Antibody-Free Quantification of Serum Chromogranin A by Targeted Mass Spectrometry
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BACKGROUND: Chromogranin A (CgA) is a 48-kDa protein that serves as a diagnostically sensitive, but non-specific, serum biomarker for neuroendocrine tumors. Immunoassays for CgA are not standardized and have a narrow dynamic range, which requires dilution of concentrated specimens. We developed and validated an antibody-free, liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based method for CgA without these limitations.

METHODS: CgA was extracted from serum using a mixed-mode anion exchange solid-phase extraction plate, digested with trypsin, and analyzed by LC–MS/MS using well-characterized CgA calibration standards. After validation, the mass spectrometry method was compared with the CISBIO immunoassay using 200 serum specimens previously submitted for CgA analysis. Specimens with discordant results were reanalyzed by high-resolution mass spectrometry- (HRMS) -based methods to assess the contribution of truncated and post-translationally modified forms of CgA.

RESULTS: The assay had a linear range of 50 to 50,000 ng/mL, recoveries between 89% and 115%, and intra- and interassay imprecision <10%. LC–MS/MS assay results showed a Pearson’s correlation of $r = 0.953$ with the CISBIO immunoassay, with CgA values being a mean 2- to 4-fold higher. Concordance for CgA between the 2 assays was 80.9% (95% CI 72.8%–89.2%), showing substantial agreement. Truncation and post-translational modification, including 2 phosphorylation sites that had not been previously observed or predicted to our knowledge, did not appear to contribute directly to discordance between the 2 assays.

CONCLUSION: Quantification of CgA by LC–MS/MS provides an analytically sensitive and reproducible alternative to commercially available immunoassays.

Introduction

Chromogranin A (CgA) is a 48-kDa, soluble, acidic glycoprotein expressed in the secretory granules of both endocrine and neuroendocrine tissue. CgA is stored in large dense-core vesicles of neuroendocrine cells and is released together with peptide hormones and biogenic amines (1, 2). Processing and posttranslational modifications vary among different neuroendocrine cell types and several biologically active CgA peptides have been identified (3, 4).

Increased CgA concentrations are observed in patients with well-differentiated neuroendocrine tumors (NETs) making CgA the preferred biomarker for neuroendocrine neoplasms (5–8). NETs are a rare class of tumors originating from a variety of neuroendocrine cell types throughout the body. Circulating concentrations of CgA serve as a prognostic marker for tumor burden, with tumor size and CgA concentrations showing an 80% to 88% concordance (9, 10).

Several commercially available immunoassay kits are available to measure serum CgA. However, diagnostic sensitivity is dependent on NET type (11), and increased CgA may also be observed in some nonendocrine and nononcological conditions (1). In addition, a lack of standardization in both the reference material and antibody-epitope recognition makes cross-kit comparison and serial monitoring of patients challenging (11–14). Proteolytic processing and posttranslational modification of CgA may impair the diagnostic sensitivity of antibody-based assays by removing, or masking, the epitopes they detect. Consequently, diagnostic sensitivities for the detection of NETs can vary substantially between kits, ranging from 67% to 93% (14, 15).

To overcome the epitope masking seen with immunoassays, a technique known as processing-independent analysis (PIA) has been used to measure total CgA concentrations (16, 17). PIA involves treatment of patient
specimens with trypsin before measurement of a representative CgA peptide by radioimmunoassay and allows quantification of total CgA irrespective of posttranslational processing. Quantification by FIA has shown up to a 500-fold increase in immunoreactivity and higher diagnostic sensitivities and specificities compared to traditional immunoassay (16, 17). This method, however, is labor-intensive, requiring fractionation of each specimen by size-exclusion chromatography.

