[APPLICATION NOTEBOOK]

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[APPLICATION NOTEBOOK]

BIOGENIC AMINES

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Automated Solid-Phase Extraction for the Analysis of Metanephrine and Normetanephrine from Plasma using ACQUITY UPLC-MS/MS for Clinical Research

Heather A Brown,[1] Lisa Calton[1]
[1] Waters Corporation, Manchester, UK

APPLICATION BENEFITS

- Reduced operator involvement through automation of SPE
- Facilitate high sample throughput using the multiwell plate format
- Reduce turnaround time and increase batch size via shortened chromatography

WATERS SOLUTIONS

ACQUITY UPLC®

Oasis® WCX µElution Plates

ACQUITY® BEH Amide Column

Xevo® TQ MS Detector

KEY WORDS

Metanephrine, normetanephrine, SPE, UPLC-MS/MS

INTRODUCTION

Analysis of plasma metanephrines is typically performed by HPLC with electrochemical detection[1] (ECD) methods which are usually labor intensive and can result in relatively low analytical specificity. Extended chromatography and time consuming sample preparation are usually needed to resolve interferences, impacting turnaround times. Relatively few automated immunoassay methods are available for plasma metanephrines, and many of the commercial methods also suffer from interference and poor analytical specificity.

The polar nature and low concentration of metanephrines in plasma pose both extraction and chromatographic separation challenges. A recent publication describes an online automated weak cationic exchanger (WCX) solid phase extraction (SPE) with hydrophilic interaction (HILIC) HPLC and analytically selective and sensitive MS detection.[2]

Here we present the further development of this method to allow automated offline SPE using Oasis WCX µElution plates utilising a Tecan liquid handling system (LHS). The extracted plate is placed onto the ACQUITY Sample Manager and is ready for analysis using ACQUITY UPLC BEH Amide Column chemistry coupled to a Xevo TQ MS Detector. Measurement of plasma metanephrine (M) and normetanephrine (NM) provides the opportunity to perform clinical research into the pathogenesis of disease states associated with catecholamine excess.

Figure 1. Tecan Freedom EVO® 100 and the Waters ACQUITY UPLC Xevo TQ MS Detector

EXPERIMENTAL

UPLC conditions

Column:	ACQUITY UPLC BEH Amide 1.7 µm , 2.1 x 50 mm (p/n 186004800)
Column temp.:	35 °C
Sample temp.:	4 °C
Injection volume:	20 µL (PLNO)
Weak needle wash:	Acetonitrile
Strong needle wash:	Water
Flow rate:	200 µL/min
Mobile phase A:	100 mM ammonium formate, pH 3.0 with formic acid (aq)
Mobile phase B:	Acetonitrile
Gradient:	2 to 35% linear gradient of Mobile Phase A over 3 minutes; 1 minute hold then step gradient to initial conditions with 1 minute re-equilibration
Run time:	5 min

MS conditions

Instrument tuned to unit resolution on MS1 and MS2 (0.7 FWHM)

System:	Xevo TQ MS Detector
Acquisition mode:	Multiple Reaction Monitoring (MRM) – see Table 2 for ion transitions
Ionization mode:	ESI positive
Capillary voltage:	0.6 kV
Dwell time:	0.04 sec

Interscan and inter channel delay 0.01 sec

Source temp.:	150 °C
Desolvation temp.:	600 °C
Data management:	MassLynx v4.1 SCN 810 with TargetLynx™ application manager.

Sample preparation

Calibrators were prepared fresh for each analysis using stripped serum spiked with solvent stocks from independently weighed solid HCl salts of metanephrine (M) and normetanephrine (NM) (Sigma-Aldrich, Dorset, UK). Quantification followed blank-correction for endogenous metanephrines.

A working solution of $^{2}H_{3}$ M, and $^{2}H_{3}$ NM internal standard (IsoSciences, King of Prussia, PA, USA) was prepared daily by 500-fold dilution of an acidified stock into LC-MS grade water.

Sample extraction

Samples, calibrators, and quality control materials were centrifuged at a minimum of 10,000 *g* to remove clots and debris. Minimum of 250 µL was transferred to barcode-labelled tubes and placed on the Tecan Freedom EVO® 100 liquid handling system (LHS). All SPE solvents, calibrators and working internal standards, Oasis WCX µElution (p/n 186002499), mixing and collection plates (p/n 186002482 and 186002481) were positioned onto the LHS.

LHS-automated steps:

- Mixing of 200 µL sample with 200 µL internal standard
- Transfer of 200 µL internal standard + sample mixture to the methanol-conditioned and water-equilibrated Oasis WCX µElution plate
- Sequential washing with 200 µL each of water, methanol, and 0.2 % (v/v) formic acid in acetonitrile
- Elution with 2 x 50 µL passes of 2% (v/v) formic acid in acetonitrile into the collection plate.

Utilizing the load-ahead feature, an injection-to-injection time of approximately 5.5 min was achieved.

Prepare samples, reagents and mobile phases	t = 0 mins
Tecan LHS mixes samples and internal standards	t = 20 mins
µElution plate conditioned and equilibrated	t = 28 mins
Samples loaded and washed on µElution plate	t = 78 mins
Samples eluted from µElution plate	t = 90 mins
Samples analysed by LC-MS/MS	t = 340 mins

Table 1. Typical workflow for the analysis of 45 samples.

Method conditions

Two blank injections were performed to allow thorough column equilibration before running sample lists. Careful attention was paid to positioning and priming of ACQUITY UPLC lines to maintain HILIC chromatography conditions.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage	Collision energy
Metanephrine	180.0	148.0	30	16
Normetanephrine	166.0	134.0	32	18
$^{2}H_3$ Metanephrine	183.0	151.0	30	16
$^{2}H_3$ Normetanephrine	169.0	137.0	32	18

Table 2. Guideline MRM parameters: Conditions optimized to facilitate in-source loss of water. Precursor ions represent the $[M+H-H_2O]^+$ species.

RESULTS

The limit of detection (LOD) and LLOQ were interpolated from mean peak to peak signal to noise ratios (S:N) from extracted ion chromatograms of 10 samples with the lowest signal to noise ratio analysed for method comparison. The calculated LLOQ S:N >10 were 45 and 127 pmol/L for M and NM, respectively. The LOD was calculated at S:N > 5, therefore, these values were half the LLOQ. Example extracted ion chromatograms in low concentration extracted plasma samples are shown in Figure 2.

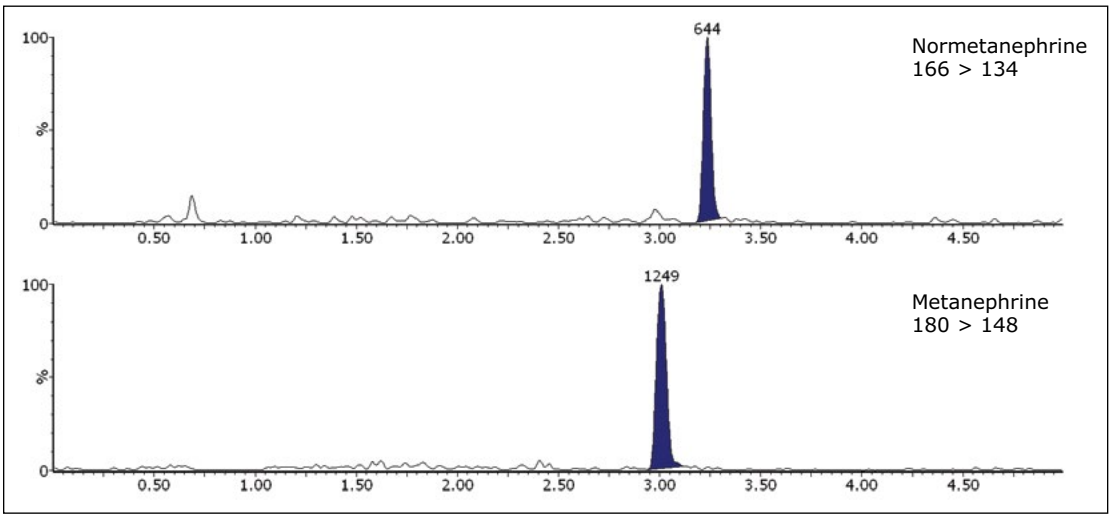

Figure 2. Normetanephrine (287 pmol/L) and metanephrine (137 pmol/L) in extracted plasma; peak to peak signal to noise ratios of 23 for both.

Within- and between-batch imprecision determined by replicate extraction and analysis over 5 days of a human plasma base pool spiked to 3 concentrations revealed mean within batch imprecision of <7.6 and <5.6 % coefficient of variation (CV) for M and NM, respectively. The same data reveals the mean between batch CV as <7.5 and <12.8 % for M and NM, respectively.

Linearity of detector response up to 24.55 nmol/L was demonstrated with the 8-point calibration curve showing a coefficient of determination >0.995 and <15 % deviation from nominal concentrations. Additionally, linearity of detector response upon extraction of an 8 nmol/L spiked sample, sequentially diluted with stripped serum, was shown.

Mean Oasis WCX µElution plate SPE efficiency was calculated as the peak area ratio of pre- to post-extraction spiked plasma (n=3) was 97 and 95 % for M and NM, respectively. Matrix effects were evaluated from the peak area of the post-extraction spiked samples (n=6) taken as a percentage of extraction solvent spiked to equivalent concentrations. Mean (range) matrix effects were 16.4 (1.8 % to 35.0) % ion enhancement for M and 8.7 (3.6 % suppression to 19.4) % enhancement for NM. Calculations using analyte: internal standard peak area response indicated matrix effect compensation by the internal standard with a mean net matrix effect (range) of 4.0 (0.5 to 6.0) % enhancement for M and 0.3 (3.3 % suppression to 7.5) % enhancement for NM.

Comparison was made using 50 plasma reference samples analysed by an online SPE LC/MS/MS method.[2] Deming regression conducted with Analyse-it® for Microsoft® Excel® for Windows® 2003 showed neither significant proportional nor constant bias across the measured range of 87–4455 pmol/L for M ($p>0.05$). Proportional bias of 15 % was found across the measured range of NM 300–14459 pmol/L ($p<0.02$), however, this became insignificant when limiting the comparison to below the upper limit of the reference sample interval (<1070 pmol/L; $p=0.48$).

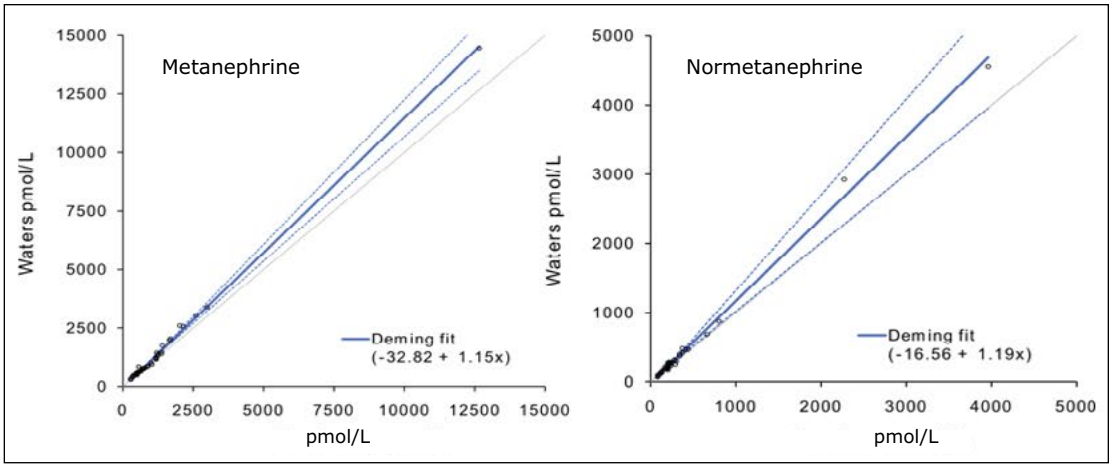

Figure 3. Deming regression comparing online SPE HPLC-MS/MS with the developed offline UPLC-MS/MS method.

Solvent standards of potential endogeneous and exogeneous isobaric interferents were injected and chromatograms were interrogated for peak and baseline interferences. Epinephrine, norepinephrine and dopamine contributed to a high baseline signal in the NM MRM. MS1 scans showed abundant NM precursor *m/z* 166 in the source. MS2 scans of potentially interfering substances under M and NM-optimised conditions indicated low levels of product ions which may pose isobaric interference with the NM 166>134 MRM transition when operating at very low resolution. For this reason, operation of MS2 at unit resolution or higher (FWHM ≤0.7) is recommended.

CONCLUSION

The use of LC-MS/MS for analysis of metanephrines in clinical research addresses the shortcomings of traditional methods such as immunoassay and HPLC with electrochemical detection. Analytically sensitive and selective quantification of low concentrations of metanephrines in plasma is possible by coupling automated SPE and ACQUITY UPLC separation with MRM analysis using the Xevo TQ MS Detector. Eluates from the Oasis WCX µElution Plate are directly compatible with the LC-MS/MS system, negating sample evaporation and reconstitution required with strong cationic exchangers.[3] Simplified processing of large numbers of samples is possible with reduced risk of preparative errors by taking advantage of the Tecan LHS.

Acknowledgement

Bob Peaston, Erin Chambers and Kendon Graham are thanked for helpful discussions regarding the development of the SPE extraction and LC method.

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Rapid and Simultaneous Analysis of Urinary Catecholamines and Metanephrines Using Mixed-Mode SPE and Hydrophilic Interaction Chromatography (HILIC) for Clinical Research

Jonathan P. Danaceau, Erin E. Chambers, and Kenneth J. Fountain
Waters Corporation, Milford, MA USA

APPLICATION BENEFITS

- Retention and baseline resolution of monoamine neurotransmitters and metanephrines without the need for ion-pairing reagents
- Rapid, simultaneous quantification of urinary metanephrines and catecholamines
- Linear, accurate and precise results from 0.5–500 ng/mL

WATERS SOLUTIONS

Oasis® WCX 30 mg 96-well Plate (p/n 186002503)

96-well Sample Collection Plate, 800 µL round well (p/n 186002481)

ACQUITY UPLC® BEH Amide Column, 1.7 µm, 2.1 x 100 mm (p/n 186004801)

ACQUITY UPLC I-Class System

Xevo® TQD

MassLynx® Software

KEY WORDS

Catecholamines, metanephrines, HILIC, SPE, LC-MS/MS, sample preparation

INTRODUCTION

Clinical researchers are often interested in measuring elevated concentrations of urinary catecholamines and their O-methylated metabolites (metanephrines). However, these compounds (in particular, norepinephrine, epinephrine, and dopamine) can be a challenge to analyze via reversed-phase LC-MS/MS due to their polarity. As a result, many research laboratories still analyze this panel using ion-pairing reagents and electrochemical detection (ECD). While reversed-phase LC-MS/MS has been used successfully, challenges still exist due to ion-suppression from urine matrix components, insufficient retention, and inadequate separation of normetanephrine and epinephrine.

Hydrophilic interaction chromatography (HILIC) is increasingly becoming a method of choice for the analysis of polar compounds.[1-6] Expanding upon an earlier published method,[6] this application note describes the extraction and analysis of monoamine neurotransmitters and metanephrines from urine. HILIC-based chromatographic separation is achieved using a Waters® ACQUITY UPLC BEH Amide Column. Waters Oasis WCX 96-well Plates are used to extract these compounds from urine. The use of mixed-mode weak cation exchange solid-phase extraction (SPE) plates, in combination with the amide column for HILIC chromatography and the Waters Xevo TQD mass spectrometer, result in a rapid, robust method with excellent linearity, accuracy and precision, as well as minimal matrix effects.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC BEH Amide Column, 1.7 µm, 2.1 x 100 mm
Column temp.:	30 °C
Sample temp.:	10 °C
Mobile phase A (MPA):	95:5 Water:ACN containing 50-mM NH_4HCOO, pH 3.0
Mobile phase B (MPB):	15:85 Water:ACN containing 30-mM NH_4HCOO, pH 3.0
Needle washes:	Strong and weak needle washes were both placed in MPB

The gradient ramp is shown in Table 1 and includes an initial hold, followed by a shallow ramp, and an increase in flow rate to re-equilibrate the column.

MS conditions

MS system:	Xevo TQD
Ionization mode:	ESI positive
Capillary voltage:	0.5 kV
Cone voltage:	Compound specific (see Table 2)
Desolvation gas:	900 L/hr
Cone gas:	0 L/hr
Desolvation temp.:	350 °C
Source temp.:	150 °C

Data were acquired and analyzed using Waters MassLynx Software (V4.1; SCN 855) and quantitated using TargetLynx.

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the methods section

Time (min)	Flow (mL/min)	%A	%B
0	0.6	0.0	100.0
1.0	0.6	0.0	100.0
2.0	0.6	10.0	90.0
2.1	1.0	10.0	90.0
2.5	1.0	30.0	70.0
2.6	1.0	0.0	100.0
3.9	1.0	0.0	100.0
4.0	0.6	0.0	100.0

Combined stock standards (10 µg/mL) of dopamine (DA), norepinephrine (NE), epinephrine (EP), 3-methoxytyramine (3-MT), metanephrine (MTN), and normetanephrine (NMT) were prepared in methanol containing 0.1% ascorbic acid to prevent oxidation. A combined internal standard stock solution composed of 10-µg/mL D3-metanephrine, D3-normetanephrine, D4-dopamine, D6-epinephrine, and D6-norepinephrine was also prepared in methanol containing 0.1% ascorbic acid. Working internal standard solutions were prepared daily in 5% MeOH with 0.1% formic acid at a concentration of 800 ng/mL.

Sample preparation

Urine samples were pre-treated with 10% (by volume) of 1-N HCl to mimic the acidic pre-treatment that is normally used for this method. 50-µL of internal standard working solution was added to a 400 µL aliquot of acidified urine, followed by 1-mL of 0.5 M NH_4CH_3COO. Pre-treated samples were loaded in individual wells of an Oasis WCX Plate that had been conditioned with 1-mL of MeOH and 1-mL of H_2O. After loading the samples, wells were washed with 1-mL of 20-mM NH_4CH_3COO, followed by 1-mL MeOH. The 96-well plate was then dried under vacuum for 30 s to remove as much methanol as possible from the sorbent bed. The target compounds were eluted from the plate with 2 x 250 µL aliquots of 85:15 ACN:H_2O containing 2% formic acid into an 800 µL 96-well Sample Collection Plate (p/n 186002481). Each aliquot was allowed to percolate through the well by gravity to maximize the contact time with the sorbent. 10-µL of the eluate was injected onto the LC-MS/MS system.

RESULTS AND DISCUSSION

The structures of all compounds are shown in Figure 1 along with their individual logP values, demonstrating the highly polar nature of many of these compounds. Table 2 shows the retention times and individualized MS parameters of each compound, including MRM transitions, cone voltage, and collision energy.

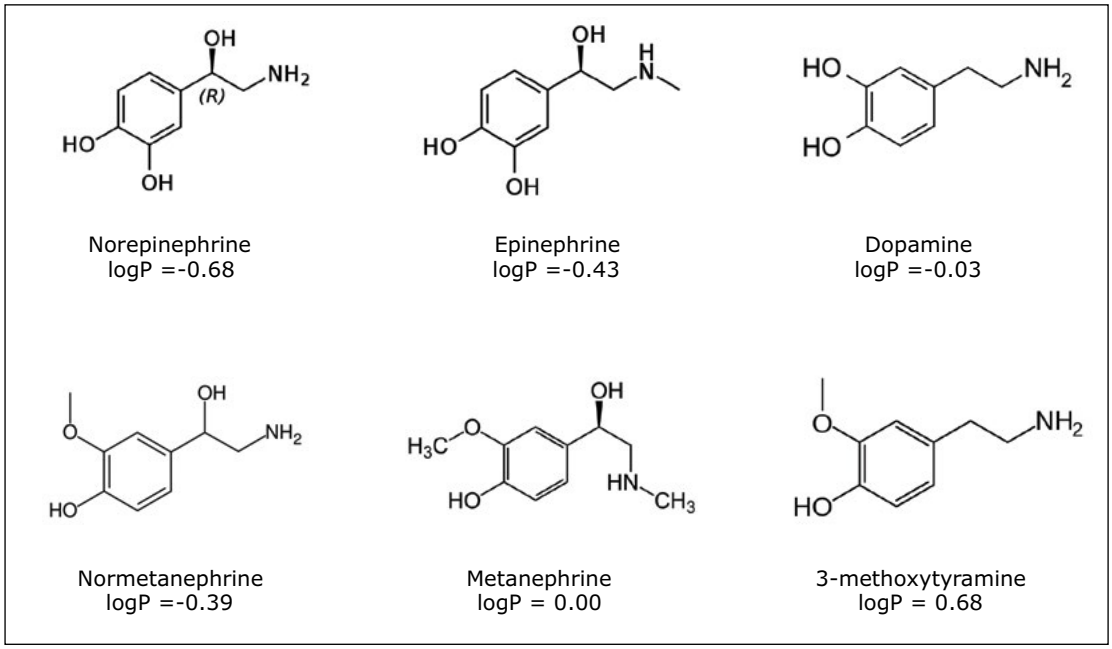

Figure 1. Names, molecular structures and logP values of catecholamines and metanephrines.

