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# Quantitative Determination of Free Calcium in Human Serum by High Performance Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS)

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## Abstract

In a novel analytical method, ultra-filterable calcium concentrations in blood were determined using liquid chromatography/tandem mass spectrometry (LC-MS/MS). Calcium present in blood serum of non-clinical adult males was analyzed following complexation with trans-1,2-diaminocyclohexane-N-N-N-N-tetraacetic acid monohydrate (DCTA). Results derived from this method demonstrated good agreement with accepted values for blood serum levels of ultra-filterable calcium, from which the concentration of physiologically-active ionized calcium may be estimated. This method provides a rapid, inexpensive means of analyzing large numbers of samples while requiring minimal sample volumes (0.1 mL), showing considerable potential for clinical and pharmacokinetic applications. This method, which can easily be used for quantitation of total calcium as well, is also applicable for assessment of calcium's isotopic distribution.

## Introduction

Accurate determination of calcium levels in blood provides vital information for assessment of an array of cardiovascular and kidney diseases. In blood serum, calcium exists in two primary forms: a protein-bound fraction and an unbound fraction. The unbound fraction, also known as free or ultra-filterable calcium, incorporates both ionized calcium and calcium which is complexed to small-molecule anions. Although ionized calcium is the physiologically active form, few methods exist for its direct detection. These methods, which typically rely on ion-specific electrodes, are limited in applicability for clinical and pharmacokinetic purposes due to high cost and sample handling restrictions [1].

Because of these concerns, most clinical calcium tests instead measure total calcium concentration, from which ionized calcium may be estimated by accounting for serum albumin levels and applying various correction formulae. However, such formulae have been shown in a number of studies to be imprecise and often inaccurate in predicting the correlation between total calcium and ionized calcium, particularly in cases of hyper- or hypocalcemia [2]. In one such study con ducted by Ladenson et al, none of the 13 commonly-used formulae evaluated proved to be more reliable in predicting hypercalcemia than unadjusted total calcium values alone [3].

One promising approach for more accurate, inexpensive quantitation of this valuable biomarker is the determination of ultra-filterable calcium, which has demonstrated strong correlation with ionized calcium in blood. Unlike total calcium levels, ultra-filterable calcium is independent of protein concentration, and prediction of the ionized fraction is therefore not reliant on the accurate estimates of albumin levels [4]. However, current methodology for quantitation of ultra-filterable calcium consumes significant time and sample volumes and in many cases requires a relatively complicated apparatus [5]. These factors limit severely applicability, especially for clinical trials and for studies in rodents and other species with low blood volumes.

In this work, we present an accurate, cost-effective method for analyzing ultra-filterable calcium concentration in blood serum using the common technology of liquid chromatography and tandem mass spectrometry de-

tection (LC-MS/MS). This method, which can also be applied for analysis of total calcium, permits high-throughput analysis for clinical and pharmacokinetic applications while requiring a fluid sample of only a fraction of a milliliter per specimen. We report results from analyses of human serum conducted using this novel method and compare with accepted values and current methodology.

## Experimental

### Chemicals and Reagents

Liquid chromatography solvents water, methanol, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA) and were high performance liquid chromatography grade or equivalent. Formic acid and ammonium hydroxide (28-30%) were reagent-grade and ACS-grade, respectively. Calcium chloride dehydrate (99%) and chelating reagent trans-1,2-diaminocyclohexane-N-N-N-N-tetraacetic acid monohydrate (DCTA, ≥99%) were purchased from Sigma-Aldrich (St. Louis, MO).

An ammonium hydroxide solution was prepared to a concentration of 0.17 M in water. DCTA solution was prepared to a concentration of 10 mM by dissolving DCTA in three parts water/methanol (85/15) and one part 0.17 M ammonium hydroxide. The 10 mM DCTA solution (pH = 8.17) was subsequently diluted by a factor of 100 with 85/15 water/methanol to yield a 0.1 mM DCTA mobile phase solution (pH = 7.2).

### Preparation of Standards and Serum

Calcium chloride dehydrate was dissolved in DCTA mobile phase solution to prepare a 10.0 mM stock standard solution. A 1:50 dilution of the stock solution with mobile phase yielded a 200 µM intermediate standard solution. Calibration standards were prepared to concentrations 0.0, 10.0, 20.0, 40.0, and 80.0 µM by parallel dilution of the intermediate solution, using DCTA mobile phase as a diluent.

For comparison, additional calibration standards were prepared in human serum matrix by the method of standard addition at concentrations of 0, 10, 20, and 40 µM above background serum calcium levels. All standards used during analysis of quality control samples were prepared in mobile phase.

Levels of free and total calcium were measured from samples of human serum purchased from Biochemed Services (Winchester, VA). Serum originated from normal, non-clinical subjects.

For each sample, a 200-µL aliquot of serum was transferred to an Ultracel YM-30 microcon filter device (Millipore, Billerica, MA) and placed in an oven at 37°C for 15 minutes. After this period, samples were centrifuged at 14000 g (13,500 rpm) at 37°C for 25 minutes using a Beckman Coulter Microfuge 22R (Brea, CA). Filters were removed and filtrates were diluted with DCTA mobile phase to a total volume of 8mL and vortex-mixed prior to analysis by LC-MS/MS.

