**Brief Communications**

**Choline in Whole Blood and Plasma: Sample Preparation and Stability**

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**BACKGROUND:** Choline is critical for a variety of biological functions and has been investigated as a biomarker for various pathological conditions including acute coronary syndrome.

**METHODS:** A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was used to quantify choline in whole blood and plasma in freshly collected samples prepared with ultrafiltration or protein precipitation. We investigated the effects of preanalytical variables including types of anticoagulants and storage temperature and time.

**RESULTS:** We observed no significant differences in whole-blood choline concentration in EDTA-anticoagulated vs heparin-anticoagulated samples: mean (SD) difference 0.9% (3.2%), P = 0.80. For plasma, choline concentrations with heparin in 5 of 12 volunteers were >10% higher than with EDTA, P = 0.01. One freeze-thaw cycle led to significant mean (SD) increases in choline concentrations in heparin whole blood, 19.3% (11.4%), P <0.01, and the effect was not significant for other sample types studied (P >0.33). For freshly collected samples stored at ambient temperature, choline concentrations in all types of samples increased with storage time. For EDTA whole blood, EDTA plasma, and heparin plasma, the choline concentration increased for the first 60 min and then stabilized. For heparin whole blood, the choline concentration continued to increase linearly with storage time for >4 h, at which time the choline concentrations were increased by approximately 50%.

**CONCLUSIONS:** Sample collection, storage, and sample preparation procedures are critical for clinical measurements of choline in whole blood and plasma. Choline is a component of phospholipids, which are critical for normal membrane structure and function. Choline is also involved in cholinergic nerve function and is a precursor to betaine, which is involved in homocysteine metabolism (1–2). Choline has been investigated as a biomarker for various pathological conditions. Increased choline concentrations in plasma and whole blood are important and independent predictors of major cardiac events (3–6). Choline is a polar, non-volatile molecule that lacks a chromophore and cannot be measured by immunoassay because of its small size and presence in all mammalian species. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for its quantification have been described (7–10). A homogeneous chemiluminescent enzyme assay for choline was described recently (11–13).

We developed an LC-MS/MS method based on previously published work (7–10). In brief, an Atlantis HILIC silica column (100 × 2.1 mm, 3 μm, Waters) was operated isocratically at 30 °C with 75/25 (vol/vol) acetonitrile/10 mmol/L ammonium formate, pH 3.0 at 0.40 mL/min for 5 min. The retention time of choline and choline-d₉ as internal standard (IS) was 2.5 min. Two transitions were monitored with multiple reaction monitoring and positive electrospray (m/z 104–60 and 58 for choline, m/z 113–69 and 66 for IS).

Both ultrafiltration (UF) and protein precipitation (PPT) were studied for sample preparation. In the PPT procedure, 50 μL sample and 50 μL IS working solution (2 μmol/L in water) were mixed with 500 μL acetonitrile before centrifugation for 10 min at 14 000g and 4 °C. In the UF procedure, 50 μL sample was mixed with 100 μL IS working solution for 5 min, then ultrafiltered at 14 000g and 4 °C for 60 min in a Microcon YM-3 UF device (Millipore).

For sample collection, whole blood was collected into a vacutainer containing EDTA or heparin through venipuncture. Blood was thoroughly mixed and placed on ice. To collect plasma, blood was centrifuged at 1600g at ambient temperature for 10 min. Plasma was transferred to polypropylene tubes and placed on ice. Samples were either processed or frozen at −80 °C immediately. In general, <15 min was required to aliquot all samples. For frozen samples, whole blood and plasma were processed in different batches because whole blood samples took longer to thaw owing to larger sample volumes. Frozen samples were processed immediately after thawing without equilibrating to ambient temperature. All studies with samples from human participants were approved by Institutional Review Board of the University of Utah.
Pooled EDTA plasma was dialyzed against 20 volumes of PBS containing 4 mmol/L EDTA at ambient temperature until choline was not detectable (7). The dialyzed EDTA plasma was used for assay calibration and negative quality control and to prepare positive quality controls by mixing with undialyzed EDTA plasma. The mean within-run, between-run, and total imprecision for 3 quality controls (6.1, 11.6, and 22.5 μmol/L) were <2.6%, <4.7%, and <5.4% (n = 24, in duplicate in each run, 12 runs in 12 different days). The method was linear from 0.1 to >100 μmol/L.

Whole blood and plasma samples (n = 104) were collected and analyzed by the described method and a previously published UF-LC-MS method (3). Choline is stable in ultrafiltrates (2). Ultrafiltrates from whole blood and plasma were obtained as previously described (3). Only ultrafiltrates were directly analyzed by the LC-MS method; whole blood and plasma and their ultrafiltrates were processed with the PPT procedure and analyzed by the LC-MS/MS method. The PPT-LC-MS/MS method produced comparable results for whole blood ultrafiltrates (y = 1.03x - 0.2, r = 0.98) and plasma ultrafiltrates (y = 1.04x - 0.1, r = 1.0). The PPT-LC-MS/MS method also produced comparable results for whole blood (y = 0.92x + 0.1, r = 1.0) and plasma (y = 1.11x - 0.2, r = 0.97), indicating that PPT and UF are essentially equivalent for sample preparation (see Supplemental Fig. 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol54/issue3). All data reported hereafter were generated with the PPT procedure. The overall time required to produce a result from sample collection could be as short as 60 min with the PPT procedure in a batch with 4 calibrators and 3 quality control samples.

