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

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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.

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1. 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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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.

2. Methods

2.1. 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.

2.2. 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.

2.3. 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.

2.4. 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:

2.4.1. 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].

2.4.2. 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).

2.5. 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]:

$$\mathbf{TEa\% = Z \times X \times CVw + B}$$

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 $\mathbf{B = 0.25 \times [8\hat{}2 + 20\hat{}2]\hat{}1/2 = 5.4\%}$.

Hence the allowable total error for 25(OH)D3 is $\mathbf{TEa\% = 1.65 \times 0.5 \times 8 \pm 5.4 = 12\%}$.

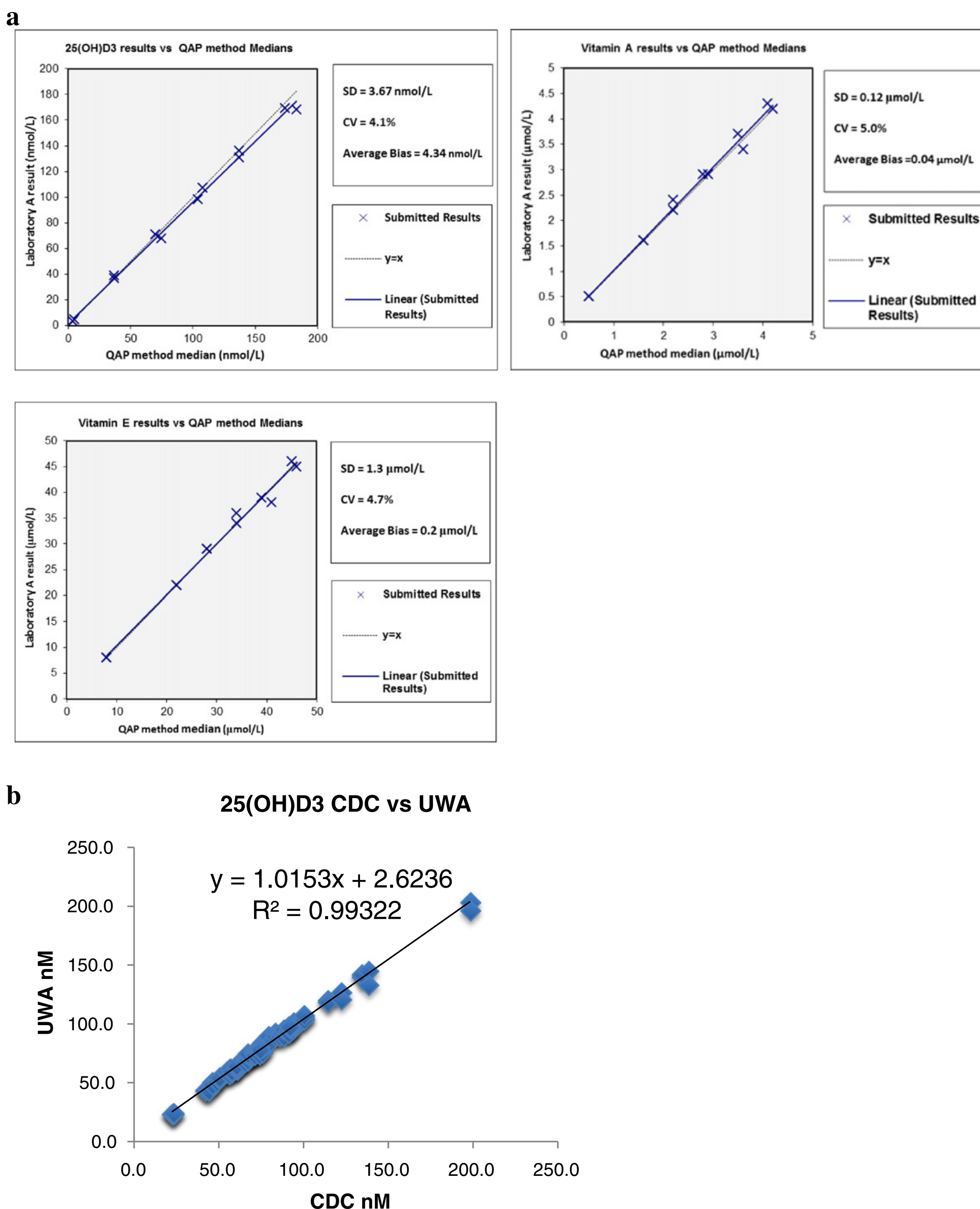

Fig. 1. a: Laboratory A external quality assurance results. RCPA Quality Assurance Programs end of cycle performance for vitamin D, A and E for the second half of 2013 [9]. Reproduced with permission. b. Laboratory B, external quality assurance results. Comparison of the CDC reference assay vs. UWA for 25(OH)D3. Data from May 2013; n = 40 [11]. Reproduced with permission.

3. Results

Twenty matched samples of UCB serum and diluted UCB plasma were analysed by two LC–MS/MS laboratories (Lab A measured vitamin D and its epimer, plus vitamins A and E; Lab B measured vitamin D and its epimer) on two consecutive occasions; Fig. 2. As part of the continued monitoring