DETERMINATION OF CYSTATIN C IN HUMAN URINE BY ISOTOPE DILUTION TANDEM MASS SPECTROMETRY

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KEYWORDS

Cystatin C; human urine; isotopically labelled peptides; monoclonal antibodies; isotope dilution; Tandem Mass Spectrometry.

ABSTRACT

This work presents the development of a methodology for the accurate and precise quantification of the renal biomarker Cystatin C in human urine by Isotope Dilution Mass Spectrometry (IDMS). The procedure is based on the addition of a known quantity of the proteotypic peptide ALDFAVG*EYNK labelled with $^{13}C_2$-glycine to the urine sample followed by protein hydrolysis using trypsin. Then, preconcentration and purification of the isotope diluted peptide was carried out by a selective monoclonal antibody bound to magnetic beads and final measurement was done after injection of the sample in a HPLC-MS/MS triple quadrupole instrument. The isotopic distribution of the isotope diluted proteotypic peptide was measured by low resolution selected reaction monitoring. Using this aquisition mode, the bandpass of the first quadrupole was widened (FWHM = 13 u) so the whole isotopic clusters for both the natural abundance and the labelled peptides entered the collision cell. The proposed acquisition mode provided similar accuracy and precision than the regular SRM mode (FWHM = 0.7 u) but a higher sensitivity was observed. The purification of the sample by antibody based enrichment of the target peptide was shown to remove interfering compounds more efficiently in comparison with a sample purification based on semipreparative liquid chromatography. Using 5 ng of the labelled peptide it was possible to quantify Cystatin C in human urine in patients with normal and impaired renal function. Recoveries from 100 to 104% were obtained in samples containing from 90 to 700 µg L^{-1} of Cystatin C with relative standard deviations from 0.5 to 6%. The stability of Cystatin C in urine samples was evaluated under different storage conditions showing that only when the urine samples were stored at room temperature during more than 10 days, a significant degradation of Cystatin C was observed.

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1. INTRODUCTION

Glomerular filtration rate (GFR) is the main indicator of normal or impaired renal function. Traditionally, GFRs is estimated by the determination of creatinine in serum using spectrophotometric or enzymatic methods [1]. However, analytical limitations due to the presence of interfering compounds have questioned the specificity of creatinine-based methods for GFR estimation [2]. Cystatin C is a low molecular weight protein (13.3 KDa) that has been identified as a GFR marker [3]. In contrast to creatinine-based GFR estimating equations, Cystatin C-based equations are not influenced by race and sex, are useful for both children and adults and predict more efficiently end-stage renal diseases [4]. The superiority of serum cystatin C as a renal biomarker has been recognized by the clinical community due to its lower concentration variability and its higher sensitivity for the early detection of renal failure [5]. However, other studies have reported that that the combined creatinine–cystatin C equation performs better than equations based on either of these markers [6].

Cystatin C has been detected in different biological fluids such as urine, serum, cerebrospinal fluid or saliva [7]. The levels of cystatin C in human serum range between 0.65 and 0.83 μg mL^{-1} for healthy individuals depending on the age and sex [8] and those levels increase by a factor of approximately two for acute renal failure [5]. According to previous works, urinary cystatin C levels in healthy individuals range from 0.033 to 0.29 μg mL^{-1} [4, 9] and may increase up to 200 fold when a renal tubular disorder occurs [7, 10]. Less invasive determination of cystatin C in human urine is proposed as a useful diagnostic and prognostic biomarker in patients with acute kidney injury [11].

Currently, PETIA (particle-enhanced turbidimetric immunoassay) [12] or PENIA (particle-enhanced nephelometric immunoassay) [13] are routinely applied in clinical laboratories for the determination of Cystatin C in serum and urine. However, significant differences in serum CysC measurement between laboratories even using the same assay by the same manufacturer have been reported leading to clinically relevant differences in GFR estimation [14]. To improve the feasibility of cystatin C as biomarker, the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) released a human serum Reference Material (ERMDA471/ IFCC) available from 2010 [15]. Since then, this material has been used to assess the accuracy of cystatin C measurements to support a worldwide standardization. In 2015, the *College of American Pathologists Chemistry* published a survey where ERMDA471/ IFCC was analyzed by several laboratories concluding that the accuracy of cystatin C measurement procedures needs to be improved if cystatin C is to achieve its full potential as a biomarker for estimating GFR [16]. Further multicenter studies carried out by the French Society of Clinical Biology also reported that most of the routine automated assays failed to meet the criteria for acceptable serum cystatin C measurements [17, 18].

In contrast to serum samples, no reference material exists for the validation of urinary cystatin C routine assays. In those cases of lack of adequate reference materials traceability to the International System of Units may be evaluated by the comparison with a reference method such as Isotope Dilution Mass Spectrometry (IDMS) [19]. IDMS currently stands out as the analytical technique for trace analysis providing results with a higher metrological quality than traditional measurement methods. During the last decade IDMS has been increasingly applied for the absolute quantification of proteins in biological samples [20-22]. In most cases isotopically labelled proteotypic

peptides are used for the absolute determination of protein levels after enzymatic digestion. Providing that the proteotypic peptide is completely released from the protein after proteolysis, concentrations traceable to the International System of units can be obtained. Therefore proteolytic experimental conditions must be carefully optimized. Also, if the labelled peptide is added before proteolysis, it must not suffer any degradation until the endogenous natural abundance analogue is completely released from the protein chain.

So far, IDMS has been scarcely applied for the quantification of Cystatin C [23-25]. We have developed and validated previously an IDMS method for the determination of cystatin C in human serum [23] using Mass Overlapping Peptides (MOPs) and we are expanding here the methodology to the determination of cystatin C in human urine where no reference methods or reference materials exist. In comparison to other published procedures on the determination of protein biomarkers by isotope dilution and mass spectrometry, our approach provides the direct quantification of the protein without performing any calibration graph. This is possible when using and internal standard characterized in terms of concentration and isotopic enrichment. Due to the lower concentration levels in urine in comparison to serum samples the application of efficient preconcentration methods are required. Therefore, we evaluate and compare here two different sample preconcentration and purification strategies: i) semipreparative cationic exchange chromatography and ii) antibody based enrichment of the target peptide [26]. The analytical figures of merit of the developed methodology and a stability study of cystain C in urine samples at different storage conditions are also presented.