Table 2. Mass spectral parameters used for analysis of catecholamines and metanephrines.

Analyte	RT (min)	MRM transitions *m/z*	Cone voltage (V)	Collision energy (eV)
3-methoxytyramine	0.83	168.1>91	22	24
		168.1>119	22	18
Metanephrine	0.89	198.1>180	18	8
		198.1>165.1	18	18
Normetanephrine	1.16	184.1>166	20	8
		184.1>134.1	12	18
Dopamine	1.24	154.0>91	22	20
		154.0>119	22	18
Epinephrine	1.38	184.1>166	20	8
		184.1>107	20	20
Norepinephrine	1.93	170>152	14	6
		170>107	14	20

Figure 2A shows the chromatography of all compounds from a 50 ng/mL calibration standard using the ACQUITY UPLC BEH Amide Column. Previous work[6] had shown that 30 mM NH_4HCOO and 15% water in MPB resulted in an ideal balance of ionic strength and solubility, enabling the resolution and peak shape seen in Figure 2A. One important feature of this separation is the resolution between NMT and EP. These two compounds have the same molecular formula and can interfere with each other if not adequately separated. Under reversed-phase conditions, the best achievable resolution between these two compounds was a separation of 0.05 min (3 s) vs. 0.22 min (13.2 s) in HILIC mode. HILIC mode allows for a more robust separation, ensuring unambiguous identification and quantification of these two compounds. Figure 2B shows the HILIC chromatography of an un-spiked urine sample, demonstrating the ability to determine endogenous concentrations of 3-MT, MTN, NMT, DA, EP, and NE (21.6, 10.6, 17.8, 6.0, 0.0, and 4.1 ng/mL, respectively). The lack of detectable EP is most likely a result of the fact that this urine sample had been stored for an extended period of time without acidic preservation.

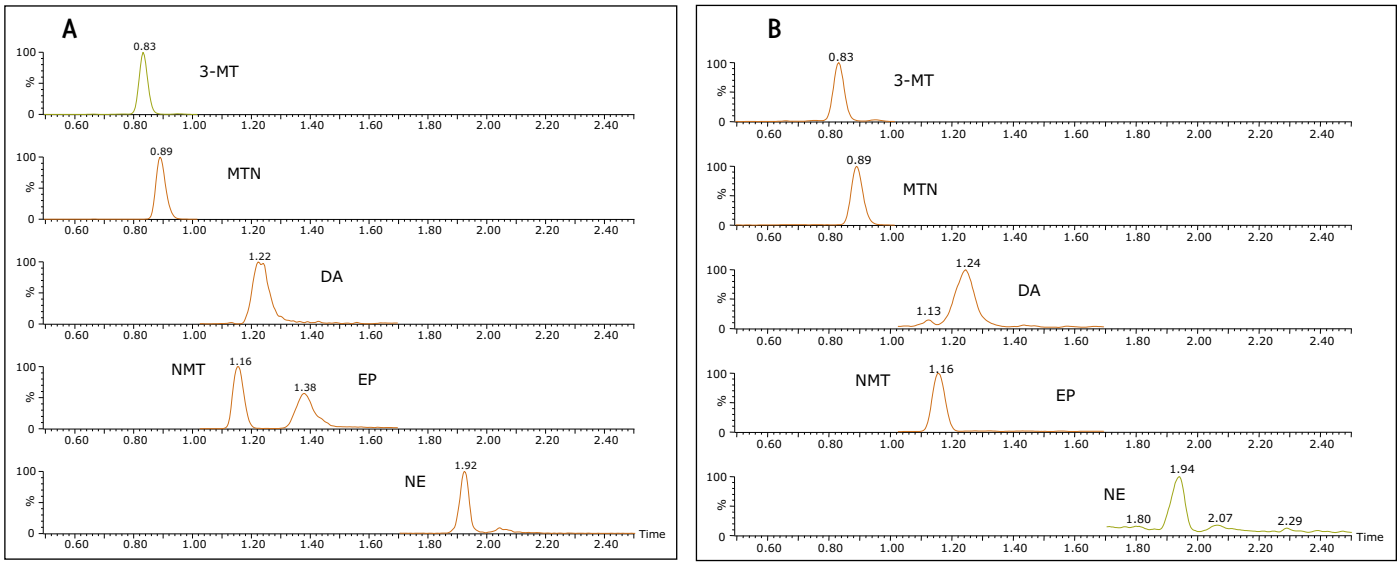

Figure 2. Chromatography of catecholamines and metanephrines on the ACQUITY UPLC BEH Amide Column, 1.7 µm, 2.1 x 100 mm.
A. Representative 50 ng/mL calibration standard.
B. Unspiked urine sample showing chromatography of endogenous catecholamines and metanephrines. Chromatographic conditions are detailed in the methods section.

Recovery and matrix effects

Extraction recoveries and matrix effects are shown in Figure 3. Recoveries ranged from 36% for NE to 98.5% for 3-MT. Reproducibility was excellent, with coefficients of variation under 5% for all compounds. Matrix effects ranged from 0% for NE to a maximum of -35% for dopamine. Most matrix effects, however, were ≤-10%, revealing another advantage of the HILIC methodology. Using the same extraction method, matrix effects were significantly larger (approximately -60%) for NE and EP under reversed-phase chromatography conditions. This is an important improvement, given the low endogenous concentrations of these two compounds.

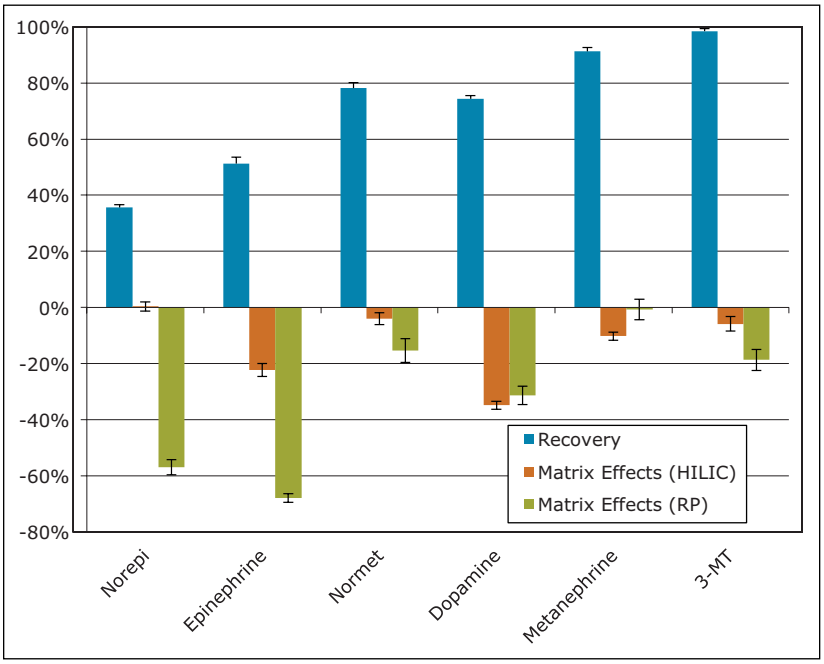

Figure 3. Recovery and matrix effects for catecholamines and metanephrines extracted from urine using Oasis WCX 30 mg 96-well Plates (N=4). The green bars represent matrix effects for reversed-phase analysis. Error bars indicate standard deviations.

Quantitative results

Calibration curves and quality control samples were prepared via the standard addition method by spiking authentic urine samples with known concentrations of analytes. After data processing, the endogenous concentrations were extrapolated from the resulting calibration curves. These data were used to correct the actual calibration concentrations. For example, the urine sample used for calibration was determined to contain 6.0 ng/mL of DA, so the calibration concentrations were changed from 0.5–500.0 to 6.5–506.0 ng/mL. The resulting calibration curves showed excellent linearity, with R^2 values of 0.992 or greater for all compounds. Figure 4 shows representative calibration curves for NMT and EP, both of which have R^2 values of 0.999. The endogenous calculated values are listed in the figure caption. R^2 values for 3-MT, MTN and DA were 0.998, 0.999, and 0.992, respectively. Quality control samples (N=4) overspiked at 1.6, 8.0, 80, and 400 ng/mL were accurate and precise (see Table 3). All QC values were within 10% of their target values, and most were within 5%. In addition, with only three exceptions, all coefficients of variation were less than 10%. This demonstrates that the method is linear, accurate, and precise over a calibration range that includes the entire scope of expected values for normal and pathologically elevated samples.

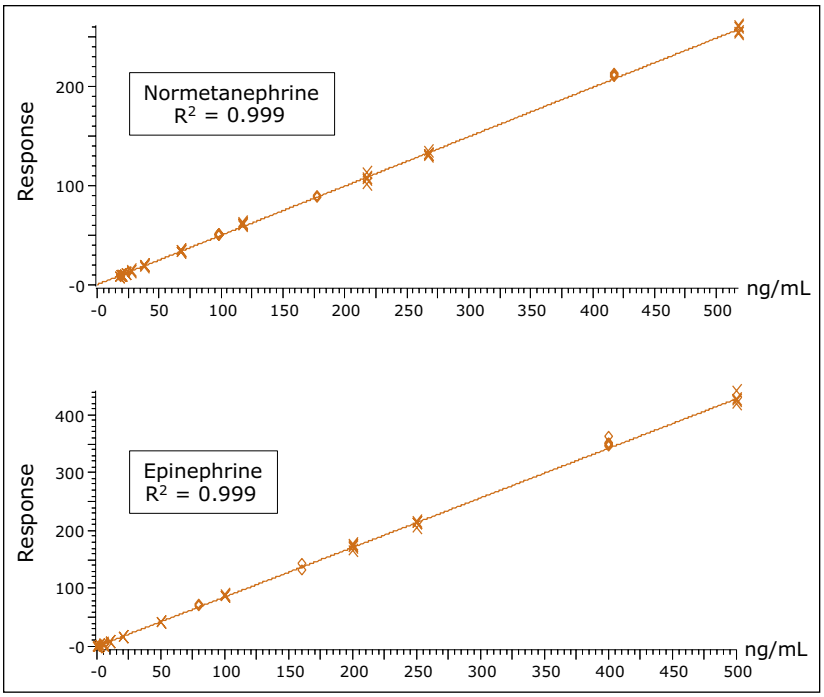

Figure 4. Calibration curves for normetanephrine (NMT) and epinephrine (EP) extracted from spiked urine samples. The data were fitted with a 1/x weighted linear fit. Basal concentrations for NMT and EP were 17.8 and 0 ng/mL, respectively.

Table 3. Quality control results for urinary catecholamines and metanephrines. Concentrations refer to the spiked concentration. Accuracies were calculated by comparing the result the sum of the spiked concentration and endogenous calculated values in the urine sample.

QC Spike	3-MT			Metanephrine			Normetanephrine		
Conc.	Acc	Bias	%CV	Acc	Bias	%CV	Acc	Bias	%CV
1.6	94.4%	-5.6%	-2.6%	98.9%	-1.1%	-2.9%	98.8%	-1.2%	-3.9%
8	95.9%	-4.1%	-3.0%	100.5%	0.5%	-1.7%	101.0%	1.0%	-4.2%
80	101.8%	1.8%	-4.3%	104.0%	4.0%	-0.9%	103.4%	3.4%	-1.6%
400	109.8%	9.8%	-5.0%	101.9%	1.9%	-2.1%	100.8%	0.8%	-0.5%
Mean	100.5%			101.3%			101.0%		

QC Spike	Dopamine			Epinephrine			Norepinephrine		
Conc.	Acc	Bias	%CV	Acc	Bias	%CV	Acc	Bias	%CV
1.6	92.1%	-7.9%	-9.5%	95.6%	-4.4%	-6.2%	94.0%	-6.0%	-7.2%
8	96.5%	-3.5%	-11.0%	95.4%	-4.6%	-3.2%	98.7%	-1.3%	-3.3%
80	102.8%	2.8%	-2.0%	103.4%	3.4%	-1.5%	99.2%	-0.8%	-14.8%
400	103.4%	3.4%	-2.4%	103.0%	3.0%	-1.9%	98.3%	-1.7%	-12.9%
Mean	98.7%			99.4%			97.5%		

CONCLUSIONS

The extraction and analysis of urinary catecholamines and metanephrines using the Oasis mixed-mode weak cation exchange (WCX) plates and an ACQUITY UPLC BEH Amide Column in HILIC mode is detailed. Extraction using the Oasis WCX Plate resulted in low matrix effects and consistent recoveries for all compounds that translated into excellent analytical precision. HILIC separation resulted in reduced matrix effects for NE and EP and improved resolution between EP and NMT, compared to optimized reversed-phase separations. Quantitative results were excellent, with linear responses from 0.5-500.0 ng/mL and excellent accuracy and analytical precision.

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Rapid and Simultaneous Analysis of Plasma Catecholamines and Metanephrines Using Mixed-Mode SPE and Hydrophilic Interaction Chromatography (HILIC) for Clinical Research

Jonathan P. Danaceau, Erin E. Chambers, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Retention and baseline resolution of monoamine neurotransmitters and metanephrines without the need for ion-pairing reagents
- Rapid, simultaneous quantification of plasma metanephrines and catecholamines
- 5x analyte concentration without the need for evaporation and reconstitution
- Linear, accurate, and precise results down to 10 pg/mL

WATERS SOLUTIONS

Oasis® WCX 96-well µElution Plate (p/n 186002499)

96-well Sample Collection Plate, 700 µL round well (p/n 186005837)

ACQUITY UPLC® BEH Amide Column 1.7 µm, 2.1 x 100 mm (p/n 186004801)

ACQUITY UPLC System

Xevo® TQ-S Mass Spectrometer

UNIFI® Scientific Information System

KEY WORDS

Catecholamines, metanephrines, HILIC, SPE, LC-MS/MS, sample preparation

INTRODUCTION

Clinical researchers are often interested in measuring elevated concentrations of plasma catecholamines and their O-methylated metabolites (metanephrines). However, these compounds (in particular, norepinephrine, epinephrine, and dopamine) can be a challenge to analyze via reversed-phase LC-MS/MS due to their polarity. As a result, many research laboratories still analyze this panel using ion-pairing reagents and electrochemical detection (ECD). While reversed-phase LC-MS/MS has been used successfully, challenges still exist due to ion-suppression from matrix components, insufficient retention, and inadequate separation of normetanephrine and epinephrine.

Hydrophilic interaction chromatography (HILIC) is increasingly becoming a method of choice for the analysis of polar compounds.[1-6] Expanding upon earlier published methods,[6-7] this application note describes the extraction and analysis of monoamine neurotransmitters and metanephrines from plasma. HILIC-based chromatographic separation is achieved using a Waters® ACQUITY UPLC BEH Amide Column. Waters Oasis WCX 96-well µElution Plates are used to extract these compounds from plasma. The use of mixed-mode weak cation exchange solid-phase extraction (SPE) plates, in combination with the amide column for HILIC chromatography and the Waters Xevo TQ-S mass spectrometer, result in a rapid, robust method with excellent linearity, accuracy and precision, as well as minimal matrix effects.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH Amide, 1.7 µm, 2.1 x 100 mm
Column temp.:	30 °C
Sample temp.:	10 °C
Mobile phase A (MPA):	95:5 Water:ACN containing 30 mM NH_4HCOO, pH 3.0
Mobile phase B (MPB):	15:85 Water:ACN containing 30 mM NH_4HCOO, pH 3.0
Needle washes:	Strong and weak needle washes were both placed in MPB

The gradient ramp is shown in Table 1 and includes an initial hold, followed by a shallow ramp and an increase in flow rate to re-equilibrate the column. The entire cycle time is 4.0 min.

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI Positive
Capillary voltage:	0.5 kV
Cone voltage:	Compound specific (see Table 2)
Desolvation gas:	900 L/hr
Cone gas:	150 L/hr
Desolvation temp.:	550 °C
Source temp.:	150 °C

Data were acquired and analyzed using UNIFI Software.

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the methods section.

Time (min)	Flow (mL/min)	%A	%B
0	0.6	0.0	100.0
1.0	0.6	0.0	100.0
2.0	0.6	10.0	90.0
2.1	1.0	10.0	90.0
2.5	1.0	30.0	70.0
2.6	1.0	0.0	100.0
3.9	1.0	0.0	100.0
4.0	0.6	0.0	100.0

Combined stock standards containing 10-µg/mL of dopamine (DA), 3-methoxytyramine, (3-MT) metanephrine (MTN), and normetanephrine (NMT) and 50-µg/mL of norepenephrine (NE) and epinephrine (EP) were prepared in methanol containing 0.1% ascorbic acid to prevent oxidation. A combined internal standard stock solution composed of 10-µg/mL D3-metanephrine, D3-normetanephrine, D4-dopamine, D6-epinephrine, and D6-norepinephrine, was also prepared in methanol containing 0.1% ascorbic acid. Working internal standard solutions were prepared daily in 5% MeOH with 0.1% formic acid at a concentration of 2.5 ng/mL.

Human plasma (sodium heparin) was obtained from Biological Specialty Corporation (Colmar, PA). Pooled plasma (6 lots) was used to prepare calibration and quality control samples.

Sample preparation

Pooled plasma samples (250 µL) were pre-treated with 250-µL of 50-mM NH_4CH_3COO and 50-µL of an internal standard working solution (2.5 ng/mL). Pre-treated samples were loaded in individual wells of an Oasis WCX 96-well µElution Plate that had been conditioned with 200-µL of MeOH and 200-µL of H_2O. After loading the samples, wells were washed with 200-µL of 20-mM NH_4CH_3COO followed by 200-µL of 50:50 ACN:IPA. The 96-well plate was then dried under vacuum for 30 s to remove as much solvent as possible from the sorbent bed. The target compounds were eluted from the plate with 2 x 25-µL aliquots of 85:15 ACN:H_2O containing 2% formic acid into an 700-µL 96-well sample collection plate (p/n 186005837). 15-µL of the eluate was injected onto the UPLC®-MS/MS System.

Figure 2A shows the chromatography of all compounds from a 20 pg/mL calibration standard using the ACQUITY UPLC BEH Amide Column. Previous work[6] had shown that 30 mM NH_4HCOO and 15% water in MPB resulted in an ideal balance of ionic strength and solubility, enabling the resolution and peak shape seen in Figure 2A. One important feature of this separation is the resolution between NMT and EP. These two compounds have the same molecular formula and can interfere with each other if not adequately separated. Figure 2A demonstrates the baseline separation of these compounds in HILIC mode, enabling their unambiguous identification and quantification. Figure 2B shows the HILIC chromatography of an unspiked plasma sample, demonstrating the ability to determine endogenous concentrations of 3-MT, MTN, NMT, DA, EP, and NE (7.0, 31.7, 70.6, 0.0, 29.4, and 360.9 pg/mL, respectively).

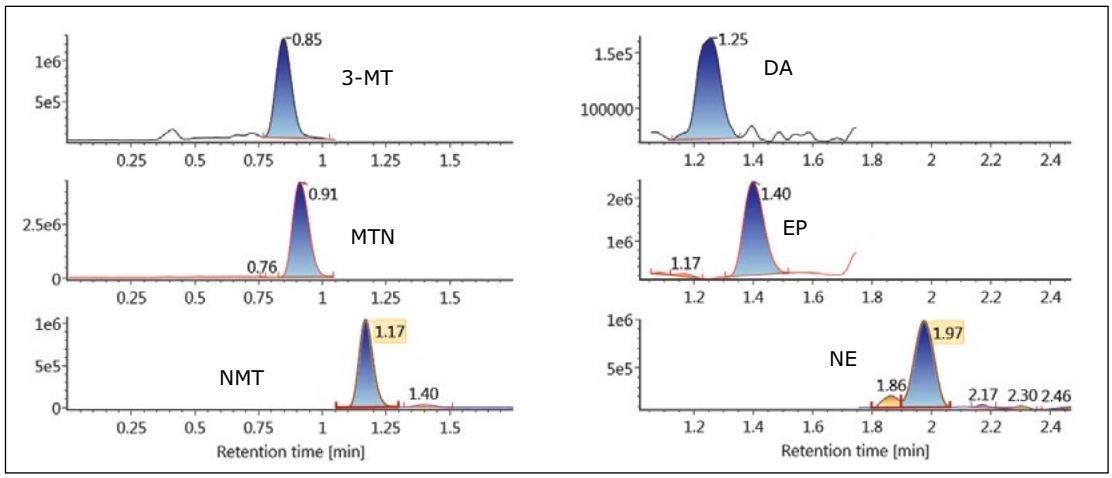

Figure 2A. Chromatography of catecholamines and metanephrines on the ACQUITY UPLC BEH Amide Column, 1.7 µm, 2.1 x 100 mm. Representative calibration standard spiked at 20 pg/mL (100 pg/mL for EP and NE).