of ion suppression by Lab A, two transition ions for phospholipids (104 → 104 and 184 → 184) were monitored for each sample that was analysed. These phospholipids could be a source of ion suppression due to their effects on the efficiency of chromatographic separation and the ionisation process. Inspection of the chromatograms from Lab A demonstrated that both serum and diluted plasma display the chromatographic separation of the target analytes as well as the intensity of the phospholipids detected in the samples. Lab A found that there were no co-eluted phospholipids with the target analytes across the UCB serum and diluted plasma with RPMI. An example chromatogram is provided in the Supplement.

3.1. Vitamin D

The results of 25(OH)D3 obtained by the two laboratories demonstrate close agreement as demonstrated by the Passing–Bablok regression

and Bland–Altman plots in Fig. 3a. Results obtained by Lab A compared with Lab B (r = 0.983, p = 0.703) with a mean difference of 0.14 nmol/L (−4.42%) [95% confidence interval (95% CI), −6.8 to 7.1]. Both methods demonstrate a close relationship between serum compared to the diluted plasma (r = 0.914, p = 0.532 for Lab A; r = 0.904, p = 0.205 for Lab B) with a mean differences of 2.2 nmol/L (6.6%) [95% CI, −9.5 to13.9] and 4.1 nmol/L (−8.5%) [95% CI, −14.5 to 6.1] for the results of Lab A and Lab B, respectively; Figs. 3b and 4a.

3.2. Epi-vitamin D

Epi-25(OH)D3 in UCB serum and diluted plasma samples were quantified by both laboratories. Epi-25(OH)D3 was detected in all serum samples, however, 40% (Lab A) and 30% (Lab B) of serum results and all diluted plasma results were below the limit of quantification (LoQ); Lab A LoQ is 3.5 nmol/L and Lab B LoQ is 2.0 nmol/L. Serum epi-25(OH)D3 results above the LOQ obtained by the two laboratories

