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Immunoprecipitation on magnetic beads and liquid chromatography–tandem mass spectrometry for carbonic anhydrase II quantification in human serum

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ABSTRACT

In this study, a magnetic bead-based platform amenable to high-throughput protein carbonic anhydrase II (CA II) capture is presented. The key steps in this approach involved immunoaffinity purification of the target protein from serum followed by on-bead digestion with trypsin to release a surrogate peptide. This tryptic peptide was quantified by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) operating in multiple reaction monitoring acquisition mode. Using a synthetic peptide standard and a structural analogue free-labeled internal standard, the resulting concentration was stoichiometrically converted to CA II serum concentration. The analytical steps, such as preparation of immunobeads, protein capture, proteolysis, and calibration, were optimized. The method was validated in terms of recovery (77%), reproducibility (relative standard deviation [RSD] < 12%), and method detection limit (0.5 pmol ml^{-1}). The developed method was applied to determining the CA II in eight healthy subjects, and the concentration measured was 27.3 pmol ml^{-1} (RSD = 65%).

Most of the studies dealing with biomarker discovery on blood are focused on the cell leakage products potentially present in that matrix; indeed, increased serum levels of various proteins are routinely used for diagnostic purposes [1–4]. The analysis of serum is challenging because of the large dynamic range of protein concentrations, spanning more than 10 orders of magnitude [1,5]. In particular, the presence of high-abundance proteins such as albumin and immunoglobulins, whose concentrations represent 60–90% of the total serum protein content, enhances the difficulty of detecting the low-abundance proteins of interest [5]. Recently, a multisite assessment of the precision and reproducibility of multiple reaction monitoring (MRM)[1]-based measurements of target proteins in plasma demonstrated that this platform may be very reproducible for proteins present at moderate to high abundance (>2 mg/ml) in nondepleted, nonfractionated plasma [6].

On the contrary, the reduction of sample complexity is an essential first step in the analysis of the serum low-abundance proteins, and it is often achieved by high-abundance protein depletion [7]. A common approach is the affinity removal method using antibody-based resins (both monoclonal and polyclonal) or affinity dye-based resins [7–9]. There are several affinity removal columns or kits commercially available for depleting up to 20 major abundant serum proteins [9,10]. Although this strategy is highly specific, even after depletion the remaining proteins are still sufficiently abundant to hamper the low-abundance protein determination. Moreover, some authors have recently pointed out the risk of losing low-abundance proteins and biomarkers when using affinity-based depletion due to the association of the targeted proteins with the abundant ones [5,9,11,12], but different results have also been published [12–15].

Another method of reducing serum sample complexity is the filtration through molecular weight cutoff membranes [9,11] for removing the high-molecular-weight proteins, including albumin. Solvent precipitation has also been employed, in particular for albumin depletion [9]; the method is nonspecific but presents the advantage of being rapid and cheap compared with the immunoaffinity-based methods. For isolation of the fraction containing the target proteins, liquid chromatographic and electrophoretic fractionation techniques have been used [9].

A promising approach is the employment of affinity reagents for specific enrichment or isolation of target proteins [5,16]. Recently, increasing attention has been given to the development and application of separation techniques employing small magnetic particles. Magnetic carriers bearing an immobilized affinity or hydrophobic ligand or ion exchange groups, or magnetic biopolymer particles

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[1] *Abbreviations used:* MRM, multiple reaction monitoring; MS, mass spectrometry; LC, liquid chromatography; CA II, carbonic anhydrase II; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; IS, internal standard; DMP, dimethyl pimelimidate dihydrochloride; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; DTT, 1,4-dithiothreitol; IAA, iodoacetamide; RT, room temperature; SPE, solid phase extraction; TISP, TurbolonSpray; MARS, Multiple Affinity Removal System; RSD, relative standard deviation; IgG, immunoglobulin G; MDL, method detection limit; MQL, method quantification limit; RP–HPLC, reverse phase high-performance liquid chromatography; LOD, limit of detection; MIL, method identification limit; MQL, method quantification limit; S/N, signal/noise.

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having affinity to the isolated structure, are mixed with the sample containing the target compound [17]. Following an incubation period in which the target compound binds to the magnetic particles, the whole magnetic complex is easily and rapidly removed from the sample using an appropriate magnetic separator. The isolated target compound can then be eluted and used for downstream applications and detection methods.

Several types of mass spectrometry (MS) techniques in conjunction with various liquid chromatography (LC) separation methods are adopted for proteomic measurements. In particular, the MRM acquisition mode is the most employed one in targeted proteomics [1,5,18–20]. For absolute quantitative analysis, labeling and label-free strategies [21] are currently carried out, both aiming to correlate the mass spectrometric signal of proteotypic peptide with the relative or absolute protein quantity directly [22].

Carbonic anhydrase II (CA II), a single polypeptide chain of 29 kDa molecular weight, is present in the cytosol of most tissues even if the highest concentration is found in erythrocytes. It catalyzes the hydration of CO_2 and the hydrolysis of esters, and its deficiency has been associated with pathological consequences such as mental retardation and cerebral calcification, osteoporosis, and renal tubular acidosis [23,24] as well as Down syndrome [25,26] and Alzheimer's disease [26]. Although serum is not the natural site of CA II, in consequence of cell release, this protein could be present at low concentration in serum. Furthermore, because CA II is involved in serious diseases, it represents an interesting model to investigate the separation, identification, and quantification of proteins present in low concentration in complex matrices.