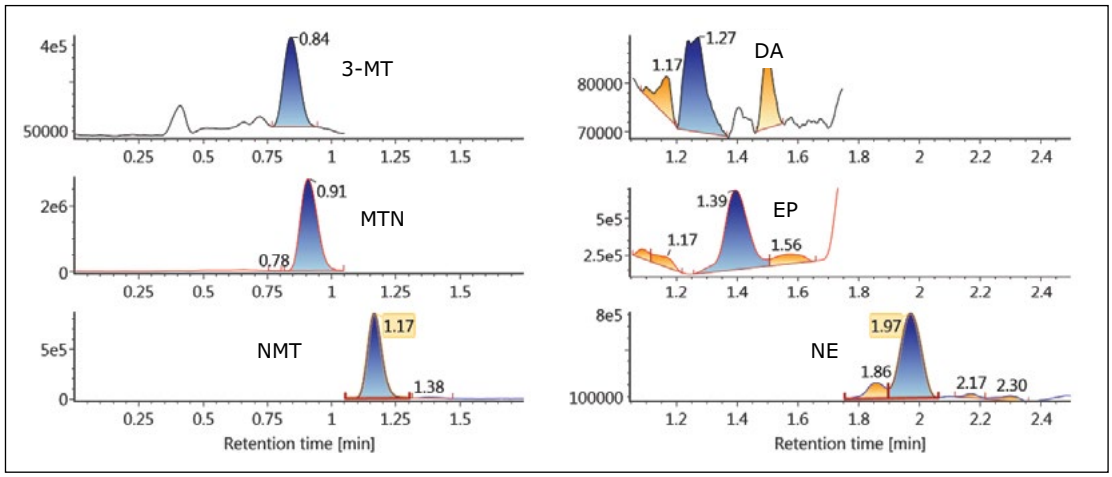

Figure 2B. Chromatography of catecholamines and metanephrines on the ACQUITY UPLC BEH Amide Column, 1.7 µm, 2.1 x 100 mm. Representative method blank. Endogenous concentrations of all compounds are listed in Table 3.

Recovery and matrix effects

Extraction recoveries and matrix effects are shown in Figure 3. Recoveries ranged from 54% for NE to 90% for DA, with an average recovery of 76.4%. Matrix effects averaged -6.9%. The largest matrix effects were -23% and -22% for NE and DA, respectively, but were negligible for all other compounds. These results highlight another advantage of HIILIC chromatography, the ability to minimize matrix effects when analyzing polar compounds.

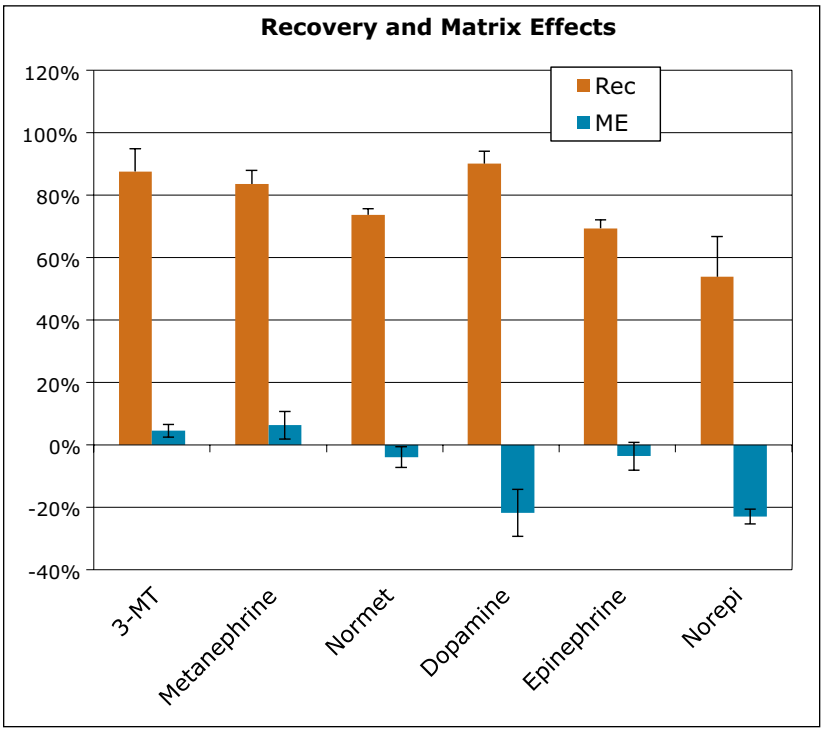

Figure 3. Recovery and matrix effects for catecholamines and metanephrines extracted from urine using Oasis WCX 96-well µElution Plates (N=4). Error bars indicate standard deviations. All compounds were spiked at 100 pg/mL into pooled human plasma.

Quantitative results

Calibration curves and quality control samples were prepared via the standard addition method by spiking pooled plasma samples with known concentrations of analytes. Two ranges of calibration curves were used, reflecting different expected concentrations of various compounds in plasma. Calibration levels for 3-MT, metanephrine, normetanephrine, and dopamine ranged from 10–2,000 pg/mL. Calibration levels for epinephrine and norepinephrine ranged from 50–10,000 pg/mL. After data processing, the endogenous concentrations were extrapolated from the resulting calibration curves. These data were used to correct the actual calibration concentrations. For example, the plasma sample used for calibration was determined to contain 31.7 pg/mL of metanephrine, so the calibration concentrations were adjusted to 41.7–2031.7 pg/mL. The resulting calibration curves showed excellent linearity, with R_2 values of 0.999 or greater for all compounds. Figure 4 shows representative calibration curves for DA and MTN, both of which have R_2 values greater than 0.999. Table 3 summarizes the calibration data for all compounds. Mean % deviations from expected calibration values were less than 1% for all analytes. In addition, the maximum % deviations from calibration values are listed and show that with the exception of epinephrine, the maximum % deviation for all calibrators was less than 10%. The calculated endogenous concentration of compounds in the pooled plasma used for calibration is also listed, along with the corrected calibration ranges for each compound.

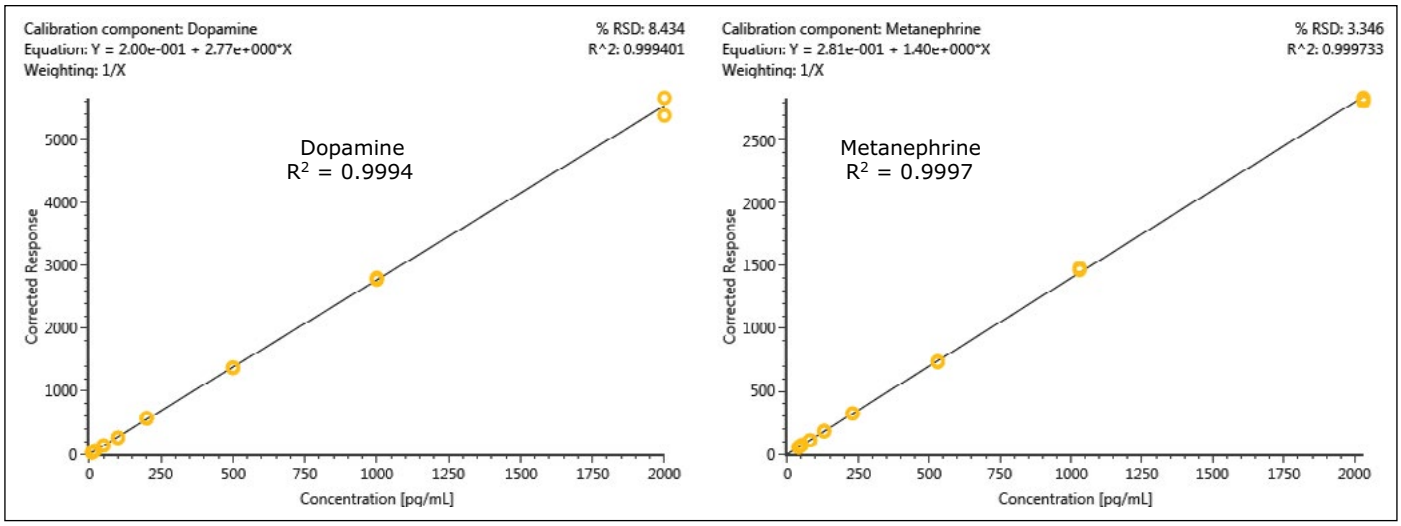

Figure 4. Representative calibration curves for dopamine (DA) and metanephrine (MTN) extracted from plasma samples. The data were fitted with a 1/x weighted linear fit. Basal concentrations for DA and MTN were 0 and 30 pg/mL, respectively.

Table 3. Summary of calibration data for plasma metanephrines and catecholamines. Mean % deviation indicates the average % deviation of all calibration points from their theoretical concentrations. The max % deviation indicates the maximum deviation over the entire calibration range. The calculated endogenous concentrations are listed and used to correct the calibration range.

	R^2	*Mean % Dev.*	Max % Dev.	Endogenous (pg/mL)	Corrected Calibration Range
3-MT	0.9993	0.25%	2.89%	7	17-2007
Metanephrine	0.9997	0.00%	2.50%	32	42-2042
Normetanephrine	0.9998	0.00%	1.72%	71	81-2081
Dopamine	0.9994	-0.33%	4.57%	0	10-2000
Epinephrine	0.9990	0.84%	11.83%	29	79-10079
Norepinephrine	0.9995	0.00%	2.59%	361	411-10411

Quality control samples (N=6) were overspiked at 200, 500, 2000, and 4000 pg/mL for EP and NE and at 40, 100, 400, and 800 pg/mL for the remaining compounds. QC results were accurate and precise (see Table 4). All QC values were within 10% of their target values, and most were within 5%. In addition, all coefficients of variation (%CV) were less than 10%. This demonstrates that the method is linear, accurate, and precise over a calibration range that includes the entire scope of expected values for normal and pathologically elevated samples.

Table 4. Quality control results for plasma catecholamines and metanephrines. Concentrations refer to the spiked concentration. Accuracies were calculated by comparing the result of the sum of the spiked concentration and endogenous calculated values in the plasma sample to the theoretical sum of these concentrations.

	QC spike concentration											
	40 pg/mL			100 pg/mL			400 pg/mL			800 pg/mL		
	Mean	S.D.	%CV	Mean	S.D.	%CV	Mean	S.D.	%CV	Mean	S.D.	%CV
3-MT	99.9%	7.4%	7.4%	99.2%	3.0%	3.1%	105.9%	1.8%	1.7%	93.9%	2.6%	2.8%
Metanephrine	99.9%	2.0%	2.0%	97.6%	0.8%	0.8%	107.3%	1.2%	1.1%	94.6%	1.7%	1.7%
Normetanephrine	99.8%	1.6%	1.6%	96.8%	1.7%	1.8%	104.6%	0.4%	0.4%	93.4%	1.0%	1.1%
Dopamine	97.0%	7.2%	7.4%	91.2%	3.4%	3.7%	103.7%	3.1%	3.0%	95.6%	2.7%	2.8%
	200 pg/mL			500 pg/mL			2000 pg/mL			4000 pg/mL		
	Mean	S.D.	%CV	Mean	S.D.	%CV	Mean	S.D.	%CV	Mean	S.D.	%CV
Epinephrine	97.3%	4.3%	4.4%	98.8%	2.2%	2.2%	100.8%	1.4%	1.4%	97.0%	2.6%	2.6%
Norepinephrine	105.1%	7.7%	7.4%	102.6%	8.2%	8.0%	96.7%	1.3%	1.3%	97.1%	4.2%	4.3%

CONCLUSIONS

The extraction and analysis of plasma catecholamines and metanephrines using Oasis WCX µElution Plates and an ACQUITY UPLC BEH Amide Column in HILIC mode is detailed. Extraction using the Oasis WCX µElution Plate resulted in low matrix effects and consistent recoveries for all compounds that translated into excellent analytical accuracy and precision. In addition, the ability to elute the samples in an extremely low volume (50 µL) enabled 5x sample enrichment without the extra time or risk associated with evaporation and reconstitution. The ACQUITY UPLC BEH Amide Column used for HILIC separation resulted in rapid and efficient separation of all compounds, with baseline resolution between normetanephrine (NMT) and epinephrine (EP). It also enabled the analysis of the monoamines, dopamine, norepinephrine and epinephrine. Quantitative results were excellent, with highly linear responses across the entire calibration range and excellent accuracy and analytical precision.

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Measurement of Urinary Serotonin for Clinical Research, Using Mixed-Mode SPE and a High-Strength Silica PFP Column

Jonathan P. Danaceau, Erin E. Chambers, and Kenneth J. Fountain
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Retention and baseline resolution of serotonin from endogenous, potentially interfering matrix components
- Minimal matrix effects
- Linear, accurate, and precise results, from 5.0 to 500 ng/mL

WATERS SOLUTIONS

Oasis® WCX 96-Well Plate, 30-mg sorbent per well, 30-µm particle size (p/n 186002503)

96-well Sample Collection Plate, 800 µL round well (p/n 186002481)

ACQUITY UPLC® HSS PFP Column, 100Å, 1.8 µm, 2.1 mm x 100 mm (p/n 186005967)

ACQUITY UPLC I-Class System

Xevo® TQD

MassLynx® Software

KEY WORDS

Serotonin, 5-HT, SPE, solid phase extraction, LC-MS/MS, quantitation

INTRODUCTION

Clinical research scientists often measure concentrations of urinary serotonin (5-HT). While serotonin is often analyzed by HPLC using electrochemical detection (ECD), the use of UPLC®-MS/MS affords greater analytical selectivity. This application note details the use of mixed-mode ion exchange solid phase extraction (SPE) to selectively isolate serotonin from urine, followed by chromatographic separation on a high-strength silica (HSS) PFP column. The method, which utilizes orthogonal sample pretreatment and chromatography, minimizes matrix effects as it promotes resolution of serotonin from potentially interfering endogenous compounds.

METHODS

A stock, standard solution of 1.0 mg/mL serotonin was prepared in methanol containing 0.1% formic acid (to discourage oxidation). A stock solution of internal standard composed of 500-µg/mL D4-serotonin (CDN Isotopes, Pointe-Claire, Quebec) was prepared in 50:50 methanol/water containing 0.1% formic acid. Working standard solutions were prepared daily in 10% methanol with 0.1% formic acid.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC HSS PFP Column, 1.7 µm, 2.1 x 100 mm
Column temp.:	30 °C
Sample temp.:	10 °C
Mobile phase A (MPA):	Water with 0.1% formic acid
Mobile phase B (MPB):	Acetonitrile with 0.1% formic acid
Purge solution:	20% Acetonitrile
Wash solution:	5% Acetonitrile

The gradient program is shown in Table 1.

MS conditions

MS system:	Xevo TQD
Ionization mode:	ESI positive
Capillary voltage:	1.0 kV
Cone voltage:	22 V
Desolvation gas:	900 L/hr
Cone gas:	0 L/hr
Desolvation temp.:	500 °C
Source temp.:	150 °C

Data were acquired and analyzed using MassLynx Software (V4.1, SCN 855). Quantification was performed using the TargetLynx™ Application Manager.

Sample preparation

Urine samples were pretreated with 1.67% (v/v) 6 N HCl, to mimic the acidic pretreatment normally used for this method. 40 µL of a 1 µg/mL internal-standard working solution was added to a 400-µL aliquot of acidified urine, followed by 1 mL of 0.5 M NH_4CH_3COO. Pretreated samples were loaded in individual wells of a WCX 96-well plate that had been conditioned with 1 mL of methanol and 1 mL of water. After loading the samples, wells were washed with 1 mL of 20 mM NH_4CH_3COO followed by 1 mL of methanol. The 96-well plate was then dried under vacuum for 30 seconds, to remove as much methanol as possible from the sorbent bed. The samples were eluted from the plate with 2 x 250 µL aliquots of 30:70 methanol/water containing 5% formic acid into an 800-µL, 96-well sample collection plate (p/n 186002481). Each aliquot was initially allowed to percolate through the well, to maximize duration of contact with the sorbent. 5 µL of the eluate was injected onto the UPLC-MS/MS system. The extraction procedure is summarized in Figure 1.

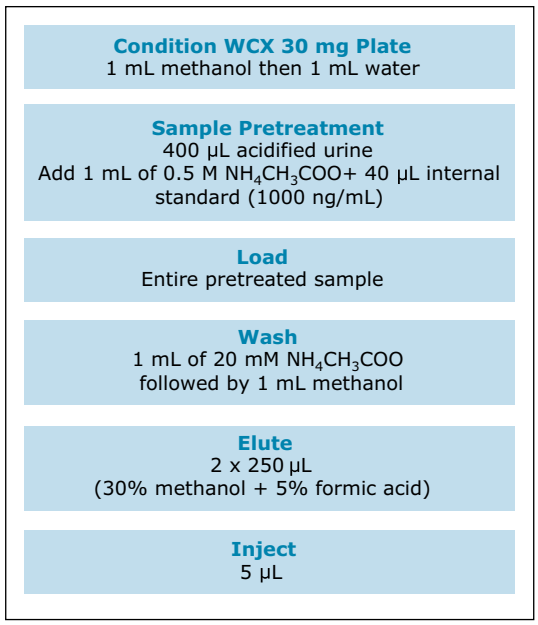

Figure 1. WCX extraction methodology for urinary serotonin.

Time (min.)	Flow (mL/min.)	%A	%B
0	0.5	98	2
0.5	0.5	98	2
2.5	0.5	70	30
2.6	0.5	98	2
3.0	0.5	98	2

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the Experimental section.

RESULTS AND DISCUSSION

Table 2 shows the retention time and individualized MS parameters of serotonin and its deuterated internal standard, including MRM transitions, cone voltage, and collision energy.

Figure 2 shows the chromatography of serotonin and its internal standard from an extracted method blank (A) and a 50 ng/mL calibration standard (B) using the ACQUITY UPLC HSS PFP Column. The serotonin peak seen in the method blank represents the endogenous concentration of serotonin (29.7 ng/mL) contained in the pooled urine used to create the calibration curve. The PFP column chemistry provided excellent retention and peak shape as well as baseline resolution from the endogenous, potentially interfering peaks that elute close to that of serotonin.

Analyte	RT (min)	MRM transitions (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
Serotonin	2.24	177.1>160	22	10
		177.1>115	22	22
d4 Serotonin (IS)	2.24	181.1>164	22	10

Table 2. Mass spectral parameters used for analysis of serotonin.

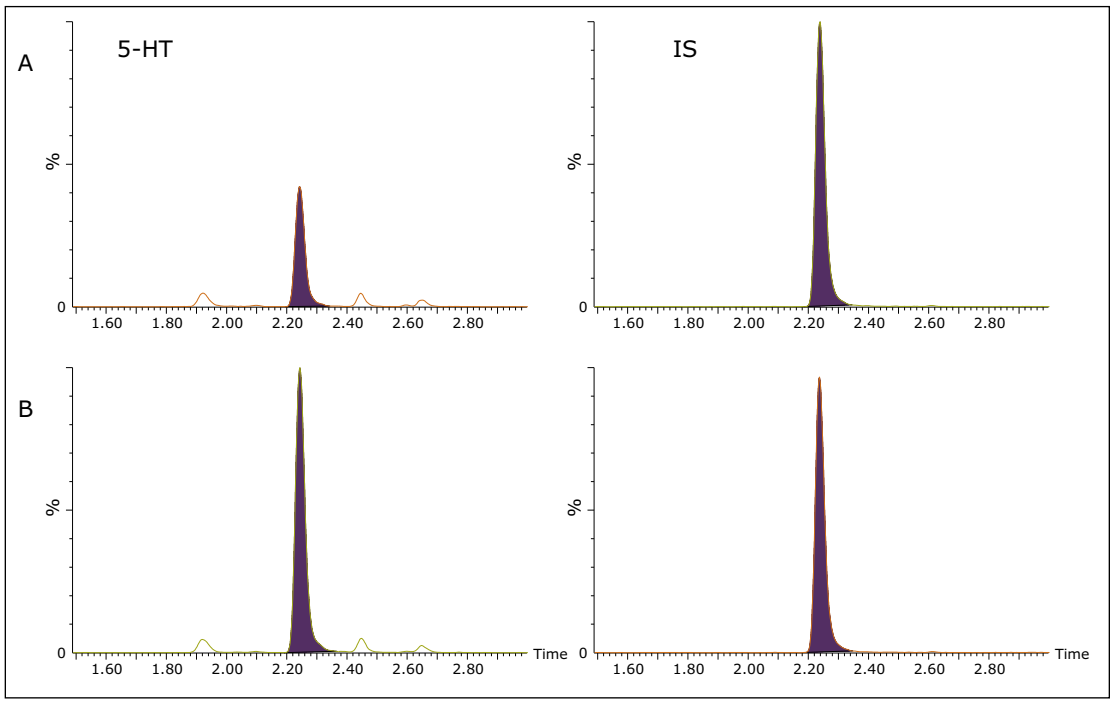

Figure 2. Chromatography of serotonin and its internal standard on the ACQUITY UPLC HSS PFP Column, 1.7 µm, 2.1 x 100 mm. A. Unspiked urine sample showing chromatography of endogenous serotonin (29.7 ng/mL). B. Representative 50 ng/mL calibration standard. Serotonin elutes at a retention time of 2.24 minutes. Chromatographic conditions are detailed in the Methods section.