2. EXPERIMETAL

2.1 Reagents and materials

The natural abundance proteotypic peptide ALDFAVGEYNK (T3, amino acids 26-36 of cystatin C, P01034, was purchased from Gene Cust (Dudelange, Luxembourg). The isotopically labelled peptide was synthesized with $^{13}C_2$-Glycine, ALDFAVG*EYNK (T3*) and was purchased from Caslo Laboratory (Lyngby, Denmark). Our IDMS methodology is based on the use of minimally labelled peptides to minimise isotopic effects. Therefore, universally $^{13}C_2$ labelled glycine was chosen for peptide synthesis due its lower cost. The isotopic enrichment of T3* peptide was calculated following the procedure described elsewhere [27] based on multiple linear regression. According to this procedure the ^{13}C isotopic enrichment of the labelled peptide was 98.2 ± 0.3%. The concentration of the T3* peptide was periodically calculated by reverse IDMS using a previously purified and characterized natural abundance peptide as described elsewhere [23]. The concentration of labelled peptide stock solution was found to be stable over more than 24 months. Rabit monoclonal antibody specific for T3 peptide was purchased from Vancouver Island Antibodies by Siscapa Assay Technologies and Dynabeads™ protein G was obtained from Invitrogen by Thermo Fisher Scientific (Waltham, Massachusetts). Stock solutions of natural (T3) and $^{13}C_2$-labelled peptide (T3*) were prepared by dissolving the standards in ultrapure Milli-Q water. All stock solutions were stored at -20 ºC and employed to prepare daily gravimetrically diluted working standard solutions in water with 0.1% of formic acid.

All solvents and reagents were of analytical reagent grade. Bovine trypsin (treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone [TPCK]), trypsin from porcine pancreas (20 µg/ampule, proteomics grade), iodoacetamide (alkylating reagent, 98%, IAA), dithiothreitol (reductant agent, >99%, DTT), urea (>99.5%), ammonium acetate (>99%), potassium dihydrogen phosphate (99.5%), potassium chloride (>99%),

ammonia (>99%), formic acid (>98%) and trifluoroacetic acid (>99%, TFA), phosphate buffered saline (PBS) and Polysorbate 20 (Tween 20) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl)-aminomethane (TRIS) and glycine were purchased from Bio-Rad Laboratories (California, USA). Sep-Pak C18 cartridges (200 mg, 3 mL) and Protein LoBind tubes of 1.5 mL were bought from Waters (Saint-Quentin, France) and from Eppendorf (Hamburg, Germany) respectively. Trypsin solutions were obtained by dissolving the enzyme in acetic acid (50 mM). DTT and IAA solutions were prepared by dissolving these reagents in Milli-Q water. A buffer solution of 100 mM was prepared by dissolving ammonium acetate in water and the pH was adjusted to 8 using a solution of 5 % NH_3 (v/v).

Five human urine samples from four healthy and one patient with renal failure were collected by the Central University Hospital of Asturias. 100 mL of first-void urine samples were collected in a sterile urine beaker with integrated urine transfer device (Greiner Bio-One GmbH, Kremsmünster, Austria). No antimicrobial additives were added to the samples. 10 mL of the sample were transferred to round base sterile Vacuete® urine tubes (Greiner Bio-One) and stored at -20ºC. All patients signed an informed consent and the work was carried out in accordance with the "World Medical Association Declaration of Helsinki" for the ethical principles for Medical Research involving human subjects.

2.2 Procedures

2.2.1 Sample treatment and enzymatic hydrolysis.

An analytical balance model MS205DU (Mettler Toledo, Zurich, Switzerland) was used for the gravimetric preparation of all solutions. An amount of 0.07 g of solid urea was

added to 0.20 mL of human urine to obtain a concentration of 6M. After that, the Eppendorf was vortexed for 30 s and 0.20 mL of ammonium acetate buffer were added to dilute the sample 1:1. Reduction of cysteine residues was carried out for 30 min at 37 °C after the addition of 0.020 mL of 200 mM DTT. Then, the alkylation of cysteine residues was carried out by adding 0.023 mL of 600 mM IAA and developing the reaction for 30 min in the dark and at room temperature. After that the excess of IAA was quenched by the addition of 0.025 mL of 600 mM DTT. Before proteolysis, the concentration of urea was reduced to 2M with the ammonium acetate buffer. Then, a known amount of T3* peptide was added and the digestion was carried out for 3 hours using a trypsin to substrate ratio (w/w) of 1:10, at pH 8 and 37ºC in accordance to our previous study [23]. The enzymatic reaction was stopped by the addition of 30 µL of TFA at 20% (v/v) to obtain a pH <2. Finally, the sample was centrifuge at 14000 g for 15 min and the supernatant was taken and evaporated to dryness.

2.2.2 Purification of the target peptide after proteolysis by semi-preparative liquid chromatography.

A semi-preparative strong cationic exchange (SCX) column was used to separate the target peptide from all other peptides and salts obtained after the proteolysis step. The purification of the urine samples by semi-preparative cation exchange liquid chromatography was carried out using a HPLC system (Agilent 1260 Infinity, Agilent Technologies) equipped with an analytical-scale fraction collector and a variable wavelength detector. The column used for this purpose was a strong cationic exchange Luna, 5 µm, SCX 100 Å column (10 × 250 mm) from Phenomenex. All samples were dried using a centrifugal vacuum concentrator (Genevac, Suffolk, UK) to remove water and organic solvents. A flow rate of 2.0 mL/min was employed using a solvent gradient

(C/D). Mobile phase C was KH_2PO_4, 5 mM, in acetonitrile/ water (1:4, v/v), pH 2.83. Mobile phase D was KH_2PO_4, 5 mM, plus KCl, 0.5 M, in acetonitrile/water (1:4, v/v), pH 2.83. The chromatographic method held the initial mobile phase composition 100% C constant for 3 min. Then, a linear gradient to 100% D up to 40 min was applied and kept for 5.0 min. Finally, the initial conditions were set up for column equilibration for 10 min. The dried extract was dissolved in 0.13 mL of phase C. Then, it was injected into the HPLC system equipped with an analytical-scale fraction collector and a variable wavelength detector. The retention time of the target peptide and the total chromatogram time were 30 and 70 min respectively. After SCX chromatography, the collected fraction, from 29.2 to 31.2 min, was evaporated, desalted by solid-phase extraction using C18 cartridges and finally dissolved in 0.1 mL of 0.1% formic acid in water before analysis by LC–MS/MS.

