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Separation and determination of cysteine enantiomers in plasma after derivatization with 4-fluoro-7-nitrobenzofurazan

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ABSTRACT

The importance of D-amino acids in mammals associated with enantio-dependent biological functions has been increasingly highlighted. In addition to naturally occurring, D-amino acid supplementation could have a positive biological impact, including cytoprotective implications. In this context, supplementation with D-cysteine has revealed beneficial effects. Quantification of cysteine enantiomers in rodent plasma has been achieved by using 4-fluoro-7-nitrobenzofurazan derivatization of the target analytes. Cystine, the main form of cysteine in the plasma, was initially reduced to cysteine using DL-dithiothreitol. Baseline enantioseparation was then achieved in less than 3 min using a (*R*,*R*)-Whelk-O 1 stationary phase and isocratic elution using CH_3OH-H_2O 90:10 (v/v) with 15 mM ammonium formate (apparent pH 6.0) at 0.5 mL/min. The derivatives were then detected using negative ESI-MS in SRM mode. An external calibration was employed for D-cysteine, while L-cysteine quantification, as an endogenous analyte, was addressed using a background subtraction strategy. The method was validated. Response functions were obtained from 0 to 300 µM and from 0 to 125 µM for D-cysteine and L-cysteine, respectively. The trueness ranged from 96% to 105% for both enantiomers with repeatability and intermediate precision lower than 8% and 15% for the D-form and the endogenous L-form, respectively. The method was successfully applied for determining D- and L-cysteine in mouse plasma after D-cysteine administration.

1. Introduction

The comprehensive analysis of metabolites within a biological sample requires the resolution of compounds that show a significant diversity of physicochemical properties and a broad range of concentrations. In addition, for a number of compounds, enantiomers result in different biological activities, and some have been reported to be physiologically active or to serve as biomarkers [1]. Most of the research related to stereoselectivity has involved xenobiotics, such as active pharmaceutical ingredients (APIs), pesticides or other compounds, whose enantiomers may behave quite differently because of different interactions within the biological systems. Thus, enantioresolution has substantial interest for numerous applications, including such recent fields as metabolomic applications [2,3]. For a long time, only L-enantiomers of amino acids (AAs) were described as naturally occurring. Such molecules are central components of life processes, acting as the building blocks of proteins and precursors of hormones and neurotransmitters. However, D-enantiomers have been increasingly reported to have specific biological functions in mammals compared to their corresponding L-forms. Few studies have been published that describe the metabolism of D-AAs. For example, D-serine has been found to be a co-agonist of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors with glycine in the brain [4], and D-aspartate has been shown to have hormonal regulatory functions in endocrine tissues [5]. D-aspartate and D-alanine were respectively downregulated in white matter and upregulated in gray matter of Alzheimer diseased brains compared to healthy ones [6]. More recently, altered D-AA concentrations have been reported in patients with cancer [7]. D-alanine was found at higher concentrations in the gastric juice of patients with both

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carcinomas and *Helicobacter pylori* infections [8]. Simultaneous determination of D- and L-AAs in human serum of patients with hepatocellular carcinoma evidenced lower levels of D-glutamate and D-glutamine [9]. Furthermore, it has been shown that in some cases, D-AA supplementation could have an inhibitory effect on tumor cell proliferation [10,11].

Cysteine is a sulfur-containing AA synthesized via methionine transsulfuration or released through protein degradation. It is a precursor of glutathione, implicated in the regulation of intracellular concentrations of reactive oxygen species and redox status. On the other hand, D-AA oxidase is a flavoenzyme with the ability to degrade D-AAs through deamidation in mammals [12]. The metabolization of D-cysteine to 3-mercaptopyruvate with D-AA oxidase is followed by the action of 3-mercaptopyruvate sulfur transferase with the production of hydrogen sulfide (H_2S). The latter is a signaling molecule that modulates synaptic activity, regulates the release of insulin and contributes to the relaxation of vascular smooth muscles through the activity of K^+ channels [13–15]. In some cases, a cytoprotective effect has been evidenced [16]. Shibuya et al. demonstrated that the administration of D-cysteine (and thus an increased level of H_2S) has a protective effect against oxidative stress in primary cultures of cerebellar neurons and reduces reperfusion injury in the kidney [17]. Souza et al. observed that increased levels of H_2S through D-cysteine supplementation have a strong protective effect on induced gastric damage in mice [18]. Lately, endogenous D-cysteine was detected in the mammalian brain along with inhibitory function of cultured mouse embryonic neural progenitor cell proliferation [19]. Serine racemase was identified as biosynthetic enzyme for D-cysteine racemization.

From an analytical point of view, the resolution of AA enantiomers in biological samples has to address the challenge of resolving enantiomers at very different concentrations, with the L-forms being physiologically much more concentrated than the D-forms. In addition, AAs are rather hydrophilic, and since most of them do not bear any chromophore moiety, derivatization is commonly employed to tune their chromatographic behavior and/or to enable their optical detection. Two main strategies have been developed: (i) indirect methods that employ chiral derivatizing reagents for the generation of diastereoisomers that might be resolvable on achiral columns, and (ii) direct methods that employ chiral stationary phases (CSPs) [20].