In contrast to immuno-based assays, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become an increasingly popular tool for protein quantification in the clinical laboratory (18, 19). Advances in instrumentation and sample preparation techniques have allowed for the detection of low nanogram concentrations of serum proteins without the need for immunofinity enrichment. Commonly used techniques for protein quantification by LC–MS/MS include enzymatic digestion followed by selected-reaction monitoring of a proteotypic peptide. One of the many benefits of protein quantification using this technique is the ability to measure the total amount of a protein, irrespective of posttranslational modifications, truncations, or oligomerizations that may hamper detection by traditional immunoassay techniques. Mass spectrometry also has the ability to quantify over a larger dynamic range than traditional immunoassays, decreasing the need for repeat analysis of specimens after dilution.

Our goal here was to develop and validate a robust and analytically sensitive LC–MS/MS method to accurately quantify total serum concentrations of CgA over a wide dynamic range. Our approach was to enrich CgA based on its high number of acidic amino acids instead of relying on more complex protein depletion or immuno-enrichment techniques. In addition, after comparing LC–MS/MS results with the CISBIO immunoassay, we tested specimens with discordant results by high-resolution mass spectrometry (HRMS) to assess the effects of differentially posttranslationally processed CgA peptides/truncation products.

Materials and Methods

A detailed description of the methods is provided in the online Data Supplement.

STANDARDS AND REAGENTS

Recombinant human CgA liquid stocks were purchased from Abcam (ab85486, Boston, MA). Purity (>95%) and the stated concentration (0.5 mg/mL) was checked by quantitative amino acid analysis (AAA Service Laboratory, Damascus, OR; Supplemental Tables 1 and 2) and adjusted accordingly (20). Isotopically labeled winged peptide internal standard (IS) EGSANR RPEDQELESL*SAIEAELEK*VAHQL (L*, K* [13C5; 15N]) was custom-synthesized by New England Peptide (Gardner, MA).

PREPARATION OF CALIBRATION STANDARDS, QUALITY CONTROL SAMPLES, AND INTERNAL STANDARD

Calibration standards and quality control samples were prepared by diluting CgA liquid stocks with defibrinated, double charcoal-stripped human serum (sample diluent), aliquoted into 1.5-mL Eppendorf tubes, stored frozen (−90°C to −70°C) until needed, and discarded after use. Separate lots of stock CgA were used for the calibration standards and quality control samples. The winged peptide IS was prepared in 0.12 M ammonium bicarbonate solution (pH 8.0) containing 0.5 mg/mL bovine serum albumin before dilution.

SAMPLE PREPARATION

All sample preparation steps were performed on a Hamilton Star Microlab liquid handling device integrated with a SPEware IP8 positive-pressure manifold.

MULTIPLE-REACTION MONITORING LC–MS/MS ANALYSIS

Analytical separation was achieved with an Aria TLX-4 Transcend 1 HPLC (Thermo-Fisher) in a staggered 4-column configuration to facilitate high throughput. Samples were first loaded onto a Waters HLB 2.1 × 20 mm, 25 μm loading column before being separated on a Phenomenex Kinetex C18 50 × 4.6 mm, 2.6 μm analytical column with a 4.0 × 2.0 mm guard column (Supplemental Fig. 1). Data acquisition and quantification were performed on a Sciex 6500+ QTrap mass spectrometer (Framingham, MA) operated in multiple-reaction monitoring mode with Analyst software (Supplemental Figs. 2 and 3 and Supplemental Tables 3 and 4).

METHOD VALIDATION

To determine intraassay precision, 20 replicates of each QC level were assayed within the same day. For interassay precision, 2 replicates of each QC level were assayed over 20 days. The limit of quantification (LOQ) was determined by serial dilution of the first calibrator until imprecision exceeded ±20%. Five replicates of each LOQ level were analyzed over 5 separate days. The limit of blank (LOB, 2 SD from the mean of 25 blanks) and limit of detection (LOD, 2 SD from LOB) were determined by running 25 diluent blanks in a single batch. Carryover was determined by running a triplicate sequence of 4 diluent blanks followed by 8 replicates of the high calibrator in succession. Recovery studies were performed by spiking 100, 250, and 1000 ng/mL of CgA into 3 separate serum pools. Recoveries on dilution were assessed by diluting patient pools containing high
concentrations of CgA (mean 4030 ng/mL) with sample diluent in ratios of 1:1, 1:4, and 1:9. Additional recovery studies were performed by mixing low and high patient serum pools together in different ratios. Recovery of the IS during SPE was evaluated by adding IS either before the SPE step or after the elution step.

