

# Determination of reduced homocysteine in human serum by elemental labelling and liquid chromatography with ICP-MS and ESI-MS detection

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**Abstract** Analytical methods allowing sensitive determination of reduced homocysteine (rHcy), one of the so-called biothiols, in human serum is a topic of growing interest due to its close relation to several human disorders, mainly cardiovascular diseases. Although most widely used analytical strategies to determine total Hcy involve derivatization by means of fluorescent labels, this work proposes the use of ebselen, a Se-containing labelling agent to derivatize the reactive sulfhydryl group of the Hcy molecule in its “free” reduced form, which is more likely to play different roles in disease pathogenesis. Optimization of the derivatization and separation conditions by high-performance liquid chromatography (HPLC) to isolate the excess of derivatizing reagent is carried out here using UV/VIS detection. Further, the study of the Se labelling reaction by electrospray ionization tandem mass spectrometry (ESI-MS/MS) provides a stoichiometry of the derivative of 1:1. The main advantage of using ebselen as a labelling agent is the presence of the Se atom in the molecule that allows the use of inductively coupled plasma mass spectrometry (ICP-MS) as a sensitive and selective Se detector. The coupling of HPLC with ICP-MS provided excellent features for the determination of Se-derivatized rHcy (detection limit of 9.6 nM) in real samples. Quantification was accomplished by using post-column isotope dilution (ID) of Se in serum samples, after

precipitation of the main serum proteins. Quantitative results for “free” rHcy turned out to be around 0.18–0.22  $\mu\text{M}$  in serum samples from healthy individuals that could be directly analyzed without sample preconcentration.

**Keywords** Reduced homocysteine · Ebselen · Labelling · LC-ICP-MS · Isotope dilution

## Introduction

Homocysteine (Hcy) is a non-protein sulfur-containing amino acid produced endogenously during the metabolism of the essential amino acid methionine. Once produced, Hcy can undergo re-methylation to methionine (which prevails in low-methionine-level conditions) or follow a *trans*-sulfuration pathway to cysteine (Cys) (favored under methionine excess or during an increased cellular demand for cysteine) that can be further incorporated into proteins [1–3]. The intracellular concentrations of Hcy are maintained with an export mechanism of homocysteine from cells to blood [4] that limits its intracellular toxicity but leaves vascular tissue exposed to the harmful effects of Hcy excess. The normal level of Hcy in plasma/serum ranges from 5 to 15  $\mu\text{M}$ , and variations are ascribed to genetic factors, age, sex, menopausal status, and other physiological and lifestyle conditions [5]. About 70 % of plasma total Hcy (tHcy) is bound to albumin, 30 % is oxidized to disulfides to form homocysteine–homocysteine or homocysteine–cysteine, and only approximately 1 % is present as “free” circulating Hcy [6]. Hyperhomocysteinemia is usually defined as a plasma tHcy >15  $\mu\text{M}$  and is considered in its moderate (15–30  $\mu\text{M}$ ), intermediate (30–100  $\mu\text{M}$ ), or severe (>100  $\mu\text{M}$ ) levels [7]; in this last case, free reduced homocysteine (rHcy) composes a substantial portion (up to 20 %) of tHcy [8]. Clinical and epidemiological studies have suggested

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that hyperhomocysteinemia is a risk factor for atherosclerotic vascular disease, arterial and venous thromboembolism, and coronary, cerebral, or peripheral arterial occlusive diseases [5, 9].

Regarding Hcy determination, most methods are focused on tHcy measurements. However, the oxidized and protein-bound Hcy forms are not biologically active [10], given that the basis of the relation of homocysteine with the endothelial cell injury produced on cardiovascular diseases is the generation of reactive oxygen species. These species are produced by autoxidation of the sulfhydryl group of the reduced homocysteine in the presence of transition metals, and this redox chemistry is only possible for the “free” circulating rHcy [11].

In this regard, of the different serum Hcy forms, the reduced form is considered the most challenging species to monitor (due to the low existing concentrations) in spite of the fact that it is the harmful form. In fact, reduced Hcy is considered a major marker of risk of cardiovascular diseases [12, 13] and its increase together with an increase in the Cys concentration has been associated with kidney disease [12, 14], vascular endothelial dysfunction [13], cerebral infarction, and ischemic stroke [15, 16].