However, the analysis and determination of cysteine enantiomers includes additional challenges due to the high reactivity of the sulfhydryl group, which is readily oxidable, causing condensation of two cysteine molecules into one single cystine. Thus, workflows generally include the use of a reducing reagent immediately followed by an alkylation step to steady the reduced analytes. In a previous work, cysteine enantiomers were derivatized with the AccQ® Tag Ultra Derivatization Kit (Waters Corporation, Milford, USA), and separated on a zwitterionic CSP derived from quinine (Chiralpak® ZWIX (+)) [30]. Although the strategy was fully validated and successfully applied to biological samples, the stability of the derivatives was limited to 6 h after the reaction before the oxidation product cystine was detected again, thus restricting the number of samples to be processed in a single batch. In order to improve the cysteine derivative stability, 4-fluoro-7-nitrobenzofurazan (NBD-F) was employed [31]. Using this approach, NBD-cysteine derivatives were found to be stable for 6 days at room temperature and after 3 freeze-thaw cycles. The NBD derivatives were resolved with a Pirkle-type CSP, (*R*,*R*)-Whelk-O 1, under reversed-phase conditions. The method was validated and applied to the quality control of L-cysteine-containing dietary supplements. Herein, we describe the development and application of such a strategy to biological samples.

Two distinct quantification strategies have been developed since the L- and D-forms are endogenous and exogenous compounds, respectively. The simultaneous quantification of molecules of both origins in the same matrix is rather challenging from a bioanalytical point of view, as it requires the simultaneous application of two methodological approaches for absolute quantification: while blank matrix can be obtained for exogenous compounds, the natural presence of the endogenous ones has to be taken into account. Thus, a background subtraction strategy was chosen for the quantification of L-cysteine. The method was then validated [23–25] and applied to the quantification of cysteine enantiomers in mouse plasma after supplementation in D-cysteine.

2. Experimental section

2.1. Chemicals

L-cysteine, D-cysteine, 4-fluoro-7-nitrobenzofurazan (NBD-F), DL-dithiothreitol (DTT), ammonium carbonate, ammonium formate and boric acid were obtained from Sigma Aldrich (Buchs, Switzerland), DL-cysteine (3,3-d_2, 98%) was purchased from Cambridge Isotope Laboratories (Tewksbury, United States), sodium hydroxide was purchased from VWR Chemicals (Dietikon, Switzerland), and sodium tetraborate decahydrate and LC-MS grade methanol and water were purchased from Fisher Scientific (Reinach, Switzerland).

Six anonymized individual human plasma samples from healthy volunteers were provided by Geneva University Hospital with written consent from the donors.

2.2. LC and MS conditions

Analyses were performed with an 1290 Infinity ultra-high performance liquid chromatograph (UHPLC) from Agilent (Santa Clara, CA, USA) equipped with a binary pump. Separations were performed with isocratic elution on a (*R*,*R*)-Whelk-O 1 column (100 × 2.1 mm ID, 1.8 µm, 100 Å pore size) from Regis Technologies (Morton Grove, IL, USA) with a 90:10 mixture of CH_3OH-H_2O (v/v) containing 15 mM ammonium formate (apparent pH fixed at 6.0) as the mobile phase. The flow rate was set to 0.5 mL/min, the column compartment was thermostated at 40 °C, and the injection volume was set to 20 µL. The UHPLC system was hyphenated to an Agilent 6490 triple quadrupole MS from Agilent Technologies equipped with an Agilent Jet Stream (AJS) ESI source. The MS was operated in SRM mode with polarity switching electrospray ionization. The precursor ions, product ions and optimized collision energies are indicated in Table S1 (supporting information). The following source parameters were used: the drying gas temperature and flow were set to 220 °C and 11 L/min, respectively. The nebulizing gas was set to 35 psi. The sheath gas temperature and flow were 250 °C and 11 L/min, respectively. The capillary voltage and nozzle voltage were set to 3000 V and 1500 V, respectively. The high-pressure ion funnel voltage was 150 V, and the low-pressure funnel voltage was 60 V. The cell accelerator voltage was set to 5 V. Instrument control, data acquisition and data treatment were performed with Agilent MassHunter software version B.08.00.

The HRMS experiments were carried out using a Waters Acquity UPLC H-class system (Milford, USA) hyphenated to a Waters Vion quadrupole-time of flight (Q-ToF) mass spectrometer (Wilmslow, UK). The chromatography system was equipped with a quaternary solvent manager, an autosampler, and a column manager composed of a pre-column eluent heater and a column oven set at 40 °C. A (*R*,*R*)-Whelk-O 1 column (100 × 2.1 mm ID, 1.8 µm, 100 Å pore size, Regis Technologies) was employed in the separation, which proceeded during 10 min in isocratic mode using a 90:10 mixture of CH_3OH-H_2O (v/v) containing 15 mM ammonium formate (apparent pH fixed at 6.0). The flow rate was 0.5 mL/min and the injection volume was 10 µL. Full scan and MS/MS experiments were used for

mass spectra acquisition in a negative electrospray mode with the source temperature at 120 °C, capillary voltage 2.5 kV, and cone voltage 20 V (offset 80 V). Nitrogen was used as a desolvation gas at 600 L/h and 300 °C and as a cone gas at 50 L/h. The acquisition mass range was *m/z* 50 – 500, scan time 0.5 s, low energy 6 eV for full scan mode. MS/MS data were collected using collision energy ramping from 10 to 50 eV at scan time 0.5 s and quadrupole setting at *m/z* 446.02 in low resolution mode (isolation width 4 Da). Internal calibration was carried out by infusion of 50 ng/mL solution of leucine-enkephalin at 20 µL/min via lock mass sprayer every 0.5 min, external calibration was conducted prior to measurements with Major Mix IMS/ToF Calibration Kit from Waters (Milford, MA, USA).

The apparent pH (pH*) was adjusted in the hydro-organic mobile phase with appropriate acid addition using a conventional pH-meter previously calibrated in aqueous buffers.