Sample stability was assessed by storing 5 serum pools at room temperature (18°C to 25°C; baseline, 1, 3, 5, and 7 days), 10 serum pools refrigerated (2°C to 8°C; baseline, 1, 3, 5, 7, and 14 days), and 5 serum pools frozen (−30°C to −10°C, baseline, 1, 3, 5, 7, 14, 21, 26, and 31 days). After each time point, samples were frozen at −80°C. All samples were subsequently thawed and analyzed on the same day. Freeze–thaw stability was assessed for 5 freeze–thaw cycles on 5 different patient serum pools. Extracted sample stability was assessed at 1, 3, and 5 days postextraction.

Hemolytic, icteric, and lipemic interference were assessed using 3 separate patient serum pools. Ion suppression was evaluated by postcolumn infusion of the proteotypic CgA quantification peptide.

Reference range studies and sample tube-type comparisons were conducted on specimens from self-reported healthy adult individuals ranging in age from 18 to 65 years (60 males, 62 females). Individuals with a previous history of cancer and individuals taking proton-pump inhibitors were excluded. Reference range probabilities were assigned.

**RESULTS**

**ANALYTICAL PERFORMANCE**

Total allowable error was determined to be 30% (or 78 ng/mL) using Tonks' Criteria with consideration of assay performance and biostatistical analysis of 122 donor specimens representative of a healthy population (23). These acceptability criteria were approved by the Medical Director overseeing the assay.

Intra- and interassay imprecision were both <10% (Table 1). The LOB was 12.1 ng/mL and the LOD was 19.9 ng/mL (Supplemental Table 9). The LOQ for the method was 50.0 ng/mL (Supplemental Fig. 4). The assay showed a linear range of 50 ng/mL to 50 000 ng/mL with inaccuracies ranging from 2.4% to 8.3% (Supplemental Table 10). Spiked recoveries ranged from 94% to 104% (Supplemental Table 11). Recoveries on dilution ranged from 100% to 107% for 1:1 dilution, 99% to 111% for 1:4 dilutions, and 97% to 117% for 1:9 dilution (Supplemental Table 12). Recoveries from patient pools containing high and low CgA levels ranged from 94% to 115% (Supplemental Table 13). Recovery of the IS during the SPE step was 95%. The assay had no carryover: mean concentrations of the matrix blanks after injection of the high calibrators were 8.9 ng/mL and below the LOD.

A limitation of the Analyst mass spectrometry software used postacquisition is the inability to generate real-time reports of ion ratios to verify analyte identity. Accordingly, we manually evaluated transition ion ratios and analyte/IS retention time alignment as shown in Supplemental Table 14. We found that relative retention times (±0.1 min) accurately reflected product ion ratios within ±25%.
**Fig. 1.** Schematic representation of the CgA sequence with the localization of the 10 peptides targeted by high-resolution-mass spectrometry-parallel-reaction monitoring (HRMS–PRM), location of the CISBIO mAb epitope-binding region, and N- and C-terminal derived CgA products. Multiple-reaction monitoring (MRM) quant peptide is in *italics*/*bold*. Identified phosphorylation sites are denoted with #. The locations of the unique phosphorylation sites are noted by *.

**Fig. 2.** (A), Extracted chromatogram for the 10 CgA peptides targeted by high-resolution mass spectrometry-parallel-reaction monitoring (HRMS–PRM). (B), Multiple-reaction monitoring (MRM) signal with targeted transitions for the quant peptide (RK-19). (C), Calibration curve for the MRM quant peptide. (D), Expansion of calibration curve showing standards 1 to 5.