2.2.3. Purification of the target peptide after proteolysis by antibody based enrichment on magnetic beads

The alternative purification procedure was based on an antibody based enrichment of the target peptide on magnetic beads. This required the use of a magnetic rack Dynamag^{TM}-2 Magnet from Thermo Fisher Scientific, a Thermomixer R for thermostatization and a centrifuge 5810R D from Eppendorf (Hamburg, Germany) for the centrifugation of the samples. 50 µL of Protein G beads were incubated with 2 µg of antibody for 10 min at room temperature under magnetic stirring. Once the antibodies were bound to the beads, the tube was placed on the magnet and the supernatant was removed. For the immunoprecipitation of the target peptide the digested extract was dissolved in 0.4 mL of PBS and 0.06 mL of Tris 1 M at pH 7 and incubated for 60 min at room temperature forming the Dynabeads-Ab-peptide complex. After that, the

mixture was placed on the magnet, and the supernatant was removed. The complex was washed three times with 0.2 mL of PBS and Tween 20. Then, the target peptide was released from the antibody adding 0.02 mL of glycine 50 mM at pH 2.8. The mixture was placed on the magnet and the supernatant was taken and evaporated to dryness. Finally, the residue was dissolved in 0.05 mL of 0.1% formic acid in water before analysis by LC–MS/MS. A flow chart of the optimized sample preparation procedure using antibodies is given in Figure 1.

2.2.4 Analysis by LC-MS/MS.

The chromatographic separation of the target peptides, natural (T3) and labeled (T3*) from the urine matrix was carried out using a high-performance liquid chromatography (HPLC) system Agilent 1290 (Agilent Technologies, Santa Clara, CA) equipped with a reversed-phase AerisPeptide XB- C18 RP column (2.1 x 100 mm, 1.7 µm particle size, 100 Å pore size, Phenomenex). The HPLC system was connected to a triple quadrupole mass spectrometer Agilent 6460 equipped with an electrospray source with a jet stream. In all cases a volume of 20 µL was injected into the HPLC system. Mobile phases A and B were water and acetonitrile respectively, both with 0.1 % of formic acid. The chromatographic method (Table S1 of the Supporting Information) held the initial mobile phase composition at 2% B for 5 min. Then, several gradients were applied. First, a linear gradient to 12% B in 7 min was applied, then from 12 to 13% B until 9 min, from 13 to 50% until 13 min and from 50 to 80% until 17 min. Finally, the initial conditions were reached and the column was equilibrated for 2 min. The HPLC system was connected to the triple quadrupole mass spectrometer through an electrospray source (ESI) working in the positive ionization mode. The ion spray voltage was 5500 V and the nebulizer pressure was set at 55 psi. The drying gas flow rate and temperature

were 7.0 L min^{-1} and 300 ºC respectively, whereas the sheath gas temperature and flow were 300 ºC and 11 L min^{-1}, respectively. The double charged ion at 613.8 m/z $(M+2H)^{2+}$ was selected as precursor ion and two fragment ions, y_6^+ ($VGEYNK^+$ at 709.3 u) and y_9^+ ($DFAVGEYNK^+$ at 1042.5 u), were measured in the second quadrupole.

Two alternative Selected Reaction Monitoring (SRM) acquisition modes were evaluated: standard SRM (Mode 1) and low resolution SRM (Mode 2). When using the standard acquisition Mode 1, the resolution of both quadrupoles was kept at a Full Width at Half Maximum (FWHM) of 0.7 u and four SRM transitions were measured for the fragment ion y_6^+ (613.8→709.3, 614.3→710.3, 614.8→711.3 and 615.3→712.3) and for the fragment ion y_9^+ (613.8 →1042.5, 614.3 →1043.5, 614.8 →1044.5 and 615.3 →1045.5). When using the low resolution acquisition Mode 2, the bandwidth of the first quadrupole was increased to a FWHM value of 13 u and in the second quadrupole reduced to 0.6 u. The transitions measured in the acquisition mode 2 were 613.8→709.3, 613.8→710.3, 613.8→711.3 and 613.8→712.3 for the fragment ion y_6^+ and 613.8→1042.5, 613.8→1043.5, 613.8→1044.5 and 613.8→1045.5 for the fragment ion y_9^+. Table S2 of the Supporting Information summarizes the acquisition parameters, Nitrogen was used as collision gas. The collision energy and the fragmentor voltage were carefully optimized to obtain the maximum sensitivity in all SRM transitions. The optimum collision energy for all SRM transitions was found to be 15 eV and the optimum fragmentor voltage was 135 V in both acquisition modes. Data acquisition and processing was carried out using the Masshunter software (version B.03.01) from Agilent Technologies.

2.2.5 IDMS calculations.

The IDMS calculation procedure applied in this work and the advantages of using minimally labelled analogues have been described previously [28]. Briefly, it is based on the fact that the isotope composition of a mixture of natural abundance and isotopically labelled peptides is a linear function of the isotope composition of the pure peptides (both natural abundance and labelled). After the measurement of the isotope composition of the peptide mixture, the molar fractions of both peptide types (natural and labelled) can be calculated by multiple linear regression. The moles of the natural abundance peptide in the sample are calculated from the ratio of molar fractions which is equal to the ratio of moles between the natural abundance and the labelled peptide. As the amount of labelled peptide added is known and the molar fractions are measured, we can easily calculate the amount of natural abundance peptide which will also correspond to the amount of natural abundance protein (in moles).