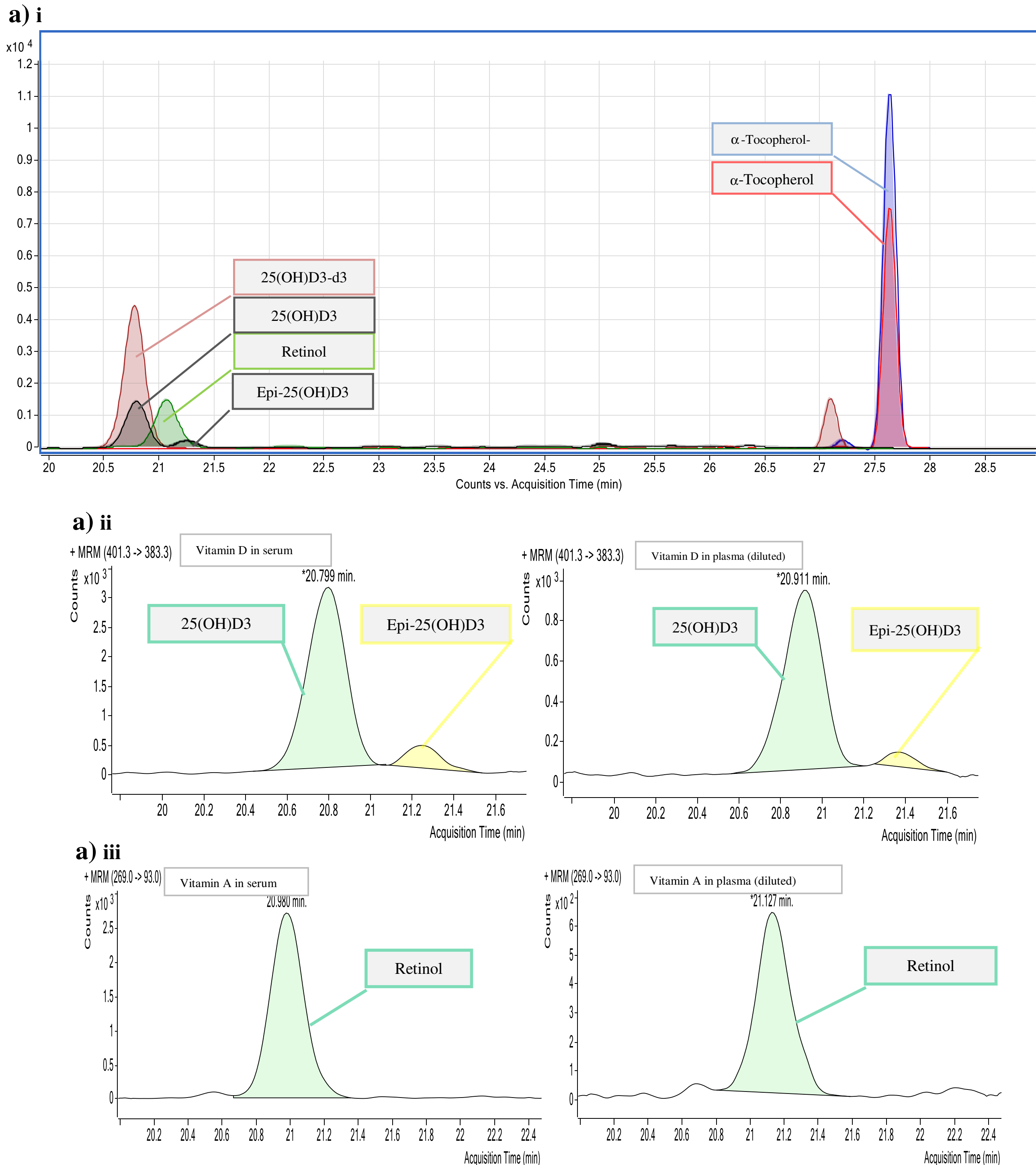

Fig. 2. a. Laboratory A method chromatograms demonstrating separation of i) fat soluble vitamins (D, A and E), ii) vitamin D in UCB serum and diluted UCB plasma sample, iii) vitamin A in UCB serum and diluted UCB plasma samples. All figures displayed are from the one subject. i) Three fat soluble vitamin chromatogram of serum sample; ii) chromatogram of vitamin D in UCB serum and diluted plasma; and iii) chromatogram of vitamin A in UCB serum and diluted plasma. b. Laboratory B method chromatograms demonstrating separation of 25(OH)D3 from epi-25(OH)D3 in UCB serum and diluted UCB plasma from the same subject; dilution factor is 0.37. Note y-axis scaled for comparison purposes.