**Table 1.** CgA assay imprecision. Inter- and intraassay for CgA QC samples.

|                    | Between run precision for chromogranin A (ng/mL) | Within run precision for chromogranin A (ng/mL) |
|--------------------|---------------------------------------------------|-------------------------------------------------|
|                    | Low QC    | Mid QC  | High QC | Low QC    | Mid QC  | High QC |
| **Target**         |           |         |         |           |         |         |
| Low QC             | 140       | 1600    | 14 000  | 140       | 1600    | 14 000  |
| Mid QC             | 141       | 1678    | 14 769  | 130       | 1705    | 13 839  |
| High QC            | 12        | 118     | 1446    | 7         | 98      | 628     |
| **Overall SD**     | 8.5%      | 7.0%    | 9.8%    | 5.3%      | 5.6%    | 4.5%    |
| **Overall CV**     |           |         |         |           |         |         |
| Low QC             | 101%      | 105%    | 105%    | 93%       | 107%    | 99%     |
| **Overall Recoveries** |         |         |         |           |         |         |
| Diff (target–mean) | –1        | –78     | –769    | 10        | –105    | 161     |
| TEa/4              | 10.5      | 120     | 1050    | 10.5      | 120     | 1050    |

TEa, total allowable error.
**STABILITY, INTERFERENCE, AND ION SUPPRESSION**

CgA was stable up to 5 freeze–thaw cycles, with mean recoveries across all cycles ranging from 99% to 103% (Supplemental Fig. 5). The protein was stable up to 5 days at room temperature and up to 31 days frozen (Supplemental Fig. 6). However, CgA was not stable when stored refrigerated at 4°C. Apparent CgA concentration increased after 24 hours when stored at refrigerated temperatures, and the increased concentrations remained consistent for up to 14 days. This increase in concentration was also observed when the same samples were analyzed with the CISBIO assay (Supplemental Fig. 7). Digested extracted samples showed stability up to 5 days when stored at 4°C. Mean recoveries were 102% with CVs ranging between 3.3% and 15.8% (Supplemental Fig. 8). Ion suppression chromatograms showed no evidence of ion suppression at the analyte retention time (Supplemental Fig. 9).

Mean recoveries ranged from 98% and 104% for slight, moderate, and grossly icteric and lipemic samples. Mean recoveries ranged from 96% and 102% for slight and moderately hemolyzed samples (Supplemental Fig. 10). Although CgA showed acceptable recoveries for grossly hemolyzed samples (mean recovery of 100%), there was noticeable ion suppression in both the analyte and internal standard in these samples (mean IS recovery of 69%) (Supplemental Fig. 11).

**REFERENCE RANGE AND SAMPLE TUBE-TYPE COMPARISON**

The reference range cutoff for healthy individuals was <311 ng/mL based on the nonparametric percentile method. No significant differences between males and females were observed (P = 0.100), and values increased slightly with age (1.4 ng/mL per year; P = 0.004). Reference range specimens were also analyzed by the CISBIO immunoassay and the cutoff was <140 ng/mL (Supplemental Fig. 12).

Serum in serum separator tubes (SST) yielded slightly higher CgA values compared with red-top serum tubes. Consequently, SST serum had a higher reference range cutoff (<336 ng/mL) and was deemed an unacceptable tube type because of this disparity, as well as the fact the red-top tubes are an established tube type for the current CgA CISBIO immunoassay.

**METHOD COMPARISON**

Results for specimens analyzed by the new LC–MS/MS assay were significantly higher when compared to the current CISBIO assay (P < 0.001). Mean CgA concentrations were 767 ng/mL (range 18–16 422 ng/mL) for the CISBIO assay compared with 1443 ng/mL (range 50–27 121 ng/mL) for the LC–MS/MS assay. Deming regression analysis gave an equation of LC–MS/MS = 0.51 + 1.88 (CISBIO), with a Pearson’s correlation of r = 0.953 (Fig. 3). There were 19 specimens that were discordant between the 2 assays: 9 specimens were increased with the LC–MS/MS assay but below the CISBIO cutoff and 10 specimens were increased with the CISBIO assay but below the LC–MS/MS cutoff (Fig. 2). Concordance after adjustment for chance agreement (Cohen’s Kappa) was 80.9% (95% CI 72.8–89.2%) showing substantial agreement (Table 2).