Thus, analytical methods to provide information on the reduced form of Hcy simultaneously to the determination of other metabolically related plasma aminothiols, such as Cys [17], are of special clinical interest. In this vein, different methods based on the use of high-performance liquid chromatography (HPLC) with different detection systems, such as mass spectrometry (MS) or fluorimetry after derivatization with different reagents (since Hcy does not have any chromophore or fluorophore group), have been proposed but for the determination of tHcy [18–20].

In the case of fluorimetric detection, the most widely used derivatization reagent is ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) which exhibits excellent sensitivity and specificity towards sulfhydryl groups and has been used for detection of tHcy in human plasma [21]. Reported HPLC–MS/MS methods involve the analysis of tHcy together with related metabolites including total cysteine (tCys) [22, 23], methionine [22], and methylmalonic acid [24]. However, in the last years, HPLC–tandem MS (MS/MS) has been also widely employed for biothiol analysis either involving or not a previous derivatization step [20]. Without the derivatization step, although the sample preparation process is dramatically simplified, it can result in excessive autoxidation of the –SH group and bring about erroneous results. Total homocysteine has been determined using HPLC–MS/MS with high specificity by multiple reaction monitoring (MRM), elevated sensitivity, and good accuracy by using isotopically labelled analogues.

In this work, we propose the absolute quantification of serum Hcy in its reduced form based on a reversed-

phase (RP)-HPLC methodology with elemental detection using inductively coupled plasma mass spectrometry (ICP-MS) after derivatization with ebselen (2-phenyl-1,2-benzisoseleazol-3(2*H*)-one). Nowadays, ICP-MS coupled with chromatography or electrophoresis provides a powerful combination for absolute quantification of heteroatom-containing molecules with high sensitivity and accuracy [25]. Since ebselen is a Se-containing reagent, which has been reported as a highly specific thiol derivatizing agent [26, 27], its application here improves the chromatographic separation of Hcy from other biological thiols like Cys and simultaneously the detectability of the sought analyte by monitoring the Se atom present in the label using ICP-MS. The robust quantification of selenium using post-column isotope dilution analysis (IDA)–HPLC–ICP-MS offers a convenient and advantageous determination of “free” reduced Hcy in human serum.

## Experimental

### Reagents, materials, and samples

Analytical reagent-grade chemicals were used throughout unless otherwise stated. All solutions and dilutions were made with high-purity deionized water (>18 M $\Omega$  Milli-Q water, Millipore, Bedford, MA, USA).

DL-Homocysteine ( $\geq 95$  %), ebselen (2-phenyl-1,2-benzisoseleazol-3(2*H*)-one), trichloroacetic acid (TCA,  $\geq 98$  %), and acetic acid ( $\geq 99.8$  %) were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-Cysteine ( $\geq 99.5$  %) and formic acid (98–100 %) were purchased by Merck (Darmstadt, Germany). HPLC-grade methanol and acetonitrile were supplied by Fisher (Waltham, MA, USA).

An isotopically enriched Se tracer with relative abundances of 98.1593 %  $^{74}\text{Se}$ , 0.2301 %  $^{76}\text{Se}$ , 0.1369 %  $^{77}\text{Se}$ , 0.4292 %  $^{78}\text{Se}$ , 0.8831 %  $^{80}\text{Se}$ , and 0.1614 %  $^{82}\text{Se}$  was obtained from Cambridge Isotope Laboratories as elemental Se powder. This powder was dissolved in a minimum volume of sub-boiled nitric acid (Merck) and then diluted with ultrapure water, as required. The concentration of Se in these solutions was established by reverse isotope dilution analysis using a natural abundance Merck certified standard.

Serum samples from healthy volunteers were kindly provided by the Hospital Central of Asturias, Laboratory for Biochemical Analysis (Oviedo, Spain). Samples were anonymous and collected in accordance with protocols approved by the relevant institutional review boards and with the Declaration of Helsinki. All serum samples were collected using BD Vacutainer<sup>®</sup> tubes (Fisher Scientific, Spain) containing gel for serum separation.