Recovery and matrix effects

Extraction recovery and matrix effects for urinary serotonin were 80% and -19%, respectively. The unique selectivity provided by the HSS PFP column not only provided the resolution of serotonin from endogenous interfering compounds, but also resulted in minimal matrix effects.

Quantitative results

Calibration and quality-control samples were prepared via the standard-addition method by spiking a urine pool with known concentrations of serotonin. After data processing, the endogenous concentration was extrapolated from the resultant calibration curve and the information used to correct the actual calibration and QC concentrations. The urine pool used for calibration was found to contain 29.7-ng/mL of serotonin, so the calibration concentrations were changed from 5.0 to 500 to 34.7 to 529.7 ng/mL. The resultant calibration curve showed excellent linearity, with an R^2 value of 0.9997. The quality-control samples (n = 6), over-spiked at 7.5, 75, and 300 ng/mL, demonstrated excellent accuracy and precision (see Table 3). All QC values were within 3% of their target values, and all coefficients of variation were less than 2%. These data demonstrate that the method is linear, accurate, and precise over a measurement range that includes the expected values for normal and elevated urine samples.

	Accuracy		
QC Level (ng/mL)	**Mean**	**S.D.**	**%RSD**
7.5	99.9%	1.6%	1.6%
75	97.9%	0.4%	0.4%
300	97.3%	1.1%	1.1%
Mean	**98.4%**	**1.3%**	**1.3%**

Table 3. Quality-control results from extracted urine samples. Concentrations refer to the spiked concentration. Accuracies were calculated by comparing the result of the sum of the spiked concentration with the endogenous, calculated values obtained from the urine sample.

CONCLUSIONS

The extraction and analysis of urinary serotonin for clinical research using the Oasis, mixed-mode, weak-cation-exchange (WCX) plates and an ACQUITY UPLC HSS PFP Column is detailed. Extraction resulted in low matrix effects and consistent recoveries that translated into excellent analytical precision. The unique selectivity provided by the HSS PFP column resulted in resolution of serotonin from endogenous, potentially interfering compounds and minimal matrix effects. Quantitative results showed a linear response across the measurement range (5 to 500 ng/mL) and excellent accuracy and analytical precision.

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[APPLICATION NOTEBOOK]

STEROID HORMONES – INCUDING VITAMIN D METABOLITES

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Analysis of 25-Hydroxyvitamin D in Serum for Clinical Research

Robert Wardle, Billy Molloy, and Lisa Calton
Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

- Facilitates high sample throughput utilizing automation in a multi-well plate format
- Oasis® µElution Plate technology eliminates time-consuming, costly evaporation and reconstitution steps
- Enables complete sample tracking from the sample barcode to data import into a LIMS from TargetLynx™ Application Manager

WATERS SOLUTIONS

ACQUITY UPLC® I-Class System

Oasis HLB µElution Plate

ACQUITY UPLC BEH Phenyl Column

Xevo® TQD

MassLynx® Software

TargetLynx Application Manager

KEY WORDS

25-hydroxyvitamin D, SPE, UPLC-MS/MS, clinical research

INTRODUCTION

The demand for serum 25-hydroxyvitamin D (25OHD) analysis has increased dramatically in recent years. While the role of vitamin D in bone metabolism is well established, comparatively little research has been performed to elucidate its role in other diseases. However, considerable time, effort, and funds are being applied to randomized, prospective clinical trials that aim to better define the link between vitamin D status and a variety of diseases – such as cancers, multiple sclerosis, heart disease, and diabetes.

Vitamin D is available in two forms: the plant-derived vitamin D_2 (ergocalciferol), and vitamin D_3 (cholecalciferol), which is formed upon exposure of the skin to ultraviolet radiation. The accepted indicator of vitamin D status – total 25OHD [that is, the sum of $25OHD_2$ and $25OHD_3$] – has been a challenge to measure accurately because the antibodies used in some immunoassays do not have 100% co-specificity for both $25OHD_2$ and $25OHD_3$. Therefore, many clinical research laboratories have now adopted LC-MS/MS based methods for measuring total 25OHD while also allowing independent quantification of $25OHD_2$ and $25OHD_3$.

Described here is a clinical research method utilizing Waters® Oasis HLB µElution Plate technology for the extraction of $25OHD_2$ and $25OHD_3$ from human serum. This method was automated on a Tecan Freedom Evo 100/4 Liquid Handler, allowing for full sample tracking from the primary tube to processed results. Chromatographic separation of extracted samples was performed on an ACQUITY UPLC I-Class using an ACQUITY UPLC BEH Phenyl Column followed by mass detection on a Xevo TQD (Figure 1).

Figure 1.
The ACQUITY UPLC I-Class and Xevo TQD.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC I-Class
Needle:	30 µL
Column:	ACQUITY UPLC BEH Phenyl, 130Å, 1.7 µm, 2.1 mm x 50 mm (P/N 186002884)
Pre-column filter:	ACQUITY UPLC Column In-line filter kit (P/N 205000343)
Mobile phase A:	Aqueous 2 mM ammonium acetate + 0.1% formic acid
Mobile phase B:	Methanol with 2 mM ammonium acetate + 0.1% formic acid
Needle wash solvent:	80% $Methanol_{(aq)}$ + 0.1% formic acid
Purge solvent:	65% $Methanol_{(aq)}$ + 0.1% formic acid
Column temp.:	35 °C
Injection volume:	20 µL
Flow rate:	0.45 mL/min.
Gradient:	See Table 1
Run time:	4.2 minutes

MS conditions

System:	Xevo TQD
Resolution:	MS1 (0.7 FWHM), MS2 (0.85 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (see Table 2 for details)
Polarity:	ESI+
Capillary:	0.80 kV
Source temp.:	120 °C
Desolvation temp.:	400 °C
Dwell time:	0.06 seconds
Inter-scan delay:	0.01 seconds
Inter-channel delay:	0.02 seconds

Data management

MassLynx v4.1 with TargetLynx Application Manager

MassLynx Tecan File Converter v2.0

MassLynx LIMS Interface v3.0

Method conditions

Time (min)	Flow Rate (mL/min)	%A	%B	Curve
Initial	0.45	35	65	Initial
2.5	0.45	20	80	6
2.7	0.45	2	98	11
3.2	0.45	35	65	11

Table 1. Gradient table for the separation of 25OHD$_2$ and 25OHD$_3$.

Operating backpressure at the initial conditions is ~7000 psi.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (kV)
25OHD$_3$ (quan)	401.3	159.1	24	24
25OHD$_3$ (qual)	401.3	365.3	24	10
[$^2H_3$]-25OHD$_3$	404.3	162.1	24	24
25OHD$_2$ (quan)	413.3	355.3	24	10
25OHD$_2$ (qual)	413.3	83.1	24	24
[$^2H_3$]-25OHD$_2$	416.3	358.3	24	10

Table 2. MRM parameters of 25OHD$_2$ and 25OHD$_3$ quantifier ions, qualifier ions, and their internal standards.

Sample preparation

25OHD$_2$ and 25OHD$_3$ certified reference solutions and their stable labeled internal standards ($^2H_3$) were purchased from IsoSciences. Calibrators were prepared in a surrogate matrix of MSG2000 stripped human serum purchased from Golden West Biologicals. The calibration range for 25OHD$_2$ and 25OHD$_3$ was 10–375 nmol/L (~4-150 ng/mL). QC materials were purchased from Recipe at ~50 and 100 nmol/L (~20 and 40 ng/mL) and UTAK at ~25, 75, and 190 nmol/L (~10, 30, and 75 ng/mL). Distilled water, methanol, ammonium acetate, zinc sulphate, and 2-propanol were purchased from Sigma-Aldrich. Formic acid was purchased from VWR.

To convert SI units (nmol/L) to conventional mass units (ng/mL) divide by 2.423 for 25OHD$_2$ and by 2.496 for 25OHD$_3$.

Semi-automated sample extraction

Extraction was performed using a Tecan Freedom Evo 100/4 Liquid Handling System (LHS). Serum calibrators, QCs, and samples were placed onto the LHS along with all reagents and consumables required. Samples were identified by barcodes and tracked through the extraction procedure. 150 µL of sample was transferred into a 96-deep well plate and 20 µL of 620 nmol/L (250 ng/mL) internal standard, 150 µL 0.2 M zinc sulphate$_{(aq)}$, and 600 µL of methanol were added. This was followed by centrifugation (off-line) for five minutes at 750 g. The samples were mixed after each reagent addition.

The Oasis HLB µElution Plate was conditioned and equilibrated with 200 µL methanol and 60% methanol$_{(aq)}$ respectively. An aliquot of each pretreated sample (600 µL) was loaded into individual wells of the plate and slowly pulled through at low vacuum. Consecutive washes with 200 µL of 5% methanol$_{(aq)}$ and 200 µL of 60% methanol$_{(aq)}$ were performed. 25OHD$_2$ and 25OHD$_3$ were eluted using 80 µL of 95:5 (v:v) methanol–IPA, followed by 50 µL of water to match the organic strength of the initial chromatographic conditions. The collection plate was sealed manually and transferred onto the ACQUITY UPLC Sample Manager (FTN) for injection onto the UPLC-MS/MS.

A MassLynx sample list was created with the MassLynx Tecan File Converter using the barcode information scanned and tracked sample positions in the 96-well plate, which allows for complete sample tracking. The MassLynx LIMS Interface may also be used to upload sample results into a LIMS.

The sample preparation time for a full plate of 96 samples is approximately two hours, requiring minimal manual intervention:

T = 0 min	Laboratory analyst locates and manually loads samples, calibrators, QCs, reagents, and consumables onto the LHS
T = 30 min	Laboratory analyst initiates the LHS pretreatment procedure
T = 1 hr 15 min	Laboratory analyst removes the protein precipitation plate for centrifugation off-line
T = 1 hr 20 min	Laboratory analyst returns the plate to the LHS and resumes the automated SPE protocol
T = 2 hr 15 min	Laboratory analyst seals the collection plate and transfers to the ACQUITY autosampler

RESULTS AND DISCUSSION

Precision was assessed by quantifying pooled human serum samples at four different concentrations in duplicate. Samples were analyzed in a fully randomized order on twenty non-consecutive days, with two analytical runs performed each day (n = 80 at each concentration level). Total precision and repeatability were ≤7.3%RSD for all runs for both 25OHD$_2$ and 25OHD$_3$ at concentrations between 20–300 nmol/L (8–120 ng/mL) (Table 3).

	Pool A		Pool B		Pool C		Pool D	
Nominal conc (nmol/L)	20		75		200		300	
	25OHD$_2$	25OHD$_3$	25OHD$_2$	25OHD$_3$	25OHD$_2$	25OHD$_3$	25OHD$_2$	25OHD$_3$
Total (%RSD)	7.3	6.6	5.8	5.4	7.3	6.1	7	5.4
Repeatability (%RSD)	6.5	6	4.3	4.6	6	4.9	6.4	4.3

Table 3. Precision Performance Summary.

Analytical sensitivity was assessed by analyzing pooled samples that were adjusted using vitamin D depleted pooled serum to span the concentration ranges 0–15 nmol/L (0–6 ng/mL). Analysis was performed over three days with two runs per day. Using a total allowable error of 35%, the LoD and LoQ were determined to be 2.56 and 3.60 nmol/L (1.06 and 1.49 ng/mL) respectively for $25OHD_2$ and 4.07 and 5.56 nmol/L (1.63 and 2.23 ng/mL) respectively for $25OHD_3$.

The method was shown to be linear over the range of 7–450 nmol/L (2.8–180 ng/mL), when low and high pools were mixed in known ratios to give 11 samples over the range. All calibration lines in spiked vitamin D depleted serum were linear with a coefficient of determination (r^2) > 0.996 over 40 occasions.

No significant carryover was observed from high concentration samples in subsequent blank samples. Recovery was assessed by supplementing each of six serum samples across the measuring range of 0–225 nmol/L (0–90 ng/mL) in addition to their endogenous levels. Samples were extracted in triplicate and the recovery for $25OHD_2$ and $25OHD_3$ ranged between 95.7%–104.6%.

An assessment of interferences was conducted by spiking the test compound at a high concentration into serum pools. Of 48 compounds tested – including endogenous and exogenous interferences, and other vitamin D metabolites – no significant interference was observed when comparing control and test sample results. Due to co-elution of C3-epi-$25OHD_2$ with $25OHD_2$ and C3-epi-$25OHD_3$ with $25OHD_3$, these were found to contribute to the overall $25OHD_2$ and $25OHD_3$ concentrations.

Chromatography conditions employed minimize interferences from oleamide, a known interference in sample tubes and phospholipids from the serum matrix. Below are example chromatograms of $25OHD_2$ and $25OHD_3$ samples (Figure 2).

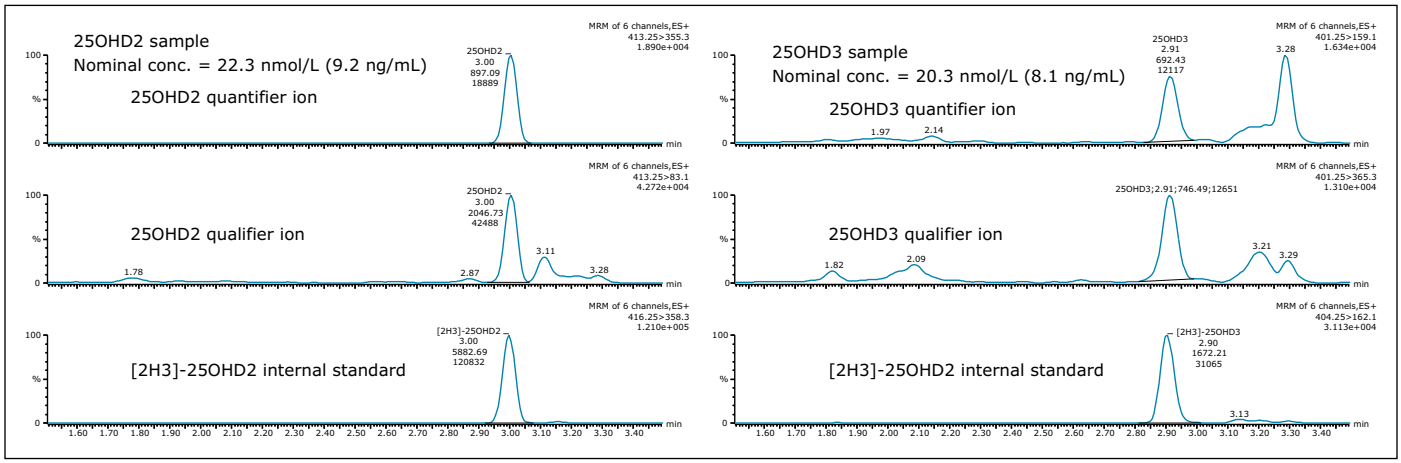

Figure 2. Chromatograms of low-level $25OHD_2$ and $25OHD_3$ samples.

Accuracy was assessed by analyzing 29 DEQAS samples with calculated concentrations compared to the nominal value. Only six samples had a percentage difference outside of ±10%, with only one sample being outside of ±15% for $25OHD_3$. The comparison between DEQAS and the Waters UPLC-MS/MS method yielded a Deming regression of y = 1.02x + 0.07 (Figure 3), which showed no significant proportional or constant bias (p values of 0.7164 and 0.9744 respectively).

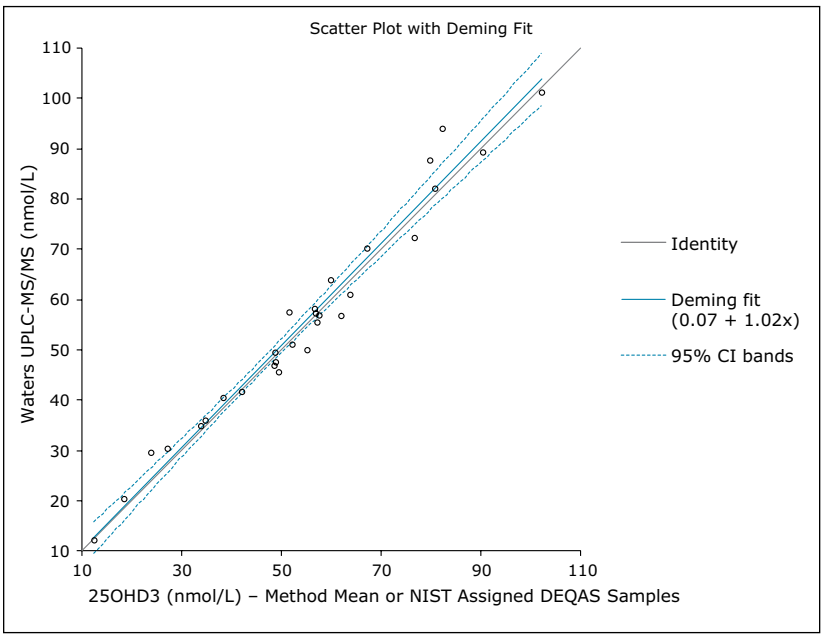

Figure 3. Deming regression agreement plot comparing the NIST assigned/method means for DEQAS samples to the Waters UPLC-MS/MS method.

CONCLUSIONS

A semi-automated clinical research method has been developed for the analysis of $25OHD_2$ and $25OHD_3$ in human serum.

The assay described demonstrates excellent precision over 20 non-consecutive days with good linearity across the required range. A sufficient level of analytical sensitivity was achieved and no significant carryover or interferences were seen. In addition, the method demonstrates good agreement with 29 DEQAS samples.

The LHS significantly reduces the manual operation steps and operator variability to ensure more consistent and reproducible results. Furthermore, the use of Oasis µElution Plate technology eliminates the need for time-consuming solvent evaporation and reconstitution steps, allowing for the analysis of at least 192 samples per work shift.

Complete sample tracking from the sample barcode through to reporting of the result in TargetLynx and transferring the data to a LIMS has been made possible with the use of the MassLynx Tecan File Converter and MassLynx LIMS Interface.

The described semi-automated method overcomes many limitations of current LC-MS/MS method. In particular, several time-consuming and labor-intensive manual sample pretreatment steps have been eliminated. This will enable a wider range of laboratories to implement UPLC-MS/MS methodology for 25OHD analysis in clinical reasearch.

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Quantitative Analysis of Dried Bloodspot 17-Hydroxyprogesterone by ACQUITY UPLC-MS/MS for Clinical Research

Heather A Brown,[1] Claudia Rossi,[2] and Lisa J Calton[1]
[1] Waters Corporation, MS Technologies Centre, Manchester, UK
[2] Department of Biomedical Science, G. d'Annunzio University Foundation, Pescara, Italy

APPLICATION BENEFITS

- Single non-derivatized liquid-liquid extraction
- Analytical selectivity for 17-hydroxyprogesterone
- Rapid result with 3.5 minute injection-to-injection time

WATERS SOLUTIONS

ACQUITY UPLC®

ACQUITY® HSS T3 UPLC Column plus VanGuard™ pre-column

Xevo® TQ MS

TruView™ Maximum Recovery Vials

MassLynx® Software

TargetLynx™ Application Manager

KEY WORDS

17-hydroxyprogesterone, dried bloodspot, adrenal steroids, UPLC-MS/MS

INTRODUCTION

Measurement of 17-hydroxyprogesterone (17-OHP) by immunoassay is prone to analytical interference arising from cross-reactivity of reagent antibodies with structurally-related steroid metabolites.[1] The dried bloodspot (DBS) has proved popularity as a sample matrix in the pharmaceutical, life sciences and clinical research arena due to simplicity of sample collection and stability of compounds within this matrix. A method for the extraction and UPLC-MS/MS analysis of DBS 17-OHP using the ACQUITY UPLC System with the Xevo TQ MS Detector (Figure 1) is described. The technique features an extended LC gradient to allow qualitative evaluation of the androstenedione (A4) and cortisol chromatographic peaks.

Figure 1. ACQUITY UPLC and Xevo TQ MS Detector

EXPERIMENTAL

Sample description

Two x 3 mm DBS were agitated for 50 minutes in 200 µL 50 : 50 acetone : acetonitrile plus 20 µL internal standard (95 nmol/L [$^2H_8$]-17-OHP in 50 : 50 methanol : water). Extract was transferred to a Waters Maximum Recovery vial, evaporated to dryness and reconstituted in 50 µL of 55 : 45 mixture of mobile phases A and B.