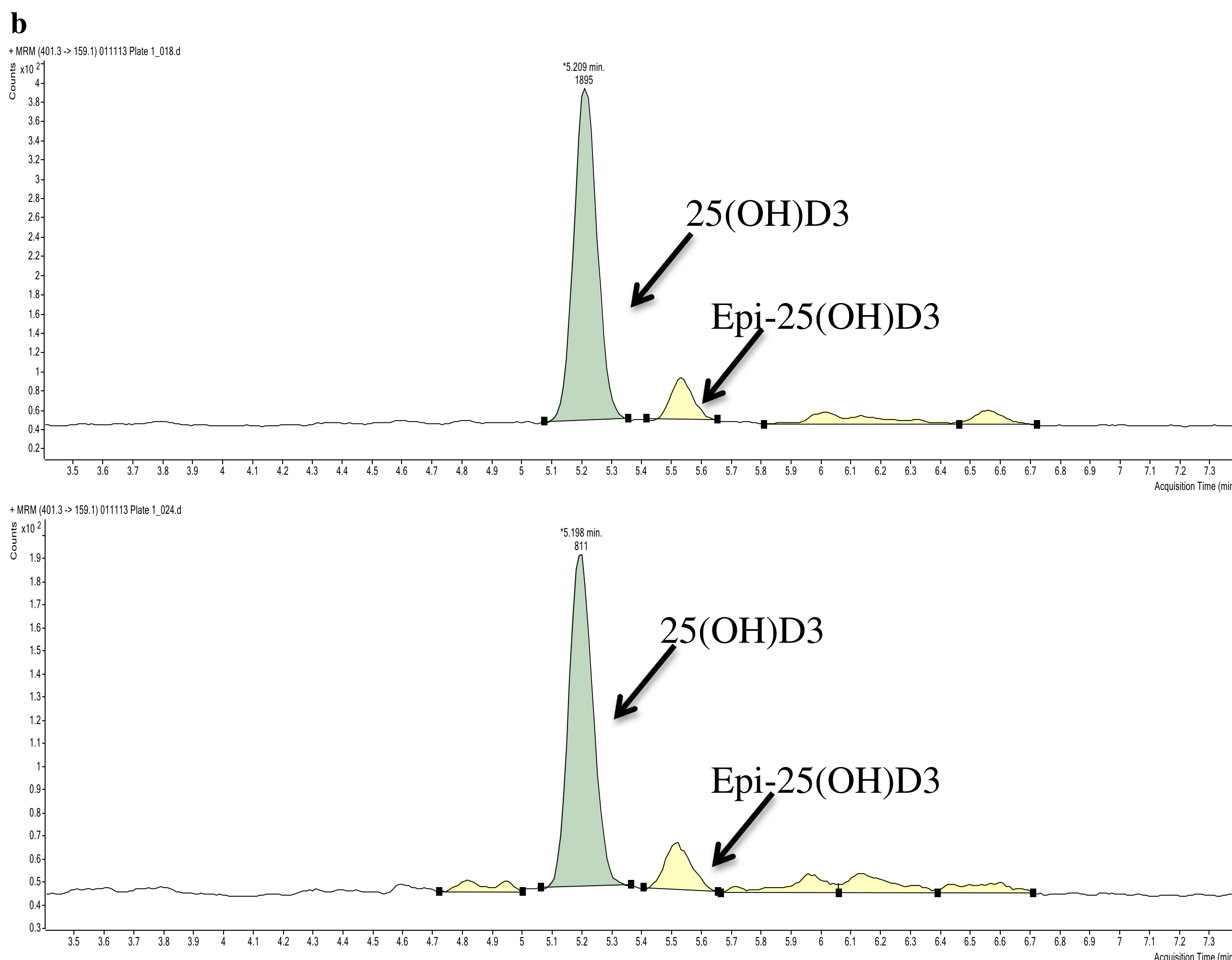

Fig. 2 (*continued*).

were correlated (r = 0.869) with a mean difference −0.76 nmol/L (−16.5%) [95% CI, −2.3 to 0.77].

