Isopropanol Protein Precipitation for the Analysis of Plasma Free Metanephrines by Liquid Chromatography–Tandem Mass Spectrometry

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BACKGROUND: High-performance liquid chromatography–tandem mass spectrometric (LC-MS/MS) analysis of plasma free metanephrines is the most diagnostically sensitive and specific screening test for the diagnosis of pheochromocytoma. We sought to develop an in-house method for this expensive test.

METHODS: We used off-line isopropanol protein precipitation of plasma to remove interfering substances before LC-MS/MS analysis. We compared the extraction efficiency and limits of quantification of protein precipitation to those of previously reported solid-phase techniques.

RESULTS: The new method had limits of quantification of 0.09 nmol/L and 0.17 nmol/L for metanephrine and normetanephrine, respectively. Method comparison with a previously described solid-phase extraction method revealed Deming regression slopes of 0.904 and 0.994, intercepts of 0.007 and 0.023, and SEs of the residuals ($S_{p}$) of 0.071 and 0.284 for metanephrine and normetanephrine, respectively. Extraction efficiency of isopropanol protein precipitation was 66% for metanephrine and 35% for normetanephrine, results that were superior to the efficiencies of 4% and 1% for our adapted solid-phase extraction method. No ion suppression was observed at the retention times for metanephrine and normetanephrine.

CONCLUSIONS: Isopropanol protein precipitation is a novel and effective off-line sample preparation method for metanephrines that offers a less expensive alternative to on-line solid-phase extraction for low-volume testing and requires a sample volume of only 200 μL. The mass spectrometric analysis time is equivalent to that of solid-phase techniques.

Measurement of plasma concentrations of unconjugated metanephrine and normetanephrine by liquid chromatography–tandem mass spectrometry (LC-MS/MS) is an effective screening test for the diagnosis of pheochromocytoma. Because of the test’s high diagnostic sensitivity, a negative screen virtually rules out the presence of a catecholamine-producing tumor (1–4). It was our goal to develop an in-house method for this expensive test.

For analysis of plasma free metanephrines by LC-MS/MS, solid-phase extraction has been the sample preparation method of choice to remove substances that cause ion suppression, such as phospholipids, salts, and proteins (5–8). We attempted to adapt previously reported solid-phase extraction methods for sample preparation into acceptable procedures (5, 6). However, we were unable to detect low levels of analyte with our relatively insensitive mass analyzer. It became obvious that unless we wanted to purchase a much more sensitive instrument, we would have to develop a more efficient method for sample preparation.

We first experimented with different equilibration, wash, and elution buffers as modifications to previously described approaches and noted some improvement in limits of detection (data not shown). We also tested acetonitrile protein precipitation, again with some benefit (data not shown), before trying more polar solvents for protein precipitation, hoping to improve recovery of the relatively polar metanephrine compounds. The most successful protein precipitation method used isopropanol. For smaller testing volumes, this method provided a good alternative to the large, expensive instrumentation necessary for on-line solid-phase extraction. In our laboratory this new method also showed significant improvements in extraction efficiency and limits of quantification, with similar imprecision and analysis time.

For isopropanol protein precipitation, we used 1.5 mL polypropylene microcentrifuge tubes to which we added 50 μL of internal standard solution (IS) containing 6.5 nmol/L metanephrine-d₃ and 6.5 nmol/L normetanephrine-d₄ (Medical Isotopes) in 100 mmol/L HCl to 200 μL of calibrator, control, or patient plasma. We then added 1 mL of isopropanol (ACS grade; J.T. Baker). After vortex-mixing for 4 min and centrifuging for 5 min at 11 300g, we transferred the supernatants to 12- × 75-mm round-bottom borosilicate glass test tubes. After air evaporation of this supernatant, we reconstituted the resulting product in 100
μL 95% acetonitrile (HPLC grade; J.T. Baker) in water. The samples were transferred to 300-μL polypropylene autosampler vials, and 10 μL of sample was injected onto the HPLC column.

