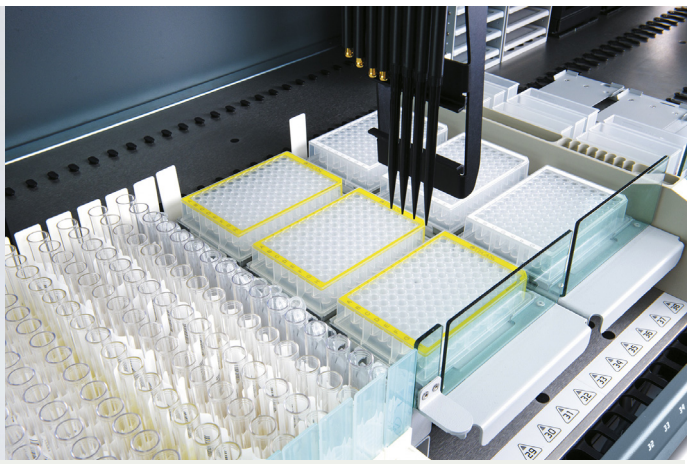

Tecan AC Extraction Plate™

Automated Sample Preparation of 25-OH-Vitamin D3/D2 from Serum with the AC Extraction Plate and the Tecan Freedom EVO® for the quantification by LC-MS/MS

Introduction

More and more laboratories are facing an increasing demand for the determination of Vitamin D status which is based on the quantification of the two metabolites 25-OH-Vitamin D3/D2. A growing number of laboratories have moved from the use of immunoassay methods to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) because of its unparalleled specificity combined with high sensitivity. Moreover LC-MS/MS offers the potential for multi-analyte determination which is required e.g. for Vitamin D status determination.

Prior to any analysis by LC-MS/MS a sample clean-up step is mandatory to remove matrix components such as proteins, lipids, carbohydrates and salts. The current methods for sample preparation include protein precipitation ('protein crash'), solvent extraction such as liquid-liquid extraction (LLE) and solid-phase extraction (SPE).

However, all these methods require at least one labor intensive and time-consuming step such as filtration, centrifugation or solvent evaporation. In addition, these steps do not allow straightforward automation of the sample preparation process which is highly desirable in order to cope with the increasing Vitamin D sample load experienced by clinical laboratories.

In this application note an approach is described which overcomes the difficulties described above. The Tecan AC Extraction Plate simplifies the sample preparation step in such a way that no centrifugation, filtration or solvent evaporation steps are needed. In fact the sample preparation procedure is reduced to simple pipetting and shaking. These pipetting and shaking steps were automated on a Tecan Freedom EVO liquid handling system. The suitability of this automated approach is demonstrated for the determination of Vitamin D in serum for LC-MS/MS analysis and with details of the workflow are described.

The Tecan AC Extraction Plate

The centerpiece of the new sample preparation method is the AC Extraction Plate using Tecan Immobilized Coating Extraction (TICE™) technology. It is a deep-well microplate with wells equipped with a proprietary coating that acts as an extraction phase for small and mainly apolar molecules. In an aqueous medium the coating absorbs analytes such as the metabolites of Vitamin D with high affinity. At the same time matrix components like proteins, phospholipids, carbohydrates and salts stay in solution which is being discarded completely. After rinsing the well with a wash solution to remove the matrix leftovers the analytes are eluted from the AC Extraction Plate using an proprietary elution solvent. The eluate can directly be utilized for LC-MS/MS analysis. Procedural variations are mitigated by adding an internal standard at the beginning of the sample preparation process. Here the isotopically labeled analyte D_6-25-OH Vitamin D3 is added directly before the extraction step.

The workflow

A mixture of modifier buffer and internal standard solution is prepared. This extraction mix is filled into the well of the AC Extraction Plate. An aliquot of the sample (e.g. serum or plasma) is added. After horizontal mixing on a shaker the liquid is removed completely from the well, leaving the analyte(s) of interest retained in the TICE™ coating of the wells of the AC Extraction Plate. A wash solution is dispensed into the well and the AC Extraction Plate is shaken again whereafter this wash solution is also removed completely. Finally an elution solvent is added and the AC Extraction Plate is again shaken. The eluate from the well is then transferred to a non-coated plate which is covered and loaded onto an HPLC autosampler for sample injection. The workflow is depicted schematically in Figure 1. Alternatively the eluate may be transferred to an HPLC vial.