## Instrumentation

**HPLC–UV/VIS and HPLC–ICP-MS** HPLC separations were carried out using a dual-piston liquid chromatographic pump (Shimadzu LC-10AD, Shimadzu Corporation, Kyoto, Japan) equipped with a simple injection valve (Rheodyne, model 7125, Cotati, CA, USA), fitted with an injection loop of 20  $\mu\text{L}$ , and a reversed-phase Europa Peptide 120  $\text{C}_{18}$  column (150 mm  $\times$  2.1 mm, 5  $\mu\text{m}$  particle size) from Teknokroma (Barcelona, Spain). The two mobile phases consisted of solvent A (0.1 % formic acid in water) and solvent B (0.1 % formic acid in methanol). The elution gradient used started with 50 % B (2 min) and then linearly increased to 100 % B in 18 min (total running time of 20 min) at a flow rate of 0.2 mL/min. A diode array detector (DAD) from Agilent Technologies (1100 Series, Tokyo, Japan) was used for UV–VIS detection at 250 nm during optimization of the derivatization and separation conditions.

Specific atomic detection of Se in the column effluent was performed using an ICP-MS model 7700 from Agilent Technologies (Agilent, Tokyo, Japan) equipped with a collision cell system (ICP-(ORS)-MS) using  $\text{H}_2$  as reaction gas at 4 mL/min. To run the instrument with organic solvents directly, we used an organic torch (1.0 mm i.d. injector). Details of the instrumental operating conditions are given in Table 1.

**HPLC–ESI-MS/MS** HPLC separations were carried out using a dual-piston liquid chromatographic pump (Agilent 1290 Infinity, Tokyo, Japan) equipped with an online degasser, a micro-well plate autosampler, a column thermostat, and a reversed-phase Zorbax Eclipse Plus  $\text{C}_{18}$  column (50 mm  $\times$  2.1 mm, 1.8  $\mu\text{m}$  particle size) from Agilent Technologies. The mobile phase consisted of solvent A (0.1 % formic acid in water) and solvent B (0.1 % formic acid in acetonitrile) delivered at a flow rate of 0.25 mL/min. The elution gradient started with 2 % B (2 min) and increased linearly to 80 % B in 13 min (maintaining 80 % B for 5 min).

Detection of the separated compounds was achieved using an Agilent 6460 Triple Quad LC/MS with a Jet Stream ESI ion source; the system was operated in the positive ion mode. For MRM measurements, the column outlet was directly connected to the ionization source. The highest abundant product ion was selected for all the analytes. Instrumental settings are shown in Table 1.

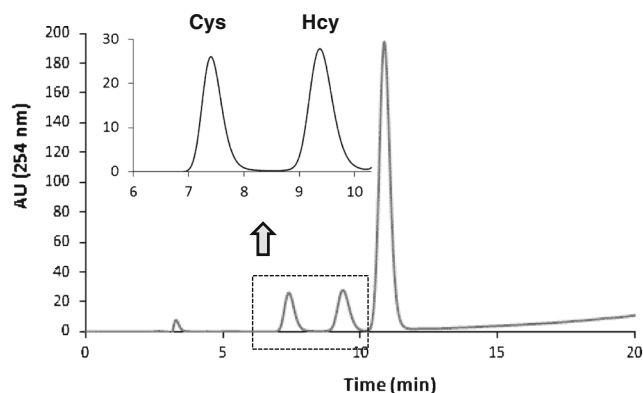
## Procedures

**Derivatization from standard solutions** Stock standard solutions of homocysteine (Hcy) and cysteine (Cys) at 1 mg/mL (7.4 mM) were prepared in a solvent mixture containing 50 % (v/v) methanol, 1 % (v/v) acetic acid, and 49 % (v/v) Milli-Q

**Table 1** Operating conditions for ESI-MS/MS and ICP-MS detection

ESI-MS/MS parameters	
Gas temp ( $^{\circ}\text{C}$ )	50
Gas flow (L/min)	5
Nebulizer (psi)	45
Sheath gas temp ( $^{\circ}\text{C}$ )	150
Sheath gas flow (L/min)	11
Capillary (V)	2500
Nozzle voltage (V)	0
ICP-MS parameters	
RF power	1550 W
Carrier gas flow rate	0.70 L/min
Collision/reaction gas	$\text{H}_2$
Gas flow	4 mL/min
Cell entrance	–40.0 V
Cell exit	–60.0 V
Isotopes monitored	$^{80}\text{Se}$ , $^{78}\text{Se}$ , $^{74}\text{Se}$
HPLC conditions	
Column	Zorbax Eclipse Plus C 18
Mobile phases	(A) 0.1 % formic acid in water (B) 0.1 % formic acid in methanol
Flow rate	0.2 mL/min
Post-column water/spike flow rate	0.4 mL/min
Injection volume	20 $\mu\text{L}$
Gradient conditions	
Time	B %
0	50
2	50
20	100
21	50