Method conditions

UPLC conditions

System:	ACQUITY UPLC System
Sample preparation plates:	V bottom polypropylene 96-well microtitre plate for extraction eg Nunc® Microwell™ 96-well plate
Sample preparation vials:	TruView LCMS Certified Maximum Recovery Vial (p/n 186005662CV)
Column:	ACQUITY UPLC HSS T3 1.8 µm, 2.1 x 50 mm (p/n 186003538) fitted with ACQUITY HSS T3 VanGuard Pre-column 1.8 µm, 2.1 x 5 mm (p/n 186003976)
Column temp.:	60 °C
Sample temp.:	8 °C
Injection volume:	20 µL (PLNO, load ahead enabled)
Weak wash:	45 % Methanol (aq) 1500 µL
Strong wash:	Equal parts water, methanol, acetonitrile and isopropanol 500 µL
Flow rate:	0.6 mL/min
Mobile phase A:	2 mmol/L ammonium acetate, 0.1 % (v/v) formic acid (aq)
Mobile phase B:	2 mmol/L ammonium acetate, 0.1% (v/v) formic acid in methanol
Gradient:	Binary system: initially 45% mobile phase B increasing linearly to 85% B over 2 min, to 98% B over 0.1 min, holding for 0.4 min before stepping down to 45% B with 1.0 min column re-equilibration.

MS conditions

System:	Xevo TQ MS Detector Tuned to unit resolution on MS1 and MS2 (0.7 FWHM)
Detection mode:	Electrospray positive ionization mode
Acquisition mode:	Multiple Reaction Monitoring (see Table 1 for ion transitions)
Capillary voltage:	0.7 kV
Collision energy:	analyte specific (see Table 1)
Cone voltage:	analyte specific (see Table 1)
Source temp.:	120 °C
Desolvation temp.:	500 °C
Inter-channel delay:	0.01 sec
Inter-scan delay:	0.02 sec

Data management

MassLynx v 4.1 incorporating TargetLynx application manager

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage	Collision energy	Dwell time (sec)	MS function (time window min)
Cortisol	363	121	26	23	0.027	2 (0-1.2)
Androstenedione	287	97	30	20	0.018	1 (1.4–2.5)
17-OHP (quantifier)	331	97	28	26	0.018	1 (1.4-2.5)
17-OHP (qualifier)	331	109	28	28	0.018	1 (1.4-2.5)
$^{2}H_{8}$ 17-OHP	339	100	28	26	0.018	1 (1.4-2.5)

Table 1. MS Parameters. Optimize precursor and product ions to 1 decimal place.

A function containing one quantifier and internal standard ion transition for both androstenedione and cortisol were added for qualitative evaluation of these compounds, with no adverse effect on 17-OHP detection. MS Function time windows were optimized for instrument duty cycle. Verify chromatogram peak retention time with a single-function MS Method prior to setting time window settings for routine use.

RESULTS AND DISCUSSION

Preparation of 9-point in-house DBS calibration series prepared from saline-washed red blood cells resuspended in spiked stripped-serum enabled linear quantification of 17-OHP between 9.9 – 1270.0 nmol/L with coefficient of determination $r^2 > 0.997$ and measurements ≤ 10 % deviation from nominal calibrator values.

Analytical sensitivity was determined from the peak-to-peak signal to noise ratio (SNR) observed in the 17-OHP chromatogram of 6 reference DBS samples with the lowest SNR (mean 0.5 nmol/L 17-OHP, range 0.5 – 2.9 nmol/L, SNR 1.6 – 4.7). The LLOQ was calculated as the concentration of 17-OHP extrapolated to give SNR > 10 and was determined to be 1.6 nmol/L. The LOD taken as the extrapolated concentration with SNR > 3 was 0.5 nmol/L. Descriptive statistical analysis was conducted using Analyse-it® in Microsoft® Excel for Windows.® The mean (95 % Confidence Interval) was 2.7 (2.2 – 3.1) nmol/L (n=22). Chromatograms from this population are shown in Figure 2A alongside a chromatogram from a separate reference population containing higher concentrations of 17-OHP shown in Figure 2B.

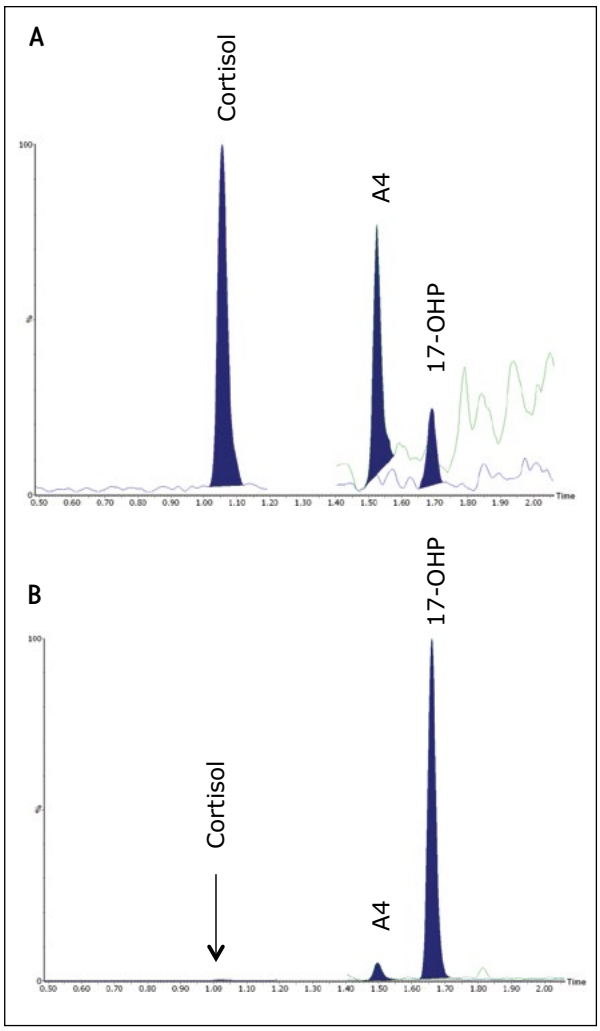

Figure 2. Vertical axis-linked extracted ion chromatograms of DBS 17-OHP at 2.69 nmol / L (A) and 511.0 nmol / L (B). 17-OHP peak to peak SNR ratio is 5.97 with area count 579 in the upper panel. Cortisol and androstenedione (A4) peaks are shown for reference.

Within- and between-batch imprecision, expressed as coefficient of variation of replicate measurements of independently-prepared quality control DBS at target mean 17-OHP concentrations 76, 151, and 303 nmol/L was < 6.7% CV (between-batch in singlicate over 5 days; within-batch n=5). The between-batch imprecision of the lowest calibrator 9.9 nmol/L was 7.3% CV (singlicate over 5 batches).

An artificial whole blood matrix was prepared for evaluation of extraction efficiency. The negative control blood matrix along with 3 positive controls of blood matrix spiked to 76, 152, and 303 nmol/L 17-OHP were spotted onto Whatman 903 filter paper. The dried residue of the extracted negative pool was resuspended in solvent standards at the maximum expected 17-OHP concentration for each level of the positive control extract, assuming total recovery of the spiked 17-OHP. The mean extraction efficiency of 17-OHP, calculated as the ratio of the peak area of the positive control: negative control post-extraction spiked sample, was 58% (57 – 59%, n = 3).

Quantitative matrix effects were assessed, expressed as the proportion of detector response suppressed or enhanced by the presence of matrix. The ratio was calculated of the peak area of post-extraction spiked negative pool: peak area of matrix-free solvent standards of equivalent concentration. The mean signal suppression due to matrix was 15% (13 – 16%, n = 3). A qualitative evaluation of signal suppression conducted by post-column infusion of a solvent standard of 17-OHP into the LC flow path of extracted samples confirmed compounds of interest do not elute within regions of signal suppression (Figure 3).

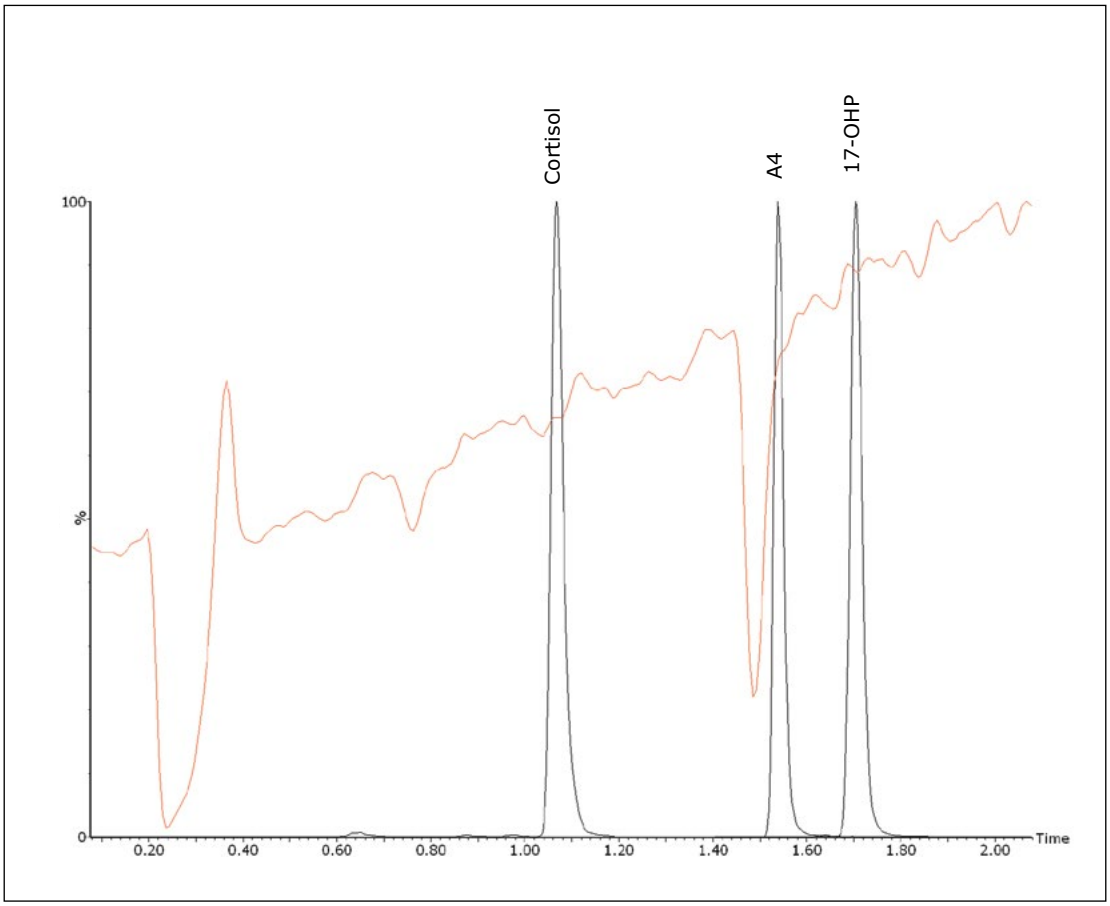

Figure 3. Qualitative evaluation of 17-OHP ionisation suppression from an extracted DBS (red), with solvent standards overlaid for reference (black). Ionisation of cortisol and 17-OHP is not suppressed by residual sample matrix.

CONCLUSIONS

The developed LC-MS/MS method enables the rapid measurement of DBS 17-OHP with analytical sensitivity and reproducibility. The simple liquid-liquid extraction technique generated a clean sample extract. This familiar and well-understood sample preparation technique can be easily adopted into routine use in a clinical research laboratory.

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References

1. Wong T *et al*. Identification of the steroids in neonatal plasma that interfere with 17α-hydroxy-progesterone radioimmunoassays. *Clin Chem* 1992;38:1830 –1837.

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Analysis of Aldosterone in Plasma for Clinical Research using the Xevo TQ-S micro

Dominic Foley

GOAL

To demonstrate the capability of Waters® Xevo® TQ-S micro mass spectrometer, to quantify low levels of aldosterone in plasma for clinical research using a highly selective sample preparation technique.

BACKGROUND

Aldosterone is a mineralocorticoid steroid hormone, that is assessed in clinical research studies to help understand the pharmacological mechanism of aldosterone synthase inhibitors (ASIs).[1] Circulating levels of aldosterone in blood are typically found at low concentrations (<100 pmol/L), which makes its analysis particularly challenging. Successfully quantifying these low levels typically necessitate the use of a mass spectrometer with high analytical sensitivity in conjunction with highly selective sample preparation techniques.

The Xevo TQ-S micro utilizes innovative StepWave™ ion source technology to improve method robustness and reduce background noise, which enable accurate and precise quantification of low level analytes such as aldosterone.

THE SOLUTION

The method for the analysis of plasma aldosterone was successfully employed using automated selective solid phase extraction sample preparation followed by LC-MS/MS analysis. The sample preparation was automated on a Tecan Freedom EVO100/4 using 96-well Oasis® MAX µElution Plates followed by analysis on the ACQUITY UPLC® I-Class System with Xevo TQ-S micro and MassLynx® Software (v4.1).

Low levels of aldosterone were successfully quantified by using automated sample preparation and the Xevo TQ-S micro.

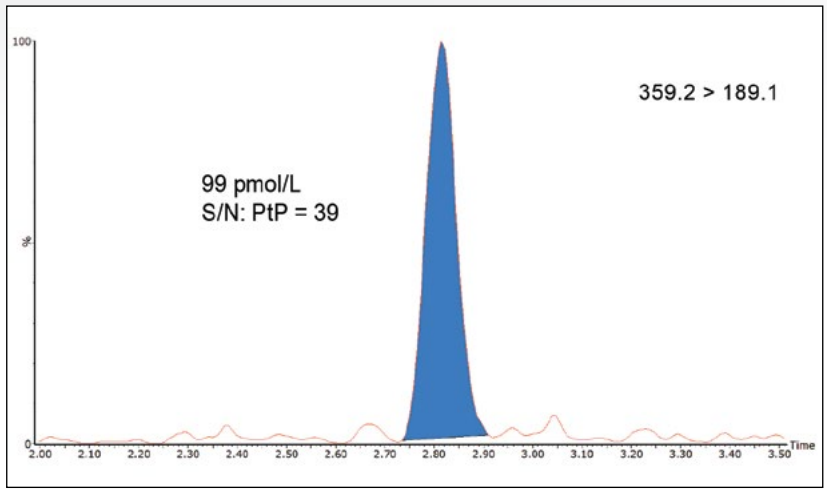

Figure 1. An extracted SPE sample of 99 pmol/L aldosterone in plasma on the Xevo TQ-S micro. S/N is calculated on the raw data using peak to peak at ±1 SD.

Sample preparation and LC-MS/MS analysis

Using the Tecan Freedom EVO100/4, plasma samples were diluted with internal standard, zinc sulphate, methanol, and phosphoric acid. Following centrifugation, sample supernatant was loaded onto the 96-well Oasis MAX µElution Plate (PN: 186001829) following conditioning and equilibration. Consecutive washes with phosphoric acid, ammonia in 10% methanol and water were performed. Samples were eluted with 70% aqueous methanol followed by water.

30 µL of each extracted sample was injected on an ACQUITY UPLC I-Class/Xevo TQ-S micro system utilizing a water/methanol gradient and an ACQUITY UPLC BEH Phenyl Column (PN: 186002884). The MRM parameters used in this analysis are shown in table 1.

Analyte	Precursor ion (*m/z*)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)
Aldosterone (Quan)	359.2	189.1	38	14
Aldosterone (Qual)	359.2	297.2	38	10
Aldosterone-d4 (IS)	363.2	190.0	38	14

Table 1. MRM parameters for both aldosterone quantifier (Quan) and qualifier (Qual) and its internal standard (IS), aldosterone-d4.

Results

As shown in table 2, a relatively low peak area at the LLOQ is obtained on the Xevo TQ-S micro using SPE extracted plasma samples. However, the signal:noise (S/N) is >20:1, which is an indication of the low background noise on the instrument resulting from the selectivity of the MRM trace and a clean SPE sample extract. In addition, the correlation coefficient demonstrates excellent linearity across a range of 28-2776 pmol/L.

MS system	Calibration curve	LLOQ (28pmol/L)	
	Correlation coefficient (r^2)	Peak area	S/N
Xevo TQ-S micro	0.9995	58	23

Table 2. Calibration curve and LLOQ performance characteristics on the Xevo TQ-S micro. S/N is calculated on the raw data using peak to peak at ±1 SD.

The additional selectivity provided by the Oasis MAX µElution Plate provides a clean sample extract for ESI MS analysis of aldosterone. This is observed in the extraction and quantification of 99 pmol/L of aldosterone in plasma using the Xevo TQ-S micro (figure 1).

Reproducibility of the method was assessed by extracting and quantifying plasma samples using six replicates at Low (99), Mid (500), and High (2000 pmol/L) concentrations. All results were ≤5.5% RSD as shown in table 3.

	Xevo TQ-S micro Repeatability (RSD%)		
Compound	Low	Mid	High
Aldosterone	5.5%	4.0%	4.7%

Table 3. Repeatability assessment for the analysis of aldosterone in plasma on the Xevo TQ-S micro.

SUMMARY

An LC-MS/MS method for the analysis of plasma aldosterone for clinical research has been developed. This method demonstrates highly efficient automated sample preparation with the Tecan Freedom EVO 100/4 and 96-well Oasis MAX µElution SPE Plates. Automated sample preparation optimizes analytical sensitivity and increases laboratory efficiency with reduced sample handling, alleviating the potential of operator error. The ACQUITY UPLC I-Class System, when combined with the robust and analytically sensitive Xevo TQ-S micro, has been shown to provide excellent repeatability (n = 6) and linearity of response across all aldosterone levels that were tested. Furthermore, the results from this clinical research method are acceptable to measure aldosterone at physiological levels.

References

1. Schumacher CD, Steele RE, Brunner HR. Aldosterone synthase inhibition for the treatment of hypertension and the derived mechanistic requirements for a new therapeutic strategy. *J Hypertens*. Oct 2013; 31(10): 2085–2093.

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[TECHNOLOGY BRIEF]

Extraction of Testosterone from Serum using Oasis Max µElution Plates for Clinical Research

Dominic Foley and Lisa Calton

GOAL

To demonstrate a highly selective solid-phase extraction (SPE) technique with Oasis® MAX µElution Plates for the extraction of testosterone from serum samples for LC-MS/MS analysis in clinical research.

BACKGROUND

To determine the pharmacokinetic, bioavailability, and safety of new formulations of testosterone gels in clinical research, accurate and precise quantification of testosterone at low physiological concentrations is required.[1]

Historically, testosterone has been analysed by direct immunoassay methods. However, it has been reported that these immunoassay methods can suffer from a lack of specificity due to the cross reactivity of structurally similar steroid hormones i.e., dihydrotestosterone. This results in greater imprecision and inaccuracy, most notably at the lower concentrations of testosterone found in serum samples. LC-MS/MS provides a solution to this problem by providing a platform to discern between similar steroids through the use of chromatographic separation and selective MRM mass spectrometry.

Additional selectivity can also be obtained through the use of sample preparation prior to analysis. The use of sample preparation can provide orthogonal selectivity to the LC-MS/MS dimension of the method. Potential interferences found in the MRM trace of the analyte of interest can be removed with selective sample preparation techniques such as mixed mode SPE.

The Oasis MAX µElution Plate enables optimized analytical sensitivity and selectivity for the analysis of testosterone in serum for clinical research.

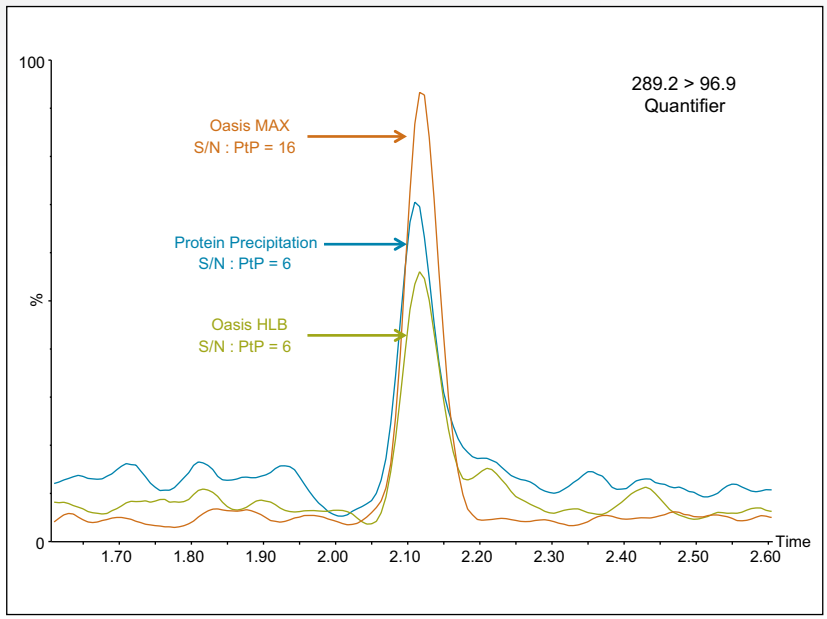

Figure 1. Testosterone quantifier MRM chromatograms of extracted serum samples using an Oasis MAX µElution Plate, Oasis HLB µElution Plate, and protein precipitation. Samples were extracted in triplicate and the mean S/N value is shown. S/N is calculated on the raw data using peak to peak at ±2 SD.

