/L and 5 mmol/L, were injected into the LC-system for evaluation of chromatographic interferences. Substances that interfered in chromatography were further evaluated using spiked samples at therapeutic concentrations. These samples were subject to the full sample preparation procedure, including solid-phase extraction, in order to evaluate if they were removed during sample preparation, and would therefore not interfere.

2.7. Stability

D/L-DFMO (50 mmol/L) and the IS (500 mmol/L) stock solutions were stored at +23 °C (room temperature) for 6 h in order to evaluate bench-top stability. The stock solutions were diluted to 10 mmol/L with Milli-Q deionized water prior to analysis. The mean value of $t = 6$ h was compared to the mean value of $t = 0$ h in order to quantitate differences, by calculating the statistic t. An F-test was run to assure there was no significant difference between standard deviations.

Stability of D-DFMO, L-DFMO and IS in the liquids used in the analytical procedure was also evaluated. Two concentrations of DFMO enantiomers (LQC and HQC) in 1000 μL human plasma were analysed. Samples were prepared, and kept at room temperature, for $t = 0$ and $t = 24$ h in each fluid (i.e., extraction, elution and reconstitution). The extraction recovery was determined and the mean value at $t = 24$ h for each fluid was compared to $t = 0$ h, using ANOVA to quantitate differences. Also, mean value at $t = 24$ h for each fluid was compared to $t = 0$ h of the quotients between D-DFMO and L-DFMO peak areas for each fluid to determine enantiomeric stability in the analytical procedure.

Short-term stability of DFMO enantiomers in human plasma was evaluated at two concentration levels (LQC and HQC). These samples were stored at room temperature for 24 h. Calibration graphs were constructed and concentrations determined. The mean value of $t = 24$ h was compared to $t = 0$ h in order to quantitate differences, by calculating the statistic t. Enantiomeric stability was also determined by comparing the D-DFMO/L-DFMO quotients of peak areas at $t = 24$ h to $t = 0$ h, by calculating the statistic t.

Freeze and thaw stability of D-DFMO and L-DFMO in human plasma was evaluated at low and high QC-level. Samples were stored at −86 °C and thawed at room temperature. All samples were analysed in triplicate after three freeze and thaw cycles. Stabilities were determined as described above for short-term stability.

Long-term stability of DFMO enantiomers in human plasma was evaluated at two concentration levels (LQC and HQC). These samples were stored at −86 °C, −17 °C, +4 °C and +23 °C (room temperature). Samples stored at −86 °C were regarded as reference samples. All samples were analysed in triplicate on day 1, 5, 15 and 30. Calibration graphs were constructed and concentrations determined. Concentrations for each temperature were compared using ANOVA to quantitate differences. Also, the limit of ±2 standard deviations (intra-assay) was used for determination of stability (i.e., added concentration ±2 standard deviations, determined during evaluation of precision). DFMO enantiomer area quotients were compared, using ANOVA, at each temperature.

Stability regarding effect of heat treatment was also evaluated at low and high QC-level in human plasma. Three samples at each concentration were stored at 50 °C for 3 h. All samples were then stored at −86 °C prior to analysis. The purpose of this was to evaluate whether DFMO enantiomers would be stable during viral deactivation, at 50 °C for 3 h, or not [30]. Samples were analysed in triplicate (i.e., heat treatment compared to no heat treatment). Calibration graphs were constructed and concentrations determined. Enantiomeric stability was determined as described above for short-term stability.

2.8. Biological application

D/L-DFMO was intravenously administered to a rat with a dosage of 375 mg/kg body weight (Sprague–Dawley rat, male, weighing approximately 300 g) [31]. The administered dose corresponds to a human dose of approximately 75 mg/kg. Recommended i.v. dosage is 100 mg/kg body weight. A venous blood sample was drawn 30 min after dosage. Plasma was analysed by the method described in this paper, at Dalarna University College, and concentration of D-DFMO and L-DFMO was determined. Prior to analysis, a partial validation was performed due to change of species as well as use of smaller sampling volume (300 μL compared to 1000 μL). Calibration standards and QC samples were prepared as described above, using human plasma. Sampling was performed at the Laboratory of Experimental Biomedicine, Gothenburg University.

