Vitamin D and Metabolites: Evaluation of Supported Liquid Extraction (SLE) prior to LC-MS/MS Analysis

Lee Williams, Rhys Jones, Helen Lodder, Geoff Davies, Steve Jordan, Richard Calverley, Claire Desbrow & Gary Dowthwaite
Biotage GB Limited, Distribution Way, Dyffryn Business Park, Ystrad Mynach, Hengoed, CF82 7TS, UK.

Introduction

Vitamin D is the collective term for a group of fat soluble pro-hormones which exist as two major forms, D_2 (ergocalciferol) and D_3 (cholecalciferol). Vitamin D is biologically inert within the body, however, two subsequent hydroxylation reactions provide the main active metabolites; 25-hydroxy-vitamin D and 1α, 25-dihydroxy-vitamin D. Humans receive vitamin D either through diet or exposure to sunlight (UVB). Deficiency can result in various disorders including osteoporosis, liver and kidney problems to increased risk of various cancers and multiple sclerosis. From this standpoint Vitamin D analysis has extremely important clinical relevance. Here we demonstrate vitamin D extraction using 96-well supported liquid extraction (SLE) prior to LC-MS/MS analysis.

Experimental Procedure

Reagents

Formic acid, ammonium hydroxide, vitamin D and metabolites were purchased from Sigma Chemical Co. (Poole, UK). Human plasma was obtained through the Welsh Blood Service (Pontyclun, UK). Vitamin D serum calibrator (25-OH-D_2/D_3) was purchased from Chromsystems (Munich, Germany). Urine was obtained from a healthy human volunteer. All solvents were HPLC grade from Fisher Scientific (Loughborough, UK).

Sample Preparation

Supported Liquid Extraction Procedure Plate: ISOLUTE SLE+ 200 Supported Liquid Extraction Plate, part number 820-0200-P01

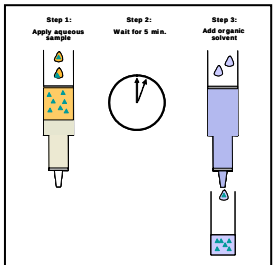


***Figure 1**. Schematic of ISOLUTE SLE+ Supported Liquid Extraction procedure. A single well of the 96-well plate is shown*

Sample pre-treatment: Plasma and urine (100 µL) were diluted 1:1 (v/v) with either 1% formic acid aq, 0.1% formic acid aq, H_2O or 0.5M NH_4OH. Spike concentrations were 100 ng for the vitamin D_2/D_3 metabolites and and 1 µg for the parent D_2/D_3.

Sample Application: The pre-treated plasma/urine (200 µL) was loaded onto the plate, a pulse of vacuum applied to initiate flow and the samples left to absorb for 5 minutes.

Analyte Elution: Addition of 1 mL of various water immiscible extraction solvents. The extraction solvents evaluated were MTBE, DCM, 95/5 DCM/IPA and EtOAc.

Post Extraction: The eluate was evaporated to dryness and reconstituted in 400 µL of 0.1% formic acid 50:50 (v/v) H_2O/ACN prior to analysis. Calibrated plasma extracts were reconstituted in 100 µL due to sensitivity issues.

HPLC Conditons

Instrument: Waters Acquity UPLC (Waters Assoc., Milford, MA, USA).
Column: Acquity UPLC BEH C18 column (1.7µ, 100 x 2.1 mm id) (Waters Assoc., Milford, MA, USA).
Mobile Phase: 0.1% formic acid aq and 0.1% formic acid/MeOH at a flow rate of 0.4 mL/min.
Gradient: The gradient conditions were set to 20%, 0.1% (v/v) formic acid aq and 80% 0.1% (v/v) formic acid MeOH increasing to 100% 0.1% (v/v) formic acid MeOH over 2 minutes. Initial starting conditions were resumed at 3.55 minutes.
Injection Volume: 5 µL.
Column Temperature: 40 °C.
Sample Temperature: 10 °C.

Mass Spectrometry

Instrument: Quattro Premier XE triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis. Initial LC-MS/MS work was conducted using ESCI, however, due to better MRM signal to noise all subsequent work was conducted using ESI.