water that was previously deoxygenated by bubbling with He. The standard working solutions, both for calibration curves and standard additions, were prepared freshly each day by diluting the stock solutions into the described solvent mixture



**Fig. 1** Chromatogram obtained by RP-HPLC using UV/VIS detection (absorbance at 254 nm) for cysteine and homocysteine standards (10  $\mu\text{M}$ ) derivatized with ebselen. Cysteine derivative (7.4 min), homocysteine derivative (9.4 min), ebselen excess (10.9 min)

to reach the suitable concentrations. Aliquots of 50  $\mu\text{L}$  of standard working solutions were mixed in plastic eppendorf tubes with 50  $\mu\text{L}$  of 150  $\mu\text{M}$  ebselen solution prepared also in the deoxygenated solvent mixture. The mixture was vortex-mixed and left at room temperature for 5 min.

**Preparation of serum samples** After being thawed at room temperature, 300  $\mu\text{L}$  of serum samples was transferred to plastic eppendorf tubes of 1.5 mL. Sample deproteinization was achieved by adding 100  $\mu\text{L}$  of cold TCA (10 %) and followed by centrifugation at  $12,000\times g$  for 15 min at 4  $^{\circ}\text{C}$ . Aliquots of 100  $\mu\text{L}$  of protein-free serum were obtained from the middle of the clear supernatant. Derivatization with

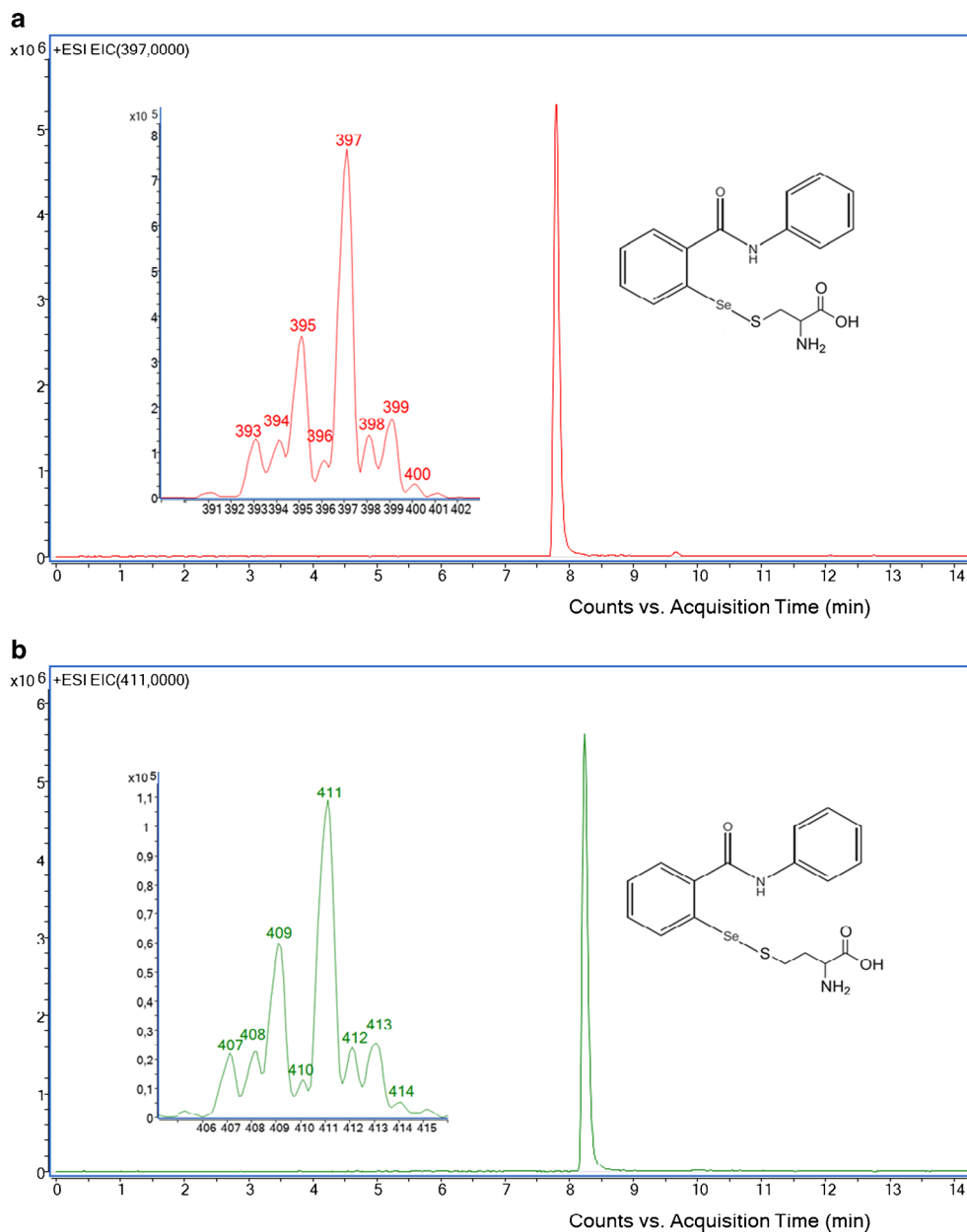
ebselen of these aliquots was conducted as in the case of the standard solutions.