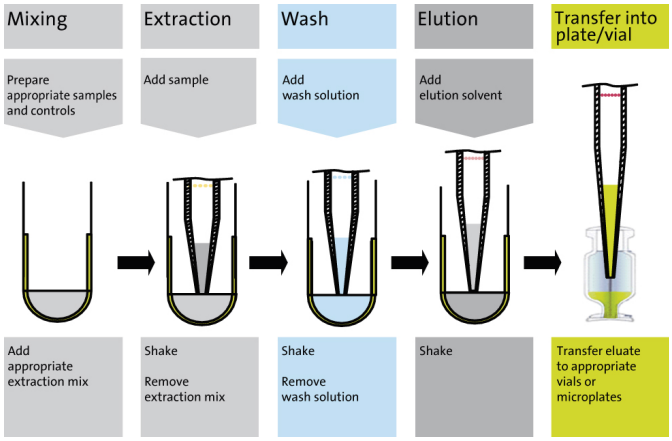

Figure 1 Typical sample preparation workflow using the AC Extraction Plate.

Materials and methods

Instruments

- Freedom EVO robotic pipetting system equipped with Te-Shake™ (Tecan)
- ACQUITY Ultra Performance UPLC® system (Waters)
- XEVO TQ-S tandem quadrupole MS/MS system (Waters)
- System operation, data acquisition and data processing by MassLynx V4.1 software (Waters).

Solvents used for sample preparation

- Internal Standard solution: D_6-25-OH-Vitamin D3 (50 ng/ml) in acetonitrile
- Modifier buffer: 0.2 M sodium carbonate/sodium hydrogen carbonate 1:1 (v/v) in water/acetonitrile 95:5 (v/v)
- Wash solution: water/methanol 90:10 (v/v)
- Elution solvent: water/methanol 10:90 (v/v)

All solvents used were LC-MS Chromasolv® grade (Sigma Aldrich, USA). Internal standard, D_6-25-OH Vitamin D3 was obtained from Cerilliant/USA.

Serum samples

Four serum calibrators and two serum quality controls (by Chromsystems), two serum quality controls (by Recipe®) and two internal serum quality controls with two defined concentrations of 25-OH-Vitamin D3 and 25-OH-Vitamin D2 metabolites were used as samples to cover the typical physiological range (Table 1).

Serum sample	25-OH-Vitamin D3 [ng/ml]	25-OH-Vitamin D2 [ng/ml]
Chromsystems Calibrator Level low	4.3	0
Chromsystems Calibrator Level 1	20.1	14.1
Chromsystems Calibrator Level 2	34.5	27.2
Chromsystems Calibrator Level 3	65.8	54.7
Chromsystems QC Level I	16.7	17.2
Chromsystems QC Level II	37.7	37.8
Recipe QC Level I	20.5	16.3
Recipe QC Level II	44.3	36.6
QC intern Level low	9.6	0
QC intern Level medium	28.8	0

Table 1 Analyte concentrations of the calibrators (Cal) and quality controls (QC) that were used in the experiments.

Automation

The sample preparation was handled in a fully walk-away protocol implemented on a Freedom EVO 100 robotic platform with the new AC Extraction Plate, using the configuration shown in Figure 2. The system used in this study was a fluid filled pipetting system, equipped with one robotic liquid handling arm (LiHa) with four channels for disposable pipetting tips. On the deck a plate carrier on a orbital shaker (Te-Shake, diameter of eccentric 3 mm) is mounted for shaking the AC Extraction Plate and a rear additional plate carrier holds the uncoated injection plate. The PosID barcode scanner is moving in the rear of the deck and scans the sample tubes.

Six sample tube trays with 16 positions each can carry up to 96 samples in total and a reagent carrier holds three troughs for the Extraction mix, Wash solution and Elution solvent. Furthermore the deck has a wash station for liquid waste and washing of the liquid channels, disposable pipetting tips (200 µL and 1000 µL) in trays and a waste for dropping used pipette tips. The pipetting system was controlled by a script-based protocol on Freedom EVOware® software.