3. RESULTS AND DISCUSSION

3.1 Selection of the acquisition mode for the measurement of the isotopic composition of the target peptide by LC-MS/MS.

Regular IDMS procedures applied for the determination of (bio)organic compounds make use of isotopically labelled compounds as internal standards and avoid mass overlap with the analyte by using multiply labelled analogues [19]. We propose the use of mass overlapping peptides labelled with only two ^{13}C atoms to minimise isotopic effects. Also, in contrast to regular IDMS procedures in which a calibration graph is required for quantification, we propose a direct quantification of the target peptide using the molar fractions of the analyte and labelled analogue obtained from the accurate measurement of the isotopic composition of the samples.

For the case of Cystatin C, two tryptic peptides could be selected for its quantification in real samples: peptides T3 (ALDFAVGEYNK) and T8 (TQPNLDNCPFHDQPHLK). However, Peptide T8 contains cysteine so it was not selected for further experiments due to the risk of artefactual modifications during the experimental workflow. Therefore, we decided to work with two fragments of the T3 peptide to check for potential bias or spectral interferences. The measurement of the isotope composition of the mixture of natural abundance (T3) and labelled peptide (T3*) by MS can be carried out measuring *n* consecutive masses using the Selected Ion Monitoring (SIM) mode with a single quadrupole. However, when working with tandem MS the isotopic distribution measured for the in-cell fragment ions obtained after collision induced dissociation (CID) does not reflect their theoretical isotope distribution because several isotopologues of the precursor molecule may contribute to the same isotopologue of the product ion. Theoretically, the calculation of the real isotope distribution of the product ion at *n* different consecutive masses would require up to n^2 transitions making this approach not practical from an experimental point of view. Alternatively, the isotope distribution of in-cell fragment ions measured by SRM can be theoretically calculated knowing the fragmentation mechanism or predicted by suitable SRM dedicated software such as IsoPatrn© [29] using a smaller number of carefully selected SRM transitions.

We have recently developed in our laboratory an alternative method for the measurement of the isotope composition of product ions which is particularly advantageous for IDMS using mass overlapping peptides (MOPs). The method is based on the adjustment of the resolution of the first quadrupole to transmit the whole precursor ion cluster to the collision cell [23]. The mass difference between a typical peptide measured at the $[M+2H^+]$ precursor ion and its $^{13}C_2$-labelled analogue is only 1

mass unit (charge +2) in the first quadrupole. Thus, by increasing the bandpass of the first quadrupole, the full isotopic distribution of an in-cell fragment ion can be directly measured with a small number of SRM transitions maintaining a standard resolution in the second mass analyzer. Using this approach, the obtained isotopic distribution can be directly compared with those calculated by standard software based on polynomial distributions of the isotope abundances of the different elements constituting the in-cell fragment ion [30]. As indicated before, this procedure is ideally suited to work with MOPs because the full transmission of the precursor ion cluster of the mixture into the collision cell is facilitated by the small mass difference between the natural abundance and labelled analogues, especially when the precursor ion is a doubly or triply charged ion.

In order to compare both acquisition modes, namely standard SRM (Mode 1) and low resolution SRM (Mode 2) a natural abundance standard of the target peptide was injected into the LC-MS/MS system to measure the isotopic distribution of the molecular fragments ions y_6^+ and y_9^+ using both acquisition modes. Figure 2 shows the comparison of the experimental with the theoretical values obtained according to reference [29] for acquisition Mode 1 and reference [30] for acquisition Mode 2 for the y_6^+ fragment . The uncertainty of the experimental values correspond to the standard deviation of n=3 injections into the LC-MS/MS system As can be seen, the experimental isotopic distributions for the y_6^+ fragment ion obtained by both acquisition modes agree well with the theoretical isotopic distributions. Similar results were obtained for the y_9^+ fragment.

In addition, Figure 3 shows the comparison of the LC-MS/MS chromatograms of the same urine sample containing 136 µg L^{-1} of cystatin C measured using the acquisition Modes 1 and 2. As observed in Figure 3 the higher ion transmission to the collision cell

achieved by acquisition Mode 2 leads to a significant increase (up to 2.5 fold) in the method sensitivity compared to the standard resolution SRM method. Therefore, providing the absence of spectral interferences, acquisition Mode 2 is more suitable when low levels of Cystatin C in urine must be measured.

3.2 Initial evaluation of the amount of labelled peptide required for urinary Cystatin C quantification in real samples

Cystatin C level in urine may increase approximately up to 200 fold in patients with kidney diseases [7, 10]. Thus, it is important to evaluate the amount of labelled T3* peptide required to accurately quantify Cystatin C in samples from both healthy and kidney failure patients for a routine basis. For this purpose, we analyzed four urine samples provided by the Central University Hospital of Asturias (Oviedo, Spain). In order to save the amount of available labelled peptide, 5 ng of T3* (0.1 g of a solution of 0.05 μg g^{-1}) were added to all samples before proteolysis. The samples were purified by semipreparative liquid chromatography as described in section 2.2.2 and the isotopic distributions of the molecular fragment ions y_6^+ and y_9^+ in urine samples were measured using acquisition modes 1 and 2. As can be seen in Figure 4 the concentration of Cystatin C in samples S1, S2 and S3 ranged from 20 to 137 ng g^{-1} while sample S4 showed a much higher concentration (from 1904 to 2083 ng g^{-1} depending the fragment ion and the acquisition mode). No significant difference was obtained between both molecular fragment ions y_6^+ and y_9^+ when using acquisition mode 1. However a significantly higher value for y_6^+ was obtained when using acquisition mode 2 in the less concentrated samples S1, S2 and S3. This could be explained by the presence of coeluting interfering compounds that are transmitted with the analyte when the mass resolution of the first quadrupole is decreased. This effect was not observed for the y_9^+

fragment and for both fragments in sample S4 probably due to the much higher Cystatin C concentration.