## Results and discussion

### Optimization of derivatization and chromatographic conditions

Typical derivatization conditions were initially taken from the literature [26] and applied to an Hcy standard solution. Reversed-phase chromatography was chosen in order to separate the derivatized Hcy from the excess of the derivatization

**Fig. 2** Extracted ion chromatograms of the reaction products of ebselen with **a** cysteine (10  $\mu\text{M}$ ) and **b** homocysteine (10  $\mu\text{M}$ ). The insets show the ESI-MS spectrums showing the isotopic distributions of the reaction products,  $m/z$  397 for cysteine and  $m/z$  411 for homocysteine



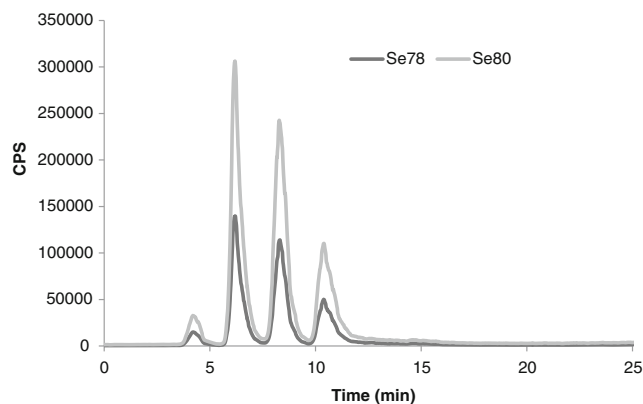
reagent (ebselen) using the conditions described in the “Experimental” section. Since Cys could be one of the species also present in serum together with Hcy, both standards (Hcy and Cys) were derivatized independently with ebselen, mixed, and separated in the chromatographic system. Figure 1 shows the chromatogram obtained by UV/VIS detection at 254 nm using the chromatographic conditions described in the “Experimental” section. Three main peaks can be observed, the first one at 7.4 min corresponding to the derivatized cysteine, the second one at 9.4 min corresponding to the derivatized homocysteine, and the peak of the remaining ebselen at 10.9 min (tested by injecting the three independent species).

The exact structure of the derivatized thiols was obtained by HPLC-ESI-MS with the conditions described in the “Experimental” section. ESI full-scan analysis showed the characteristic selenium isotope pattern for both derivatized thiols. Figure 2 shows the ESI-MS spectra of the reaction of 10  $\mu\text{M}$  cysteine (Fig. 2a) and 10  $\mu\text{M}$  homocysteine (Fig. 2b), respectively, with ebselen (40  $\mu\text{M}$ ) in deoxygenated methanol/water (1:1) containing 1 % (v/v) acetic acid. The observed reaction products contained a molecular ion at  $m/z$  397 for the cysteine derivative and at  $m/z$  411 for the homocysteine derivative, confirming the expected 1:1 stoichiometry described in the literature for the derivatization products of thiol compounds with ebselen [26]. In addition, the extracted ion chromatograms for both derivatives (Fig. 2) show a single peak at 7.8 and 8.3 min, respectively, with a good signal to noise ratio and chromatographic profile.