In a recent study by our laboratory [27], CA II quantification in serum was performed by automated LC chip technology coupled with an electrospray ionization (ESI) source and a triple quadrupole mass spectrometer, operating in MRM acquisition mode, after purification by reversed phase LC, enzymatic digestion, and surrogate peptide selection. The highly sensitive nanoelectrospray chip technology was necessary because only 1 μl of serum could be submitted to the LC isolation step without column overloading.

In the current study, a Protein G magnetic bead-based antibody platform amenable to high throughput is proposed for the selective enrichment of CA II in human serum, followed by its accurate and reliable quantification by a label-free procedure performed on a less technologically sophisticated LC–ESI–tandem mass spectrometry (MS/MS) system. Magnetic beads bound to polyclonal anti-CA II antibodies as affinity probes to isolate the specific target protein, and a conventional LC–ESI–MS/MS apparatus amenable to small molecule quantitation, were used for the determination of the CA II proteotypic peptide produced through proteolysis.

Materials and methods

Materials

Anti-CA II polyclonal antibody was purchased from U.S. Biological (Swampscott, MA, USA), and Dynabeads Protein G were obtained from Invitrogen (Carlsbad, CA, USA). Synthetic peptide standards (5 mg each, certified title $\geqslant$95%), corresponding to GGPLDGTYR (CA II proteotypic peptide) and GGPLEGTYR (internal standard [IS]), were purchased from CRIBI Center (Padova University, Italy). The human CA II standard (<80%), dimethyl pimelimidate dihydrochloride (DMP), triethanolamine, Tween 20, Bradford reagent, ammonium bicarbonate, phosphate-buffered saline (PBS, pH 7.2), and trifluoroacetic acid (TFA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane, 1,4-dithiothreitol (DTT), iodoacetamide (IAA), urea, and protease inhibitor mix were purchased from GE Healthcare (Uppsala, Sweden). All organic solvents were of the highest grade available from Carlo Erba Reagents (Milan, Italy) and were used without any further purification. Ultrapure water was produced from distilled water by a Milli-Q system (Millipore, Billerica, MA, USA). Modified porcine trypsin, sequencing grade, was commercialized by Promega (Madison, WI, USA).

Peptide stock solutions were prepared in 0.1% TFA at 1 g L^{-1} and stored at −20 °C. The human CA II standard was reconstituted with 0.1% TFA at 1 g L^{-1} and stored at −80 °C. Its actual title was tested by the Bradford assay and was found to be 85 ± 5%.

Samples

Serum samples were obtained from the Department of Experimental Medicine at Sapienza University of Rome (Italy) by venipuncture of healthy volunteers (20–40 years of age), with collection done in a BD P100 Blood Collection System (Becton Dickinson, Franklin Lakes, NJ, USA) with K_2EDTA anticoagulant and protease inhibitors mix (GE Healthcare). After clot formation, the sample was centrifuged at 1000*g* for 15 min. The serum was removed, and aliquots were stored at −80 °C. All serum samples were checked to verify the absence of hemolysis.

Immobilization of antibody on magnetic beads

The antibody solution (400 μl of 1 μg μl^{-1} anti-CA II) was added into an Eppendorf microcentrifuge tube containing 200 μl (6 mg) of prewashed Dynabeads Protein G and incubated by gentle mixing at room temperature (RT) for 2 h to allow time to attach onto the surface of the beads. Following incubation, residual unbound antibody was removed by washing two times with 0.5 ml of citrate phosphate buffer (pH 5.0) with 0.05% Tween 20. Approximately 10 μg of antibody was bound per milligram of beads. For cross-linking of antibody to beads, the Dynabeads Protein G with immobilized polyclonal antibody were washed with 1 ml of 0.2 mol L^{-1} triethanolamine (pH 8.2) and resuspended in 1 ml of freshly prepared cross-linking buffer (20 mmol L^{-1} DMP in 0.2 mol L^{-1} triethanolamine, pH 8.2). The mixture was incubated under gentle rotation for 30 min at RT and placed on a magnet, and then the supernatant was discarded. To stop the reaction, the beads were resuspended in 1 ml of 50 mmol L^{-1} Tris (pH 7.5) and incubated for 15 min, and then the beads were rinsed three times with 1 ml of 100 mmol L^{-1} PBS with 0.05% Tween 20 before use.

CA II capture and digestion

To capture CA II, the Dynabeads Protein G with immobilized antibody were incubated at 4 °C for 1 h, under gentle mixing, with 20 μl of serum diluted with 100 mM PBS (1:49, v/v), and then the beads were recovered from the sample and washed three times with 1 ml of 100 mmol L^{-1} PBS. Immunocomplex was resuspended in 40 μl of 6 mol L^{-1} urea solution in 25 mmol L^{-1} ammonium bicarbonate and 2 μl of 10 mmol L^{-1} DTT and incubated at 37 °C for 1 h, under slight agitation, to denature captured antigen. Then 8 μl of 10 mmol L^{-1} IAA was added, and the immunocomplex was incubated at RT for 1 h in the dark. Subsequently, 8 μl of 10 mmol L^{-1} DTT was added and incubated at 37 °C for 1 h, under slight agitation, to consume any leftover alkylating agent and to avoid trypsin alkylation. The mixture was then diluted with 25 mmol L^{-1} ammonium bicarbonate to obtain a final urea concentration of 1 mol L^{-1}. Reconstituted trypsin solution (20 μg ml^{-1} in 25 mmol L^{-1} ammonium bicarbonate) was added to a final concentration of 5.8 μg ml^{-1}. The samples were allowed to digest under gentle mixing overnight at 37 °C, the digestion was quenched by adding 4 μl of formic acid, and then 50 μl of 3 pg ml^{-1} IS solution was added. The supernatant (∼400 μl) containing the peptides coming from both antigen and antibody digestion was recovered

using a magnet, and an aliquot was subjected to LC–MS/MS analysis.