3.3 Recovery studies in fortified human urine samples using preparative liquid chromatography

The accuracy and precision of the proposed procedures were evaluated performing recovery studies in a fortified human urine sample. First, the endogenous concentration of Cystatin C was determined analyzing, six replicates of the same urine sample. For this purpose, 7 ng of the labelled peptide T3* were added before proteolysis. Then, the sample was treated according to the Experimental section and purified applying the semipreparative liquid chromatography procedure and measured by both acquisition modes. The average concentration obtained in the sample from all measurements was 82.85 ± 2.10 ng g^{-1} of protein or 7.61 ± 0.32 ng g^{-1} of tryptic peptide. Then, several aliquots of 0.2 mL of the same urine sample were fortified adding different amounts of natural abundance T3 peptide to yield theoretical Cystatin C concentrations of ca. 100, 130 and 670 ng g^{-1} and cover the expected range of cystatin C levels for healthy patients and for patients with renal failure. Also, a variable amount of the isotopically labelled peptide T3* was added to each replicate to obtain a similar concentration than that of the natural abundance peptide.

Three independent replicates for each concentration level were analyzed using both acquisition modes. The sample preparation was based on a tryptic proteolysis and a clean-up step applying semi-preparative strong cation exchange (SCX) liquid chromatography as described in the Experimental section. Table 1 and Table S3 of the Supporting Information show the recovery values obtained for each concentration level in human urine using both acquisition modes. As can be seen, the recoveries ranged

from 89 to 98% using Mode 1 and from 97 to 100% using Mode 2. The relative standard deviations were always lower than 5%. We did not observe worsening of the accuracy and precision of the recovery values when the concentration decreased from 700 to 100 ng g^{-1} with both acquisition modes for the fragment ions, y_6^+ and y_9^+. In contrast to the low concentrated urine samples analyzed in section 3.2 by acquisition mode 2, we did not observed at the studied concentration range any spectral interference from the reagents or from the sample matrix that could affect the accuracy and precision of the recovery values.

3.4 Recovery studies in fortified human urine samples using antibody based enrichment on magnetic beads.

In order to improve the sample purification we evaluate another strategy based on an antibody based enrichment of the target peptide on magnetic beads. To do that, it was necessary to optimize several parameters such as the amount of monoclonal antibody added to the sample (1, 2 and 5 µg), sample incubation time (1 to 23 h) and sample volume (0.2 and 0.4 mL). The best results were obtained with 2 µg, 1 h and 0.2 mL of sample, respectively. These conditions were used to carry out recovery studies in the same fortified urine sample analysed in section 3.3 at 90, 120 and 700 ng g^{-1} of putative cystatin C. Three independent replicates for each level were analyzed. As in the previous section, the minimally labelled peptide was added to each replicate before the proteolysis step. Due to the high efficiency of this purification step we measured the samples using only acquisition Mode 2 to obtain the highest sensitivity. Table 1 shows that, using this purification strategy, recoveries from 100 to 104%, with relative standard deviations lower than 6% were obtained. These results demonstrate that the enrichment of the target peptide using a monoclonal antibody can be successfully

employed to quantify Cystatin C in urine without worsening the analytical figures of merit of the method. Our results demonstrate that this strategy is able to recover significant and equal amounts of both natural and labelled peptide from the hydrolysed sample in a much shorter time compared to the purification based on semipreparative liquid chromatography. Figure 5 shows a LC-MS/MS chromatogram of a urine sample purified by semipreparative liquid chromatography and measured with Mode 2 overlaid with an LC-MS/MS chromatogram of the same sample purified by antibody based enrichment and also measured by Mode 2. As can be observed in Figure 5, the purification by semipreparative liquid chromatography provides a 2 fold higher sensitivity. However, when applying the purification based on inmunoafinity the sample preparation is much faster and a complete removal of spectral interferences is achieved. For example the total analysis time required for ten samples from sample collection to data treatment is reduced from 3 days when using semipreparative liquid chromatography to 8 hours when using the enrichment with the antibody on magnetic beads. The only drawback of the antibody sample preparation procedure is the high cost of the monoclonal antibody.

3.5 Stability of Cystatin C in human urine

Using the optimized conditions with the antibody-based sample preparation procedure we studied the stability of Cystatin C in urine samples under different storage conditions. A homogeneous urine sample of a healthy patient was divided in 24 aliquots of 0.2 mL. Twelve different storage conditions were evaluated combining three different temperatures in the dark (-20°C, 4°C and room temperature) and four different times (1, 8, 10 and 34 days). Two different aliquots of the sample were stored in each condition. Figure 6 and Table S4 of the Supporting information shows the Cystatin C

levels obtained for the y_6^+ fragment ion obtained in each aliquot (ng g^{-1}). In comparison to previous works, Herget-Rosenthal and coworkers [9] reported that Cystatin C was not stable in urine at -20ºC after 28 days whereas Ji et al [25] reported stability for 15 days at -20ºC. Our results show that cystatin C in urine seems to be stable at -20ºC after 34 days. When storing the samples at room temperature, Herget-Rosenthal and coworkers [9] encountered stability problems after three days but in our study the levels of Cystatin C only decreased significantly after 10 days.