Positive ions were acquired in the multiple reaction monitoring (MRM) mode. ***Table 1***. shows the various scan functions and transition details for the quantifier ions used in this study.

Desolvation Temperature: 450 °C

Ion Source Temperature: 150 °C

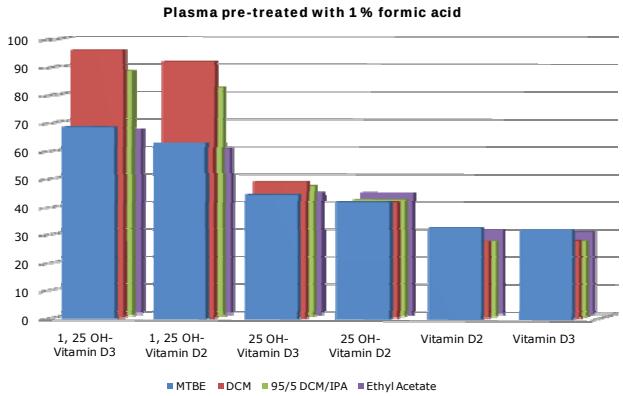
Collision Gas Pressure: 3.46 x 10^{-3} mbar

Scan Function	Analyte	Transition	Cone Voltage V	Collision Energy eV
1	1,25-OH Vitamin D_2	411.4 > 151.0	25	23
	1,25-OH Vitamin D_3	399.4 > 135.0	25	20
2	25-OH Vitamin D_2	395.4 > 119.0	20	27
	25-OH Vitamin D_3	401.4 > 365.4	20	11
3	Vitamin D_2	397.3 > 379.3	20	9
	Vitamin D_3	385.3 > 259.3	25	13

Table 1. *Quattro Premier XE mass spectrometer parameters*

Results

Figures 1-4. illustrate the recovery profiles obtained after spiking various vitamin D analytes into both plasma and urine. At all loading pHs the 25-OH vitamin D_2/D_3 and parent D_2/D_3 exhibited higher extraction efficiencies from spiked urine samples.



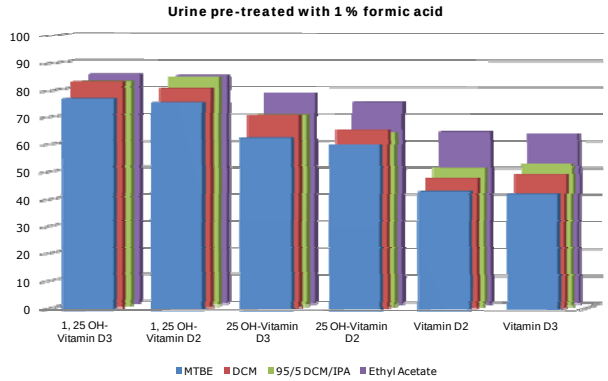
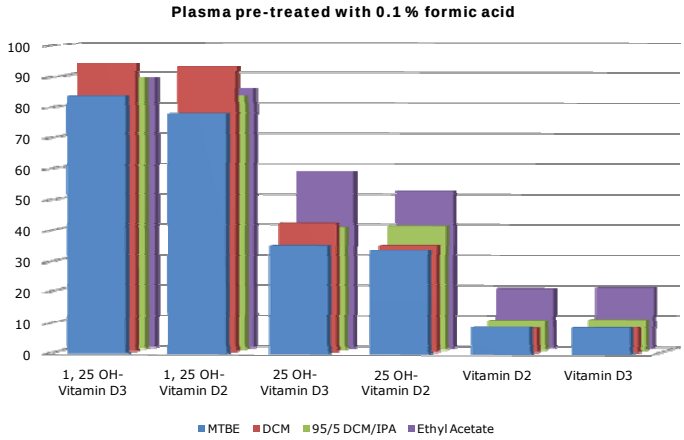


Figure 1*. Vitamin D recovery profile from plasma and urine using 1% formic acid pre-treatment.*