3.3. Vitamin A

Retinol was measured in UCB serum and diluted samples using Lab A method. Vitamin A was quantifiable in all serum samples and 65% of diluted plasma samples; with 35% of diluted plasma results below the method LoQ. Quantified results of vitamin A in serum and diluted plasma samples demonstrated a medium correlation and mean difference of −9.9% across the analytical runs; Figs. 3c and 4b.

3.4. Vitamin E

α-Tocopherol levels were quantified in both serum and diluted plasma; however, all of the diluted plasma results were below the LoQ (3 μmol/L) and hence a reliable comparison could not be made.

4. Discussion

This study examined the suitability of diluted UCB plasma, compared with undiluted UCB serum, and provides the first report on its utility for the quantification of vitamins A and D by LC–MS/MS. This study also demonstrates the agreement between results obtained across two LC–MS/MS laboratories for vitamin D, plus the continued challenges faced in the quantitation of epi-25(OH)D3.

The appropriate selection of sample matrix is an important issue in the assessment of a number of blood analytes in clinical laboratories. Although serum and plasma are commonly used as blood specimen type, they are not equivalent biological matrices; for example, serum has less protein concentration than plasma as a result of blood clotting process [15]. Whilst, evidence based recommendations are in place supporting serum and plasma (undiluted) for the quantification of vitamins A and E [6], similar recommendations are not currently in place for vitamin D. The results presented here support the reliable use of serum and RPMI 1640 diluted plasma for the quantification of vitamins A (retinol) and D (25(OH)D3), with a wide dilution range of 26–43%.

Accuracy of results generated is important for clinical decision making, especially when clinical practice guidelines quote absolute finite numbers for interpretation. This point has been hotly debated in recent years in relation to vitamin D results. Reassuringly, the 25(OH)D3 results obtained by Lab A and Lab B demonstrate close agreement, even with the use of different commercial calibrators; with a mean difference between labs of 4.4%. This further supports the important efforts of standardisation of methods to improve clinical utility of results, with both commercial calibrators being traceable to the one standard reference material; NIST 972. In addition, this standardisation is further supported by the ongoing peer review of both laboratories through their participation in an external quality assurance programme.

Although the clinical role of epi-25(OH)D3 is still unclear, epi-25(OH)D3 is reportedly detectable serum levels in approximately 90% of adults and 93% of children [16]. Consequently, chromatographic separation and detection of the epimer are important for accurate

quantification of 25(OH)D3 to avoid over-estimation. In the current study, epi-25(OH)D3 was detected in all samples of UCB serum, of which 60% (Lab A) and 70% (Lab B) of results were higher than the LoQ of the methods. Previously, we have found, from studies conducted in Lab B, that the diluted epimer value can be reported typically where the initial serum value is greater than 6 nmol/L [unpublished data]. Of note