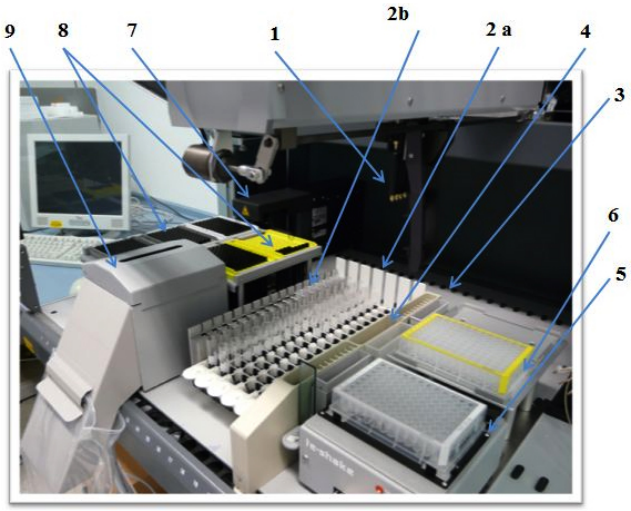

Figure 2 The Tecan Freedom EVO® 100 platform used for sample preparation with the AC Extraction Plate fits on a standard lab bench Deck layout: 1 Liquid handling arm with four channels with adapter for disposable pipetting tips, 2 a six 16-position sample tube carriers, 2 b Eppendorf carrier for Calibrator and Control tubes 3 Reagent carrier, 4 Wash station, 5 Orbital shaker Te-shake™ (diameter of eccentric 3 mm) with 6 combined carrier for injection plate, 7 PosID 2 barcode scanner, 8 disposable pipetting tips 1000 µL and 200 µL, 9 Waste for dropping the used pipette tips.

Sample preparation and analysis

The modifier buffer was mixed with internal standard solution (50 ng/ml) at a ratio of 2:1 (v/v). This mixture represents the actual extraction mix used for sample preparation procedure.

The exact procedure for the sample preparation from start to finish is as outlined in Figure 3.

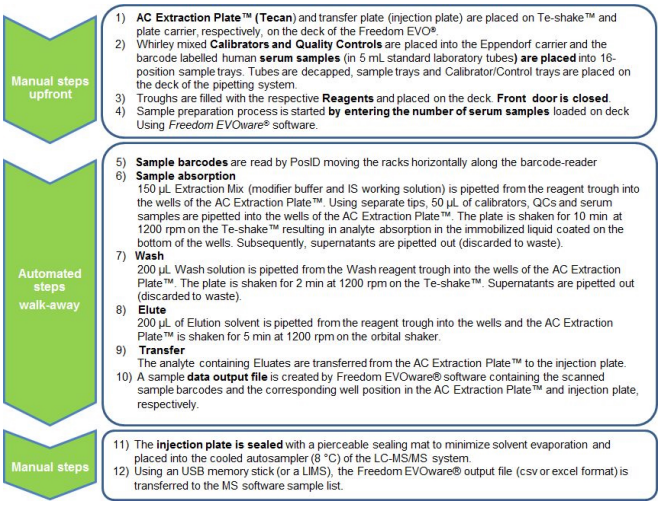

Figure 3 Detailed workflow of the automated sample preparation process with the AC Extraction Plate on the Freedom EVO® 100.

HPLC parameters

Eluent A: Water / Methanol (90:10, v/v) + 0.1 % formic acid
Eluent B: Methanol / Acetonitrile (80:20, v/v) + 0.1 % formic acid
Column: ACQUITY UPLC BEH C18 column, 2.1 x 50 mm; 1.7 µm (Waters)

Column temperature: 45 °C
Injection volume: 20 µl
Flow rate: 0.4 ml/min

Gradient:

Start: 70 % B
From 0.1 min to 1.1 min: 70 % to 98 % B
From 1.1 min to 2.6 min: 98 % B
From 2.7 min to 5.0 min: 70 % B

25-OH-Vitamin D3 and the internal standard eluted at 1.40 min while 25-OH-Vitamin D2 eluted at 1.44 min.

Total HPLC run time: 5 min

LC-MS/MS parameters

Ionization and gases

- Atmospheric Pressure Chemical Ionisation (APCI)
- Positive mode
- Nitrogen (N2) used as desolvation gas
- Argon (Ar) used as collision gas

MS-parameters

- Corona discharge current: 2.0 µA
- Probe temperature: 550 °C
- Cone gas: 900 L/h / 2.5 bar
- Nebulizer gas: 150 L/h / 2.5 bar
- Source temperature: 100 °C
- Collision gas (Ar): 0.15 mL/min.

A summary of the MRM-parameters used is shown in Table 3.

Performance parameters

All experimental data was obtained with automated pipetting on Freedom EVO with the AC Extraction Plate using the chemicals and workflow as described above.

1. Linearity

Linear regression analysis showed linearity for 25-OH-Vitamin D3 and 25-OH-Vitamin D2 over their calibration range of 4.3 – 65.8 ng/mL and 14.1 – 54.7 ng/mL, respectively, with coefficients of determination (r^2) >0.997 and >0.994, respectively.

2. LLOQ

The LLOQ (CV <20% and S/N >10) was 4.3 ng/mL for 25-OH-Vitamin D3 and 14.1 ng/mL for 25-OH-Vitamin D2.