Chromatographic and mass spectrometric conditions

The LC apparatus consisted of a series 200 binary LC micropump, a series 200 binary LC pump, two vacuum degassers, an autosampler (PerkinElmer, Norwalk, CT, USA), and an eight-port Valco valve equipped with a 100-μl peek loop (VICI AG International, Schenkon, Switzerland). The LC separation was carried out on a Jupiter Proteo 4-μm C12 column (150 × 1 mm i.d., 4 μm average particle size, 90 Å pore size) equipped with a 2.1-mm i.d. guard column from Phenomenex (Torrance, CA, USA). A C18 trap column (4.0 × 2.0 mm i.d.) supplied by Phenomenex was used for on-line solid phase extraction (SPE) with 100 μl of samples being injected.

ESI–MS was carried out on an API 3000 triple quadrupole instrument equipped with a TurboIonSpray (TISP) interface and with a built-in software-controlled eight-port valve (Applied Biosystems/MDS Sciex, Concord, ON, Canada). The LC–MS system, data acquisition, and processing were managed by Analyst software (version 1.4.2, Applied Biosystems/MDS Sciex).

The scheme of the on-line system is shown in Fig. 1. The two alternate positions of the software-controlled eight-port valve allowed flow switching in the trap column. The LC binary pump (pump 1) was used to deliver a 300-μl/min flow rate through the trap column for loading and washing the injected sample, whereas the LC binary micropump (pump 2) was used to deliver a 70-μl/min flow rate for eluting the analyte from the trap column in back flushing mode, for carrying out the chromatographic run, and subsequently for flushing and equilibrating the column. The mobile phases used for sample loading and washing (pump 1) were water (A) and acetonitrile (B), with both containing 1% (v/v) formic acid. Mobile phases for analyte elution (pump 2) consisted of water (C) and acetonitrile/methanol (60:40, v/v) (D), with both containing 0.1% (v/v) formic acid.

The sample was loaded onto the trap column and desalted by washing with 2% B for 2 min. Elution was performed by switching the eight-port valve; after an isocratic step at 10% D for 1 min, D was linearly increased to 40% within 7 min and then the eight-port valve was resettled to the starting position and B and D were brought to 95% within 1 min and held constant for 4 min to rinse the column and trap column. Finally, the D content was lowered

