Development and Applications of Methods for Assessing Fat Soluble Micronutrients (FSMs) in Dried Blood Spots and Plasma by HPLC and HPLC-MS/MS

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Abstract

Epidemiological studies have shown that deficiencies in vitamin A and vitamin E are very high in developing countries, whereas vitamin D deficiency is a global health concern. Deficiencies in fat soluble vitamins have been associated with negative health consequences, and are most severe when they occur during pregnancy, where they may lead to complications in both the mother and the offspring. In order to better understand the status of populations and associations with diseases, the establishment of analytical methods for determining fat soluble micronutrient (FSM) concentrations is essential. Therefore, the central aim of this thesis was to develop simple and reliable assays for FSMs in human blood and plasma, which would be suitable for application in large-scale clinical studies.

The process of analytical method development in this thesis included: i) establishing and validating a dried blood spot (DBS) test for the measurement of retinol (biomarker for vitamin A) concentrations; ii) an assay to simultaneously determine all FSM (retinol, α-tocopherol, γ-tocopherol, β-tocopherol, 25OHD3, 25OHD2, lutein, zeaxanthin, lycopene and β-carotene) concentrations using a minimal volume of plasma; and iii) to validate these methods through the measurement of concentrations of FSMs in a sub-set of plasma samples in an ongoing clinical study (n = 103 women at two time points, one of which is first and the other the second trimester). This enabled me to evaluate the impact different anti-coagulants on FSM concentrations, and to obtain pilot data on the concentrations of FSMs in pregnant women in South Australia, and how they change across gestation.

The DBS method that I established for measuring retinol showed good reliability and robustness when extracted using acidic conditions. Additionally, using this method, retinol in DBS was shown to be structurally stable at room temperature for 10 weeks, when stored

in the dark and with desiccants. The measurement of all FSMs in plasma also showed reliable and sensitive results including high precision for all analytes (coefficient of variation < 13%), high accuracy when compared to standard reference materials (82% - 112%), and excellent linearity in the plasma matrix ($R^2 \geq 0.99$).

The evaluation of the impact of anti-coagulant type on FSM levels showed significant differences in concentrations for retinol, lycopene and β-carotene between EDTA and heparin-treated plasma samples. Additionally, FSM concentrations decreased by up to 20% for some analytes when they were stored at room temperature for > 20 h before processing.

The assessment of FSMs in the group of pregnant women in South Australia indicated vitamin A, E and carotenoid sufficiency, as well as low prevalence of vitamin D insufficiency (< 10%). Significant changes in concentrations of vitamin D, E and carotenoids between the first trimester and second trimester during pregnancy were also identified.

In conclusion, this thesis reports the successful development of methods for measuring vitamin A in DBS and FSMs in plasma using HPLC, which are reliable, accurate and robust. The DBS method will be a valuable tool for clinical studies and studies in remote areas where sample collection and transportation are difficult.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Publications

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Y. Huang, J. Carragher, D. Cozzolino, Measurement of Fructose, Glucose, Maltose and Sucrose in Barley Malt Using Attenuated Total Reflectance Mid-infrared Spectroscopy, *Food Anal. Methods*, (2015) 1-7.

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Y. Huang, P.R. Clements, R.A. Gibson, Simple and robust measurement of vitamin A on dried blood spots by HPLC, *Australian Society for Medical Research: South Australian Scientific Meeting*, South Australia.3 June 2015.

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Abbreviations

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25OHD3	25-hydroxycholecalciferol; or 25-hydroxyvitamin D3
25OHD2	25-Hydroxyergocalciferol; or 25-Hydroxyvitamin D2
ANOVA	analysis of variance
BHT	butylated hydroxytoluene
CI	confidence interval
DAD	diode array detector
DAPTAD	4-(4'-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione
DBS	dried blood spot
DEQAS	vitamin D External Quality Assessment Scheme
DHA	docosahexaenoic acid
EDTA	ethylene diamine tetraacetic acid
FLD	fluorescence detector
FSM	fat soluble micronutrient, including vitamin A, D, E and carotenoids
HPLC	high performance liquid chromatography
HPLC-MS/MS	high performance liquid chromatography coupled with tandem mass spectrometry
IoM	Institute of Medicine, US
IQR	interquartile range
IS	internal standards
LoA	limits of agreement (in Bland-Altman tests)
LOD	limit of detection
LOQ	limit of quantification

MTBE	methyl *tert*-butyl ether
MPA	mobile phase A
MPB	mobile phase B
MRM	multiple reaction monitoring
MS	mass spectrometry
m/z	mass to charge ratio
NHMRC	National Health and Medical Research Council of Australia
NIST	National Institute of Standards and Technology
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
QC	quality control
RAE	retinol active equivalents
RBP	retinol binding protein
RDI	recommended dietary intakes
SD	standard deviation
S/N	signal to noise ratio
SPE	solid phase extraction
SRM	standard reference material
THF	tetrahydrofuran
UPLC	ultra performance liquid chromatography
UV	ultra violet
WHO	World Health Organisation