The choline concentrations for samples from 12 volunteers are listed in Table 1 (see detailed data in Supplemental Table 1 in the online Data Supplement). Among all volunteers whole blood produced higher choline results than plasma with both EDTA and heparin as anticoagulants, especially for subjects 4 and 8. Higher choline concentrations in whole blood could be explained by intracellular choline production in blood cells, positive choline transport into blood cells, and choline removal from plasma by cellular uptake into some tissues, especially under certain cardiovascular conditions (3, 4, 6). For fresh whole blood samples from all volunteers, there were no significant differences in choline concentrations between EDTA and heparin: mean (SD) difference 0.9% (3.2%), P = 0.80 by paired Student t-test for 2-tailed distribution. With plasma, mean (SD) choline concentrations in heparin plasma were significantly different (P = 0.01) from

<table>
<thead>
<tr>
<th>V</th>
<th>EDTA WBb</th>
<th>LH WB</th>
<th>%Diffc</th>
<th>EDTA PL</th>
<th>LH PL</th>
<th>%Diff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD)d</td>
<td>14.86 (11.49)</td>
<td>14.89 (11.20)</td>
<td>0.9% (3.2%)</td>
<td>7.82 (1.48)</td>
<td>8.33 (1.37)</td>
<td>7.2% (7.4%)</td>
</tr>
<tr>
<td>P</td>
<td>0.80d</td>
<td>0.01d</td>
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</tbody>
</table>

Table 1. Choline concentrations in freshly collected samples after collection and after 1 freeze/thaw cycle.a (Continued)

<table>
<thead>
<tr>
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<th>After 1 freeze/thaw cycle</th>
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<tbody>
<tr>
<td>EDTA WB</td>
<td>LH WB</td>
</tr>
<tr>
<td>-0.2% (4.2%)</td>
<td>19.3% (11.4%)</td>
</tr>
<tr>
<td>0.46*</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

*a All samples were analyzed in triplicate. See Supplemental Table 1 in the online Data Supplement for a complete data set. EDTA, potassium EDTA; LH, lithium heparin; WB, whole blood; PL, plasma.
*b WB and PL choline concentrations are mean (SD) in μmol/L for triplicate results.
*c Percentage differences (%Diff) between LH and EDTA.
*d P values obtained with paired Student t-test and 2-tailed distribution for samples after 1 freeze/thaw cycle vs fresh samples.
EDTA plasma [8.33 (1.37) vs 7.82 (1.48) μmol/L, respectively], and were >10% higher for 5 of 12 volunteers, possibly owing to sample instability with heparin during the plasma separation step. Table 1 also shows that 1 freeze/thaw cycle led to significant increases in choline concentrations in heparin whole blood: mean (SD) difference, 9.3% (11.4%), $P < 0.01$, and the effect was not significant for EDTA whole blood, EDTA plasma, and heparin plasma ($P > 0.33$). Additionally, 1 freeze/thaw cycle led to smaller increases in choline concentrations in heparin whole blood samples from subjects 4 and 8, which had much higher choline concentrations compared to samples from other study participants.

At ambient temperature, choline concentrations in all types of samples increased with storage time (Fig. 1). The data shown are means for 3 volunteers at each time point. For EDTA whole blood, EDTA plasma, and heparin plasma, the choline concentration increased for the first 60 min and then leveled off. For heparin whole blood, the choline concentration continued to increase linearly with storage time for at least 4 h, at which time the choline concentrations were increased by approximately 50% compared to time 0. In a study with 1 EDTA whole blood sample, the choline concentration increased at a rate of 15% per day (95% confidence limits 11% to 18%) when stored at 4 °C and showed no change when stored at −80 °C. An earlier
study of patient samples stored for more than 5 years at −80 °C and analyzed with the UF-LC-MS method (3) showed no systematic increases of choline concentration, results that exclude relevant sample instability at this temperature (data not shown).

Holm et al. (7) previously reported on choline sample stability in EDTA plasma and serum. They found that EDTA plasma was stable up to 72 h at 0 and 25 °C; serum was stable up to 72 h at 0 °C; and choline in serum increased linearly as a function of time, reaching a 7-fold increase after 72 h at 25 °C. Their report does not indicate if the EDTA plasma used was collected fresh, however. Our results for EDTA plasma were similar except for the approximately 10% increases in the choline concentrations during the first hour.

Overall, 3 variables (anticoagulant type, freeze/thaw cycle, and storage temperature/time) were found to affect choline sample stability. EDTA, the preferred anticoagulant for both whole blood and plasma choline, has been previously proved to be a more efficient anticoagulant in preventing clotting formation in plasma samples (14). A few reported cases of drug analysis (15, 16) have shown matrix-related irreproducibility to be more pronounced with heparin as the anticoagulant than with EDTA. The explanation could include both clot formation and sample instability. Heparin and EDTA have different anticoagulant mechanisms. Heparin as a polysaccharide that accelerates the inactivation of thrombin (an enzyme that promotes clotting) and EDTA chelates calcium ions and interrupts the clotting cascade at multiple points. Conceivably, in vitro enzymatic formation (e.g., enzymatic cleavage of choline esters) of choline may occur during the time interval when samples are stored under conditions at which enzymes have significant activity. Further, the in vitro process could be inhibited by EDTA because phospholipases are calcium dependent, whereas heparin may not be inhibitory.

In summary, sample collection, storage conditions and sample preparation procedures are critical for clinical measurements of choline in whole blood and plasma. We found that choline samples are more stable in EDTA than in other anticoagulants. Rapid and simple sample preparation procedures are necessary to minimize sample degradation.

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References


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