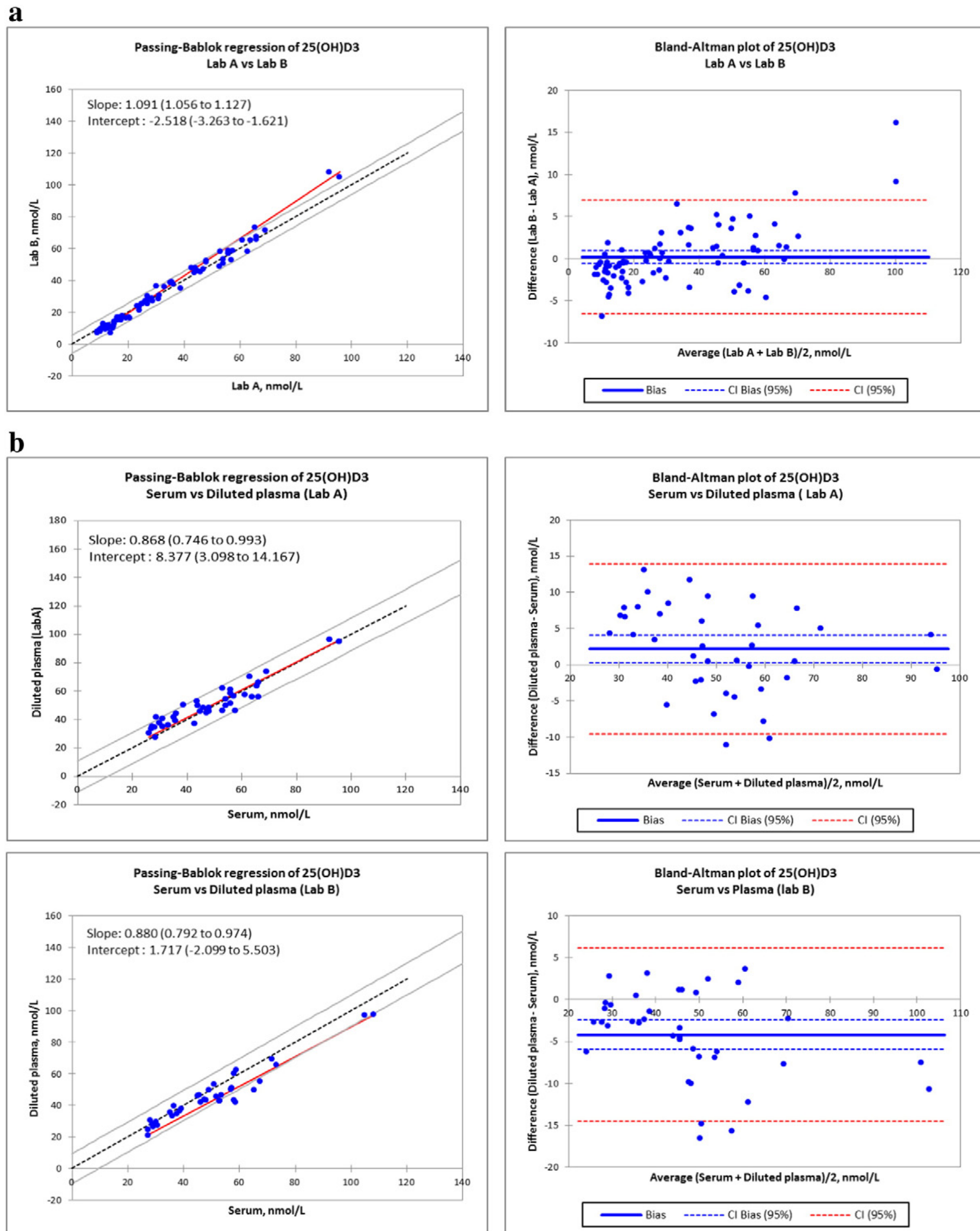

Fig. 3. a: Passing–Bablok regression plots and Bland–Altman plot demonstrating the agreement in 25(OH)D3 results obtained from Lab A compared with Lab B for all the samples analysed i.e., both serum and diluted plasma. Results obtained by Lab A compared with Lab B ($r = 0.983$, $p = 0.703$) with a mean differences of 0.14 nmol/L (−4.42%) [95% confidence interval (95% CI), −6.8 to 7.1]. b: Passing–Bablok regression plots and Bland–Altman plot demonstrating the agreement in 25(OH)D3 results obtained from UCB serum and diluted UCB plasma. Both methods demonstrate a close relationship between serum compared to the diluted plasma ($r = 0.914$, $p = 0.532$ for Lab A; $r = 0.904$, $p = 0.205$ for Lab B) with a mean differences of 2.2 nmol/L (6.6%) [95% CI, −9.5 to13.9] and 4.1 nmol/L (−8.5%) [95% CI, −14.5 to 6.1] for the results of Lab A and Lab B, respectively. Both these percentage mean differences are within the limit for the desirable specification for allowable total error (TE%) for 25(OH)D3 (12.0%). c: Passing–Bablok regression plots and Bland–Altman plot demonstrating the agreement in vitamin A results obtained from UCB serum and diluted UCB plasma. Results above method LoQ (0.16 μmol/L) were only plotted in the graphs. Quantified results of vitamin A in serum and diluted plasma samples demonstrated the mean difference of a −0.07 μmol/L [95% CI, −0.41 to 0.28] representing a mean change of −9.9% across the analytical runs. Results of UCB serum versus diluted UCB plasma showed a medium correlation ($r = 0.45$, $p = 0.224$).