3. Intra-day precision and inter-day precision

CV values for intra-day (repeatability) and inter-day precision (intermediate precision) for Chromsystems Quality Controls (QC) Level 1 and 2 for 25-OH-Vitamin D3 and 25-OH-Vitamin D2 were <6% in all cases. Additional QC samples from Recipe® and internal QCs provided CV values of <7% in all experiments (Table 2).

Samples	Target (ng/ml)	Intra-day precision CV (%)	Inter-day precision CV (%)	Accuracy %
25-OH-Vitamin D3				
Chromsystems:				
QC Level I	16.7	3.7	4.2	99.4
QC Level II	37.7	3.7	5.5	96.9
Recipe:				
QC Level I	20.5	1.1	5.7	99.2
QC Level II	44.3	1.4	4.0	99.8
QC intern:				
Level low	9.6	2.7	5.2	99.0
QC intern:				
Level medium	28.8	4.1	5.0	102.2
25-OH-Vitamin D2				
Chromsystems:				
QC Level I	17.2	4.9	5.8	101.8
QC Level II	37.8	3.5	5.8	95.4
Recipe:				
QC Level I	16.3	2.2	6.9	112.7
QC Level II	36.6	4.3	4.3	107.3
QC intern:				
Level low	n.d.	n.d.	n.d.	n.d.
QC intern:				
Level medium	n.d.	n.d.	n.d.	n.d.

Table 2 Target/Range concentrations of 25-OH-Vitamin D3 and 25-OH-Vitamin D2 on Chromsystems, Recipe and internal QC samples as well as validation parameters of the automated TICE™ UPLC method. n.d. not determined; a) n = 5; b) n = 4 x 5.

4. Accuracy

Accuracy for 25-OH-Vitamin D3 and 25-OH-Vitamin D2 of the Chromsystems QCs (Level 1 and 2) target values ranged from 95.4 – 101.8%. Accuracy for 25-OH-Vitamin D3 and 25-OH-Vitamin D2 of the Recipe QCs (Level 1 and 2) and of internal QCs (low and medium) target values ranged from 99.0% to 112.7% (Table 2). In Figure 4 the results of the total 25-OH-Vitamin D concentration determined by the AC Extraction Plate were compared to

- **DEQAS** (Vitamin **D** **E**xternal **Q**uality **A**ssessment **S**cheme) results of LC-MS/MS participants
- DEQAS **ALTM** (**A**ll-**L**aboratory **T**rimmed **M**ean) results
- **NIST** (**N**ational **I**nstitute of **S**tandards & **T**echnology) reference measurement procedure

All methods provided comparable results for the total 25-OH-Vitamin D concentrations.

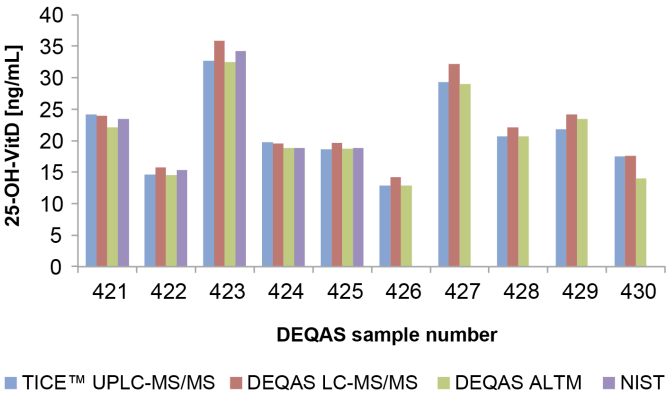

Figure 4 Comparison of total 25-OH-Vitamin D concentrations of DEQAS samples 421 – 425 (October 2012 distribution) and samples 426 – 430 (January 2013 distribution) measured by TICE™ UPLC-MS/MS (in blue) with reported results from DEQAS survey: DEQAS LC-MS/MS participants (n = 139) in red, DEQAS ALTM (n = 1064) in green and NIST reference measurement procedure in violet.

5. Extraction efficiency / Recovery

Extraction efficiency of the automated sample preparation method with the AC Extraction Plate was 84% for 25-OH-Vitamin D3 and 80% for 25-OH-Vitamin D2.

6. Matrix effects

For both analytes negligible ionization enhancement was observed: 105% for 25-OH-Vitamin D3 and 109% for 25-OH-Vitamin D2. A value of >100% indicates ionization enhancement and a value of <100% indicates ionization suppression [1].

7. Ion suppression

Ion suppression testing showed no signal suppression at the typical retention times for any of the analytes under investigation.