2.3. Calibration solutions and quality control samples

L-cysteine is a naturally present in plasma. Thus, a background subtraction strategy had to be used for its quantitation, whereas an external calibration was employed in the case of the D-form. For both enantiomers, six individual plasma samples were pooled, aliquoted for storage at −80 °C and extemporaneously unfrozen to prepare the calibrators. L- and D-cysteine calibration solutions were prepared at three concentration levels (k = 3) starting from non-doped pooled plasma (25 µL per solution) and following the standard addition method for the L-form.

For D-cysteine, the three calibrators were set as (1) non-doped pooled plasma, (2) pooled plasma doped with a final concentration of 150 µM D-cysteine, and (3) pooled plasma doped with a final concentration of 300 µM D-cysteine. For L-cysteine, the three calibrators were set as (1) non-doped pooled plasma, (2) pooled plasma doped with 75 µM L-cysteine, and (3) pooled plasma doped with 125 µM L-cysteine.

Three quality control (QC) samples were chosen for each cysteine enantiomer, corresponding to pooled plasma (25 µL per sample) doped with final concentrations of 50, 100 and 225 µM for D-cysteine and to pooled plasma doped with added concentrations of 25, 50 and 100 µM for L-cysteine. Then, 75 µM DL-cysteine-3,3-d_2 was added as an internal standard in all the calibration solutions and QC samples. Each sample was treated with 2 volumes of cold CH_3CN for protein precipitation. The samples were mixed with a Thermomixer (Vaudaux-Eppendorf AG, Schönenbuch, Switzerland) for 15 min at 1200 rpm and 4 °C and then centrifuged for 10 min at 4 °C and 15,000 G. The supernatants were collected and treated with DTT (as described in Section 2.4) and then derivatized with NBD-F (as described in Section 2.5).

2.4. Reduction of cystine with DL-dithiothreitol

After protein precipitation, the supernatants were collected and 3 volumes were diluted with 7 volumes of 0.2 M aqueous $(NH_4)_2CO_3$ before a reduction step with DTT at a final concentration of 2.5 mM. The reduction was performed by mixing the samples for 40 min at 300 rpm and 50 °C. The samples were then cooled at room temperature before derivatization.

2.5. Derivatization with 4-fluoro-7-nitrobenzofurazan

For derivatization with NBD-F, 1 vol of the reduced samples was diluted with 2 volumes of borate buffer (100 mM, pH 9.0) and 1 vol of NBD-F in CH_3CN at a final concentration of 0.2 mM. The samples were incubated for 2 min at 40 °C and then cooled in ice-cold water. The reaction with NBD-F was eventually stopped with 0.5 vol of 2% trifluoroacetic acid in H_2O (v/v) before injection.

2.6. Method validation

The selectivity was assessed by monitoring the respective retention times of both NBD-cysteine derivative enantiomers in plasma with LC/Q-ToF HRMS (see Section 2.2).

For both enantiomers, trueness and precision (i.e., repeatability (S_r^2) and intermediate precision ($S_{I.P.}^2$)) were evaluated with three determinations (n = 3) of the three QC samples (k = 3) prepared as described in Section 2.3. The matrix effect (ME) was assessed by building calibration curves in triplicate (n = 3) as described in Section 2.3 using either pooled plasma or H_2O and computed with ME = [1-(mean slope in matrix/mean slope in water)] × 100, for three separate days (j = 3).

The limit of quantification (LOQ) was examined as the minimum concentration that could be quantified with acceptable precision, thus corresponding to the lowest tested amount of both cysteine enantiomers during method validation. The limit of detection (LOD) was estimated for a signal-to-noise ratio (S/N) value of 3 from the S/N value of the lowest concentration of the corresponding cysteine enantiomer in the calibration solutions. Carryover was assessed by injecting a blank sample after the most concentrated QC and during all acquisition sequence. In all cases, negligible carryover effect was found.

2.7. Biological samples

The validated method was applied to the quantification of cysteine enantiomer derivatives in mouse plasma after the animals were administered D-cysteine. 11-weeks-old athymic female Nude-*Foxn1nu* mice (Envigo, Huntingdon, UK) (n = 40) were anaesthetized with isoflurane. Malignant pleural mesothelioma (model 1) or lung carcinoma human cells (model 2) were injected subcutaneously (1 million tumor cells suspended in 200 µL of HyStem-C Cell culture matrix, Sigma Aldrich) on the right and the left dorsa using a 22-gauge needle. 500 mg/kg/day of D-cysteine or vehicle (CT) were administered via gavage (OR) for 10 days. Afterward, the mice were euthanized and their blood was collected. The plasma was extracted and stored at −80 °C until further processing. All the experiments involving animals were revised and approved by the Ethical Committee for Animal Experimentation of the Canton of Geneva (file GE-61–20; GE-142–20). To determine the cysteine enantiomers, 75 µM DL-cysteine (3,3-d_2, 98%) was added as an internal standard in all the samples (25 µL of plasma per sample). Each sample was treated with 2:1 cold CH_3CN for protein precipitation (as described in Section 2.3) and then with DTT (as described in Section 2.4) and derivatized with NBD-F (as described in Section 2.5).