CONCLUSIONS

This work presents the development of a methodology for the Cystatin C quantification in human urine by isotope dilution mass spectrometry. The proposed methodology comprises: i) the addition of a minimally ^{13}C-labelled proteotypic peptide before protein hydrolysis ii), the purification of the sample after proteolysis based on the affinity of the proteotypic peptide to a monoclonal antibody and iii) the measurement of the isotopic distribution of the proteotypic peptide by low resolution selected reaction monitoring (SRM). We demonstrated that the proposed low resolution SRM mode (Mode 2) provides a significant increase (up to 2.5 fold) in the method sensitivity compared to the standard resolution SRM method (Mode 1) and enabled the quantification of Cystatin C in human urine without the need of extra calibration runs of standards. We have demonstrated that using a fixed amount of labelled peptide (5 ng) it is possible to quantify Cystatin C in human urine in patients with normal and impaired renal function. Our results show that the purification of the sample by antibody based enrichment of the target peptide on magnetic beads removes more efficiently interfering compounds in comparison with a sample purification based on semipreparative cation exchange liquid chromatography. In addition, the total analysis time for ten samples decreases from 3

days to 8 hours. Quantitative recoveries from 100 to 104% were obtained in samples containing from 90 to 700 µg L-1 of Cystain C with relative standard deviations from 0.5 to 6 %. When purifying the sample by antibody based enrichment the precision obtained ranged from 0.8 to 4.8% (% RSD) and the recovery values ranged from 100 to 102% at differenty concentration levels. When analysing peptide standards the presence of interferences in our method was assessed by the comparison of the experimental isotopic distributions with the theoretical values. When analysing real samples the presence of interference was evaluated by performing recovery experiments at different concentration levels. In addition, our methodology allows an additional way to check for spectral interferences in real samples by examining the residuals of the multiple linear regression. In case of interferences, a lack of normality in the residuals and an increased squared sum of residuals would be observed. Finally, our results show that when the urine samples are stored during more than 10 days at room temperature, a significant degradation of Cystatin C is observed but when the samples are stored at -20ºC cystatin C in urine seems to be stable for 34 days.

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TABLES

Table 1. Recovery studies obtained in fortified human urine samples for the molecular fragment ions y_9^+ and y_6^+ using two different SRM acquisition modes (1 and 2) and two different sample purification strategies (semipreparative liquid chromatography and peptide enrichment using a monoclonal antibody). Uncertainty is expressed as the standard deviation of n=3 independent replicates. Values in brackets correspond to the Relative Standard Deviation (% RSD) of the recovery values.

Acquisition mode	Purification method	Cystatin C concentration (ng g^{-1})	Recovery (%) y_6^+	Recovery (%) y_9^+
Mode 1	Semipreparative liquid chromatography	136	94 ± 3 (3%)	91 ± 5 (5%)
		160	93 ± 3 (3%)	89 ± 3 (3%)
		700	98 ± 2 (2%)	98 ± 1 (1%)
Mode 2	Semipreparative liquid chromatography	136	99 ± 3 (3%)	97 ± 3 (3%)
		160	99 ± 4 (4%)	99 ± 1 (1%)
		700	100 ± 1 (1%)	100 ± 2 (2%)
Mode 2	antibody based enrichment	90	100 ± 4 (4%)	103 ± 2 (2%)
		120	102 ± 1 (1%)	104 ± 0.4 (0.5%)
		700	102 ± 6 (6%)	103 ± 5 (5%)

FIGURES

Figure 1. Sample preparation procedure applied in this work.

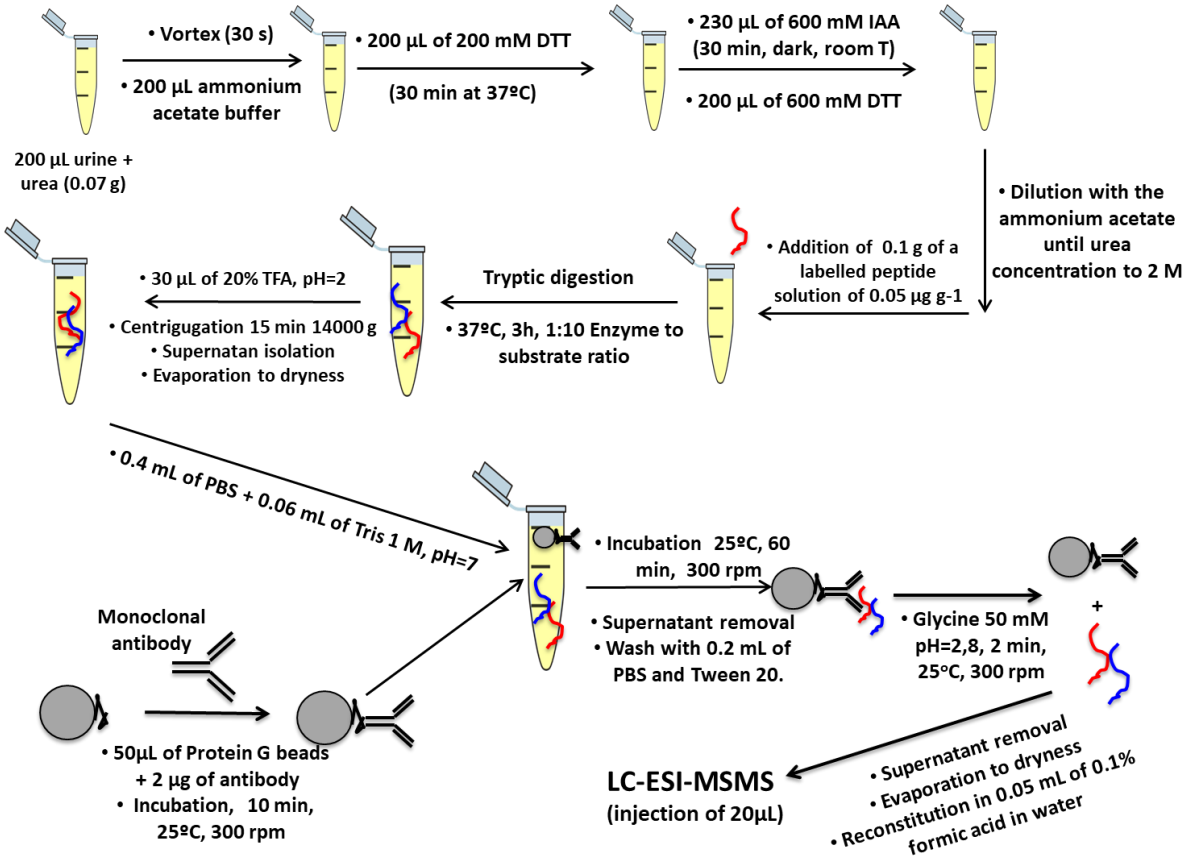

Figure 2. Comparison of the theoretical (dark grey) and experimental (clear grey) isotopic distributions obtained for the natural abundance molecular fragment ion y_6^+ using acquisition Modes 1 and 2. The theoretical isotopic distributions were obtained according to reference [29] for acquisition Mode 1 and reference [30] for acquisition Mode 2. The uncertainty of the experimental values represents the 1s standard deviation of n=3 independent injections into the LC-MS/MS system.