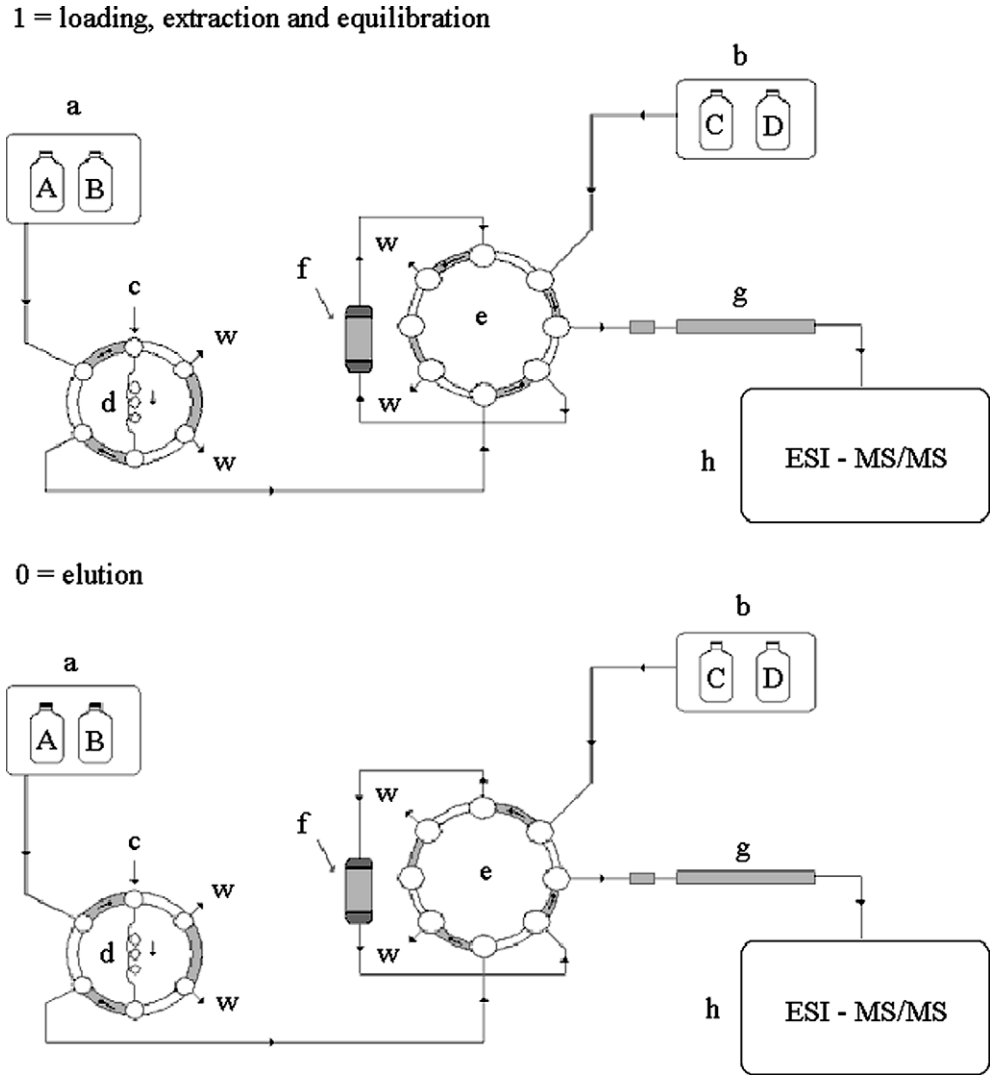

Fig. 1. On-line LC–MS/MS CA II proteotypic peptide analysis after off-line immunoextraction and tryptic digestion. Analytical system scheme: 1, position used for sample loading, extraction, analytical column equilibration, and cartridge regeneration; 0, position used for peptide elution from the extraction/enrichment trap column and peptide separation by the analytical column. Mobile phases: A, water; B, acetonitrile (containing 1% [v/v] HCOOH); C, water; D, acetonitrile/methanol (60:40, v/v, both containing 0.1% [v/v] HCOOH). a, pump 1; b, pump 2; c, autosampler; d, 100-μl loop; e, eight-port valve; f, extraction/concentration trap column; g, analytical column; h, mass spectrometer; w, waste.

to 10% and the B content was lowered to 2% over 1 min, and both the column and trap column were reequilibrated for 15 min.

The mass spectrometer was calibrated using polypropylene glycol as standard (Applied Biosystems). Ionization and mass spectrometric conditions were optimized for both of the peptides (CA II-specific peptide and IS peptide) by infusing at a 5-μl min^{-1} flow rate a 0.1-ng μl^{-1} solution prepared in water/acetonitrile/formic acid (70:30:0.1, v/v/v). TISP interface was operated in the positive ionization mode by applying to the capillary a voltage of 5500 V. Nitrogen was used as curtain, nebulizing, and turbo spray gases (heated at 300 °C), and the gas pressures were set at 20, 30, and 40 psi, respectively. Nitrogen, kept at medium pressure (arbitrary units), also served as collision gas.

Diprotonated molecules ($[M + 2H]^{2+}$) were mass selected by the first quadrupole and fragmented. In the product ion scan mode, the range of m/z 200–1000 was monitored for both CA II proteotypic peptide and IS.

A flow chart of the analytical procedure is shown in Fig. 2.

Calibration and recovery studies

Standard solutions for calibration were prepared by drawing the appropriate volume of the CA II-specific peptide GGPLDGTYR working standard solution so as to obtain nine concentration levels in the range of 0.05–100 pg μl^{-1} and 50 μl of a 3-pg μl^{-1} IS peptide GGPLEGTYR working standard solution. The solvent was evaporated at 37 °C under a gentle N_2 stream, and then the solution was reconstituted with 400 μl of 25 mmol L^{-1} ammonium bicarbonate acidified with formic acid (25 mmol L^{-1}). Volumes of 100 μl were injected. The ion current profiles of the selected transition pairs for the two peptides were extracted from the LC MRM dataset, the resulting peak areas were measured, and the plot of the ratio between the peak areas of the CA II synthetic peptide and the IS peptide versus concentration was obtained. The matrix-matched calibration line was also constructed by using a serum pool obtained by mixing serum aliquots from the eight healthy subjects. Nine 20-μl aliquots were processed as reported above. Next, to obtain the same concentrations as for the external calibration, the samples were transferred into vials spiked with the suitable volume of standard and IS solutions after solvent evaporation. All samples were run in triplicate interleaving three blank samples before the next series of injections, and results were averaged.

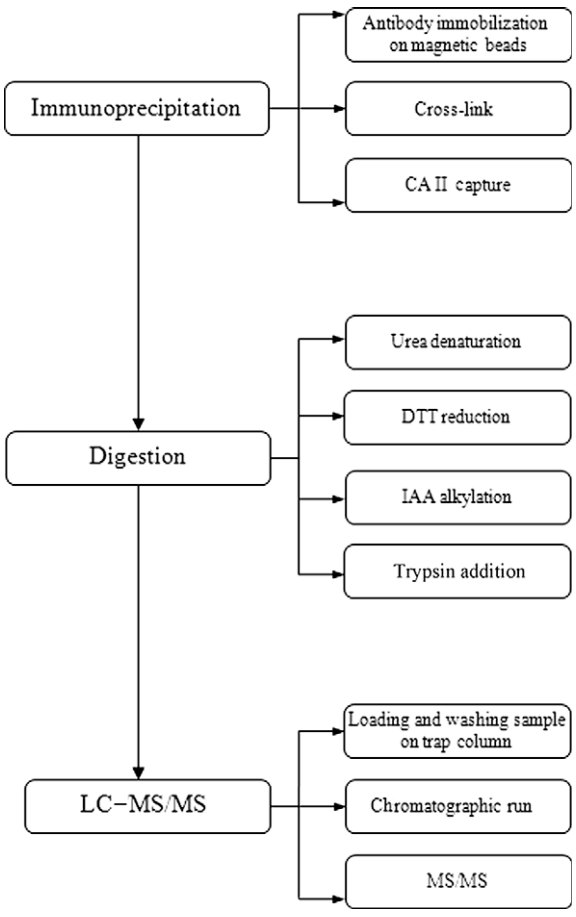

Fig. 2. Flow chart of the analytical procedure.