3. Results and discussion

Cysteine is a sulfur-containing AA whose metabolism is responsible for the generation of essential low molecular weight compounds implicated in redox homeostasis. In some cases, the degradation of cysteine has been evidenced with stereoselective pathways [17]. The sulfhydryl function of cysteine is also highly reactive. Cystine is generated through the formation of a disulfide bridge between the sulfhydryl groups of two cysteine molecules. Thus, in most cases complex or biological samples are submitted to a reduction step to convert all cystine into cysteine and to prevent it from downstream reoxidation. An additional derivatization step might be employed for added stability and sensitivity of detection [26]. One-pot derivatization of cysteine enantiomers with NBD-F was previously applied to the quality control of L-cysteine-containing dietary supplements [22]. The use of a fluorogenic reagent has been repeatedly described for highly sensitive detection of amino acids, peptides or proteins, since NBD-F reacts with both amino and sulfhydryl groups (Fig. 1) [27,28]. In the case of cysteine,

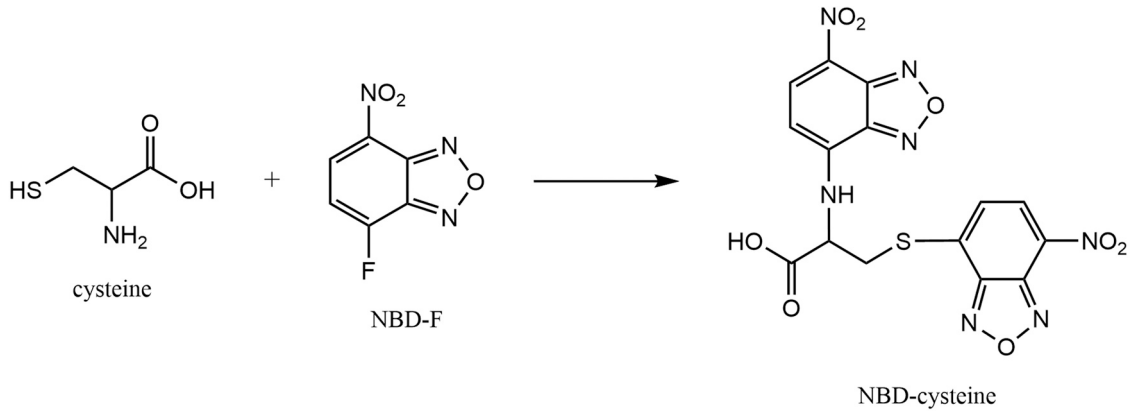

Fig. 1. Derivatization reaction of cysteine with 4-fluoro-7-nitrobenzofurazan (NBD-F).

the reaction with sulfhydryl groups renders them unavailable for oxidation. Moreover, the benefits of this reagent include a fast reaction under mild conditions, a high stability of the generated derivatives and a suitable retention with reversed-phase chromatography [20,29]. In the previous work, no reduction step was performed before derivatization. However, capsule dietary supplements were only composed of L-cysteine, cellulose, silica, and magnesium stearate. In the biological samples, the redox status of cysteine is expected to be altered by the local environment [30]. To the best of our knowledge, to date no method allows for obtaining a representative and stable snapshot of the cysteine/cystine ratio for a further reliable analysis of both forms. Thus, an additional reduction step with DL-dithiothreitol (as described in Section 2.5) was performed to displace the redox status of the cysteine/cystine pool toward the reduced form before enantiomeric quantification in plasma. Therefore, quantitative results were obtained as the total resulting L- or D-cysteine enantiomeric values.

The enantioselective analysis of cysteine derivative was performed with the very well-known Pirkle-type (*R*,*R*)-Whelk-O 1 CSP. This CSP operates according to a π-donor-acceptor mechanism, in which π-π-stacking interactions are the dominating forces accompanied by other interactions including H-bonds, steric and dipole-dipole. Enantioseparation is obtained when at least one of these interactions occurs stereoselectively. Whelk-O 1 CSPs exist as both enantiomeric forms (CSPs 1 and 2), allowing for a reversal of the elution order, with this feature being particularly useful for enantioselective analyses in complex matrices [31]. (*R*,*R*)-Whelk-O 1 is based on 1-(3,5-dinitrobenzamido)−1,2,3,4-tetrahydrophenanthrene as the chiral selector. Therefore, *π-π*-stacking interactions can occur between the tetrahydrophenanthrene region and the benzofurazan group of the NBD-cys derivatives. Moreover, the free carboxylic group of the derivatives can be active in H-bonding and dipole-dipole stacking with the spare groups of the chiral selector. The mobile phase was a 90:10 mixture of CH_3OH-H_2O (v/v) containing 15 mM ammonium formate (pH* 6.0), as it was demonstrated to afford favorable thermodynamic and kinetic properties (i.e., number of theoretical plates and resolution) [22]. With regard to the previous study, herein the dimensions of the column were reduced to the following: 100 × 2.1 mm ID, 1.8 µm, 100 Å pore size. Thus, analysis time and solvent consumption dropped while ensuring similar retention factor (k) of 3.1 and 4.0 for D-cysteine and L-cysteine, respectively. Under these conditions, the baseline resolution of cysteine enantiomers was obtained in less than 2.5 min with the D-form eluting before the L-form (Fig. 2).

Bi-derivatized NBD-cysteine derivative identity was confirmed by LC/Q-ToF HRMS measurements of derivatized D/L-cysteine standards in (−)-ESI mode, where deprotonated molecular ion ($[M-H]^-$, *m/z* 446.01578; $C_{15}H_8N_7O_8S$, -0.61 ppm) and *in-source* fragment ($[M-C_9H_6N_4O_5]^-$, *m/z* 195.98152; $C_6H_2N_3O_3S$, −0.85 ppm) of both enantiomers were detected in full scan mode at 2.06 (D-form) and 2.42 min (L-form) respectively (Fig. S1, supporting information). Fragmentation spectra collected at these retention times with precursor setting of 446.02 Da in collision-induced dissociation (CID) mode under collision energy ramp of 10–50 eV demonstrated complete absence of the parent ion and two main fragments (Fig. S2, supporting information). First fragment represented NBD-thiolate ion, identical to the *in-source* fragment observed in full scan mode (*m/z* 195.98152). Its formation can be rationalized as a consequence of ß-lactamization, where thiolate is eliminated after an attack of negatively charged COO^- on carbon atom neighboring sulphur (Fig. 3a). Identity of the second ion at *m/z 149.98934* was deduced from a calculated molecular formula ($[M-C_9H_6N_4O_5-^{\bullet}NO_2]^{\bullet-}$; $C_6H_2N_2OS$, 0.1 ppm) as radical detachment of NO_2 group from NBD-thiolate, a fragmentation commonly observed for nitroaromatic compounds (Fig. 3b) [32].