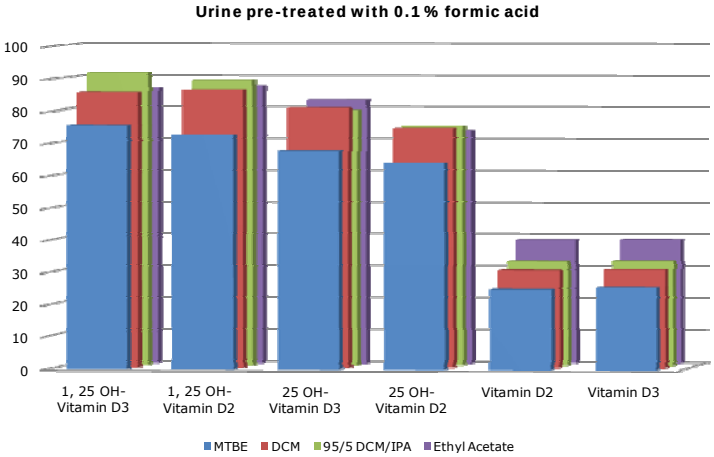


Figure 2*. Vitamin D recovery profile from plasma and urine using 0.1% formic acid pre-treatment.*

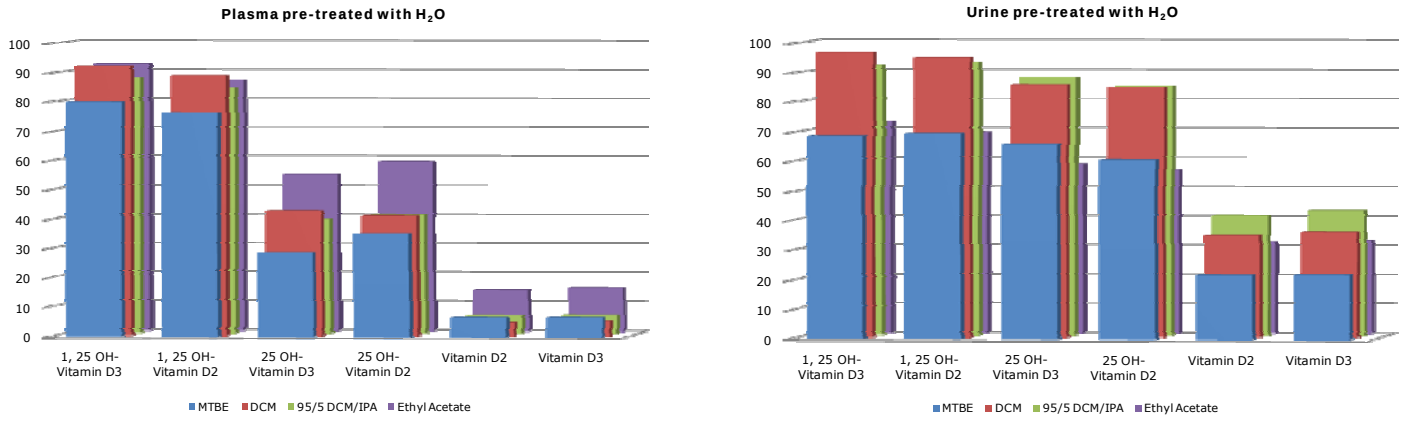


Figure 3. *Vitamin D recovery profile from plasma and urine using H_2O pre-treatment.*