From the initial set of candidates, two suitable transition pairs were chosen for acquisition in MRM mode for both peptides. The declustering, entrance, and focusing potentials were maintained at the optimal values of 28, 7, and 370 V, respectively, whereas collision energy was optimized for each ion. The LC–ESI–MS/MS parameters are summarized in Table 1.

Recovery studies were conducted by spiking human serum samples with different amounts of CA II standard at different procedure steps while maintaining the IS amount constant.

Results and discussion

Abundant protein depletion

To avoid significant interference due to nonspecific interaction of the most abundant serum proteins with the antibody capture of CA II, in a first attempt, immunoaffinity depletion of serum samples was carried out as the first analytical step before immunoprecipitation. Depletion was performed on the Agilent Human-14 Multiple Affinity Removal System (MARS) immunoaffinity spin cartridge according to the recommendations of the manufacturer. After depletion, the sample was handled as described in Materials and methods. Although recoveries from spiked samples were 80–90%, quantitative determinations on unspiked individual serum samples gave both lower concentrations and lower between-sample standard deviations (16.3 pmol ml^{-1} and relative standard deviation [RSD] = 18%, respectively, $n = 8$) compared with the previous results obtained in our laboratory [27]. This result suggested that serum depletion may lead to loss of CA II to an unpredictable extent. Probably, the CA II is concomitantly removed during depletion due to the nonspecific binding to depleted proteins [28]. Thus, immunoprecipitation of CA II was optimized on whole serum.

Development of magnetic separation technique

Among the different commercially available magnetic bead systems tested, characterized by a variety of surface chemistries, the Dynabeads Protein G were chosen for the CA II antigen capture procedure. Indeed, this magnetic bead system showed high antibody coupling efficiency, good functional orientation of antibody for antigen capture, low background binding of serum peptides and proteins, and appropriate specificity for the succeeding MS analysis.

During method development, each step was optimized to maximize the final recovery of CA II. An increasing amount of anti-CA II polyclonal antibody (25, 50, 100, and 150 μg) was incubated with a fixed amount of Dynabeads Protein G (50 μl, corresponding to 1.5 mg of beads) in a fixed sample volume of 100 μl, keeping the incubation time constant (5 h). An estimate of the antibody amount bound to Protein G magnetic beads was performed by comparing protein concentrations measured by Bradford assay by using an immunoglobulin G (IgG) standard. The supernatants

Table 1
Instrumental parameter settings under MRM conditions for the two peptides: CA II proteotypic peptide and IS peptide.

Peptide	Retention time (min)	Precursor ion	MRM transitions (m/z)	Ion type	Relative collision energy (%)[a]	Relative abundance (%)
GGPLDGTYR	4.2	$[M + 2H]^{2+}$	468.3 → 411.2	y^{2+}	20	30
CA II peptide			468.3 → 496.3	y	17	100
GGPLEGTYR	4.0	$[M + 2H]^{2+}$	475.3 → 418.2	y^{2+}	21	90
IS peptide			475.3 → 625.3	y	16	100

[a] Relative collision energy is expressed as percentage with respect to the maximum voltage difference value between the high-pressure entrance quadrupole and the collisional cell quadrupole (± 130 V) permitted by the instrument.

containing the unbound antibody were discarded, and the complex was washed twice with the washing buffer to remove unbound antibody. Then the antibody was recovered after complex dissociation with citrate buffer (pH 2.5), and its concentration was determined spectrophotometrically. The bonded amount of anti-CA II polyclonal antibody increased up to 14.9 ± 0.5 μg for 50 μl of bead solution using 100 μg of antibody. This ratio (6.7 μg antibody/mg beads) is very similar to that reported by Berna and coworkers [29] for a different antibody (8 μg antibody/mg beads). Next, keeping constant the amount of anti-CA II polyclonal antibody (100 μg) and the amount of Dynabeads Protein G (50 μl), the efficiency of the Dynabeads Protein G/anti-CA II polyclonal antibody binding reaction was tested by increasing the incubation time (1 h, 2 h, 4 h, 8 h, and overnight). No significant increase in the binding reaction was observed from 2 h to overnight incubation times. The effect of cross-linking between Dynabeads Protein G and anti-CA II polyclonal antibody was also investigated. In all cases in which the anti-CA II polyclonal antibody was covalently bound to the magnetic beads by a cross-linking reaction, the LC–MS/MS analysis of the peptide at m/z 468.2 ($[M + 2H]^{2+}$) exhibited a significant peak area increase (>30%). Probably, when cross-linking was not performed, a fraction of the immunocomplex was lost during washing. After the best conditions for obtaining the complex antibody beads had been determined, we optimized the incubation volume and the incubation time for serum samples. The same amount of magnetic beads with immobilized antibody (200 μl) was used for extracting 20 μl of serum spiked with 0.5 pmol of CA II standard and diluted in different volumes (200, 500, 750, and 1000 μl) of PBS. The samples were incubated overnight at 4 °C. The best LC–MS/MS result in terms of recovery was obtained using 20 μl of serum diluted to 500 μl with PBS buffer. Moreover, the effect of sample incubation time was investigated (30 min, 1 h, 2 h, 4 h, 8 h, and overnight) using 20 μl of diluted serum. Recovery did not further increase after 1 h of incubation, whereas for higher incubation times an increase of background signal in the MRM mass chromatogram was noted, probably due to the extraction of other proteins by means of nonspecific interactions.