The reaction mechanism of thiol compounds with ebselen is based on the cleavage of the Se–N bond of ebselen by the SH group of the thiol and the formation of a selenenyl sulfide Se–S bond between ebselen and the thiol molecule, as previously described [26]. In order to optimize the derivatization reaction, different conditions were evaluated by using neutral or acidified mixtures of methanol/water at different ratios, deoxygenation of all solvents, different ebselen/thiol ratios (from 1:1 to 10:1), incubation time (from 5 to 30 min), and temperature (from ambient temperature to 37 °C). Best results were obtained using deoxygenated methanol/water (1:1) containing 1 % acetic acid as reaction medium, and an ebselen/thiol ratio of 4:1. Higher molar ratios generated an important excess of ebselen that produced chromatographic overlap of the peaks (data not shown). Incubation time and temperature did not affect the derivatization reaction.

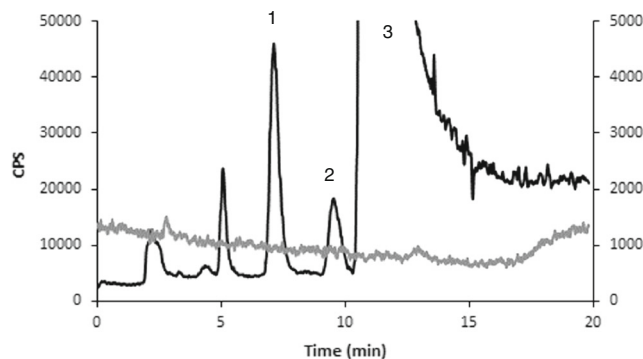
#### Determination of derivatized reduced homocysteine by HPLC-ICP-MS

Since the ebselen–thiol derivatization products contain a Se heteroelement in their structure, we can take the advantage of the features of an element selective detector, ICP-MS, for specific monitoring after the HPLC separation. Figure 3 shows a typical Se chromatogram obtained in this way where



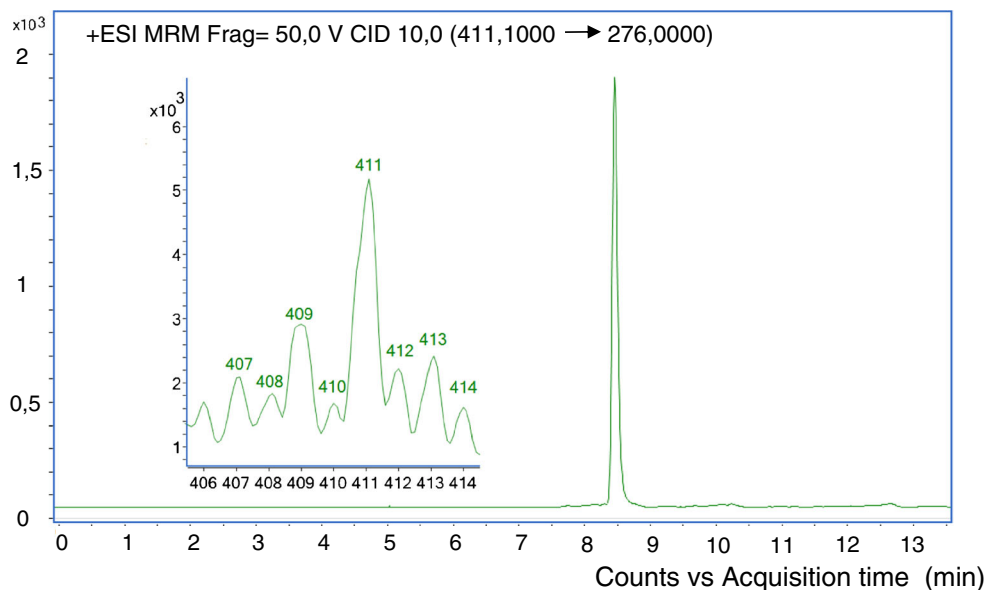
**Fig. 3** Illustrative HPLC-ICP-MS chromatogram showing the profiles of  $^{80}\text{Se}$  (grey trace) and  $^{78}\text{Se}$  (dark trace) obtained for Cys and rHcy standards (10  $\mu\text{M}$ ) after derivatization with ebselen. Cys derivative (7.4 min), rHcy derivative (9.4 min), ebselen excess (10.9 min)