**SEMIQUANTIFICATION OF 10 CGA PEPTIDES BY HRMS–PRM**

All targeted 10 CgA peptides (Table 3) showed acceptable linearity with R² ≥ 0.98, but differed in their LOQ when analyzed by HRMS–PRM (Supplemental Fig. 13). In general, peptides from the central region of the CgA protein within the epitope-binding region of the CISBIO assay (position 142–412) showed lower recoveries than N- and C-terminal derived peptides. Samples with increased concentrations previously analyzed by HRMS-DDA showed the presence of phosphoserines in 2 of these mid-region peptides, SK-21 (142–162), and SR-10 (322–331) (Supplemental Figs. 14 and 15).

**ANALYSIS OF DISCORDANT PATIENT SPECIMENS BY HRMS–PRM**

Four patient specimens with discordant results between the CISBIO and LC–MS/MS assay were reanalyzed by HRMS–PRM (Fig. 3). Measured CgA concentrations were 5 to 9 times higher in these specimens when measured by LC–MS/MS. Mean concentrations were

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**Table 2.** Assay concordance between the CISBIO ELISA assay and the LC–MS/MS assay for 200 serum specimens. Cohen’s Kappa = 0.809.

| CISBIO ELISA | LC–MS/MS | CgA concentrations | Below cutoff (<140 ng/mL) | Above cutoff (≥140 ng/mL) | Total |
|-------------|--------|--------------------|--------------------------|--------------------------|-------|
| Below cutoff (<311 ng/mL) | | | 87 (0.90) | 10 (0.10) | 97 (0.48) |
| Above cutoff (≥311 ng/mL) | | | 9 (0.09) | 94 (0.91) | 103 (0.52) |
| Total | | | 96 (0.48) | 104 (0.52) | 200 |

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100 ng/mL (range 72–126 ng/mL) for the CISBIO assay and 669 ng/mL (range: 567–920 ng/mL) for the LC–MS/MS assay. When reanalyzed by HRMS–PRM, CgA concentrations measured in these 4 discordant patient specimens displayed increased concentrations across all 10 peptides. Mean concentrations ranged from 445 to 854 ng/mL across the 10 peptides, above the LC–MS/MS cutoff point of 311 ng/mL (Table 3). Of the 4 discordant specimens, absence of peptides corresponding to specific regions of the CgA sequence indicates possible N- and C-terminal truncation (discordant specimen D2), C-terminal truncation (discordant specimen D3), and internal phosphorylation (discordant specimens D2 and D3). However, all these modifications are well outside the CISBIO epitope.

ANALYSIS OF PATIENT SPECIMENS WITH INCREASED CONCENTRATIONS BY HRMS–PRM

Four specimens that were increased with both the CISBIO and LC–MS/MS assay but differed in their magnitudes of increase were reanalyzed by HRMS–PRM (Fig. 3). Measured CgA concentrations were 6 to 17 times higher in these specimens when measured by LC–MS/MS. Mean concentrations ranged from 445 to 854 ng/mL across the 10 peptides, above the LC–MS/MS cutoff point of 311 ng/mL (Table 3). Of the 4 samples with increased concentrations, the absence of the C-terminal peptide in increased specimen E1 indicated possible C-terminal truncation. In increased specimen E4, a 2-fold increase in both N-terminal peptides indicated possible contribution from the CgA-derived vasostatin-1 (19–94 in the CgA sequence) or vasostatin-2 (19–131 in the CgA sequence) peptide. Possible phosphorylation or CgA processing was indicated by the absence of peptide ER-13 in the same sample. In both cases, phosphorylation/truncation was outside the CISBIO epitope region.