Digestion efficiency

The enzymatic digestion protocol was optimized to obtain the best peptide formation yield. For this purpose, the digestion efficiency was checked by spiking immunocomplex sample after immunoprecipitation with a known amount of CA II standard (2 nmol) while maintaining a constant volume (400 μl), and the recovery of surrogate peptide by increasing the trypsin concentration was evaluated by LC–MS/MS. Experiments were done in quadruplicate, and the results are reported in Table 2. A recovery of 97 ± 3% was obtained by adding to approximately 250 μl of sample 100 μl of a trypsin solution (20 ng μl^{-1}), suggesting that in these conditions the proteotypic peptide released from CA II digestion could stoichiometrically represent the absolute amount of its parent protein in serum. This concentration of enzyme is higher than that usually adopted in other protocols, including those adopted by our laboratory in a previous study [27,29].

Table 2
Hydrolysis yield.

Trypsin (mg)	Peptide (%)	RSD (%)
0.5	48	18
1.0	83	8
2.0	97	3
3.0	95	5

Note. CA II standard added: 2 nmol, total volume = 400 μl.

Method validation

To determine the performance characteristics of the magnetic beads-based capture system coupled to LC–ESI–MS/MS analysis, we assessed linear range, method detection limit (MDL), method quantification limit (MQL), recovery, accuracy, and precision.

In addition to ionization efficiency and reproducibility, the selected proteotypic peptides also need to meet the proteotypic selection criteria described in literature [30,31]. In our previous work based on protein isolation by reverse phase high-performance liquid chromatography (RP–HPLC) before protein digestion, the peptide GGPLDGTYR was selected as an appropriate peptide of CA II and the peptide GGPLEGTYR, having very similar chemical and physical properties, was selected as the relative IS. The benefits and limits of a nonlabeled peptide as IS have been discussed previously [32]. For this reason, they were also the first candidates for this method. From the product ion MS/MS spectrum of diprotonated GGPLDGTYR peptide at m/z 468.3 (shown in Fig. 3) and diprotonated GGPLEGTYR at m/z 475.3, the two most intense transitions were chosen for MRM acquisition. The supernatant containing the tryptic cleaved peptide mixture was separated from magnetic beads, and an aliquot was injected into the LC–ESI–MS/MS system. The mass chromatograms relevant to the two selected transitions for CA II-specific peptide are shown in Fig. 4A and B, and we see that background compounds present in the sample give, for the transition 468.3 → 212.3 (Fig. 4A), a signal poorly resolved from that relevant to the proteotypic peptide. This problem was solved by changing the selected transition 468.3 → 212.3 to 468.3 → 496.3 (Fig. 4C) that did not show background peaks. The IS was eluted approximately 0.2 min earlier and did not show any background interference.

Peptide adsorption is an important factor that may affect accuracy and reproducibility. To reduce peptide adsorptive processes, protein low-retention tubes were used. Surface adsorption phenomena are ruled by the adsorption constant, so the adsorbed compound amount decreases as the solvent volume increases. In addition, salt removal by means of C18 mini-columns is a time-consuming critical step in the peptide analysis protocols. Bearing in mind these considerations, we devised an on-line injection system that permitted us to inject by an autosampler a relatively large volume (100 μl) into a small bore (1 mm i.d.) column. Our system is more complicated than that previously used for a similar study [29], but the current system has some advantages: (i) the injection and trap washing with water can be made during analytical column equilibration, (ii) highly retained compounds did not enter