Since no D-cysteine could be natively detected in this biological matrix, the D-form could be considered an exogenous compound. In this case, the development of an absolute quantification strategy using LC-MS/MS generally requires establishing a standard calibration curve undergoing the same matrix effect and extraction efficiency as the compound of interest. This usually means that standard curves have to be built in the same matrix as the targeted analyte.

On the other hand, the naturally occurring L-form is endogenous and needs to be estimated with another methodological approach, since no blank matrix is available. For the quantitative analysis of endogenous compounds, several strategies have been proposed, including standard addition, background subtraction, a surrogate matrix and surrogate analyte methods [33]. In the case of L-cysteine quantification using NBD-F derivatization, the background subtraction strategy was employed. In detail, a pooled matrix of volunteer plasma (n = 6) was used to build the calibration for both cysteine enantiomers. For L-cysteine, the endogenous signal (y_0) was subtracted from the measurement of each concentration level (y; k = 3). Thus, the calibration curve represented $y - y_0$ versus the added standard concentration. This approach was selected since volunteer plasma was available to a very large extent, thus ensuring the use of a single batch of pooled biofluids throughout all experiments.

To highlight the value of using a similar matrix as biological samples for calibration, an evaluation of matrix effects (ME) was performed. Determining ME with a chromatographic analysis coupled to MS is a substantial part of determining the reliability of experimental results. This might be achieved using various strategies, including linear regression, a comparison of the slope values of calibration curves built with standards in neat solvent or matrix-matched, post-column infusion or post-extraction addition. In this case, because no blank or stripped matrix was available for

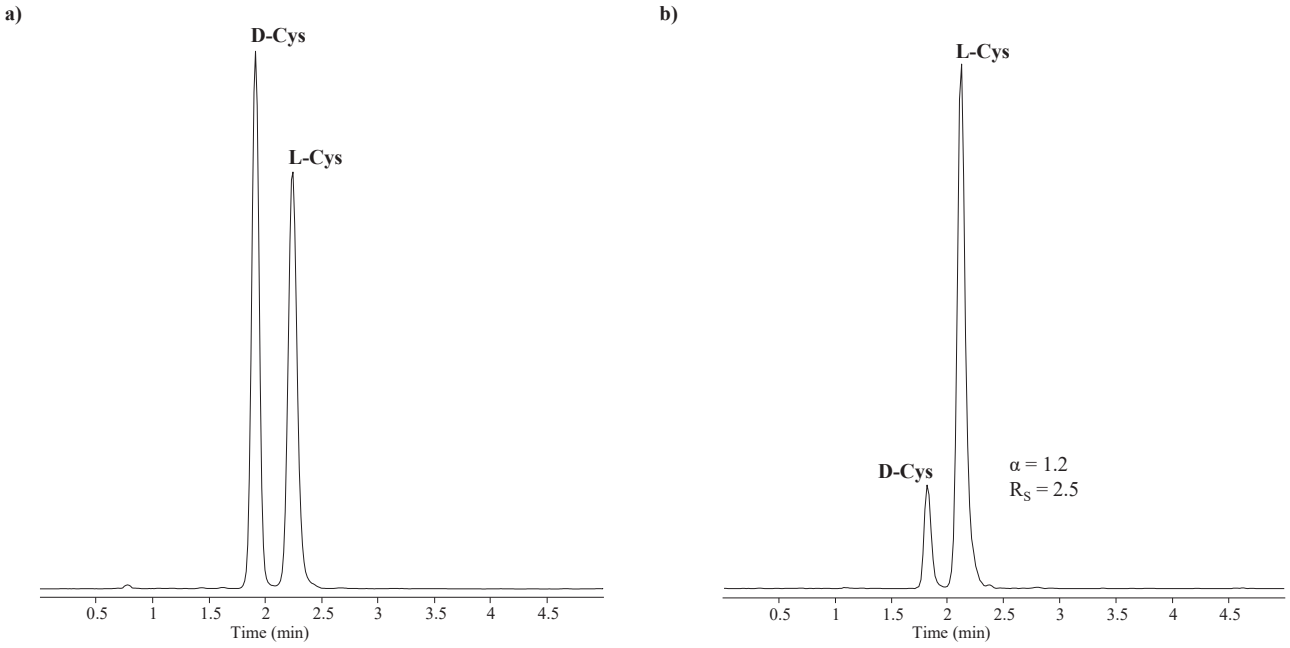

Fig. 2. SRM chromatogram of NBD-cysteine enantiomers a) in human plasma b) in mouse plasma obtained with triple-quadrupole mass spectrometry operated in negative ESI mode. Experimental conditions: (*R,R*)-Whelk-O 1 column (250 × 2.1 mm ID, 1.8 µm, 100 Å pore size) with a 90:10 mixture of CH_3OH-H_2O (v/v) containing 15 mM ammonium formate (pH* 6.0) as the mobile phase. The flow rate was set to 0.5 mL/min, and the column compartment was thermostated at 40 °C. The injection volume was set to 20 µL. The *m/z* 446.0 -> 196.0 channel is shown, α refers to the separation factor, and R_S is the resolution factor.