THE SOLUTION

In this technology brief, an Oasis MAX µElution Plate was successfully employed for the selective extraction of testosterone from serum. This chemistry provides mixed-mode interaction which utilizes both hydrophobic and anion exchange functionality. The added selectivity of this sorbent is more proficient at removing interferences than conventional reversed-phase SPE.

Sample preparation and LC-MS/MS analysis

Serum samples were diluted with internal standard, ammonia, zinc sulphate, methanol, and water. Following centrifugation, sample supernatant was loaded onto the 96-well Oasis MAX µElution Plate (PN:186001829) following conditioning and equilibration. A high pH wash was performed with ammonia in 20% methanol and samples were eluted with methanol followed by water.

20 µL of each extracted sample was injected on an ACQUITY UPLC® I-Class/Xevo® TQD utilizing mobile phases consisting of 2 mM ammonium acetate and 0.1% formic acid in water and methanol. Separation was performed on an ACQUITY UPLC HSS C_{18} SB Column (PN:186004118). The MRM transitions used for this analysis were *m/z* 289.2 for the testosterone precursor ion and *m/z* 96.9 and 109.0 for the quantifier and qualifier product ions respectively. Testosterone-$^{13}C_3$ was used as the internal standard.

Results

The additional selectivity provided by the Oasis MAX chemistry compared to other extraction techniques, provides enhanced analytical sensitivity for ESI MS analysis of testosterone. This is observed in the extraction and quantification of testosterone in serum using optimized Oasis MAX µElution, Oasis HLB µElution, and protein precipitation extraction protocols. The largest S/N ratio observed for the quantifier ion of testosterone was obtained using the Oasis MAX µElution Plate for the sample preparation (Figure 1).

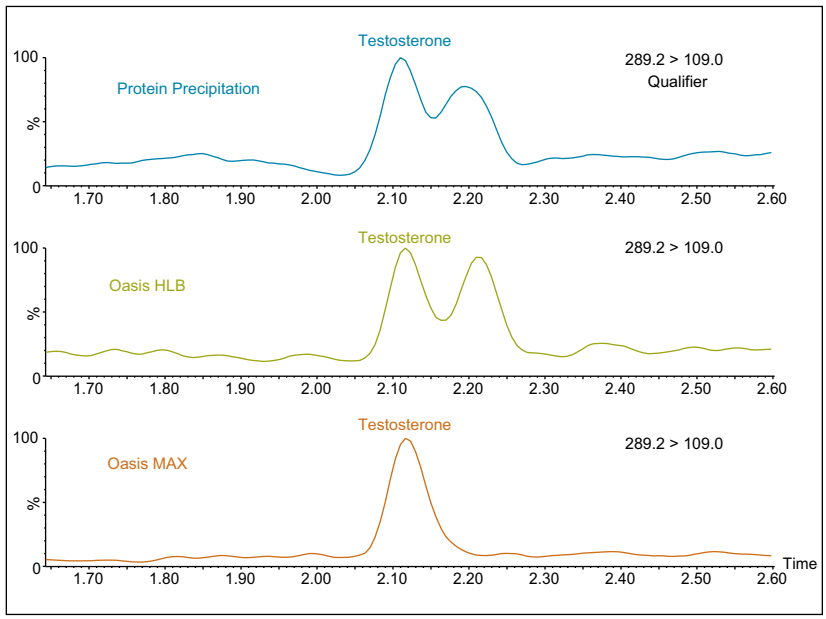

Figure 2. Qualifier MRM chromatograms for extracted serum testosterone samples using Oasis MAX µElution Plate, Oasis HLB µElution Plate, and protein precipitation.

The Oasis MAX µElution Plate also removes an interference clearly present in the qualifier trace of the same serum samples, as can be seen in Figure 2. The same interference is also evident in Figure 1. This selectivity improves the accuracy of the ion ratios observed between the quantifier and qualifier ions as well as peak integration reproducibility.

Over a calibration range of 0.17–51 nmol/L for testosterone the precision of the method was determined by extracting and quantifying three replicates of tri-level QC material on two occasions per day over five separate days (n = 30). The results of these experiments are seen in Table 1, where total precision and repeatability is ≤5.0% RSD.

	Total QC precision (RSD)			QC repeatability (RSD)		
Compound	Low	Mid	High	Low	Mid	High
Testosterone	5.0%	4.4%	4.3%	3.1%	3.0%	1.4%

Table 1. Total precision and repeatability for the analysis of testosterone in serum at low (0.51 nmol/L), mid (3.4 nmol/L), and high (34 nmol/L) concentration points.

SUMMARY

A LC-MS/MS method for the analysis of testosterone for clinical research has been developed. Sample preparation was performed using an Oasis MAX 96-well µElution Plate for the extraction of testosterone from serum. The benefits of this method include:

- Highly selective sample preparation, leading to a nearly 3 fold improvement in S/N relative to other evaluated techniques.
- Removal of interferences observed at low physiological concentrations of testosterone, which enables analytically sensitive analysis of testosterone in serum (LLOQ 0.17 nmol/L).
- Excellent reproducibility across the calibration range (≤5.0% RSD).

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1. Miller J et al. Pharmacokinetics and Relative Bioavailability of Absorbed Testosterone After Administration of a 1.62% Testosterone Gel to Different Application Sites in Men With Hypogonadism. *Endocr Pract*. 2011 Jul-Aug;17(4):574-583. doi: 10.4158/EP10192.OR.

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Analysis of Serum 17-Hydroxyprogesterone, Androstenedione, and Cortisol by UPLC-MS/MS for Clinical Research

Heather A Brown,[1] Claudia Rossi,[2] and Lisa J Calton[1]
[1]Waters Corporation, Wilmslow, UK
[2]Department of Biomedical Science, G. d'Annunzio University Foundation, Pescara, Italy

APPLICATION BENEFITS

- Single liquid-liquid extraction (LLE), without derivatization
- Analytical sensitivity for 17-hydroxyprogesterone, from limited-volume samples
- Chromatographic resolution of isobaric steroid intermediates

WATERS SOLUTIONS

ACQUITY UPLC® System

ACQUITY UPLC HSS T3 UPLC Column

ACQUITY UPLC HSS T3 VanGuard™ Pre-column

Xevo® TQ MS

TruView™ Maximum Recovery Vials

MassLynx® Software

TargetLynx™ Application Manager

KEY WORDS

17-hydroxyprogesterone, androstenedione, cortisol, deoxycortisol, corticosterone, adrenal steroid, UPLC-MS/MS

INTRODUCTION

Measurement of 17-hydroxyprogesterone (17-OHP) by immunoassay is prone to analytical interference arising from cross-reactivity of reagent antibodies with structurally-related steroid metabolites. A method for the extraction and UPLC®-MS/MS analysis of serum 17-OHP and two additional adrenal steroids, androstenedione (A4) and cortisol, using the ACQUITY UPLC System with the Xevo TQ MS (Figure 1) is described.

Figure 1. ACQUITY UPLC with Xevo TQ MS.

EXPERIMENTAL

Calibrators and quality-control material (QC) were prepared in double charcoal-stripped serum over the stated measurement range. Samples, 50 µL and 20 µL, of internal standard ($[^2H_8]$ 17-OHP, $[^2H_7]$ A4, and $[^2H_4]$ cortisol) were extracted into 1 mL of methyl-tert-butyl-ether. The evaporated organic layer (800 µL) was reconstituted in 50 µL 45% (v/v) methanol (aq).

LC conditions

System:	ACQUITY UPLC
Sample preparation vials:	TruView LCMS Certified Maximum Recovery Vial (p/n 186005662CV)
Column:	ACQUITY UPLC HSS T3, 1.8 µm, 2.1 x 50 mm (p/n 186003538) with an ACQUITY HSS T3, VanGuard Pre-column, 1.8 µm, 2.5 x 5 mm (p/n 186003976)
Column temp.:	60 °C
Sample temp.:	8 °C
Injection volume:	20 µL
Flow rate:	600 µL/min
Injection mode:	Partial loop, with needle overfill (PLNO); Load ahead feature enabled (5 min injection-to-injection)
Mobile phase A:	2 mmol/L ammonium acetate, 0.1 % (v/v) formic acid (aq)
Mobile phase B:	2 mmol/L ammonium acetate, 0.1 % (v/v) formic acid in methanol
Gradient (binary system):	Initial conditions of 45% mobile phase B were increased linearly to 47% over 1 minute and then, over an additional minute, to 57%. Thereafter, mobile phase B was again increased, this time to 98% over 0.5 minutes. The composition was then maintained at 98% B for 0.5 minutes before reverting to 45% B followed by column re-equilibration for 1.5 minutes. The overall run time was 4.5 minutes.

MS conditions

System:	Xevo TQ (0.7 FWHM on MS1 and 0.8 FWHM MS2)
Ionization mode:	Electrospray positive
Capillary voltage:	0.7 kV
Collision energy:	Analyte specific (see Table 1)
Cone voltage:	Analyte specific (see Table 1)

Data management

MassLynx Software v4.1 with TargetLynx Application Manager

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage	Collision energy	Dwell time (s)	MS function (time window, in min)
Androstenedione (quantifier)	287	97	30	20	0.02	1 (2.35–3.50)
Androstenedione*	287	109	30	20	0.02	
17-OHP (quantifier)	331	97	28	26	0.02	
17-OHP*	331	109	28	28	0.02	
Cortisol (quantifier)	363	121	26	23	0.20	2 (0.00-1.70)
Cortisol*	363	97	26	30	0.20	

Table 1. MS Parameters. Optimize precursor and product ions to 1 decimal place.

**Denotes optional, qualifier ion transition. MS function-time windows were optimized for the instrument's duty cycle. Verify chromatogram-peak retention time using a single-function MS method before establishing time-window settings for routine use.*

RESULTS AND DISCUSSION

The limit of detection (LOD) and lower limit of quantification (LLOQ) were determined as the lowest concentration of steroid spiked into charcoal-stripped serum to produce a signal-to-noise ratio (SNR) >3 or >8, respectively. The LLOQ was confirmed by demonstrating ≤15% imprecision and deviation from assigned values following replicate analysis (n =10), a percentage value that surpasses the imprecision targets of ≤20% CV prescribed by the United States Food and Drug Administration (USFDA) in its Guidance for Industry.[1] The LOD and LLOQ were 0.25 and 0.5 ng/mL, respectively, for all analytes. Mass (ng/mL) to nanomolar-unit conversion factors are x3.03 for 17-OHP, x2.76 for cortisol, and x3.49 for A4.

The correlation between analyte concentration and response ratio was linear from the LLOQ to 300, 500, and 200 ng/mL for 17-OHP, cortisol, and A4, respectively. The coefficient of determination (r^2) for all analytes was >0.997. Figure 2 shows a calibration curve for 17-OHP. Calculated concentrations of calibrators were ≤10% of assigned values, with the exception of the lowest concentration calibrator at the LLOQ, for which a deviation of ≤15% from the nominal value was accepted.

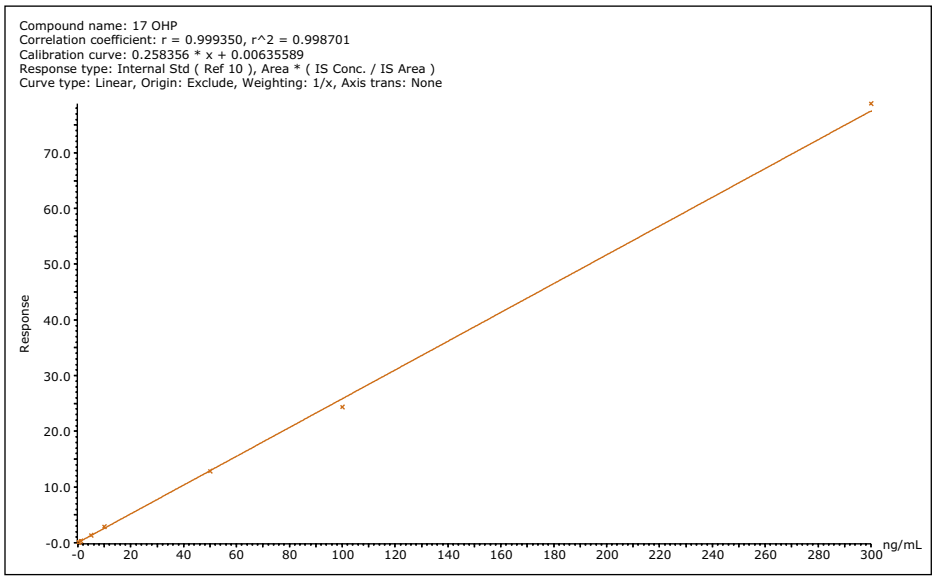

Figure 2. Double charcoal-stripped serum calibration curve for 17-OHP.

The within-batch imprecision was determined from replicate (n = 5) analysis of multiple levels of QC. The maximum between-batch imprecision was evaluated by replicate (n = 5) analysis of the QC over five consecutive days. The findings are summarized in Table 2. The determined concentrations were <7.5% of nominal values.

Analyte	QC (ng/mL)	CV (W) %	CV (B) %
17-OHP	2.5	6.3	7.9
	25.0	3.5	9.0
	75.0	2.8	6.6
	250.0	3.4	4.4
Cortisol	2.5	2.8	5.9
	25.0	4.3	6.8
	75.0	2.3	1.4
	250.0	1.0	2.1
A4	2.5	4.9	2.3
	35	2.1	6.1
	75	3.5	2.5

Table 2. Within (W) and Between (B) batch imprecision determined from replicate analysis of QC. QC is categorized by nominal/weighed in concentration.

Recovery was evaluated as the ratio of detector response in pre- to post-extraction-spiked serum samples. Recovery was calculated as >70% for all steroids. The magnitude of electrospray ionization suppression from residual sample interferences was estimated by calculation of the matrix factor for each analyte. The matrix factor was defined as the mean ratio of the peak-area response in presence to absence of residual sample matrix (n = 6, human whole serum). These studies indicated approximately 10% of the detector signal for cortisol and A4, with 50% of the signal for 17-OHP was suppressed by residual matrix ions. The use of stable isotope-labeled internal standards compensated for the observed matrix effects.

Further interference studies were conducted whereby serum was supplemented with a selection of adrenal steroid intermediates, extracted and analyzed by the UPLC-MS/MS method described herein. A subset of intermediates, isobaric for the MRM transition 347 > 121 were identified, namely: 11- and 21-deoxycortisol and corticosterone. Inspection of the total-ion chromatograms of the three isobars showed these steroids were resolved from one another (Figure 3). Interferences were not observed at or around the retention time of the compounds of interest in the 347 > 121 total-ion chromatograms, confirming the analytical selectivity for the detection of 17-OHP, cortisol, and A4 with respect to the subset of steroid intermediates described herein.

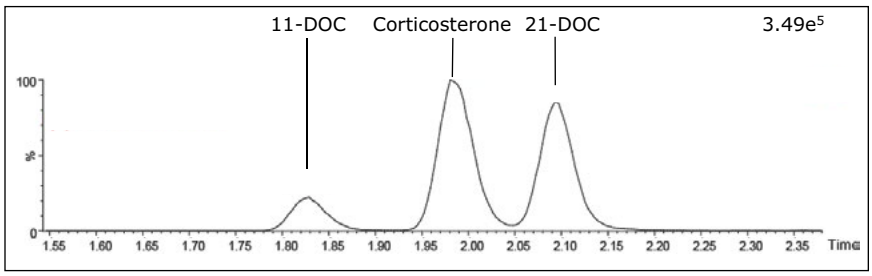

Figure 3. Total-ion chromatogram of 11-deoxycortisol (11-DOC), corticosterone, and 21-deoxycortisol (21-DOC) in a 10 ng/mL aqueous standard. For reference, the retention times of cortisol, A4, and 17-OHP were 1.51, 2.57, and 2.70 minutes, respectively.

CONCLUSIONS

A rapid method for quantification of 17-OHP, cortisol, and A4 was developed using UPLC-MS/MS. Using MRM qualifier and quantifier ion transition ratios allows analytical sensitivity with added confidence in compound identity when clinical research requires a high level of analytical selectivity. The research method demonstrates good linearity, analytical sensitivity, and precision. The selectivity of the UPLC method enables chromatographic resolution of isobars for added confidence in the measured result.

References

1. Guidance for Industry for Bioanalytical Method Validation. (2001). U.S. Department of Health and Human Services, Food and Drug Administration.

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UPLC-MS/MS Analysis of Aldosterone in Plasma for Clinical Research

Dominic Foley and Lisa Calton
Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

- Analytical selectivity improves reproducibility through removal of interferences
- Analytical sensitivity enables the quantification of low physiological levels of aldosterone
- Facilitates high sample throughput utilizing automation of the multiwell plate format

WATERS SOLUTIONS

ACQUITY UPLC® I-Class System

Oasis® MAX µElution Plate

CORTECS® UPLC® C_{18} Column

Xevo® TQ-S Mass Spectrometer

MassLynx® Software

KEY WORDS

Aldosterone, SPE, UPLC-MS/MS

INTRODUCTION

Aldosterone is a mineralocorticoid steroid hormone that plays a central role in the regulation of blood pressure. Traditionally, aldosterone has been analysed by radioimmunoassays. However, these methods involve the use of hazardous radioisotopes. In addition, they can suffer from a lack of specificity due to the cross reactivity of structurally similar steroid hormones and metabolites, which may result in greater imprecision and inaccuracy. To minimize specificity issues, radioimmunoassay methods employ time-consuming manual extraction protocols. UPLC-MS/MS combined with automation of the sample preparation with sample tracking capabilities provides an alternative means of aldosterone analysis for clinical research. An integrated workflow solution enables selective and analytically sensitive characterisation of aldosterone with a reduction in sample handling time.

In this application note we describe a clinical research method utilizing an Oasis MAX µElution Plate for the extraction of aldosterone from plasma, which has been automated on the Tecan Freedom Evo 100/4 Liquid Handler. Chromatographic separation was performed on an ACQUITY UPLC I-Class System using a CORTECS UPLC C_{18} Column followed by detection on a Xevo TQ-S Tandem Quadrupole Mass Spectrometer (Figure 1).

Figure 1. The Waters ACQUITY UPLC I-Class System and Xevo TQ-S Mass Spectrometer.

EXPERIMENTAL

UPLC conditions

System:	ACQUITY UPLC I-Class
Needle:	30 µL
Column:	CORTECS UPLC $C_{18,}$ 2.1 x 100 mm, 1.6 µm (Waters p/n 186007095)
Mobile phase A:	Water
Mobile phase B:	Methanol
Needle wash solvent:	80% methanol$_{(aq)}$
Purge solvent:	40% methanol$_{(aq)}$
Column temp.:	45°C
Injection volume:	25 µL
Flow rate:	0.40 mL/min
Gradient:	See Table 1
Run time:	4.0 minutes

MS conditions

System:	Xevo TQ-S
Resolution:	MS1 (0.7 FWHM)
	MS2 (0.5 FWHM)
Acquistion mode:	Multiple reaction monitoring (MRM) (see Table 2 for details)
Polarity:	ESI negative
Capillary:	2.40 kV
Source temp.:	150 °C
Desolvation temp.:	600 °C
Dwell time:	0.1 seconds
Inter-scan delay:	0.01 seconds
Inter-channel delay:	0.02 seconds

Data management

MassLynx® v4.1 with TargetLynx™ Application Manager

Sample preparation

Aldosterone certified reference solution and its stable labeled internal standard ($^4H_2$) were purchased from Cerilliant (Round rock, TX) and IsoSciences (King of Prussia, PA), respectively. Calibrators were prepared in a surrogate matrix of MSG4000 stripped human serum purchased from Golden West Biologicals (Temecula, CA). The calibration range for aldosterone was 42-4161 pmol/L (15-1500 pg/mL). QC materials were prepared using pooled plasma purchased from SeraLab (Haywards Heath, UK) at 99, 500 and 2000 pmol/L (36, 180 and 720 pg/mL).

To convert SI units (pmol/L) to conventional mass units (pg/mL) divide by 2.774.

Sample extraction

Extraction was performed using a Tecan Freedom Evo 100/4 Liquid Handler. To 200 µL of sample; 25 µL of 4000 pmol/L internal standard, 200 µL 0.1 M zinc sulphate in 50% methanol and, 450 µL of 0.05% (v/v) phosphoric acid were added. The samples were mixed between each reagent addition. The samples were centrifuged for 5 minutes at 1000g.