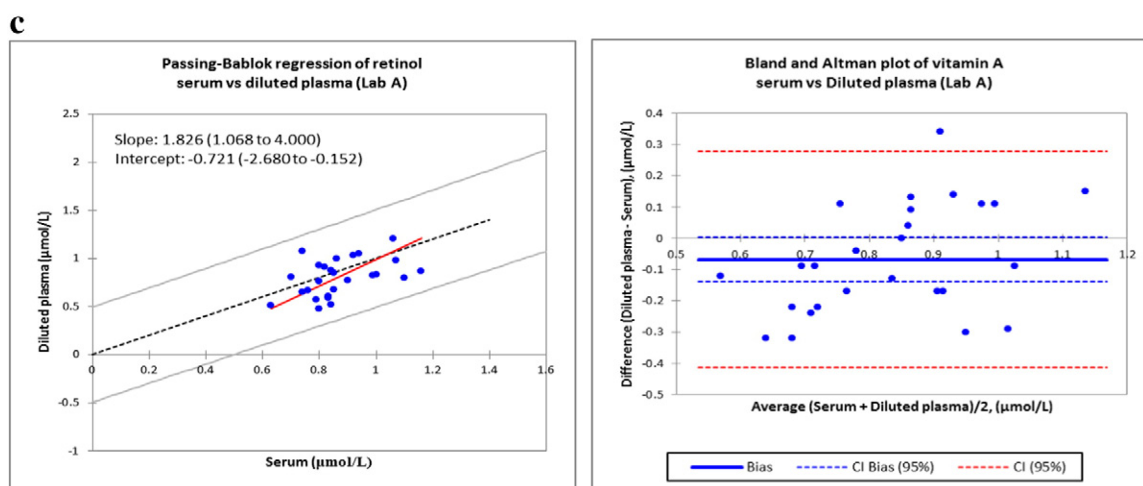

Fig. 3 (*continued*).