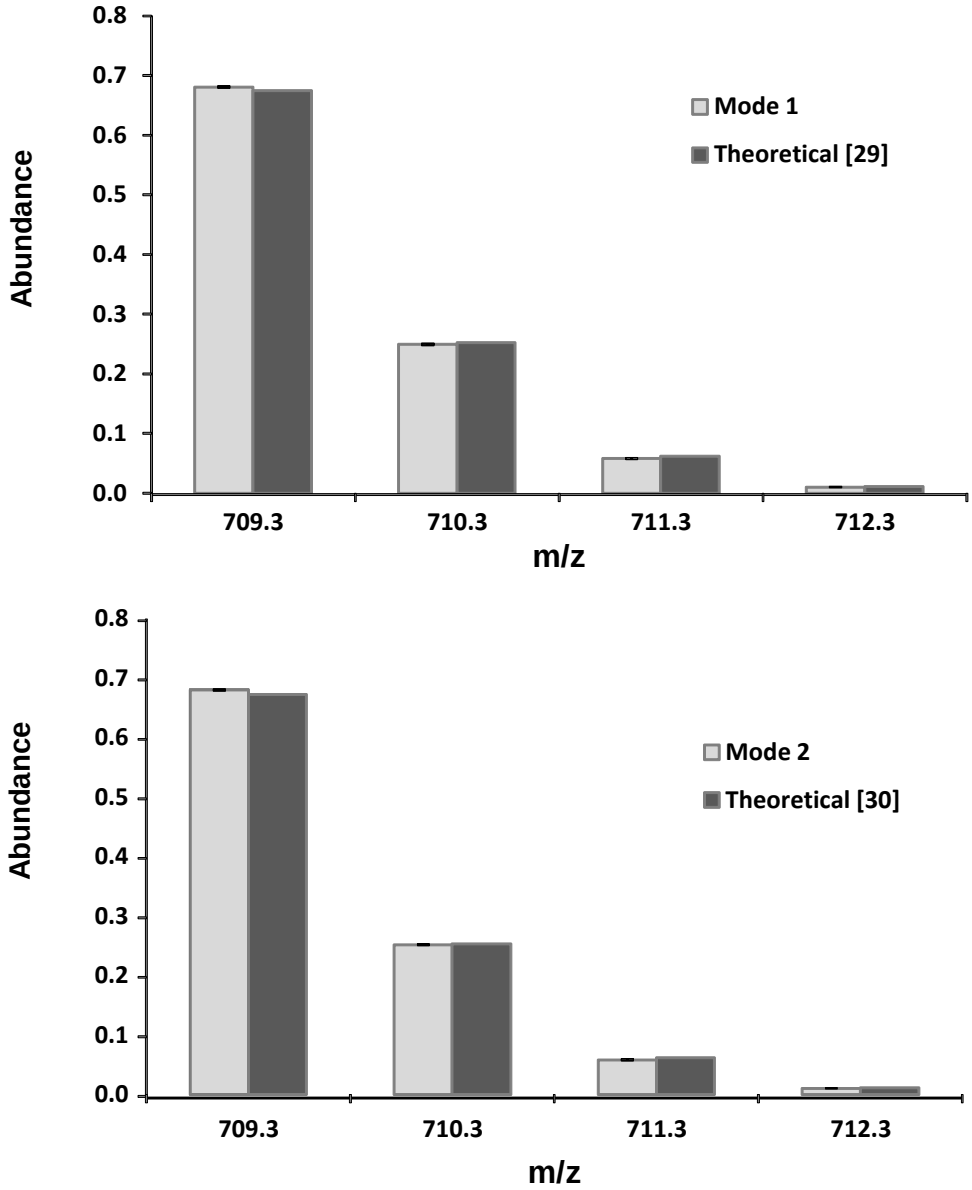

Figure 3. Comparison of LC-MS/MS chromatograms of a urine sample of 136 µg L^{-1} measured using the acquisition modes 1 (red) and 2 (black) for the molecular fragment ions y_6^+, (dashed line) and y_9^+, (solid line).

