Clinical Biochemistry xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Vitamins D and A can be successfully measured by LC–MS/MS in cord blood diluted plasma

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ARTICLE INFO

Article history:
Received 16 January 2015
Received in revised form 4 April 2015
Accepted 17 April 2015
Available online xxxx

Keywords:
Cholecalciferol
Retinol
Neonates
Mass spectrometry
Umbilical cord blood diluted plasma

ABSTRACT

Objectives: In widely used protocols for the collection and isolation of cord blood mononuclear cells, investigators are left with substantial volumes of diluted plasma which could be used for other measurements. The aim of this study was to ascertain the validity of umbilical cord blood (UCB) diluted plasma samples for vitamin D, A and E analysis compared to UCB serum samples.

Design & methods: Twenty UCB matched samples of diluted plasma and serum were collected. The samples were analysed by two liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods on two separate occasions.

Results: The results of 25(OH)D3 obtained by the two laboratories demonstrated close agreement with a mean difference of 0.14 nmol/L [95% confidence interval (95% CI), −6.8 to 7.1]. Both methods demonstrate close agreement for 25(OH)D3 in UCB serum versus diluted UCB plasma; mean difference 2.2 nmol/L [95% CI, −9.5 to 13.9] and 4.1 nmol/L [95% CI, −14.5 to 6.1] for the results from Lab A and Lab B, respectively. Vitamin A was quantified by Lab A in UCB serum and diluted UCB plasma; mean difference 0.07 μmol/L [95% CI, −0.41 to 0.28]. Results of 25(OH)D3 epimer and vitamin E in the diluted UCB plasma were below the limit of quantification, and could not be compared with UCB serum.

Conclusions: Diluted UCB plasma can be used for the quantification of retinol and 25(OH)D3 by LC–MS/MS. By contrast, quantification of 25(OH)D3 epimer and vitamin E in diluted UCB plasma is not supported by this study due to limitations in analytical sensitivity.

Introduction

Fat soluble vitamin deficiency is classically associated with complications of diseases presenting in neonates [1]. Of the four vitamins in this group, vitamins A, D and also K have pleiotropic actions whilst vitamin E has important anti-oxidant activity. Of these, vitamin D has received a lot of attention recently as a result of the meteoric rise in the number of publications showing that this secosteroid plays a crucial role in a plethora of physiological functions and is associated with many acute and chronic illnesses. In particular, there is mounting interest in the potential importance of vitamin D status, and to a lesser extent vitamin A, during early life for a wide range of health outcomes [2].

Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) quantification of each of these fat soluble vitamins, including separation of epi-25(OH)D3, is now established [3–5]. Serum, and also undiluted plasma, are the validated matrixes for analysis of vitamins A (retinol), D (25(OH)D3) and E (α-tocopherol). However the diluted plasma matrix, which is widely used in protocols for the collection and isolation of viable mononuclear cells, has not been validated for use in the LC–MS/MS analysis of small molecules. Given the limited volumes of blood available in birth cohort studies, and the implicit value of these in the context of a research intensive large-scale epidemiological projects, it is of interest to determine whether vitamins D, A and E may be adequately measured in diluted plasma from umbilical cord blood (UCB).

Abbreviations: 25(OH)D3, 25-hydroxy vitamin D3; BIS, Barwon Infant Study; LoQ, limit of quantification; LC–MS/MS, liquid chromatography coupled with mass selective detection; MNC, mononuclear cell; MRM, multiple reaction monitoring; PFP, pentafluorophenyl; RCPAQAP, Royal College of Pathologists of Australasia Quality Assurance Programs; TM, transport medium; 25(OH)D3-d3, tri-deuterated 25-hydroxy vitamin D3; UCB, umbilical cord blood.

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http://dx.doi.org/10.1016/j.clinbiochem.2015.04.014
0009-9120/

The aim of this study was to validate the measurement of vitamins D plus vitamins A and E using LC–MS/MS in diluted UCB plasma versus UCB serum.

Methods

Subjects

Twenty participants, recruited as part of the Barwon Infant Study (BIS), were randomly selected for comparison of matched serum and diluted plasma of UCB samples. BIS is a population derived birth cohort study conducted in south-eastern Australia that has been designed to investigate the early life origins of immune dysregulation. UCB was collected and stored as part of the BIS protocol. The project was approved by the Barwon Health Human Research Ethics Committee (10/24) and written informed consent was obtained prior to collection.

Sample collection

The primary aim in collection of UCB was to isolate a large number of viable mononuclear cells (MNC) that could be cryopreserved for future immune studies. To this end, two separate samples of UCB were collected using a 50 mL syringe inserted into the umbilical cord vein. Where there was an adequate volume of UCB, the majority of the sample was added to a sterile tube containing exactly 20 mL of sterile Transport Medium (RPMI-1640) with 10 IU/mL preservative-free heparin (DBL Heparin Injection BP (porcine mucous) 5000 IU/5 mL), and the remaining blood added directly to a serum collection tube. Samples of serum were collected and aliquoted after the tube was centrifuged (2700 *g*, 10 min at 20 °C). In addition, the volume of anti-coagulated diluted UCB was accurately measured, and the tube centrifuged (2700 *g*, 10 min at 20 °C). The diluted plasma samples were aliquoted and stored with the matched serum samples at −80 °C.

Dilution of UCB plasma

Once the blood cells were pelleted, the diluted plasma volume was estimated (= total volume anti-coagulated diluted UCB − volume of pelleted blood cells), and then the dilution factor was calculated (=(diluted plasma volume − 20) mL/diluted plasma volume (mL)). Depending on the volume of UCB collected, samples ranged in dilution from 0.26 to 0.43 (mean ± SEM 0.32 ± 0.01) of neat plasma.