### Liquid Chromatography and Mass Spectrometry

Samples were analyzed using a Series 200 high-performance liquid chromatography system by PerkinElmer (Waltham, MA) with triple-quadrupole mass spectrometry detection performed on an API 4000 by Applied Biosystems (Foster City, CA) using Analyst 1.4.1 software. A Phenomenex C18 3-µm Gemini 50 x 2.0 mm column was used for chromatography. The method utilized isocratic elution with a mobile phase composition of 85/15 water/methanol with 0.1 m M DCTA. A volume of 5 µL was used for all injections, and flow rate was constant at 0.25 mL/min. Analysis time was approximately 2.5 minutes, with a target analyte retention time of approximately 1.5 minutes. The chromatography system was interfaced with mass spectrometry detection via Turbo Ion Spray (TIS). The instrument was run in the negative ion detection multiple reaction monitoring (MRM) mode and tuned to monitor the double charged transition of m/z 191 → m/z 169. Detailed method parameters are shown in Table 1.

| | | | |
|---|---|---|---|
| Source Temperature | 600°C | Resolution Q1 | Unit |
| Interface Heater | On | Resolution Q3 | Unit |
| Ionization Voltage | -3700 V | Dwell | 200 |
| Curtain Gas | 10 | Entrance Potential (EP) | -10 |
| Nebulizer Gas (G1) | 40 | Declustering Potential (DP) | -30 |
| Turbo Gas (G2) | 50 | Collision Energy (CE) | -20 |
| Collision Gas (CAD) | 4.0 | Collision Exit Potential (CXP) | -6.0 |

## Results

Human serum samples were analyzed for ultra-filterable calcium content and quantitated against a series of calibration standards prepared in mobile phase solution. Comparison of the calibration curves derived from calibration standards prepared in DCTA mobile phase versus calibration standards prepared in human serum demonstrated slope differences of less than 2% for all three comparison trials, as well as overall. This finding confirmed the absence of significant off-target matrix effects and validated the use of mobile phase for preparation of standards and quality control samples as a substitute for unattainable calcium-free serum matrix. All sets of calibration standards demonstrated strong linearity over a

concentration range of 0.0 µM to 100 µM calcium, consistently yielding calibration curves with correlation coefficients (r) of 0.999 or greater (Figure 1).

Quality control samples composed of mobile phase spiked at 0 µM, 20 µM, 40 µM, and 80 µM were included in each analytical set. Analyte recoveries for quality control samples ranged from 94.6% - 110%, with an average recovery of 99.5% and relative standard deviation of 4.6% (N=18) (Table 2).

**Table 2.**

| Sample | Peak Area | Detected µM | Spiked µM | % recovery | Average µM | SD | % RSD |
|---|---|---|---|---|---|---|---|
| LQC1 | 1.46E+05 | 22.0 | 20.0 | 110 | 20.5 | 1.1 | 5.5 |
| LQC2 | 1.34E+05 | 19.9 | 20.0 | 99.7 | | | |
| LQC3 | 1.45E+05 | 21.8 | 20.0 | 109 | | | |
| LQC4 | 1.40E+05 | 21.0 | 20.0 | 105 | | | |
| LQC5 | 1.33E+05 | 19.8 | 20.0 | 98.9 | | | |
| LQC6 | 1.35E+05 | 20.1 | 20.0 | 100 | | | |
| LQC7 | 1.27E+05 | 18.9 | 20.0 | 94.6 | | | |
| MQC1 | 2.53E+05 | 39.6 | 40.0 | 99.0 | 38.9 | 1.1 | 2.8 |
| MQC2 | 2.60E+05 | 40.6 | 40.0 | 102 | | | |
| MQC3 | 2.48E+05 | 38.7 | 40.0 | 96.7 | | | |
| MQC4 | 2.47E+05 | 38.5 | 40.0 | 96.4 | | | |
| MQC5 | 2.41E+05 | 37.6 | 40.0 | 94.0 | | | |
| MQC6 | 2.45E+05 | 38.2 | 40.0 | 95.5 | | | |
| HQC1 | 4.85E+05 | 77.8 | 80.0 | 97.2 | 78.5 | 2.2 | 2.8 |
| HQC2 | 5.13E+05 | 82.4 | 80.0 | 103 | | | |
| HQC3 | 4.82E+05 | 77.3 | 80.0 | 96.6 | | | |
| HQC4 | 4.84E+05 | 77.5 | 80.0 | 96.9 | | | |
| HQC5 | 4.85E+05 | 77.7 | 80.0 | 97.1 | | | |

This Method was applied to detect free Calcium of Dog Serum. The method precession was evaluated in this measurement by 10 parallel samples. The results are shown below in Table 3. Good method precession was achieved.

**Table 3.** Free Calcium Detected in Dog Serum (µM)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1.11 | 1.05 | 1.18 | 1.19 | 1.11 | 1.09 | 1.10 | 1.04 | 1.09 | 1.10 |
| Average = 1.11 | | | | SD = 0.048 | | | %RSD = 4.3 | | |

Analysis of non-fortified serum against calibrants prepared in mobile phase demonstrated good agreement (within 1%) with results derived from matrix-matched standards, yielding an average ultra-filterable calcium concentration of 1.55 mM. This concentrations agree with literature values of 1.48 – 1.62 mM ultra-filterable calcium in blood derived from non-clinical humans [6,7].

These findings indicate that this method is applicable for determination of ultra-filterable calcium concentration in blood serum. Apart from the selection of a highly effective chelating reagent in DCTA, the unique and critical step of this method is the addition of chelating reagent to the mobile phase. By preventing dilution of the reagent during liquid chromatography, this step prohibits dissociation of the calcium metallochelate complexes, ensuring that all chelated calcium can be detected by mass spectrometry.

Subsequent to this work, this method was successfully applied for quantitation of ultafilterable calcium in plasma samples from adult beagles for pharmacokinetic analyses (unpublished data). This method may also be adapted for determination of total calcium concentrations by adding DCTA solution to the biological sample prior to filtration. Though this work focused on blood samples, the method holds promise for applications across a wide range of biological samples, thus providing a valuable tool for clinical and pharmaceutical analyses.

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