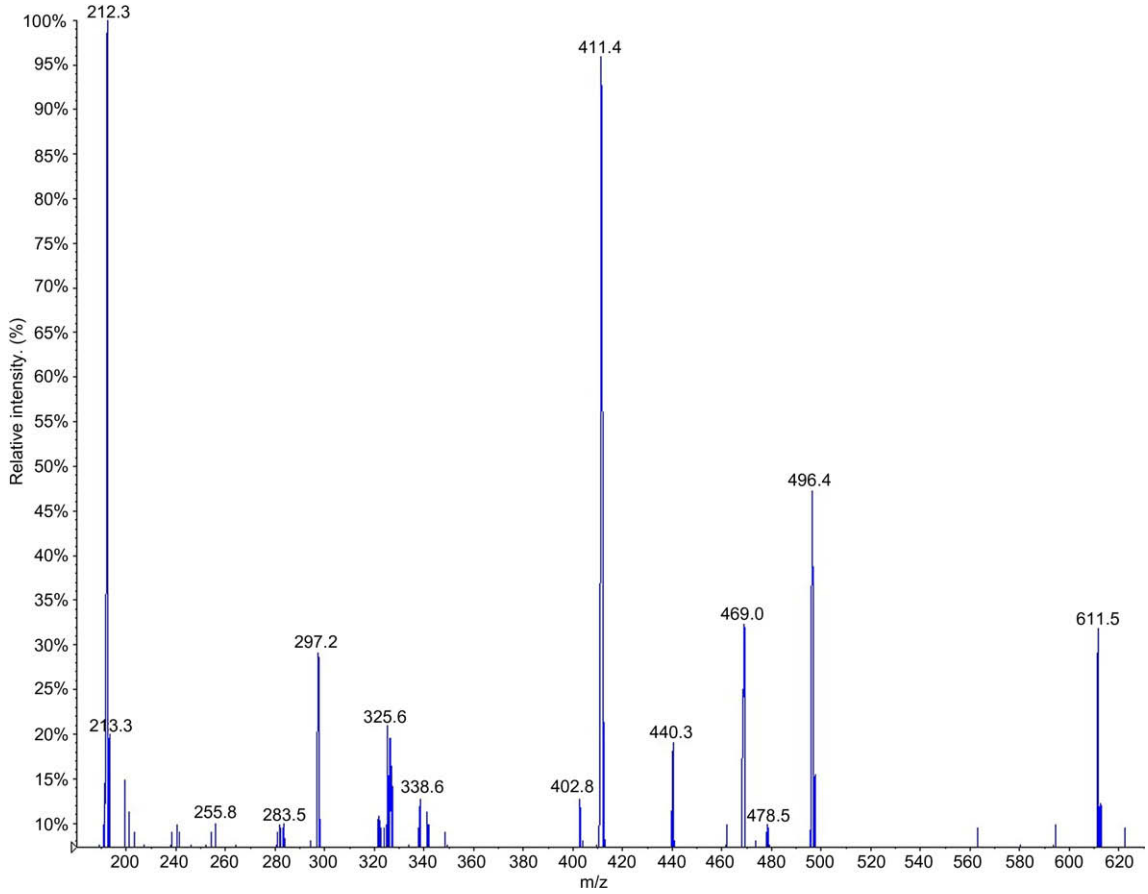

Fig. 3. Product ion spectrum of the diprotonated molecule of CA II-specific peptide GGPLDGTYR (m/z 468.3) acquired at 24% relative collision energy, obtained by analyzing a 10-pg μl^{-1} standard solution in infusion mode, at a 5-$\mu l\ min^{-1}$ flow rate.

the analytical column, and (iii) the trap washing with strong solvent can be made during sample separation.

Calibration graphs were constructed as described in Materials and methods. Two different regression lines were considered: external calibration and matrix-matched calibration. When the areas of the standard peaks were not normalized for the IS response, the ratio between the slopes (b_{mm}/b_{ext}) was 0.73 ± 0.6, whereas after normalization it was 1.04 ± 0.05. These data showed that ion suppression due to matrix compounds is moderate but not negligible and that the unlabeled IS was adequate. The memory effect was nearly 100% when the most diluted standard solution (0.05 pg μl^{-1}) was injected after the most concentrated one (100 pg μl^{-1}) and became negligible only after three blank injections. The response was found to be linear for the calibration range used, with R^2 = 0.9990 and 0.9863 for the external and matrix-matched calibrations, respectively.

Recovery, accuracy, and reproducibility of the method were calculated by analyzing six 20-µl aliquots of a serum pool unspiked and spiked with CA II at three concentration levels during 3 weeks, with three LC–MS/MS analyses being performed for each immunoprecipitated sample. Results are shown in Table 3. If we consider that the tryptic digestion regarding the representative peptide recovery is quantitative, the roughly 23% analyte loss should be due to an incomplete capture by the antibody. The RSD (<12%) was comparable to the RSDs of similar studies [29,33,34].

Instrumental limit of detection (LOD), calculated as three times the intercept of the external calibration regression line, was 2 pg of the injected synthetic peptide (~2 fmol). The method identification limit (MIL) and method quantification limit (MQL) were estimated from the serum samples, with samples being run in triplicate and results being averaged. MIL (signal/noise [S/N] = 3 for the second most intense transition in MRM) and MQL (S/N = 10 for the sum of the two selected transitions), expressed as pmol ml^{-1} CA II in serum, were 0.3 and 0.5, respectively.

Although the performances of the method based on immunoprecipitation by magnetic beads were not superior to those based on reversed phase LC fractionation, it present two advantages: (i) many samples can be treated contemporaneously and (ii) due to the higher selectivity and loadability, a less sophisticated MS/MS platform can be used.

CA II quantitation in human serum samples

Eight human serum samples from apparently healthy subjects (seven males and one female, 20–40 years of age) were analyzed, and for each sample three RP–HPLC runs were done. For CA II-specific peptide quantification, external calibration was employed and recovery correction was done. The concentration of the endogenous CA II in serum was found to be 27.3 pmol ml^{-1} (RSD = 65%).