three well-resolved peaks can be observed corresponding to a mixture of derivatized cysteine (~7.2 min), derivatized homocysteine (~9.3 min), and the excess of ebselen (~11 min). It is noteworthy that the peak profile is slightly broader than in the case of UV/VIS detection (Fig. 1). This can be ascribed to the instrumental setup used for this application which includes an online dilution system of the mobile phase (1:1.5) eluting from the column before entering the ICP-MS, in order to decrease the methanol content in the plasma (down to ~40 % at 20 min) which can produce a decrease on the Se ionization efficiency [28]. Under these conditions, the detection limit of the HPLC-ICP-MS technique for rHcy was calculated using a freshly prepared standard calibration curve (peak height versus concentration) as three times the standard deviation of the background divided by the sensitivity (as the slope of the calibration curve) and turned out to be 2.9 nM (0.4 ng/mL). Linearity was also evaluated by the determination coefficient ( $r^2$ ), which was higher than 0.999 (obtained with concentrations ranging from 0.04 to 6.8  $\mu\text{M}$ ). The HPLC-ICP-



**Fig. 4** Illustrative chromatogram obtained by IDA-HPLC-ICP-MS for a serum sample (dark trace) with a rHcy concentration of 0.179  $\mu\text{M}$ . Peaks for Cys derivative (1), rHcy derivative (2), and ebselen excess (3) can be observed. The grey trace shows the chromatogram obtained for the same serum sample but without derivatization with ebselen

**Fig. 5** Chromatogram obtained in ESI-MRM mode for the 411 → 276 *m/z* transition in a human serum sample with a rHcy concentration of 0.179 μM. The *inset* shows the isotopic distribution of the homocysteine derivative by ESI full-scan analysis



MS method precision, expressed as intra-day relative standard deviation (RSD), was assessed using three replicates at a concentration level fixed in the high range of the calibration curve and turned out to be around 7 %.

Further on, quantitative studies were undertaken by means of an online isotope dilution (ID) method. Instrumentally, this was conducted by using a post-column addition of an isotopically enriched aqueous Se solution ( $^{74}\text{Se}$ , 98.1593 %) with known concentration that was continuously pumped by the T-piece (used simultaneously for dilution of the methanol content of the mobile phase) and mixed with the eluent coming from the chromatographic column. After calculating the isotope ratio at each point of the chromatogram and applying the online isotope dilution equation [29], it was possible to calculate the total Se in each peak of the chromatogram. Taking into account the Se/homocysteine stoichiometry (observed by ESI-MS) of the derivative selenenyl sulfide (1:1), the final homocysteine concentration could be calculated from the Se measurements. By comparing the theoretical homocysteine concentration (in the standard solution) with the obtained homocysteine concentration obtained by measuring Se in the peak by ID-HPLC-ICP-MS, the recovery for the whole process could be estimated and turned out to be  $101.6 \pm 7.5$  %.

**Table 2** Recoveries of reduced homocysteine in human serum

Added rHcy (μM)	Found rHcy (μM)	Recovery (%)
0	0.179	–
0.215	0.354	90
0.568	0.734	98
1.110	1.271	99

### Validation of the ID-HPLC-ICP-MS method

In order to evaluate the level of trueness of the proposed ID method for the determination of rHcy in human serum, recovery experiments were performed. For this aim, four aliquots of the same serum sample were spiked with four different concentrations of homocysteine standard (0, 0.2, 0.6, and 1.1 μM, respectively). All serum aliquots were treated as described in the “Experimental” section, and then they were analyzed by ID-HPLC-ICP-MS. The chromatogram obtained for the serum sample without spiking can be observed in Fig. 4. In the chromatogram, several Se-containing peaks can be observed that are not present in the chromatogram of the same serum sample without ebselen labelling (Fig. 4). These peaks have been identified according to their retention times and correspond to derivatized Cys (7.1 min) and rHcy (9.5 min) together with the excess of ebselen (at about 11 min). Other unidentified Se-containing peaks at 2 and 5 min can be also observed and could be ascribed to other low molecular weight species (e.g., Se-methionine) present in serum samples. The identity of the labelled rHcy peak was confirmed by HPLC-ESI-MS/MS using single reaction monitoring mode using the transition