L-cysteine, the standard addition approach was used as described before and compared to the solvent calibration. The same concentration levels (k = 3) were used to build the calibration curves of both cysteine enantiomers in water. The calibration curves were built in triplicate (n = 3), and the matrix effect was evaluated as ME = [1-(mean slope in matrix/mean slope in water)] × 100. The resulting ME was 56% for L-cysteine and 70% for D-cysteine. To correct for the ME, a deuterated internal standard (IS), L-cysteine-3,3-d_2, was used. The IS was added at a fixed concentration in all the calibrators. For each concentration level of the calibration curves, the standard signal (y) was divided by the IS signal (y_{IS}). Thus, the corrected calibration curves were built with y/y_{IS} versus the added concentration. After a correction with IS, the ME for L-cysteine was − 7% and − 0.9% for D-cysteine. ME values of approximately ± 20% were considered close to the repeatability values [34].

3.1. Method validation

The developed method for cysteine enantiomer quantification was validated [23–25]. Selectivity, response function, trueness, precision (repeatability and intermediate precision), LOD and LOQ were evaluated. For both enantiomers, the selectivity was assessed for any potential interferences by monitoring the corresponding retention time of NBD-cysteine derivative enantiomers in plasma using LC/Q-ToF HRMS measurements. As a result, the presence of two major coeluting compounds was evidenced with *m/z* 360.9981 for the D-form (2.0 min) and *m/z* 342.0231 for the L-form (2.4 min). Thus, the latter were considered not detrimental for quantitative analysis since both do not overlap with quadrupole isolation window for Qual/Quan. transitions.

3.1.1. D-cysteine

A three-point calibration was performed using pooled plasma as a representative matrix (as described in Section 2.3). Three QC level samples were selected as described in Section 2.3. The calibration solutions and the QC samples were prepared in triplicate (n = 3) for three consecutive days (j = 3), allowing for three independent determinations of the QC values for each day. A linear calibration curve between the peak area corrected with the internal standard (D-cysteine/D-cysteine-3,3-d_2) versus the concentration was built. The results are listed in Table 1. The trueness was considered the mean value of the recovery for the three concentrations. For D-cysteine, the recovery values were between 101% and 103%. The precision was expressed as coefficients of variation (CV) for both intraday variability (repeatability: CV_W) and interday variability (intermediate precision: CV_{IP}). CV_W and CV_{IP} were lower than 8% for all investigated concentrations (Table 1). The accuracy profile was established thanks to a β-expectation tolerance interval (ß-ETI) with a probability of 80%. The acceptability limits were set at ± 30% (Fig. 4a). The accuracy profile representing the total analytical error (TAE) was comprised into the acceptability limits, meaning that D-cysteine can be quantified over the tested concentration range (50, 100 and 225 µM). Moreover, the LOQ, defined as the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy, was confirmed as the lowest tested concentration in this case (50 µM). The LOD was estimated from the S/N value of the lowest calibration point with the addition of D-cysteine (i.e., 150 µM). The corresponding concentration of 0.25 µM on average was estimated for an S/N value of 3. Finally, the use of β-ETI is an easy and straightforward method for estimating the method uncertainty (MU). This can be easily used by analysts without any extra experimental effort [35]. The uncertainty interval illustrated here is assumed to contain 95% of all possible future measurements, regardless of the concentration. The uncertainty was approximately 7% for 50 and 100 µM, and below 12% for 225 µM (Fig. 5a).

3.1.2. L-cysteine

Similar to D-cysteine, a three-point calibration was employed for measuring L-cysteine as described in Section 2.3. To evaluate the trueness and precision, three concentration levels (k = 3) were