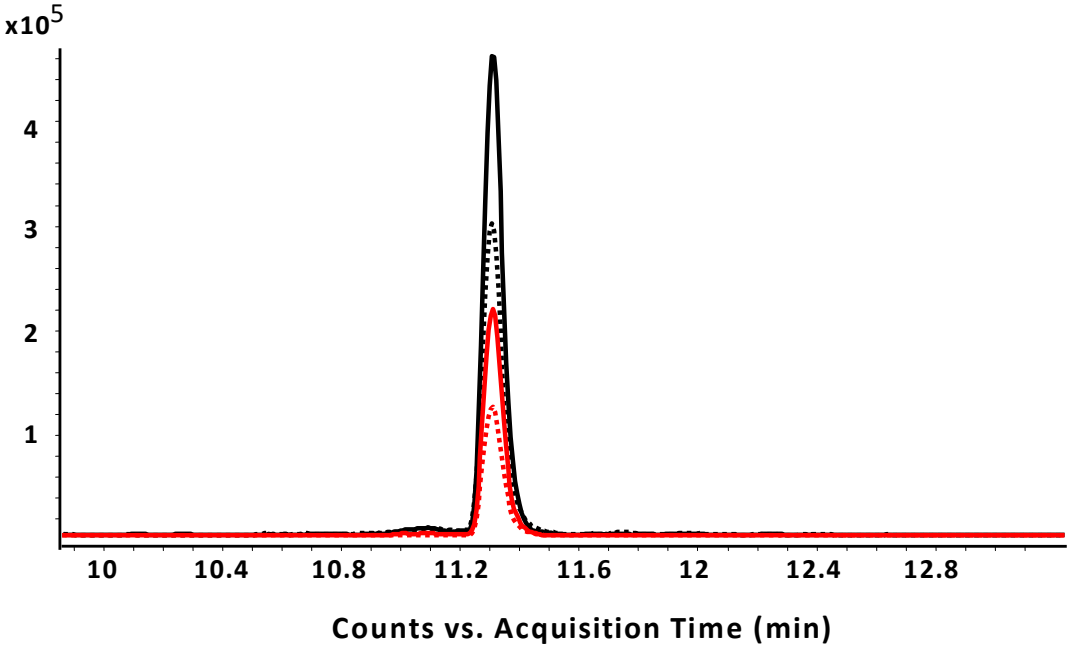

Figure 4. Cystatin C concentrations (ng g^{-1}) obtained in four human urine samples (S1, S2, S3 and S4) for molecular fragment ions y_6^+ and, y_9^+ using acquisition modes 1 and 2.

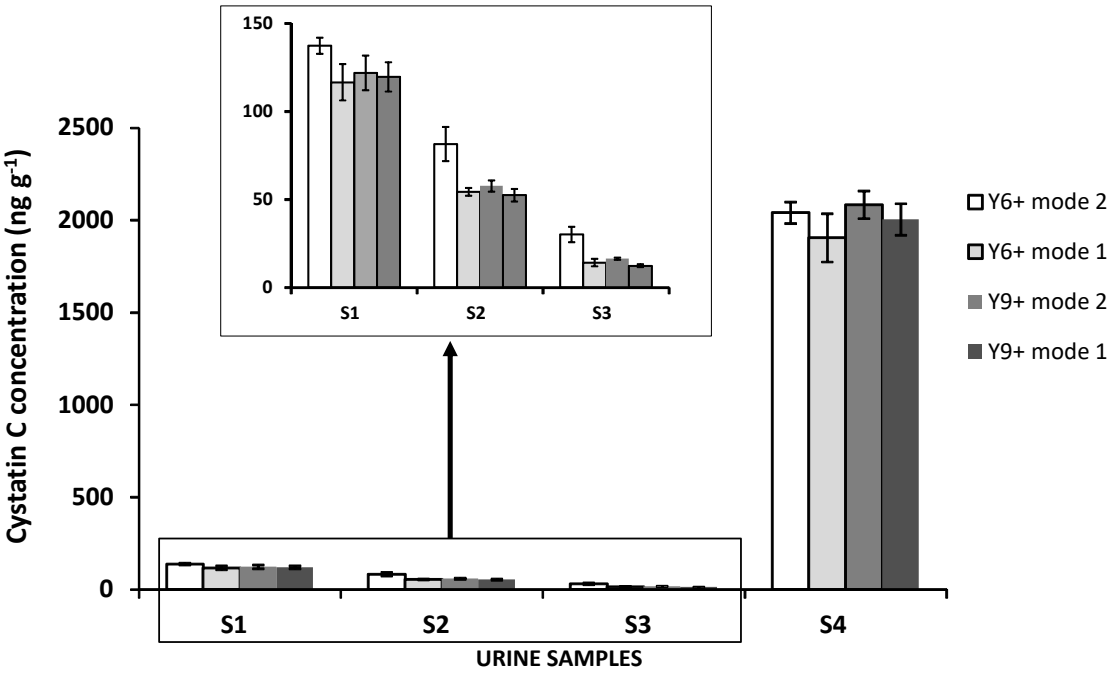

Figure 5. LC-MS/MS chromatograms (Total Ion Count of all transitions measured for y_6^+ and y_9^+ using acquisition Mode 2) of a human urine sample of 90 ng g^{-1} of Cystatin C. The black line corresponds to the sample purified by semipreparative liquid chromatography and the red line corresponds to the same sample purified by antibody based enrichment.

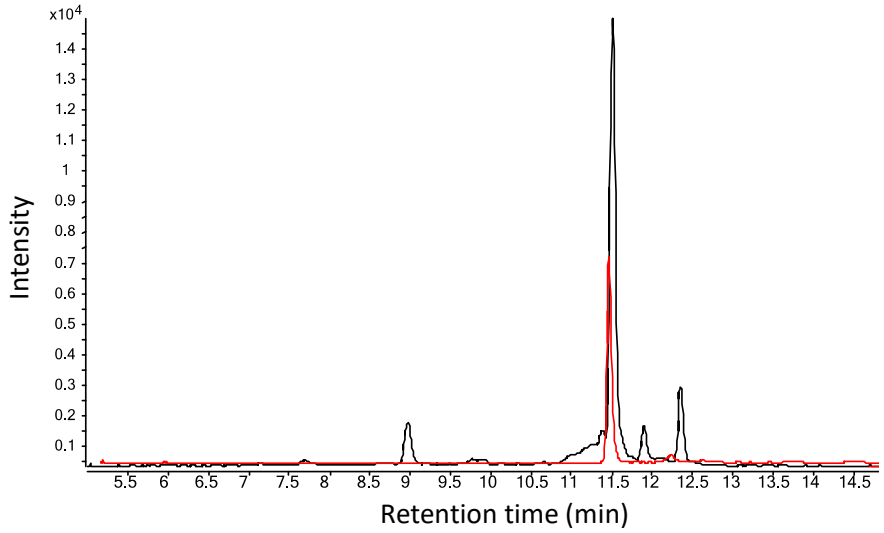

Figure 6. Duplicate values (1 and 2) of Cystatin C (ng g^{-1}) obtained in 24 aliquots of the same human urine sample stored at different temperatures (room temperature of ca. 25ºC, 4ºC and -20 ºC) for 1 (black), 8 (white), 10 (grey) and 34 (dark grey) days. Error bars correspond to the standard deviation of three independent injections into the LC-MS/MS system.