**Table 3** Reduced homocysteine in human serum

Sample	rHcy (μM) (mean ± SD, <i>n</i> =3)	rHcy (ng/mL) (mean ± SD, <i>n</i> =3)
1	0.187 ± 0.019	25.2 ± 2.6
2	0.186 ± 0.020	25.1 ± 2.6
3	0.223 ± 0.023	30.1 ± 3.1
4	0.208 ± 0.022	28.1 ± 2.9
5	0.179 ± 0.019	24.2 ± 2.5

of the parent ion to the product ion ( $m/z$  411  $\rightarrow$  276) which corresponds to the fragmentation of the ebselen moiety. The HPLC-ESI-MS/MS (MRM) chromatogram for this transition is displayed in Fig. 5 and shows a single peak eluting at the same time than that obtained for the homocysteine standard.

After the assessment of the species identity in the serum sample, the quantitative step was taken. The standard addition calibration curve acquired during the analysis of the spiked serum samples by IDA-HPLC-ICP-MS was found to exhibit good linearity with a determination coefficient ( $r^2$ ) of 0.9977. The average recovery calculated from this calibration curve (Table 2) displays values ranging from 90 to 99 %. Therefore, no matrix effects seem to affect the trueness of the analytical data using the developed ID method.

The intra-day precision of the entire method was determined by three replicate analyses of a serum sample. The concentration of rHcy found in this sample was 0.186  $\mu$ M with a relative standard deviation of 10 % which is adequate for the determination of the low levels of rHcy present in human serum.

Finally, the procedural limit of detection (LOD) was estimated based on three times the standard deviation of the mean concentration of rHcy determined in ten replicated measurements of blank reagents spiked with the analyte (3 nM, minimum detectable concentration) following the same analytical protocol used for the analysis of samples. A value of 9.6 nM (1.3 ng/mL) was obtained. This value is one of the lowest reported in the literature for homocysteine determinations and directly compares with those obtained using fluorescence detection [30] or ESI-MS in MRM mode [31].

### Quantification of reduced homocysteine in human serum samples

The proposed strategy was applied to the quantification of reduced homocysteine in human serum samples, the lowest abundant of the possible forms of this amino acid in human serum. All serum samples ( $n=5$ ) were treated as described in the “Experimental” section. After deproteinization, derivatization with ebselen was performed and the reaction product injected in the chromatographic system. Quantification was conducted by post-column isotope dilution as stated for the homocysteine standards. Table 3 shows the obtained results. As can be observed, there are no big differences among serum samples and the determined concentrations are in the nanomolar (ng/mL) range, in agreement [15] with the values obtained from the relatively scarce techniques that permit this sensitive analysis that report a concentration of 0.18–0.24  $\mu$ M being in the free reduced form [15].

### Conclusions

A new method for the determination of reduced homocysteine in human serum has been proposed and evaluated based on the labelling of the free –SH group with ebselen. The formation of the derivative has proved to be quantitative, providing a 1:1 stoichiometry (obtained by ESI-MS) that could be preserved after the chromatography necessary to separate the excess of the labelling reagent. Since ebselen contains a Se atom in its structure, it was also possible to address the detection of the cysteine and homocysteine derivatives of ebselen by ICP-MS using Se as heteroelement after HPLC separation. The excellent figures of merit of this coupling permitted the direct determination of free reduced homocysteine in serum samples by applying post-column isotope dilution analysis as quantification strategy. The obtained results revealed homocysteine concentrations in the analyzed samples in the nanomolar range that could be directly attained without any analyte preconcentration step.

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**Conflict of interest** The authors declare that they have no competing interests.

**With regard to the statement of informed consent** The samples were collected anonymously by the Hospital Central of Asturias. In any case, samples were collected in accordance with protocols approved by the relevant institutional review boards and with the Declaration of Helsinki.

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