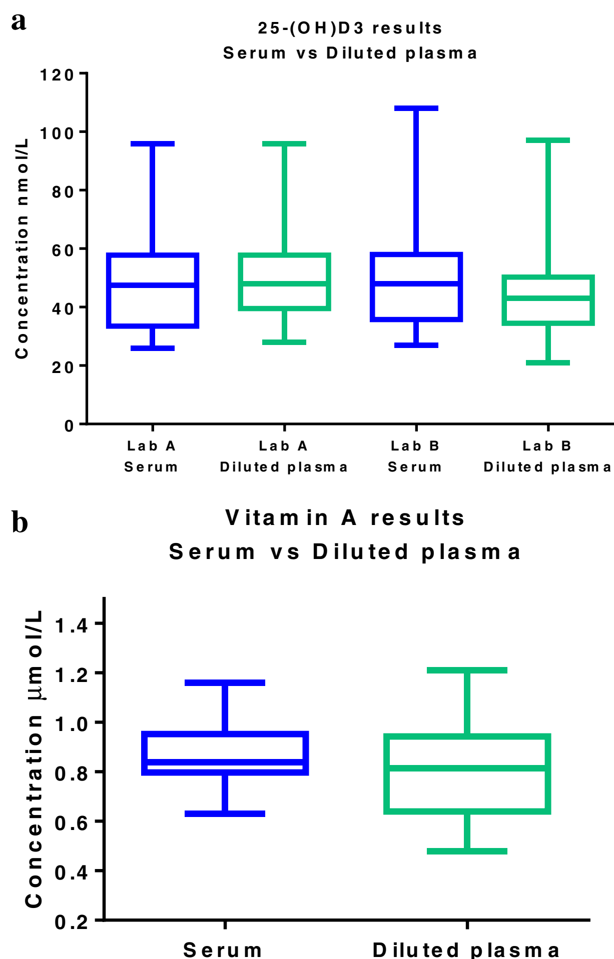

Fig. 4. a: Box plot demonstrating the minimum, first quartile, median, third quartile, and maximum of 25(OH)D3 results of UCB serum versus diluted UCB plasma obtained by laboratories A and B. Method LoQ is 3.5 nmol/L (Lab A) and 2.0 nmol/L (Lab B) for both 25(OH)D3 and its epimer. b: Box plot demonstrating the minimum, first quartile, median, third quartile, and maximum of vitamin A results of UCB serum versus diluted UCB plasma obtained by laboratory A. The box plot was created based the results, which were higher than method LoQ (0.16 μmol/L).

in this current study, in contrast to the close agreement demonstrated for 25(OH)D3 is the percentage mean difference between the C3-epimer results for Labs A and B (−16.5%).

Quantitation of the C3-epimer form of 25(OH)D3 remains challenging by LC–MS/MS irrespective of the matrix. Whilst chromatographic separation is readily achievable, there is a lack of biological variation data for epi-25(OH)D3, the allowable TE% could not be calculated. Further confounding quantitation was the absence of a commercial calibrator for epi-25(OH)D3; therefore in this study, the multiple level commercial calibrators used to create the 25(OH)D3 standard curve were applied for the quantification of epi-25(OH)D3. In addition, most reported UCB results for epi-25(OH)D3 were close to the LoQ levels (3.5 nmol/L for laboratory A and 2.0 nmol/L for laboratory B), and this could be an additional source of variation between the two laboratory results.

Vitamin A (retinol) has previously been reported in UCB serum samples and low levels (<0.7 μmol/L) have been correlated with low birth weight [17]. Our study demonstrates a favourable comparison of UCB serum and diluted plasma utilising LC–MS/MS methodology. The percentage mean difference between the results of the UCB serum and diluted plasma was −9.9%, which is less than the allowable TE% of 17.1% [13]. However, the results between the two groups showed a medium correlation ($r = 0.451$). This observation might be related to the unexplained reported difference in the biological variation for serum (13.6%) versus plasma (6.2%) retinol [6,18,19].

Finally, in the current study, vitamin E levels in all diluted UCB plasma results were below the method's LoQ (3 μmol/L). Our observation of the low vitamin E in UCB is in agreement with previous findings by Didenco and colleagues who demonstrated that cord blood α-tocopherol levels were significantly lower than the maternal blood level by 80% [20]. It is hypothesized that this is due to selective transfer of α-tocopherol by the placenta. Therefore, UCB diluted plasma is not suitable for the quantification of vitamin E.

5. Conclusion

Diluted UCB plasma can be used for the quantification of 25(OH)D3 and vitamin A by LC–MS/MS. By contrast, measurement of the 25(OH)D3 epimer and vitamin E in diluted UCB plasma is not supported by this study due to the current limitation of analytical sensitivity for quantification. Potentially this limitation could be addressed in the future through the use of increased sample volume.

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Financial disclosure

The authors have no financial relationships relevant to this article to disclose.

Conflicts of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of this manuscript.

Authorship statement

All authors listed contributed to this work. Professor Allen, Professor Ponsonby and Dr Vuillermin developed the initial study concept. Dr Collier organised and distributed the de-identified samples. Mr Albarhani and Dr Clarke analysed the samples in the respective laboratories and performed the statistical analysis in conjunction with Dr Collier. Dr Greaves and Dr Roche supervised and guided Mr Albarhani's work, which formed part of his PhD candidature. Mr Albarhani wrote the first draft of this manuscript and all authors critically reviewed the manuscript, assisted in data interpretation, approved the final manuscript as submitted and agree to be accountable for all aspects of this work.

Data sharing statement

Additional information including analytical protocol and raw data may be obtained by contacting the corresponding author.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2015.04.014.

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