Using a technique previously reported by de Jong et al. (6), we resolved the analytes by using normal-phase liquid chromatography on an Atlantis HILIC Silica 3-μm 2.1 × 30-mm column (Waters) at a flow rate of 0.3 mL/min, with a 2-min gradient from buffer A (95% acetonitrile, 3% methanol, 2 mmol/L ammonium formate in water, pH 3) to buffer B (80% acetonitrile, 20 mmol/L ammonium formate in water, pH 3). We quantified the analytes by using isotope-dilution multiple reaction–monitoring on a Waters Quattro Micro mass spectrometer (transitions: metanephrine, 180.20/148.15; metanephrine-d3, 183.25/151.20; normetanephrine, 166.18/134.10; normetanephrine-d3, 169.25/137.15). Total instrument analysis time was 4.6 min for each sample, enabled by use of column switching on an HPLC instrument (Waters Alliance HT 2795) equipped with 2 columns that can be washed and developed in parallel.

Calibrators and controls were prepared in charcoal-stripped serum (SeraCare). The 5 calibrators contained metanephrine (purchased from Sigma) at 0.1, 0.5, 1.0, 2.0, and 5.0 nmol/L and normetanephrine (Sigma) at 0.3, 0.5, 1.0, 2.0, and 5.0 nmol/L, respectively. Low and high controls contained metanephrine at 0.2 and 0.6 nmol/L and normetanephrine at 0.6 and 1.2 nmol/L, respectively. Stock solutions of analytes were prepared in 100 mmol/L HCl and stored at −70 °C under nitrogen.

Isopropanol precipitation of plasma proteins led to a method with the following interassay imprecision (CV) values: metanephrine, 17.3% at 0.13 nmol/L, 6.4% at 0.50 nmol/L; normetanephrine, 11.1% at 0.5 nmol/L, 5.7% at 1.0 nmol/L (n = 20). Intraassay imprecision (n = 20) was 5.6% for metanephrine and 7.0% for normetanephrine, each at 0.3 nmol/L. Mean recoveries were 96.1% for metanephrine and 106.3% for normetanephrine, with no interference from hyperproteinemia (total protein 80 g/L), hyperlipidemia (triglycerides >3.4 mmol/L), or heparin anticoagulation (n = 6 for each). The limit of quantification (LOQ; the concentration at which the CV = 20%) was 0.09 nmol/L for metanephrine and 0.17 nmol/L for normetanephrine, and the lower limit of detection (2 SD at the LOQ plus the average signal from charcoal-stripped serum) was 0.02 nmol/L for metanephrine and 0.03 nmol/L for normetanephrine. The signal-to-noise ratio was 20 at 0.04 nmol/L for metanephrine and 0.01 nmol/L for normetanephrine. The assay was linear to 20 nmol/L for both compounds.

Method comparison with the new extraction method was performed by an outside reference labora-

tor by using 1-mL bed volume hydrophilic-lipophilic–binding (HLB) columns (Waters) (Fig. 1A and B), as described by Lagerstedt et al. (5). Deming regression analysis (Analyze-It) revealed an agreement between the 2 assays for normetanephrine, with a slope (95% CI) of the regression line of 0.994 (0.978–1.010), an intercept of 0.023 (–0.092 to 0.137), and an SE of the residuals (S_yx) of 0.284. Method comparison for metanephrine revealed a slope of 0.904 (0.624–1.185), an intercept of 0.007 (−0.073 to 0.0875), and S_yx of 0.071.