The Oasis MAX µElution Plate (Waters p/n 186001829) was conditioned and equilibrated with 200 µL methanol and water, respectively. An aliquot of each of the pretreated samples (625 µL) was loaded into individual wells of the plate and slowly pulled through at low vacuum. Consecutive washes with 200 µL of 0.05% (v/v) phosphoric acid, 200 µL 0.1% ammonia in 10% methanol, and 200 µL water were performed. Aldosterone was eluted using 50 µL of 70% methanol$_{(aq)}$, followed by 40 µL water.

Method conditions

On instrument setup ensure the mobile phase lines are purged for a minimum of 10 minutes to remove additives, which could negatively impact sensitivity for aldosterone.

Time (min)	Flow Rate (ml/min)	%A	%B	Curve
Initial	0.400	60	40	Initial
1	0.400	60	40	6
2	0.400	40	60	6
2.3	0.400	5	95	11
2.8	0.400	60	40	11

Table 1. Gradient table for the separation of aldosterone. Operating backpressure at the initial conditions is 12000 psi.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage	Collision energy
Aldosterone (Quan)	359.2	189.2	55	18
Aldosterone (Qual)	359.2	297.2	55	16
Aldosterone-$^{4}H_2$	363.2	190.2	55	18

Table 2. MRM parameters for both aldosterone quantifier and qualifier and its internal standard, aldosterone-$^{4}H_2$.

RESULTS

No interferences were observed at the retention time of aldosterone when nine structurally related compounds were examined (18-hydroxycorticosterone, cortisol, cortisone, 11-deoxycorticosterone, corticosterone, 11-deoxycortisol, 21-deoxycortisol, prednisolone, and prednisone). In Figure 2, the chromatographic selectivity of the column is demonstrated through the baseline resolution of aldosterone and its metabolic precursor, 18-hydroxycorticosterone. Separation of 18-hydroxycorticosterone is necessary due to the detection of its isotopic transition in the aldosterone-$^{4}H_2$ internal standard MRM trace, when present in the sample at 60 nmol/L. In addition, separation of prednisone was required due to ion suppression of aldosterone at prednisone concentrations of 1 µmol/L. This baseline resolution allows for the independent quantification of aldosterone.

Analytical sensitivity investigations reveal that the analytical sensitivity of this method would allow precise quantification (<20% RSD) at 42 pmol/L for aldosterone. Signal:noise (S/N) of the 42 pmol/L calibration standard was >10:1 on ten separate occasions.

Total precision was determined by extracting and quantifying three replicates of tri-level QC material on two occasions per day over five separate days (n = 30). Repeatability was assessed by analyzing three replicates at each QC level. The results of these experiments are shown in Table 3, where total precision and repeatability at the low (99 pmol/L), mid (500 pmol/L), and high (2000 pmol/L) concentrations is ≤9.8% RSD for aldosterone.

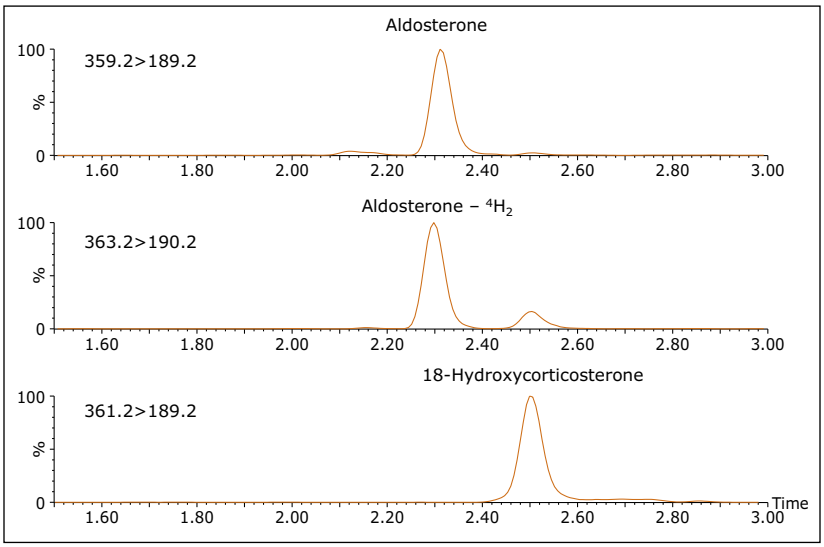

Figure 2. UPLC separation of aldosterone, aldosterone-$^{4}H_2$, and 18-hydroxycorticosterone using a CORTECS UPLC C_{18} Column.

	Total QC precision (RSD)			QC repeatability (RSD)		
Compound	Low	Mid	High	Low	Mid	High
Aldosterone	9.8%	7.1%	4.8%	8.2%	7.0%	3.9%

Table 3. Total precision and repeatability for the analysis of aldosterone.

The method was shown to be linear over the range of 42–4161 pmol/L when different ratios of high and low concentration pools of aldosterone were combined and analysed. In addition, calibration lines in spiked serum were linear with coefficient of determinations (r^2) >0.996 on 10 separate occasions.

No system carryover was observed from high concentration samples at 10400 pmol/L into subsequent blank injections. A 1:2 dilution was successfully performed on an over-range sample with a concentration of 7606 pmol/L, providing a mean accuracy of 99% with an RSD of 4.4%.

Matrix effects were evaluated as the peak area of extracted post spiked aldosterone plasma samples taken as a percentage of extraction solvent samples spiked to equivalent concentrations. Mean (range) matrix effects were 1.10 (1.03–1.20) with RSD of 5.9% for aldosterone. Calculations using analyte:internal standard response ratio indicated compensation for signal enhancement by the internal standard, providing a mean (range) net matrix effect of 1.00 (0.90–1.10) with RSD of 7.7% for aldosterone.

Samples were selected (n = 59) for comparison against an independently developed LC-MS/MS method for aldosterone. A selection of samples from this comparison having quantified values <100 pmol/L are shown in Figure 3. These chromatograms demonstrate the selectivity of the extraction protocol and the sensitivity of the analytical method.

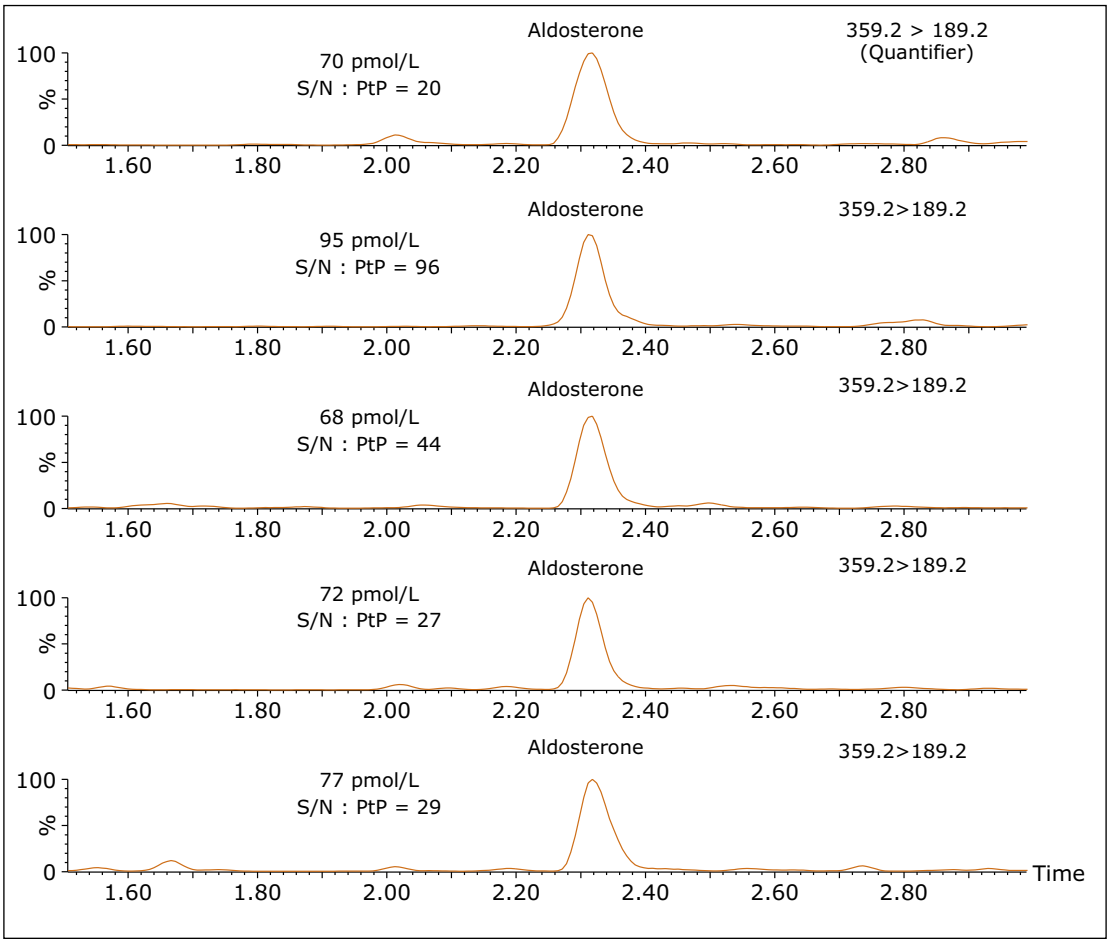

Figure 3. Five representative chromatograms from plasma samples having quantified aldosterone values <100 pmol/L. The signal-to-noise ratio was calculated on the raw data using peak to peak at ± 2 SD.

Comparison data were processed using Analyse-it Software v2.3. The comparison between the two independent LC-MS/MS methods yielded a Deming regression of y = 1.07x - 22.94 (Figure 4A), which showed no significant proportional or constant bias (p>0.05). Altman-Bland agreement between the methods demonstrates a mean negative bias of -4.9% (95% CI -10.3 to 0.5%) (Figure 4B) for the UPLC-MS/MS method described herein.

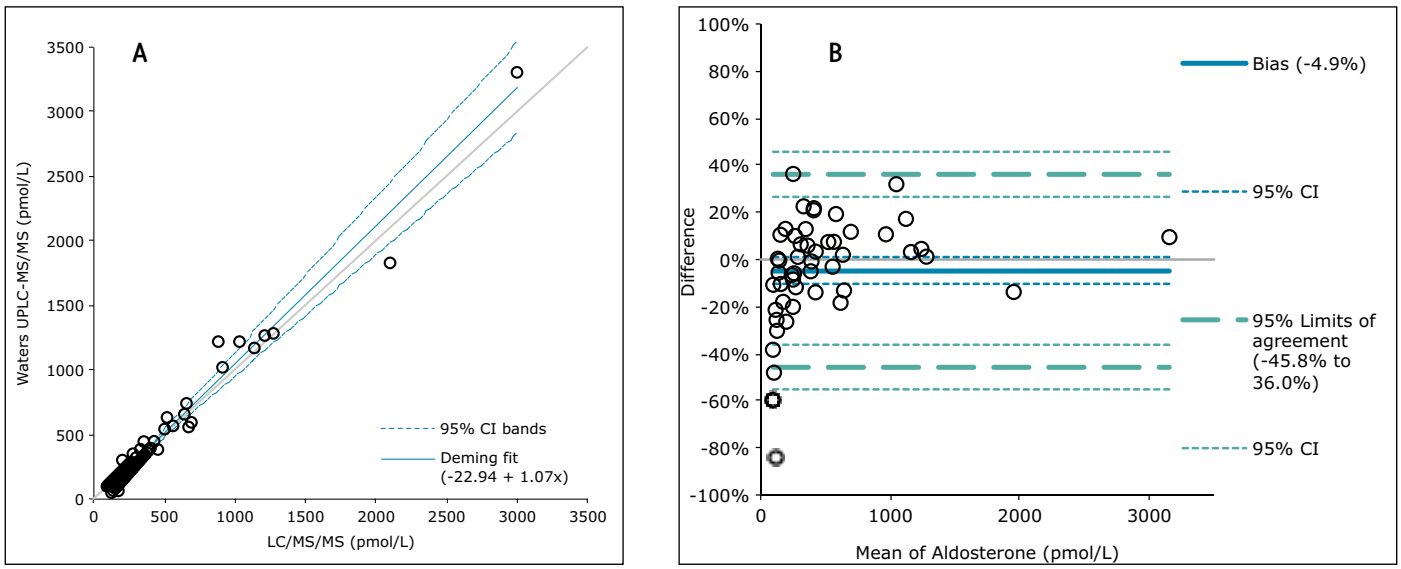

Figure 4. A) Deming regression comparing the Waters UPLC-MS/MS method to another LC-MS/MS method for aldosterone analysis, B) Altman-Bland agreement showing the % difference between the Waters UPLC-MS/MS method and another LC-MS/MS method for aldosterone analysis.

CONCLUSIONS

An analytically sensitive and selective clinical research method has been developed for the analysis of plasma aldosterone.

Using only 200 µL sample volume, this analytical method provides sufficient analytical sensitivity to analyze low physiological levels of aldosterone (42 pmol/L). Selective chromatography and SPE using anion exchange to remove interferences provides clean chromatograms, which enables accurate and precise quantification of aldosterone across the concentration range. Automation of the analytical method in combination with sample tracking capabilities improves laboratory workflow and reduces sample handling, which alleviates the potential for operator error.

Acknowledgement

Professor Brian Keevil and his colleagues at the Department of Clinical Biochemistry, University Hospital of South Manchester, Wythenshawe, UK, are thanked for the provision of anonymized plasma samples for the analysis.

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[TECHNOLOGY BRIEF]

Analysis of Serum Adrenal Steroids Using the ACQUITY UPLC I-Class with Xevo TQD for Clinical Research

Heather A. Brown, Stephen Balloch, and Lisa J. Calton
Waters Corporation, Wilmslow, UK

GOAL

To demonstrate the analytically sensitive and precise measurement of adrenal steroids from limited-volume clinical research serum samples. To evaluate the Xevo® TQD with the ACQUITY UPLC® I-Class System (FTN) (Figure 1) as a solution for what is considered a challenging application.

BACKGROUND

Precise measurement of low concentrations of steroids in limited-volume complex matrices such as serum or plasma poses an analytical challenge which previously called for a high performance mass detector such as the Xevo TQ-MS. This work evaluates the utility of combining improved chromatographic resolution, column efficiency, and reproducibility afforded by the ACQUITY UPLC I-Class System (FTN) with the entry-level Xevo TQD.

THE SOLUTION

Analytes were extracted from limited-volume serum samples using a non-derivatized, single liquid-liquid-extraction procedure. Analytically sensitive quantification was achieved by the superior resolution of the ACQUITY UPLC I-Class System (FTN) in combination with the robust simplicity of the Xevo TQD.

Deuterated internal standard-spiked serum (50 µL) was mixed with methyl-tert-butyl-ether (1 mL). Steroids were extracted into the organic

The challenge of serum 17-hydroxyprogesterone, cortisol, and androstenedione measurement is made routine with UPLC-MS/MS for clinical research.

Figure 1. Waters ACQUITY UPLC I-Class System with Xevo TQD Mass Spectrometer.

Analyte	Precursor ion (*m/z*)	Qualifier (quantifier) product ion (*m/z*)	Cone voltage (V)	Collision energy (V)
A4	287.1	97.1 (109.1)	46	22
Cortisol	363.1	121.1 (121.1)	42	24
17-OHP	331.1	97.1 (109.1)	44	23

Table 1. Instrument settings for MRM analysis. Optimize m/z to 1 decimal place.

layer which was transferred to a maximum recovery vial, evaporated to dryness and reconstituted in 50 µL of 45% (v/v) aqueous methanol. Chromatographic separation of the injected 20-µL sample was achieved using an ACQUITY UPLC HSS T3, 2.1 x 50 mm Column (p/n 186003538) fitted with a chemistry-matched VanGuard™ 2.1 x 5 mm Pre-column (p/n 186003976). A three-stage gradient separation over 2.5 minutes enabled the resolution of the compounds of interest, in addition to a number of isobaric steroid intermediates. The injection-injection time was 5.0 minutes to permit adequate LC column washing and re-equilibration.

The performance of the ACQUITY UPLC I-Class System (FTN) with Xevo TQD was evaluated in comparison with the ACQUITY UPLC System with Xevo TQ-MS. Analysis of multiple levels of in-house prepared stripped-serum quality controls (QC) highlighted the superior precision achievable with the ACQUITY UPLC I-Class (FTN) with Xevo TQD (n = 5, over 5 occasions, 2.5 – 75 ng/mL for A4 and to 250 ng/mL for 17-OHP and cortisol). Calculation of 17-OHP internal standard chromatogram peak widths using TargetLynx™ Application Manager processing confirmed the improved reproducibility of peak widths using the ACQUITY UPLC I-Class (FTN). Example overlaid chromatograms of 17-OHP internal standard from extracted QCs are shown in Figure 2.

Replicate analyses of stripped-serum calibrators enabled like-for-like comparison of the analytical sensitivity of the two systems. The peak-to-peak signal-to-noise ratio (S/N) of calibrator 1 (0.5 ng/mL) was comparable between the two systems for 17-OHP. Further method optimization is required to match the analytical sensitivity of the two systems for cortisol and A4 (Table 2).

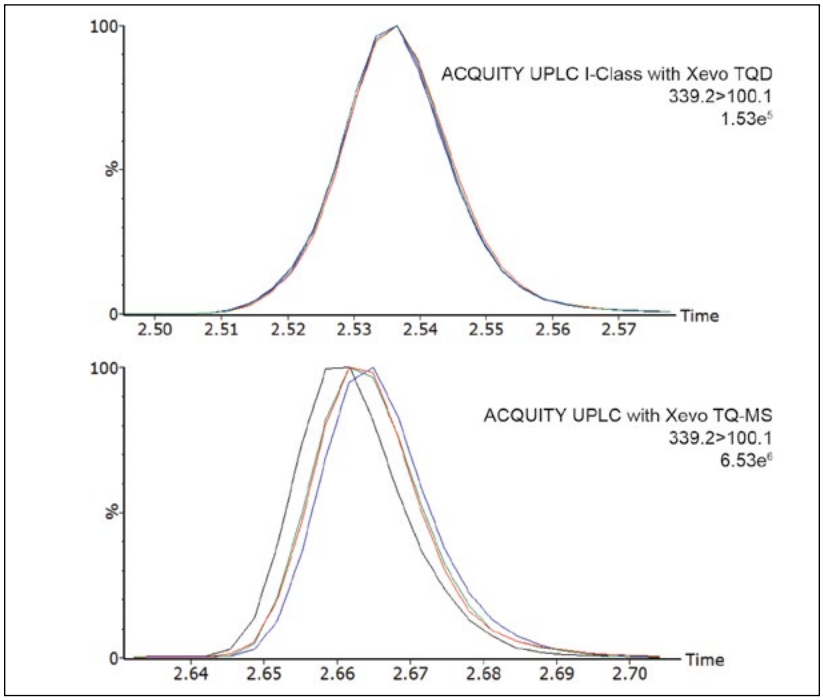

Figure 2. Superior peak width and retention time reproducibility is observed with the ACQUITY UPLC I-Class (FTN) with Xevo TQD compared with the ACQUITY UPLC with Xevo TQ-MS. Peak width imprecision was improved on the ACQUITY UPLC I-Class (FTN) compared with the ACQUITY UPLC (0.78 versus 2.14 % CV).

System	Analyte	Maximum imprecision (% CV)	Cal 1 (0.5 ng/mL)	
			Peak area	S/N
ACQUITY UPLC I-Class/ Xevo TQD	A4	5.6	7002	65
	Cortisol	4.4	350	36
	17-OHP	5.3	2118	31
ACQUITY UPLC/ Xevo TQ-MS	A4	6.1	9864	409
	Cortisol	6.8	1008	148
	17-OHP	9.1	4178	35

Table 2. Summary method characteristics comparing two Waters LC-MS/MS systems. Peak-to-peak signal to noise ratio (S/N) was calculated using MassLynx® Software with TagetLynx Application Manager ignoring zeros and the worst 5% of scans. Mass to molar conversion units for A4, cortisol, and 17-OHP are x 3.49, 2.76, and 3.03, respectively.

SUMMARY

The data presented indicates the ACQUITY UPLC I-Class (FTN) with Xevo TQD is capable of meeting the analytical precision necessary for measurement of physiologically-relevant concentrations of serum 17-OHP for clinical research.

The narrow diameter tubing of the ACQUITY UPLC I-Class System creates a low-dispersion system allowing for greater LC peak capacity and precision than achieved before with the ACQUITY UPLC System (Figure 2). The inclusion of active pre-column heating of mobile phase brings further advantages in terms of column efficiency, improving reproducibility of peak area integration. Precision at low concentrations contributes to overall analytical sensitivity (Table 2). With further optimization of LC methods to improve detection of A4, this configuration can be used in laboratories with low to moderate detector sensitivity needs.