LC–MS/MS ANALYSIS OF CGA PHOSPHORYLATION

For the specimens with highly increased CgA, sequence coverage was ≥63% for all 3 samples. A total of 9 phosphorylation sites were identified across all 3 samples, 7 of which have previously been reported in UniProt. We identified 2 novel phosphorylation sites not previously reported in UniProt at S112 and S136 (Supplemental Figs. 16 and 17).
Table 3. Results for 8 patient samples analyzed by high-resolution mass spectrometry-parallel-reaction monitoring (HRMS–PRM). Four discordant samples and 4 increased outlier samples were selected for reanalysis by HRMS–PRM.

| Assay                                      | LOQ and linear range (ng/mL) | Position in CgA protein sequence | Phosphorylation detected by HRMS–DDA | Discordant specimens | Increased specimens |
|--------------------------------------------|------------------------------|----------------------------------|---------------------------------------|----------------------|---------------------|
| CISBIO ELISA assay                         | 16 (16–820)                 | N/A                              |                                      | Specimen D1: 126     | Specimen E1: 213    |
| MRM LC-MS/MS assay                         | 50 (50–50 000)              | N/A                              |                                      | Specimen D2: 98      | Specimen E2: 299    |
| HRMS–PRM Assay:                            |                              |                                  |                                      | Specimen D3: 72      | Specimen E3: 247    |
| CIVEVISDTLSKPSPMPV SQECFETLR (CR-27)       | 500 (500–50 000)            | 35–61                            | No                                   | Specimen D4: 103     | Specimen E4: 211    |
| ELQDLALQGAK (EK-11)                        | 100 (100–50 000)            | 78–88                            | No                                   |                      |                     |
| HSGFEDELESEVLENQ SSQAELK (HS-22)           | 250 (250–50 000)            | 97–118                           | Yes                                  | Specimen D1: 561     | Specimen E1: 4700   |
| EAVEEPSSK (EK-9)                           | 250 (250–50 000)            | 119–127                          | No                                   | Specimen D2: 0       | Specimen E2: 1481   |
| SGEATDGARPOALPEP MQESK (SK-21)             | 100 (100–50 000)            | 142–162                          | Yes                                  | Specimen D3: 587     | Specimen E3: 1593   |
| YPGPQAEQDSGLSQQ GLVDREK (YK-22)            | 250 (250–50 000)            | 194–215                          | No                                   | Specimen D4: 1143    | Specimen E4: 7188   |
| SGELEGTEER (SR-10)                         | 50 (50–50 000)              | 322–331                          | Yes                                  | Specimen D1: 665     | Specimen E1: 3845   |
| EDSLAEGLPLQVR (ER-13)                      | 250 (250–50 000)            | 400–412                          | Yes                                  | Specimen D2: 595     | Specimen E2: 2801   |
| RPEDEGSLESLASIE AELKE (RK-19)              | 50 (50–50 000)              | 428–446                          | No                                   | Specimen D3: 460     | Specimen E3: 1581   |
| VAHQLQALR (VR-9)                           | 250 (250–50 000)            | 447–455                          | No                                   | Specimen D4: 709     | Specimen E4: 6070   |

HRMS–PRM, high-resolution mass spectrometry-parallel-reaction monitoring; HRMS–DDA, high-resolution mass spectrometry-data-dependent acquisition.
Discussion

The use of LC–MS/MS for the quantification of serum CgA provides several analytical advantages over standard immunoassays, including the ability to quantify over 4 orders of magnitude. Eliminating the need to reanalyze samples after dilution allows for a greater than 2-fold increase in sample throughput per batch compared to the current CISBIO immunoassay. In addition, an advantage of this LC–MS/MS assay is that it does not rely on antibodies for CgA enrichment and thus has greater potential for standardization.

Digestion of CgA with trypsin yielded over 40 unique peptides, 3 of which were suitable for quantification based on optimal sensitivity, digestion reproducibility, and absence of known posttranslational modifications. One peptide, RPEDQELESLSAIEAELEK (428–446 in the CgA sequence), demonstrated the best assay performance, with digestion variability corrected by using an isotopically labeled IS with extended flanking sequences (i.e., a winged version of the sequence).