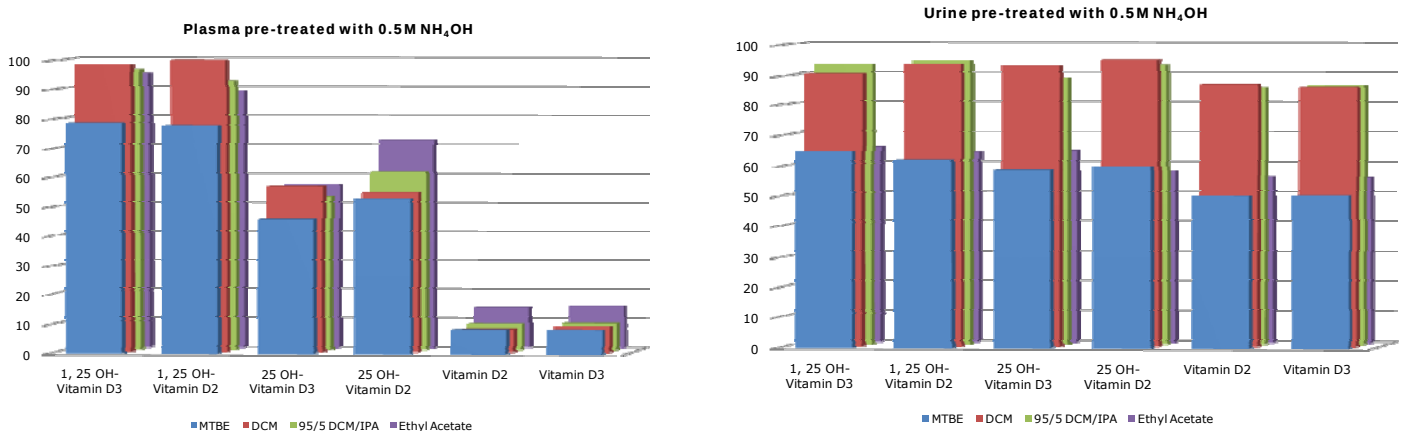


Figure 4. *Vitamin D recovery profile from plasma and urine using 0.5M NH_4OH pre-treatment*

Figure 5. illustrates the comparison of peak areas for a calibrated 25-OH vitamin D_3 plasma sample (37.5 ng/mL). Standard SLE+ protocols were used for extraction and compared with methanol and acetonitrile protein precipitated samples. The highest peak areas were returned using 0.5M NH_4OH pre-treatment and DCM as the extraction solvent. The majority of RSDs were in the range of 5-15%. This concentration of extraction was far lower than previously spiked samples and showed generated signal to noise levels just above 10:1. 25-OH Vitamin D2 demonstrated a signal to noise ratio of approximately 3:1 and as a result the samples were not quantifiable.

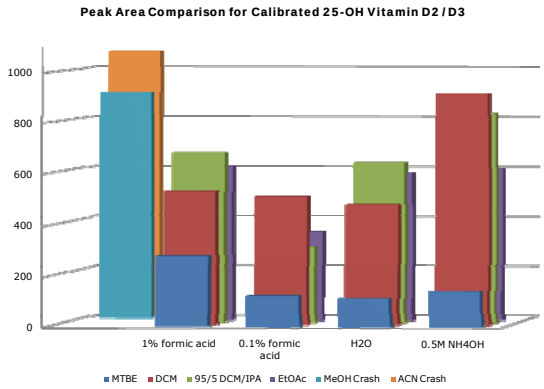


Figure 5. *25-OH Vitamin D_3 recovery profile from calibrated plasma*

Conclusions

1. The results demonstrate that in most cases recoveries were higher from spiked urine compared to plasma, suggesting that protein binding could be the difference.
2. The trends for both matrices illustrates lowering extraction efficiency as the lipophilicity of the analyte increases 1,25 di-OH > 25-OH > D_2/D_3.
3. Urine extraction using 0.5M NH4OH pre-treatment and DCM or 95/5 DCM/IPA extraction are the only protocols to show recoveries > 80% for all vitamin D and metabolites.
4. 25-OH vitamin D_3 demonstrated some high reproducible peak areas using 0.5M NH4OH pre-treatment in combination with DCM or 95/5 as the extraction solvent. 25-OH vitamin D_2 sensitivity was insufficient to obtain meaningful results.

Future Research:-

We will investigate the kinetics of protein disruption on column by increasing contact time of the organic solvents. We will also examine the use of protein disrupting agents such as trichloroacetic acid (aq), MeOH and ACN in addition to high salt concentrations in an attempt to disrupt protein binding prior to SLE+ extraction.

www.biotage.com

United States and Canada
Tel: +1 704 654 4900
Toll-Free: +1 800 446 4752
ordermailbox@biotage.com

United Kingdom, EIRE
Biotage
Tel: +44 1992 501535
order@eu.biotage.com

Sweden
Biotage
Tel: +46 18 56 59 00
order@eu.biotage.com

Japan
Biotage
Tel: +81 422 281233
order@biotage.co.jp