3. Results and discussion

3.1. Method development

3.1.1. Regression analysis

Data was found to be normally distributed. Heteroscedasticity was confirmed. Percentage RE plots were constructed of all models and limits of ±20% and ±15% were set for the lowest calibration standard and the other calibration standards, respectively. Also, deviations of QC samples were used

Table 2
Accuracy, intra- and inter-assay precision, and extraction recovery for the determination of DFMO enantiomers in 1000 μL human plasma

$n = 15$, $k = 5$	Added (μmol/L)	Found, mean (μmol/L)	CV (%)	Accuracy (%)	Recovery (%)	SD (%)
D-DFMO in human plasma						
Intra-assay	75	66	5.8	−11.3	46.1	4.4
Inter-assay			6.5			
Intra-assay	375	387	3.9	3.2	56.2	5.5
Inter-assay			6.6			
Intra-assay	750	727	2.4	−3.1	61.9	2.5
Inter-assay			5.8			
L-DFMO in human plasma						
Intra-assay	75	68	8.5	−8.9	48.6	6.2
Inter-assay			10.4			
Intra-assay	375	385	2.5	2.7	54.2	4.6
Inter-assay			6.5			
Intra-assay	750	734	2.1	−2.1	61.2	2.9
Inter-assay			5.0			

for determination of the most suitable regression model. Percentage RE plots showed that linear regression, without and with weighting, were unsuitable for DFMO enantiomers, due to large deviations of LLOQ samples as well as other calibration standards. Non-weighted quadratic regression was not satisfactory due to deviations of LLOQ samples. Quadratic regressions with weighting appeared useful, as well as logarithmic transformation of responses and concentrations, and Box–Cox transformation of responses. When examining accuracy of LQC samples, quadratic regressions with weights as well as Box–Cox transformation, resulted in larger deviations compared to double logarithmic transformation followed by linear regression. Hence, log–log transformation and linear regression was found to be the most suitable regression model for both D-DFMO and L-DFMO in human plasma.

3.2. Method validation

3.2.1. Enantioseparation

Enantioseparation of D-DFMO and L-DFMO was verified by direct injections of each enantiomer. Resolution of enantiomers was 1.3 for the highest calibration standard.

3.2.2. Accuracy, precision, lower limit of quantification

Precision and accuracy is shown in Table 2 for D-DFMO and L-DFMO in human plasma. LLOQ was 25 μmol/L in human plasma where the precision was 4.3% and 5.7% and the deviation was −7.1% and −5.6% ($n = 5$) for D-DFMO and L-DFMO, respectively. In Table 3, precision and accuracy is shown for D-DFMO and L-DFMO determination without the use of IS. As can bee seen, the IS present improves method performance at the LQC concentration level.

3.2.3. Linearity

Linear calibration graphs (25–1000 μmol/L) were obtained for DFMO enantiomers with coefficients of determination $r^2 > 0.997 \pm 0.003$ (mean ± SD, $n = 5$) for both enantiomers in human plasma. Slopes were 1.566 ± 0.014 and 1.640 ± 0.065 for D-DFMO and L-DFMO, respectively, and intersects were 0.103 ± 0.056 and 0.093 ± 0.053 (mean ± SD, $n = 5$). Back-calculated concentrations of the calibration standards had deviations of no more than ±14%. For LLOQ see above (accuracy, precision, lower limit of quantification).

3.2.4. Extraction recovery

The extraction recovery of D-DFMO and L-DFMO in human plasma is found in Table 2. For IS the extraction recovery was only 11.9 ± 0.9% for the IS ($n = 3$). Norvaline was used, although poor extraction recovery, due to difficulties finding a suitable internal standard. In Table 3, accuracy and precision is found when determined without the use of IS. As can be seen, that the use of IS improve accuracy and precision at LQC concentration level, although no large differences were noticed for the other QC levels. Therefore, the IS was used for determinations of DFMO enantiomers, although poor extraction recovery.

3.2.5. Selectivity

No interfering peaks from endogenous compounds were noticed in the human plasma from the six healthy volun-

Table 3
Accuracy, intra- and inter-assay precision, for the determination of DFMO enantiomers in 1000 μL human plasma, without IS

$n = 15$, $k = 5$	Added (μmol/L)	Found, mean (μmol/L)	CV (%)	Accuracy (%)
D-DFMO in human plasma, without IS				
Intra-assay	75	65	6.0	−13.8
Inter-assay			6.2	
Intra-assay	375	385	6.6	2.5
Inter-assay			8.5	
Intra-assay	750	728	3.6	−2.9
Inter-assay			5.5	
L-DFMO in human plasma, without IS				
Intra-assay	75	67	8.8	−11.2
Inter-assay			13.9	
Intra-assay	375	383	5.7	2.1
Inter-assay			8.8	
Intra-assay	750	736	3.6	−1.8
Inter-assay			4.5	