Experimental

Twenty UCB sera and 20 diluted plasma de-identified samples were thawed and 150 μL aliquots delivered in a Styrofoam container to laboratory A (Lab A) [LC–MS/MS laboratory, Clinical Biochemistry Mass Spectrometry Laboratory, RMIT University, VIC, Australia] and to laboratory B (Lab B) [UWA Centre for Metabolomics, Metabolomics Australia, University of Western Australia, WA, Australia]. Both laboratories were blinded to the sample pairs for analysis and results were returned to the BIS coordinator (FC) for pair identification. Samples were analysed in two non-consecutive runs (R1 and R2) in random order to consider between run effects in the two laboratories.

The two LC–MS/MS methods are briefly described below:

Laboratory A

This LC–MS/MS method was established for the simultaneous quantification of fat soluble vitamins [25(OH)D3, vitamin A (retinol) and E (α-tocopherol)] and utilised an Agilent-1200 LC coupled with an Agilent-6410 Triple Quadrupole Mass Spectrometer (Agilent Technology Inc., VIC, Australia).

Samples (100 μL) were prepared using a routine liquid–liquid hexane extraction which incorporated tri-deuterated 25-hydroxy vitamin D3 (25(OH)D3-d3) and hexa-deuterated α-tocopherol as the internal standards (IsoSciences LLC, PA, USA). A pursuit pentafluorophenyl (PFP) column (150 mm × 2 mm, 3 μm) (Agilent Technology Inc., VIC, Australia), with matching guard column was used to separate the fat soluble vitamins; this included clear separation of 25(OH)D3 from its epimer (epi-25(OH)D3).

Electrospray ionisation (positive-mode) in association with multiple reaction monitoring (MRM) was utilised to quantify 25(OH)D3 and its isomer (401 → 383), retinol (269 → 93) and α-tocopherol (431 → 165). The 25(OH)D3-d3 (404 → 386) was used as the internal standard for 25(OH)D3 and retinol (its match stable internal standard was unavailable, thus, and 25-(OH)D3-d3 was used as the closest retention time to retinol) whilst hexa-deuterated α-tocopherol (437 → 171) was the internal standard for α-tocopherol [6]. Vitamin D was calibrated using a Recipe Calibrator set (Recipe, Munich, Germany) which is reported to be traceable to NIST-SRM972. Vitamins A and E were calibrated using the Bio-Rad Calibrator (Bio-Rad Laboratories, Munich, Germany) which is traceable to NIST-SRM968e [7].

Method imprecision for 25(OH)D3 is 2.6%, 3.1% and 4.7% at 150, 68 and 25 nmol/L, respectively; for vitamin A 2.9%, 3.8% and 4.7% at 3.4, 1.7 and 0.5 μmol/L, respectively; and for vitamin E is 4.4%, 4.0% and 5.5% at 54, 22 and 6 μmol/L, respectively. The LOQ was 3.5 nmol/L for 25(OH)D3 and its epimer, 0.16 μmol/L and 3 μmol/L for vitamins A and E, respectively [8]. Independent ongoing peer review of this method is conducted through participation in the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) (Fig. 1a) [9].

Laboratory B

This LC–MS/MS method is for the analysis of 25(OH)D3 and it epimer. Analysis was performed on an Agilent-6460 coupled to a 2-dimensional 1290 UPLC system. The method uses 50 μL of serum and has a run time of 8 min. Vitamin D was calibrated using a Chromsystems Calibrator set (Chromsystems, Munich, Germany) which is reported to be traceable to NIST-SRM972. The imprecision of the method for 25(OH)D3 at 75 nmol/L and 18 nmol/L is 0.5% and 2.2%, respectively. The LOQ for 25(OH)D3 is 2 nmol/L [10]. Independent ongoing peer review of this method is conducted through participation in the Vitamin D standardisation programme run by the CDC and NIH [11] (Fig. 1b).

Statistical analysis

Passing–Bablok regression and Bland–Altman difference plots were used to compare the results of vitamin measurements in UCB serum and diluted UCB plasma. Spearman correlation was used to examine the group of results. A p-value was calculated using the Mann–Whitney two-tailed test, and $p < 0.05$ was considered statistically significant. Percentage mean differences were calculated based on the average percentage differences of the overall peer results. All statistical calculations and comparison plots were conducted using XLSTAT software [12].

Allowable total error (TEa) for vitamins A and E was taken from the Ricos Biological Variation database [13]. TEa for vitamin D was calculated as follows [14]:

TEa% = Z × X × CVw + B

Where: Z = 1.65; X = 0.5; B = desirable specification for inaccuracy (bias).

Bias can be calculated from: $\mathbf{B = 0.25 \times [CVw_2 + CVg_2]^{1/2}}$.

From reference [14]: Within subject biological variation (CVw) = 8%; and between subject biological variation (CVg) = 20%.

Then **B = 0.25 × [8^2 + 20^2]^1/2 = 5.4%**.

Hence the allowable total error for 25(OH)D3 is **TEa% = 1.65 × 0.5 × 8 ± 5.4 = 12%**.

Please cite this article as: Albarhani AA, et al, Vitamins D and A can be successfully measured by LC–MS/MS in cord blood diluted plasma, Clin Biochem (2015), http://dx.doi.org/10.1016/j.clinbiochem.2015.04.014