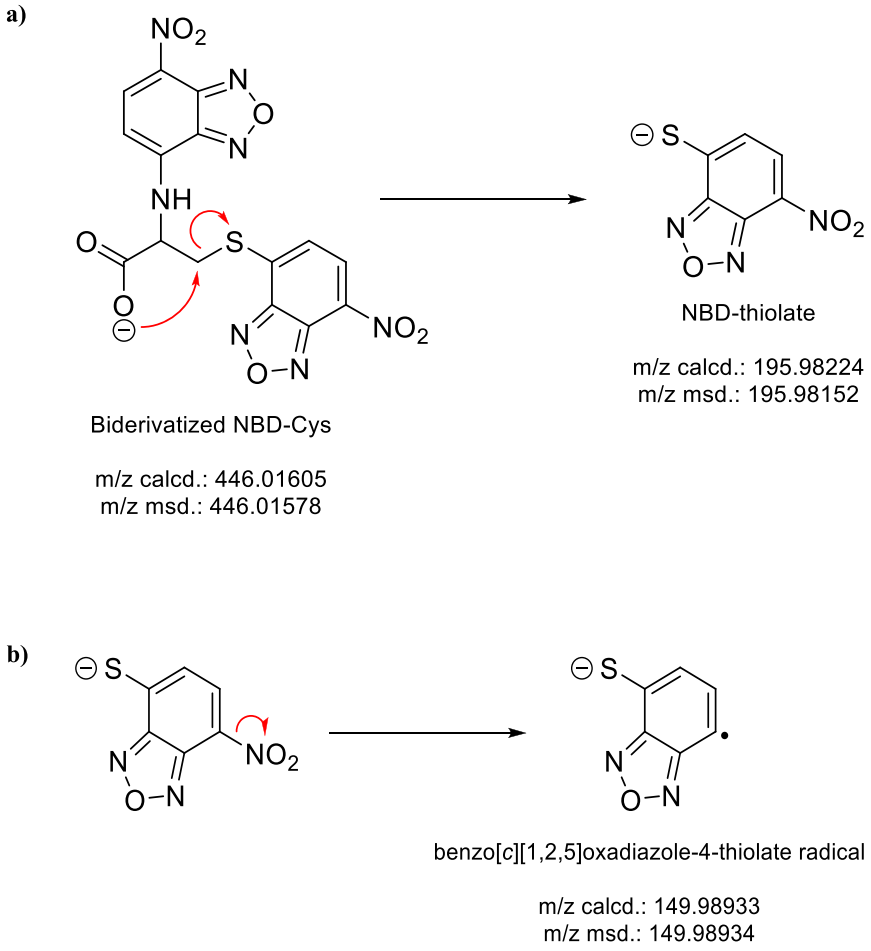

Fig. 3. Proposal for 4-fluoro-7-nitrobenzofurazan cysteine derivative fragmentation mechanisms based on LC/Q-ToF HRMS data; a) fragment at *m/z* 195.98152 b) fragment at *m/z* 149.98934.

chosen for validation (QC). The calibration solutions and QC samples were prepared in triplicate (n = 3) for three days (j = 3). After evaluating the response function by several calibration models, a quadratic least-square curve built based on the ratios of the analyte peak area over that of the corresponding internal standard, applying the discussed background subtraction (L-cysteine$_{added}$/L-cysteine-3,3-d_2 – L-cysteine$_{endogenous}$/L-cysteine-3,3-d_2) versus the concentration, was found to yield the best performance. For L-cysteine, the trueness expressed as recovery values was between 96% and 105%. CV_W and CV_{IP} were both lower than 15% for all evaluated concentrations. As previously described, the accuracy profile was established with the acceptability limits set at ± 30% (Fig. 4b). 80% β-ETI was within the acceptability limits, meaning that L-cysteine can be measured with an appropriate error over the investigated concentration range (25, 50 and 100 µM). The LOD was estimated from the S/N value of the lowest calibration point corresponding to the endogenous concentration of L-cysteine in plasma. The corresponding concentration of 1 µM on average was estimated at an S/N value of 3. The uncertainty was around 20% for 25 and 100 µM and below 30% for 50 µM (Fig. 5b).

3.2. Application to biological samples

There is evidence that H_2S can be produced either from L- or D-cysteine with distinct pathways for each enantiomer [17]. This might be the case for other unknown functions of D-cysteine; thus, the understanding of D-cysteine-specific pathways is of interest. The development of a quantitative LC-MS/MS method for the enantioresolution of cysteine in biological samples is a promising tool paving the way to such a knowledge. The developed and validated method for cysteine enantiomers was applied to their quantification in the plasma of immunocompromised mice after D-cysteine

Table 1
Summary of validation criteria for cysteine enantiomer determination in plasma.

Analyte	Theoretical added concentration (µM)	Trueness (%)	Repeatability (%CV_W)	Intermediate precision (%CV_{IP})	Lower tolerance limit (% of theoretical added concentration)	Upper tolerance limit (% of theoretical added concentration)
D-cysteine	50.0	103.1	4.5	4.5	96.4	109.8
	100.0	102.2	3.5	4.3	95.3	109.1
	225.0	101.0	7.3	7.9	89.0	112.9
L-cysteine	25.0	105.0	7.2	12.0	83.4	126.5
	50.0	98.6	8.5	15.0	71.4	125.9
	100.0	96.4	12.2	12.2	78.3	114.4

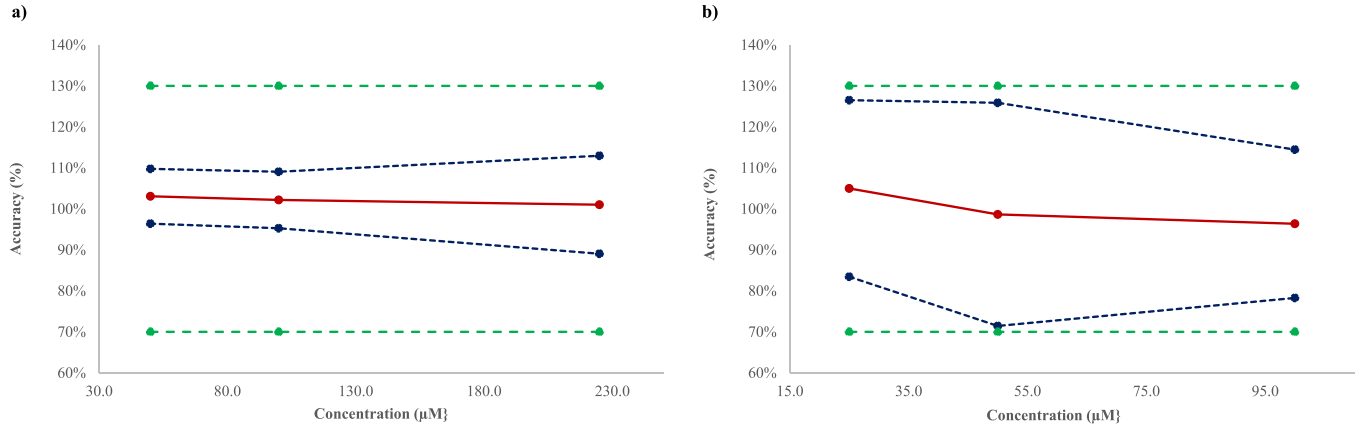

Fig. 4. Accuracy profiles of a) D-enantiomer and b) L-enantiomer of cysteine. The red line represents the accuracy (%), the blue dotted lines represent the tolerance interval limits (%) and the green dashed lines represent the acceptability limits (%).