In comparing assay results, we observed a positive bias for our LC–MS/MS assay compared with the CISBIO assay; CgA values were 2 to 4 times higher. Consequently, the cutoff for increased CgA was higher in the LC–MS/MS assay. The bias may reflect different sample preparation techniques and different source material used for the calibrators. Our calibrator material consisted of recombinant full-length CgA with protein content confirmed by quantitative amino acid analysis. In contrast, the CISBIO assay used a proprietary recombinant form of CgA that we could not characterize. The protein did not yield any identifiable proteotypic peptides when run by both HRMS–PRM/DDA and our LC–MS/MS assay. Nevertheless, despite the bias toward higher LC–MS/MS values, there was substantial agreement between the 2 assays in terms of assay concordance (Table 2).

For the specimens exhibiting discordance, we explored the possibility of epitope masking or loss causing lower CgA values in the CISBIO assay. For example, several CgA-derived peptides have been described, including the C-terminally derived GR-44 peptide (including the C-terminally derived GR-44 peptide (428–446 in the CgA sequence, Fig. 1), which contains the proteotypic peptide used in our LC–MS/MS assay but lacks the CISBIO epitope (24, 25). In addition, the epitope contains several predicted or confirmed phosphorylation sites that could theoretically impact quantification by affecting binding affinity, effectively masking the immunoassay (26). To investigate assay discordance in representative specimens, we reanalyzed 8 outlier specimens by HRMS–PRM (targeting 10 unique peptides near the N-terminus showed higher concentrations than those in the central or C-terminal regions, indicating possible processing to the vasostatin-1 and -2 peptides (19-131 in the CgA sequence). For peptides derived from or near the central epitope-binding region of the CgA protein, increases were not as high as those observed in peptides from the N- and C-terminal ends (Fig. 1, Table 3). This may be the result of phosphorylation (e.g., SR-10, SK-21) or incomplete protein digestion. Processing of CgA to a major known endogenous peptide GV-19 (position 393–411, Fig. 1) (24) may explain the absence of peptide ER-13 (position 400–412) in 3 or the 8 samples reanalyzed by HRMS–PRM. Tryptic peptide ER-13 is almost completely contained within the endogenous GV-19 peptide sequence.

Preliminary data from 3 patients with very highly increased CgA showed the presence of 9 phosphorylation sites, 3 of which fell within the mid-region peptides targeted by HRMS–PRM (Supplemental Figs. 15–17). Importantly, 1 phosphorylation site was within the epitope-binding region (Serine 218, Fig. 1), possibly impacting the CISBIO assay, although there was no evidence of this modification impacting the results for the discordant or other increased outlier specimens (Table 3). Interestingly, we identified 2 novel phosphorylation sites not described in UniProt (last modified, December 2, 2020; Accessed December 15, 2020) at S112 and S136. While the clinical significance of these 2 phosphorylation sites are unknown, previous studies have shown the presence of phosphorylated CgA in individuals with carcinoid tumors (26).

Conclusion

We have developed a high-throughput, analytically sensitive, and robust LC–MS/MS assay to quantify CgA in...
serum. This method was validated according to CLSI guidelines and showed acceptable performance for use in a clinical laboratory. Quantification over 4-orders of magnitude in dynamic range in the LC–MS/MS assay increased sample throughput by avoiding the need to perform duplicate analysis on dilution. While LC–MS/ MS results are higher than corresponding CISBIO immunoassay results, substantial concordance exists between the 2 methods. Where discordance did occur, we reanalyzed peptides spanning the full length of the CgA protein but were unable to confirm whether it was due to direct epitope masking or other factors. However, we did identify several phosphorylation sites, 1 of which fell within the epitope-binding site for the CISBIO assay. One limitation to our study was the lack of knowledge about clinical samples with known NETs. In the future, we will use the LC–MS/MS assay to study CgA concentrations in individuals with known NETs from different origins.

Supplemental Material

Supplemental material is available at Clinical Chemistry online.

Nonstandard Abbreviations: CgA, chromogranin A; IS, internal standard; SPE, solid-phase extraction; MRM, multiple-reaction monitoring; HRMS, high-resolution mass spectrometry; DDA, data-dependent acquisition; LOB, limit of blank; LOD, limit of detection; PRM, parallel-reaction monitoring.

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