Both the mean concentration and RSD were very different from those found in a previous study by our laboratory (56 pmol ml^{-1} and 21%, respectively). Because both methods were validated in a similar way and the sampled population was different but similar, a possible explanation may be that the external addition of standard CA II was not similar to the endogenous CA II situation at the isolation step in both cases. The chromatographic isolation used in the previous work fractionated denaturated samples, whereas the immunologic capture is performed in native samples. If the protein were present in different forms (modified or associated in complex structures), the affinity for the antibody would be

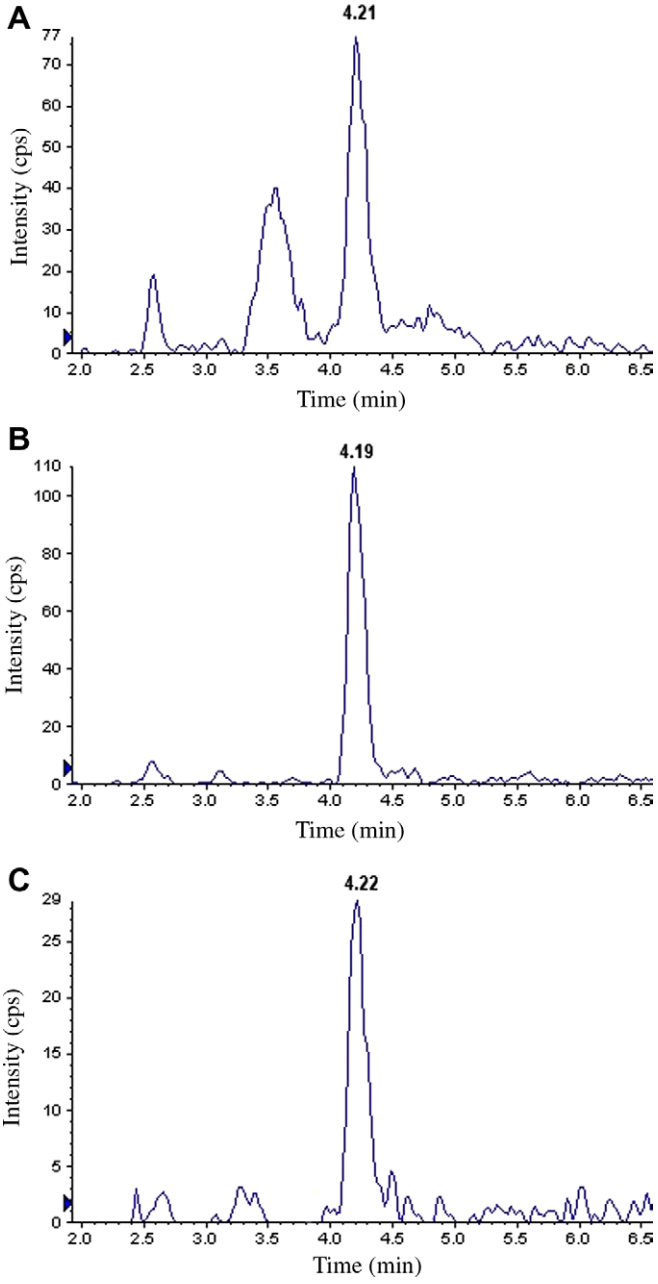

Fig. 4. LC–ESI–MS/MS extracted ion current in MRM mode deriving from the analysis of an unspiked sample relative to 468.3 → 212.3 transition (A), 468.3 → 411.2 transition (B), and 468.3 → 496.3 transition (C).

Table 3
Method recoveries and precision.

CAII added (pmol ml^{-1})	–	30	100	150
CAII found (pmol ml^{-1})	15.5	37.7	95,5	132.5
Recovery	–	74	80	78
RSD% (n = 6)	12	7	9	5

different, so the recovery of the standard may give erroneous indications.

Conclusions

As demonstrated by the first attempts to conduct a multilaboratory assessment of the precision and reproducibility of MRM-based measurements of proteins in plasma published recently [6,35], the preclinical validation of candidate biomarker by LC–MS/MS is becoming a hot topic.

In the method described in this work, the protein of interest, CA II, was isolated from serum by immunoprecipitation. A proteotypic peptide, produced stoichiometrically through proteolysis with trypsin, was ultimately quantified, using a synthetic peptide and a structural analogue free-labeled synthetic peptide as IS, by LC–ESI–MS/MS in MRM acquisition mode. An analytical column having 1 mm internal diameter and a triple quadrupole instrument were employed. A column switching system was used for on-line SPE sample cleanup. The assay was validated by recovery studies of both intact proteins and proteotypic peptide. Good precision and MDL as low as 0.5 pmol ml^{-1} were obtained.

This strategy, based on isolation of target protein by immunoaffinity and its absolute quantification by quantifying one of its proteotypic products, could represent a very specific and sensitive analytical approach to reach the analytical goal of low-abundance protein determination in clinical samples such as serum. Nevertheless, a comparison of physiological CA II concentrations in sera of eight apparently healthy subjects obtained by using this approach with those found in a previous study by using chromatographic isolation of the fraction containing the target protein followed by a more technologically advanced platform, such as chip LC–MS/MS [27], showed very different values. If our data are not affected by an unrecognized error, these results pose a question. A protein may be present in a certain biological specimen in different forms (e.g., free and involved in a complex with other molecules). Is the analysis of spiked samples ever a correct way for making validation? When trying to isolate the target from the most abundant proteins, what form is enriched?

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