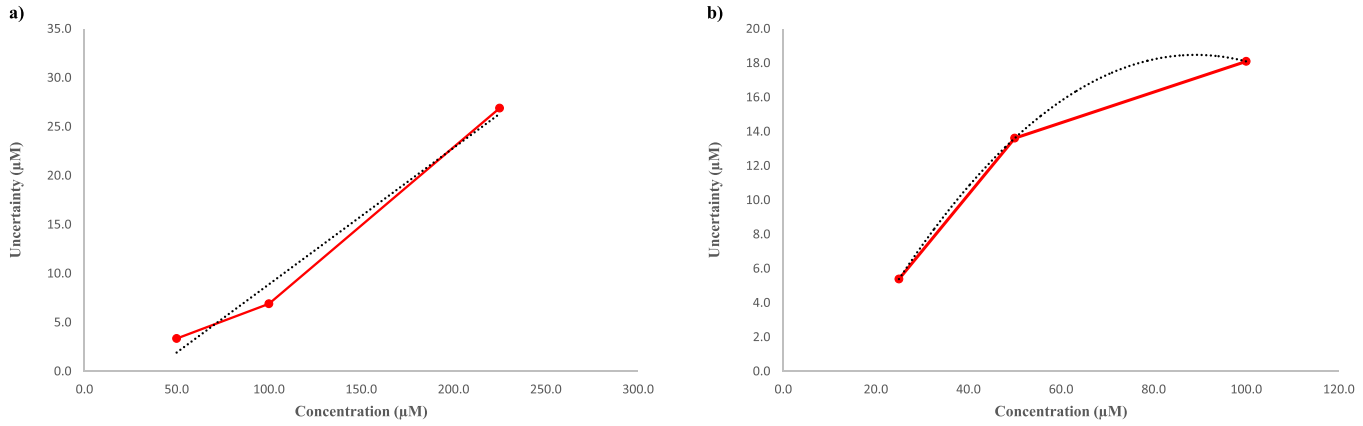

Fig. 5. Absolute uncertainty profiles for a) D-enantiomer and b) L-enantiomer of cysteine.

Table 2
Concentrations of cysteine enantiomers in plasma for malignant pleural mesothelioma (model 1) or lung carcinoma human cell (model 2) subcutaneously injected mouse.

Sample	D-cysteine in plasma ± uncertainty (µM)	L-cysteine in plasma ± uncertainty (µM)
Model 1 – D-cysteine (CT)	Not detected	97.6 ± 18.2
Model 1 + D-cysteine – mouse 1	223.2 ± 26.1	58.2 ± 15.4
Model 1 + D-cysteine – mouse 2	95.2 ± 8.2	46.4 ± 12.7
Model 1 + D-cysteine – mouse 3	273.3 ± 33.0	44.9 ± 12.3
Model 2 – D-cysteine (CT)	Not detected	107.3 ± 17.3
Model 2 + D-cysteine – mouse 1	129.4 ± 13.0	78.9 ± 18.1
Model 2 + D-cysteine – mouse 2	163.3 ± 17.8	72.6 ± 17.6
Model 2 + D-cysteine – mouse 3	203.4 ± 23.3	60.1 ± 15.8

Table 3
Relative and absolute uncertainty functions for D- and L-cysteine.

Analyte	Relative uncertainty function	Absolute uncertainty function
D-cysteine	$y = 0.0003x + 0.0451$	$y = 0.1394x - 5.0493$
L-cysteine	$y = -5E\text{-}05x^2 + 0.0064x + 0.0902$	$y = -0.0032x^2 + 0.5685x - 6.8326$

supplementation through the diet. Both enantiomers of cysteine were measured to check whether the L-cysteine values corresponded to standard values after D-cysteine supplementation or if an alteration of the endogenous concentration could be evidenced. The biological samples were subjected to protein precipitation, reduced with DL-dithiothreitol and derivatized with NBD-F, as described in Section 2.7. The concentrations of L- and D-cysteine are presented in Table 2, with the relative and absolute uncertainty functions presented in Table 3 for L- and D-cysteine. The absolute uncertainty functions of each enantiomer were used to compute the respective value of each of the obtained results. The uncertainty values were below 30% and 15% for the L- and D-forms, respectively, with the experimental concentrations.

4. Conclusion

In this study, the enantioseparation of cysteine enantiomers using an (*R*,*R*)-Whelk-O 1 stationary phase with NBD-F derivatization was successfully applied to biological samples. Derivatization with NBD-F was employed to improve the cysteine stability

compared to previous work [21]. D-cysteine was not detected in native plasma, whereas L-cysteine is a naturally occurring compound. Thus, our strategy approached the challenge of simultaneously quantifying an exogenous and an endogenous compound with a unique sample preparation. A background subtraction strategy was used for quantifying L-cysteine using a similar matrix as the study sample, allowing for related recovery and matrix effects between the latter and the calibration samples. Moreover, the matrix effect was assessed, and a deuterated internal standard was used to significantly reduce it. Satisfactory results were obtained for the selectivity, trueness, and precision. The method was validated for both enantiomers and eventually applied to mouse plasma after D-cysteine supplementation.

CRediT authorship contribution statement

Sabrina Ferré: Conceptualization, Formal analysis, Investigation, Methodology, Data curation, Visualization, Validation, Writing – original draft, Writing – review & editing **Víctor González-Ruiz:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing **Joséphine Zangari:** Conceptualization, Resources **Sergey Girel:** Formal analysis, Investigation, Writing – review & editing **Jean-Claude Martinou:** Conceptualization, Resources, Writing – review & editing **Roccaldo Sardella:** Methodology, Writing – review & editing **Serge Rudaz:** Conceptualization, Methodology, Visualization, Validation, Funding acquisition, Resources, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2021.114539.

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