8. Specificity

Using Muliple Reaction Monitoring (MRM) for each analyte as well as for the internal standard two very specific mass transitions were defined (Table 3). In combination with the upstream chromatography top-level specificity was reached satisfactorily.

Analyte	Precursor Ion [m/z]	Product Ion [m/z]	Cone voltage [V]	CE [eV]	dwell time [sec]	Rt [min]
25-OH-VitD3 (quantifier ion)	383.3	257.2	25	15	0.025	1.40
25-OH-VitD3 (qualifier ion)	383.3	229.2	25	18	0.025	1.40
25-OH-VitD2 (quantifier ion)	395.3	269.2	25	15	0.025	1.44
25-OH-VitD2 (qualifier ion)	395.3	251.2	25	18	0.025	1.44
D6-25-OH-VitD3 (quantifier ion)	389.3	263.2	25	15	0.025	1.40
D6-25-OH-VitD3 (qualifier ion)	389.3	229.2	25	18	0.025	1.40

Table 3 MRM parameters used for MS/MS analysis (CE: collision energy; Rt: retention time)

9. Method comparison

The results generated by automated TICE™ technology based sample preparation followed by UPLC-MS/MS analysis with one analytical column were compared to a standard method relying on protein precipitation (PPT) and two-dimensional UPLC-MS/MS. A set of 77 human serum samples covering the clinically relevant concentration range of 25-OH-Vitamin D3 was processed with both for methods. In one sample even a significant amount of 25-OH-Vitamin D2 was determined with by both sample preparation methods. For method comparison four samples were excluded because their values were below the LLOQ. Finally 73 samples which were analysed with Bablock Passing regression (Figure 5) and yielded a very good correlation:

[Automated TICE™ LC-MS/MS] = 1.02 x [Manual On-line SPE LC-MS/MS] + 0.06

Pearson coefficient (r) = 0.988

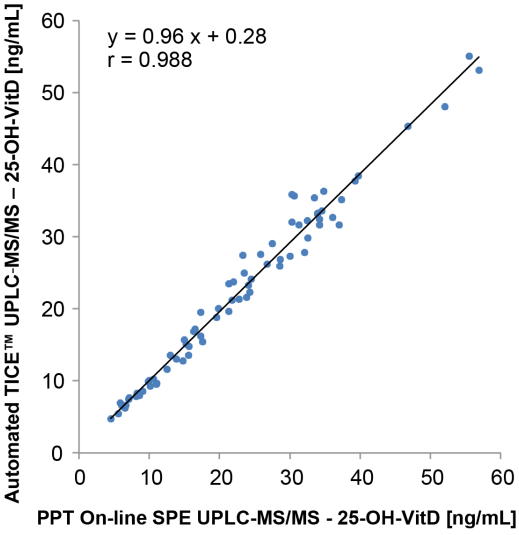

Figure 5 Comparison of total 25-OH-Vitamin D concentrations resulting from automated TICE™ UPLC-MS/MS method with a method employing manual protein precipitation followed by on-line SPE (2-dimensional UPLC-MS/MS)

Conclusion

During past years there has been much progress in automation of LC-MS/MS methods for laboratory medicine particularly concerning sample preparation [2]. The best solution should represent sample preparation directly connected to an LC-MS/MS system without any further manual intervention. A big advancement to fully automated LC-MS/MS front-end modules is the automated TICE™ technology based LC-MS/MS proceduce with almost fully automated sample preparation and minimal manual intervention.

The performance of the automated TICE™ technology based UPLC-MS/MS approach combining practicability with high sample throughput yielded very satisfactory results. This sample preparation technique using the Freedom EVO liquid handling system in combination with the Tecan AC Extraction Plate showed to be straightforward applicable for routine analysis of 25-OH-Vitamin D from serum. It may be easily adapted for other small molecule analytes in routine clinical analysis.

In summary, the Tecan AC Extraction Plate provides a fast, robust and easy-to-automate consumable for sample preparation requiring minimal sample pretreatment. Time consuming and difficult to automate procedures including protein precipitation, filtration, centrifugation or reconstitution get obsolete thus accelerating and simplifying the entire sample preparation workflow.

References

1. Matuszewski BK, Constanzer ML, Chavez-Eng CM. *Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS.* Analytical chemistry. 2003;**75**(13):3019-30. Epub 2003/09/11.
2. Vogeser M, Kirchhoff F. *Progress in automation of LC-MS in laboratory medicine.* Clinical biochemistry. 2011;**44**(1):4-13. Epub 2010/07/06.

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