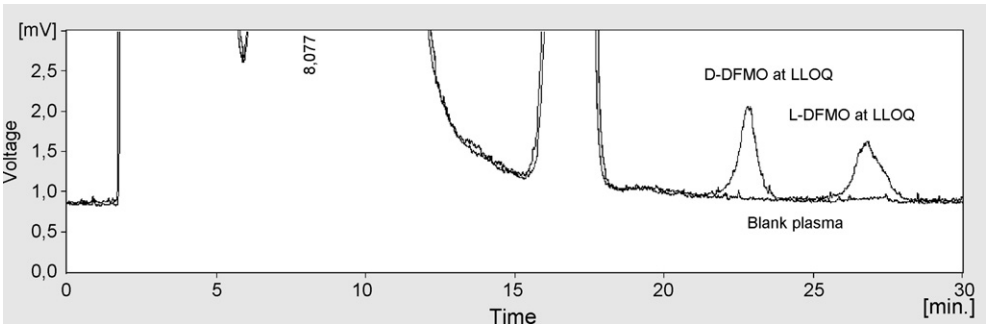

Fig. 2. Chromatogram of D-DFMO and L-DFMO at LLOQ in human plasma, compared to blank plasma sample.

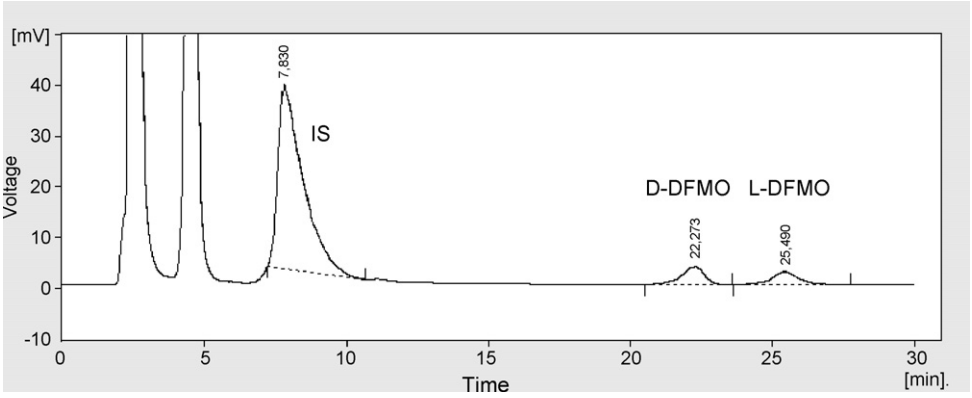

Fig. 3. Determination of D-DFMO and L-DFMO in male rat 30 min after i.v. administration of D/L-DFMO. D-DFMO was determined to 511 μmol/L and L-DFMO to 475 μmol/L.

teers. Fig. 2 shows a chromatogram of DFMO enantiomers in human plasma at LLOQ and a chromatogram of a blank plasma sample. When performing direct injections of solutions, chromatographic interferences were found for acetaminphen (1000 μmol/L injected), metronidazole (500 μmol/L and 5000 μmol/L), sulfadoxine (1000 μmol/L), sulfamethoxazole (1000 μmol/L) and suramine (4000 μmol/L). Spiked solutions of interfering substances were evaluated throughout the full sample preparation procedure, including solid-phase extraction. All but sulfadoxine were completely removed. Extraction recovery was 0.6% for sulfadoxine, but since the C_{max} is relatively high for this drug, there is still interference with respect to the second eluting DFMO enantiomer. Hence, the described method is not suitable for determination of L-DFMO, when patients received sulfadoxine treatment.

3.3. Stability

The D/L-DFMO stock solution and the IS stock solution were found to be stable at room temperature for at least 6 h. DFMO enantiomers were stable in all liquids used in the analytical procedure, short-term stability, freeze and thaw stability and during heat treatment. D-DFMO and L-DFMO were stable for at least 30 days at all tested temperatures. No significant decrease in mean values was found for the three tested temperatures (−17 °C, +4 °C, and +23 °C), compared to concentrations obtained the first day of analysis. Significant increase at 95% confidence level was found for high QC samples at day 5, compared to the other days, for all temperatures (including the reference temperature −86 °C) but this was not due to DFMO instability. Also, significant decrease was found for high QC samples stored at +23 °C, but this decrease was not noticed for low QC samples, and the decrease was within method precision. Stability was verified for DFMO enantiomers during heat treatment at 50 °C for 3 h. No indication of conversion from one enantiomer to another was noticed.

3.4. Biological application

D-DFMO was determined to 511 μmol/L and L-DFMO to 475 μmol/L. A chromatogram is shown in Fig. 3.

4. Conclusion

A bioanalytical method for the chiral determination of DFMO in human plasma, by solid-phase extraction and liquid chromatography with evaporative light scattering detection, has been developed and validated. The method has proven to be accurate, reproducible and selective, and without the need of derivatization. It has been shown that this method is sensitive enough and suitable for the chiral determination of D-DFMO and L-DFMO in human plasma for pharmacokinetic studies in humans.

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