Given the favorable comparison of isopropanol precipitation with solid-phase extraction, we attempted to evaluate extraction efficiency to determine why we observed poor limits of detection when we used off-line adaptations of the previously published methods. For this experiment, we added 50 μL IS either to patient sample (preextraction addition) or to the solid-phase eluates or the isopropanol supernatants after extraction (postextraction addition). We processed samples by using either the HLB solid-phase extraction method of Lagerstedt et al. (5), modified slightly to use 1 mL patient sample and 50 μL IS, or a weak cation exchange method similar to that described by de Jong et al. (6). Briefly, samples (200 μL plasma, 50 μL IS, and 800 μL water) were applied to 1-mL bed volume WCX columns (Waters) conditioned with acetonitrile and water, washed with water and 90% acetonitrile in water, and eluted with 1 mL 95% acetonitrile in ammonium formate (pH 3). Eluates from solid-phase extraction cartridges were dried and reconstituted in parallel with supernatants from protein precipitation. For the unextracted IS, 50 μL IS was dried down and reconstituted in 100 μL 95% acetonitrile.

Extraction efficiency was then calculated 2 ways (Table 1). First, efficiency was calculated as the IS peak area from preextraction addition divided by the IS peak area from postextraction addition. The results of this calculation demonstrated high recoveries with the use of isopropanol precipitation (approximately 105%) and low recoveries with HLB solid-phase extraction (approximately 55%), consistent with a previous report (5). When we calculated efficiency as the IS peak area from preextraction addition divided by the IS peak area from unextracted IS, the recovery for HLB solid-phase extraction was poor (4% for metanephrine, 1% for normetanephrine), but there was still improvement with the protein precipitation approach (66% for metanephrine, 35% for normetanephrine). Neither analyte was detectable using our off-line adaptation of the on-line weak cation exchange method.

To determine if ion suppression could account for the higher limits of detection of the HLB solid-phase extraction method, we performed a postcolumn infusion experiment (8), in which a constant rate of IS was infused into the postcolumn mobile phase using a T-
junction, while reconstituted extracts of normal, pooled specimens were chromatographed. The results show no substantial ion suppressive interference from protein precipitation or solid-phase HLB extracts at the retention times for the 2 analytes (Fig. 1C and D).

Isopropanol protein precipitation of plasma metanephrines has better extraction efficiency, similar imprecision, comparable mass spectrometric analysis time, and better limits of detection than previous solid-phase extraction techniques. Because low extraction ef-

Table 1. Extraction efficiency.

| Extraction method  | Pre/postpercentagea |  | Pre/unextracted percentageb |
|--------------------|---------------------|  |  |  |
|                    | Metanephrine | Normetanephrine | Metanephrine | Normetanephrine |
| Protein precipitation | 111 | 102 | 66 | 35 |
| HLB column         | 55 | 53 | 4 | 1 |
| WCX column         | 0 | 0 | 0 | 0 |

a Percentage preextraction addition compared to postextraction addition  
b Percentage preextraction addition compared to unextracted internal standard.
ficiency from solid-phase extraction is not due to ion suppression, the analytes must have unacceptably high or low affinity for the columns previously used, or the analytes are complexed with another molecule not removed by solid-phase extraction. Although our results for HLB solid-phase extraction agreed well with previously published results [Table 1 and Ref (5)], the off-line adaptation of a weak cation exchange solid-phase extraction method had poorer recovery than the online extraction from which it was derived (6). The reason for this discrepancy is unclear.

Method comparison of the isopropanol protein precipitation with the previously described HLB solid-phase extraction technique was very good for normetanephrine; however, it was unexpectedly worse for metanephrine. This result was surprising given that both methods employ MS/MS. An important observation was that the solid-phase extraction method had higher imprecision at the concentrations tested, a finding that may explain the slight bias and increased scatter seen for metanephrine (5).

Our data demonstrate that off-line solid-phase extraction may not be an ideal method for use with less sensitive mass analyzers. Indeed, with simple protein precipitation results were superior to those of solid-phase extraction with respect to limits of detection and analyte recovery, similar to what we have demonstrated previously for centrifugal clarification of urine specimens (9). Fortunately, the new approach allowed us to use our current mass spectrometers to quantify plasma metanephrines, and in the future this method may facilitate better recovery of other polar analytes.

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References


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