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A Clinical Research Method for the Analysis of Serum Testosterone and Androstenedione

Dominic Foley and Lisa Calton
Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

- Analytical selectivity improves reproducibility through removal of interferences
- Analytical sensitivity enables the quantification of low physiological levels of testosterone and androstenedione
- Facilitates high sample throughput utilizing automation of the multi-well plate format

WATERS SOLUTIONS

ACQUITY UPLC® I-Class

Oasis® MAX µElution Plates

ACQUITY® HSS SB C_{18} Column

Xevo® TQD Mass Spectrometer

MassLynx® Software

TargetLynx™ Application Manager

KEY WORDS

Testosterone, androstenedione
SPE, UPLC-MS/MS

INTRODUCTION

Testosterone and its precursor, androstenedione, are androgenic steroid hormones that are involved in the development and maintenance of sexual characteristics. Analysis of these structurally similar steroid hormones using UPLC®-MS/MS provides three levels of selectivity: 1) sample preparation, 2) liquid chromatography, and 3) mass spectrometric detection using multiple reaction monitoring (MRM). LC-MS/MS, while analytically sensitive and selective, has been reported to suffer from a lack of agreement between laboratories when methods are independently developed using different calibration materials and employing manual extraction techniques that may introduce operator variability. The availability of a voluntary Hormone Standardization (HoSt) Program, co-ordinated by the United States Centers for Disease Control and Prevention (CDC), has begun to help address these harmonization issues.

Here we describe a clinical research method utilizing Oasis MAX µElution Plate Technology for the extraction of testosterone and androstenedione from serum, which has been automated on the Tecan Freedom Evo 100/4 Liquid Handler. Chromatographic separation was performed on an ACQUITY UPLC I-Class System using an ACQUITY UPLC HSS SB C_{18} Column followed by detection on a Xevo TQD Tandem Quadrupole Mass Spectrometer (Figure 1), utilizing MassLynx Software v4.1 with TargetLynx Application Manager. In addition, the method has employed CDC HoSt testosterone samples to evaluate the accuracy and therefore suitability of the method for analysing testosterone for clinical research.

Figure 1. Waters ACQUITY UPLC I-Class System and Xevo TQD Mass Spectrometer.

EXPERIMENTAL

UPLC conditions

System:	ACQUITY UPLC I-Class
Needle:	30 µL
Column:	ACQUITY UPLC HSS SB C_{18}, 2.1 x 50 mm, 1.8 µm (p/n 186003532)
Mobile phase A:	water with 2mM NH_4Ac + 0.1% formic acid
Mobile phase B:	methanol with 2mM NH_4Ac + 0.1% formic acid
Needle wash solvent:	80% methanol + 0.1% formic acid
Purge solvent:	50% methanol + 0.1% formic acid
Column temp:	60°C
Injection vol.:	20 µL
Flow rate:	0.60 mL/min
Gradient:	See Table 1
Run time:	3.3 minutes

MS conditions

System:	Xevo TQD
Resolution:	MS1 and MS2 (0.7 FWHM)
Acquistion mode:	Multiple Reaction Monitoring (MRM) (see Table 2 for details)
Polarity:	ESI positive
Capillary:	0.50 kV
Source Temp:	140 °C
Desolvation Temp:	450 °C
Dwell Time:	0.05 seconds
Inter-scan delay:	0.01 seconds
Inter-channel delay:	0.02 seconds

Data management

MassLynx Software v4.1 with TargetLynx Application Manager

Sample preparation

Testosterone, androstenedione, and their $^{13}C_3$ labeled internal standards were purchased from Cerilliant (Round Rock, TX). MSG4000 stripped human serum was purchased from Golden West Biologicals (Temecula, CA). Using these materials, calibrators were prepared over the range of 0.17 – 52 nmol/L (0.05 – 15 ng/mL), with quality controls (QCs) at 0.52 nmol/L, 3.5 nmol/L, and 35 nmol/L (0.15, 1, and 10 ng/mL) for both testosterone and androstenedione.*

** To convert SI units (nmol/L) to conventional mass units (ng/mL) divide by 3.470 for testosterone and 3.494 for androstenedione.*

Sample extraction

Extraction was performed using a Tecan Freedom Evo 100/4 Liquid Handler. To 200 µL of sample; 25 µL of internal standard, 40 µL of 2% ammonia (aq), 150 µL 0.2M zinc sulphate, and 250 µL of methanol were added. 300 µL of water was added prior to centrifugation for 5 minutes at 1000 g. The samples were mixed after the addition of each reagent.

The Oasis MAX µElution SPE Plate (p/n 186001829) was conditioned and equilibrated with 200 µL methanol and water, respectively. An aliquot of each of the pretreated samples (600 µL) was loaded into individual wells of the plate and slowly pulled through at low vacuum. The plate was washed with 200 µL of 0.1% ammonia in 20% methanol and dried. The analytes were eluted using 2 x 25 µL methanol, followed by 50 µL water.

Method conditions

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.600	50	50	Initial
1	0.600	50	50	6
2.25	0.600	40	60	6
2.26	0.600	5	95	6
2.8	0.600	50	50	11

Table 1. Gradient table for the separation of testosterone and androstenedione. Operating backpressure at the initial conditions is 6500 psi.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage	Collision energy
Testosterone (quan)	289.2	97.0	38	25
Testosterone (qual)	289.2	109.0	38	28
Testosterone –$^{13}C_3$	292.2	100.0	38	25
Androstenedione (quan)	287.2	97.0	38	25
Androstenedione (qual)	287.2	109.0	38	28
Androstenedione –$^{13}C_3$	290.2	100.0	38	25

Table 2. MRM parameters for testosterone, androstenedione, and their internal standards.

RESULTS

No interferences were observed at the retention time of both testosterone and androstenedione when eight structurally related compounds were examined (17-hydroxyprogesterone, epitestosterone, dihydrotestosterone, DHEA, DHEAS, 11-deoxycorticosterone, corticosterone, and 21-deoxycortisol). The chromatographic selectivity of the column is demonstrated through the baseline resolution of testosterone and its epimer; epitestosterone (Figure 2). Separation of androstenedione, 17-hydroxyprogesterone, and DHEA from testosterone is necessary because these analytes or their isotopes produce signals in the testosterone MRM trace at concentrations of 1 µmol/L.

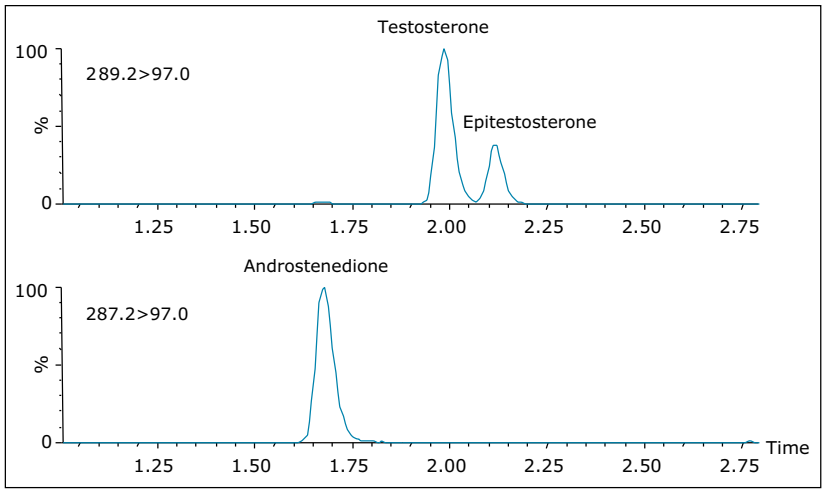

Figure 2. UPLC separation of testosterone, epitestosterone, and androstenedione using an AQCUITY UPLC HSS SB C_{18} Column.

No system carryover was observed in blank injections following measurement of high concentration samples (52 nmol/L) for both analytes. A 1:4 dilution was successfully performed on an over-range sample (102 nmol/L) with a mean accuracy of 98% (2.0% RSD) and 100% (3.1% RSD), for testosterone and androstenedione, respectively.

The method was shown to be linear over the range of 0.17 - 52 nmol/L when different ratios of high and low concentration pools of testosterone and androstenedione were combined and analysed. Calibration lines in spiked serum were linear with coefficient of determinations (r^2) >0.994 over 10 separate occasions.

Analytical sensitivity investigations revealed that this method would allow precise quantification (<20% RSD) at 0.085 nmol/L for both testosterone and androstenedione. The lowest calibrator was established at 0.17 nmol/L. At this concentration the signal:noise (S/N) was consistently greater than 10:1, while maintaining <20% precision performance for both analytes.

Total precision was determined by extracting and quantifying three replicates of tri-level QC material on two occasions per day over five separate days (n=30). Repeatability was assessed by analyzing three replicates at each QC level. The results of these experiments are seen in Table 3, where total precision and repeatability at the low (0.52 nmol/L), mid (3.5 nmol/L), and high (35 nmol/L) concentrations is ≤4.0% RSD for testosterone and ≤4.7% RSD for androstenedione.

	Total QC precision (RSD)			QC repeatability (RSD)		
Compound	Low	Mid	High	Low	Mid	High
Testosterone	4.0%	2.8%	4.0%	3.1%	2.6%	4.0%
Androstenedione	4.0%	2.7%	4.7%	3.5%	2.7%	4.7%

Table 3. Total precision and repeatability for the analysis of testosterone and androstenedione in serum.

Matrix effects were evaluated as the peak area of endogenous testosterone and androstenedione samples taken as a percentage of extraction solvent samples spiked to equivalent concentrations. Mean (range) matrix effects were 0.67 (0.55–0.78) for testosterone and 0.77 (0.69–0.86) for androstenedione. Calculations using analyte:internal standard response ratio indicated compensation for signal suppression by the internal standard, providing a mean (range) net matrix effect of 0.99 (0.88–1.18) for testosterone and 1.03 (0.89–1.22) for androstenedione.

Phase I Hormone Standardization (HoSt) samples (CDC, Atlanta, GA) were used to evaluate the method accuracy for testosterone. Samples (n=40) were analysed in duplicate on two occasions over five separate days. Excellent correlation between the calculated values and assigned values for the CDC HoSt samples was demonstrated with a coefficient of determination (r^2) of 0.999 (Figure 3). Phase 1 evaluation from the CDC demonstrated a 3.3% mean bias for this method which is within their ±6.4% bias acceptance limit.

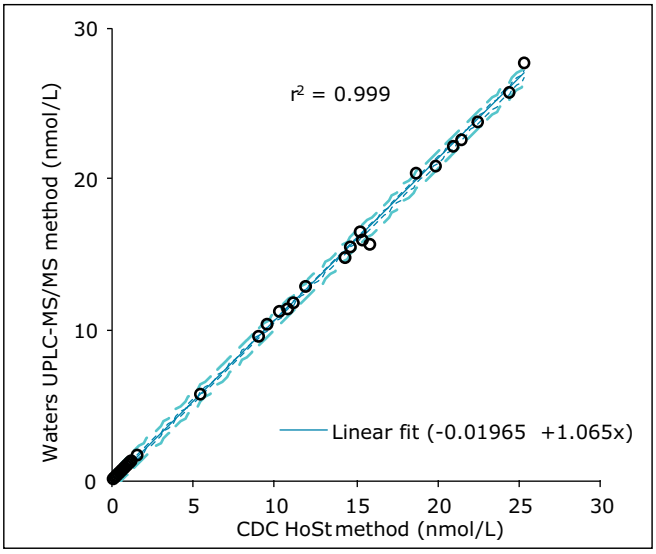

Figure 3. A simple linear regression of the UPLC-MS/MS method and CDC reference values for testosterone HoSt samples.

A set of anonymized serum samples were selected (n=35) for comparison against an independently developed LC-MS/MS method for testosterone and androstenedione. Comparison data were processed using Analyse-it Software v2.3. The comparison between the two independent LC-MS/MS methods yielded a Deming regression of y = 1.07x + 0.04 for testosterone (Figure 4A), showing statistically significant proportional bias but no significant constant bias, and y = 0.96x + 0.08 for androstenedione (Figure 4B), showing no significant proportional or constant bias.

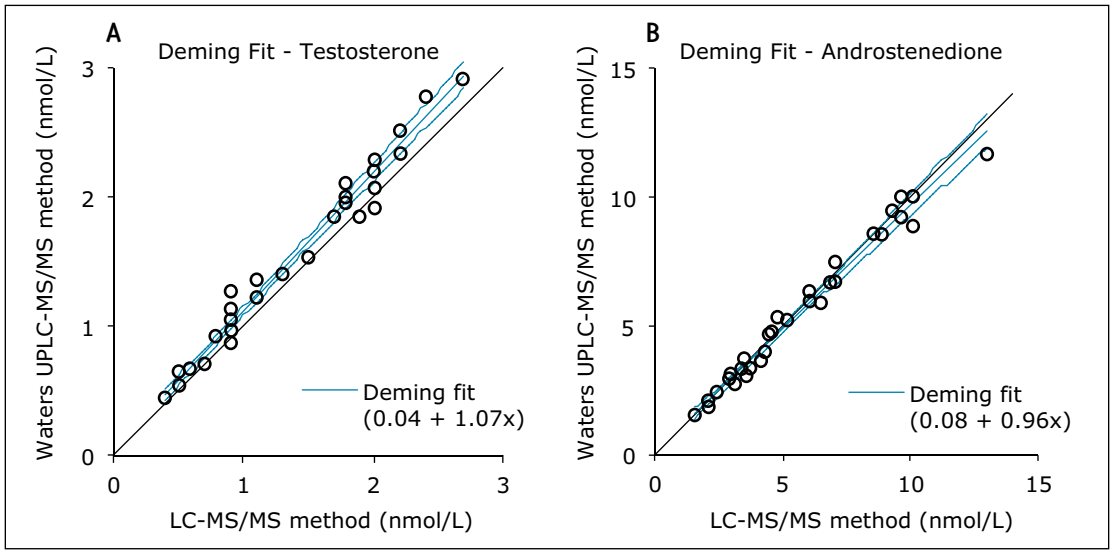

Figure 4. Deming regression of testosterone (A) and androstenedione (B) comparing the Waters UPLC-MS/MS method to a liquid-liquid extraction LC-MS/MS method.

Using the same samples, a comparison was performed between this automated method and the same method but using manual SPE sample preparation for the analysis of testosterone and androstenedione. Comparison of the sample set yielded a Deming regression of y = 1.01x + 0.01 for testosterone, showing no significant proportional bias or constant bias, and y = 0.97x + 0.17 for androstenedione, showing no significant proportional or constant bias. This indicates equivalency of the manual and automated extraction methods, which allows for flexibility in sample preparation options for the LC-MS/MS analysis of both analytes.

CONCLUSION

An analytically sensitive and selective clinical research method has been developed for the analysis of serum testosterone and androstenedione.

Excellent method precision was achieved through the use of analytically sensitive and selective sample preparation, chromatography, and MRM mass spectrometry on the ACQUITY UPLC I-Class UPLC System and Xevo TQD. In addition, method accuracy for the analysis of testosterone has been demonstrated through evaluation of CDC HoSt samples.

The sample preparation has been automated using the Tecan Freedom Evo 100/4 Liquid Handler, significantly reducing sample handling time and improving laboratory efficiency with sample tracking capabilities. Both a manual and the automated extraction methods have been shown to be equivalent, providing the user with flexibility in sample preparation options.

Acknowledgement

Professor Brian Keevil and his colleagues at the Department of Clinical Biochemistry, University Hospital of South Manchester, Wythenshawe, UK, are thanked for the provision of anonymized plasma samples for this analysis.

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Analysis of Testosterone, Androstenedione, and Dehydroepiandrosterone Sulfate in Serum for Clinical Research

Dominic Foley, Michelle Wills, and Lisa Calton
Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

- Analytical sensitivity enables the quantification of low physiological levels of the steroids
- Analytical selectivity improves reproducibility through removal of interferences
- LC-MS/MS enables high sample-throughput when utilizing multi-well plate automation

WATERS SOLUTIONS

Oasis® PRiME HLB µElution Plate

ACQUITY UPLC® HSS T3 Column

ACQUITY UPLC HSS T3 VanGuard™ Pre-column

ACQUITY UPLC I-Class System (FTN)

Xevo® TQD

MassLynx® Software

TargetLynx™ Application Manager

KEY WORDS

Testosterone, androstenedione, DHEAS, SPE, LC-MS/MS

INTRODUCTION

Testosterone, androstenedione, and dehydroepiandrosterone sulfate (DHEAS) are androgenic steroid hormones that are involved in the regulation of sexual characteristics. Analysis of these structurally similar steroid hormones by LC-MS/MS provides three levels of selectivity; sample preparation, liquid chromatography, and detection by multiple reaction monitoring (MRM) mass spectrometry. A selective sample preparation method has previously been developed which demonstrates excellent analytical sensitivity for testosterone and androstenedione using the Oasis MAX µElution SPE Plates.[1] However, with the inclusion of DHEAS into the analysis, this SPE is unsuitable, as it irreversibly binds DHEAS through an anion exchange mechanism. Therefore, a more suitable sample preparation protocol for this panel of steroids is required.

Here we describe a clinical research method utilizing Oasis PRiME HLB µElution Plate technology for the extraction of testosterone, androstenedione, and DHEAS from serum, which has been automated on a Tecan Freedom Evo 100/4 Liquid Handler. Chromatographic separation was performed on an ACQUITY UPLC I-Class System using an ACQUITY UPLC HSS T3 VanGuard Pre-column and ACQUITY UPLC HSS T3 Column, followed by detection on a Xevo TQD Mass Spectrometer (Figure 1). In addition, we have evaluated External Quality Assessment (EQA) samples for testosterone, androstenedione, and DHEAS to evaluate the bias and therefore suitability of the method for analyzing testosterone, androstenedione, and DHEAS for clinical research.

Figure 1. The Waters ACQUITY UPLC I-Class and Xevo TQD.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC I-Class (FTN)
Needle:	30 µL
Column:	ACQUITY UPLC HSS T3 2.1 x 50 mm, 1.8 µm (Waters P/N 186003538)
Pre-column:	ACQUITY UPLC HSS T3 VanGuard 2.1 x 5 mm 1.8 µm (Waters P/N 186003976)
Mobile phase A:	Water with 2 mM ammonium acetate + 0.1% formic acid
Mobile phase B:	Methanol with 2 mM ammonium acetate + 0.1% formic acid
Needle wash solvent:	80% methanol(aq)
Purge solvent:	40% methanol(aq)
Column temp.:	50 °C
Injection volume:	15 µL
Flow rate:	0.60 mL/min
Gradient:	See Table 1
Run time:	4.7 minutes

MS conditions

System:	Xevo TQD
Resolution:	MS1 (0.75 FWHM) MS2 (0.75 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (see Table 2 for details)
Polarity:	ESI positive/negative
Capillary:	0.4 kV
Source temp.:	150 °C
Desolvation temp.:	450 °C
Inter-scan delay:	0.01 seconds
Inter-channel delay:	0.02 seconds

Data management

MassLynx v4.1 Software with TargetLynx Application Manager

Sample preparation

Testosterone, androstenedione, and DHEAS certified reference solutions and their stable labeled internal standards were purchased from Sigma Aldrich (Poole, UK). Calibrators were prepared in a surrogate matrix of MSG4000 stripped human serum purchased from Golden West Biologicals (Temecula, CA). Testosterone and androstenedione calibrators were prepared over the range of 0.17–69 nmol/L, with quality controls (QCs) at 0.52 nmol/L, 5.2 nmol/L and 35 nmol/L. DHEAS calibrators were prepared over the range of 0.14–54 µmol/L with QCs at 0.41 µmol/L, 4.1 µmol/L, and 27 µmol/L.

To convert SI units to conventional mass units divide by 3.470 for testosterone (nmol/L to ng/mL), 3.494 for androstenedione (nmol/L to ng/mL) and 2.716 for DHEAS (µmol/L to µg/mL).

Sample extraction

Extraction was performed using a Tecan Freedom Evo 100/4 Liquid Handler. To 100 µL of sample; 25 µL of 28 nmol/L testosterone-$^{13}C_3$ /androstenedione-$^{13}C_3$, and 1.4 µmol/L DHEAS-$^{2}H_5$, 200 µL methanol and 550 µL water were added. The samples were mixed after each reagent addition. Samples were centrifuged for 5 minutes at 4000 *g*.

An aliquot of each of the pre-treated samples (600 µL) was loaded into individual wells of the Oasis PRiME HLB µElution Plate (Waters P/N 186008052) and slowly pulled through at low vacuum (100 mbar). Consecutive washes with 200 µL of 0.1% (v/v) formic acid in 35% (v/v) methanol(aq) and 200 µL 0.1% (v/v) ammonia in 35% (v/v) methanol(aq) were performed. Analytes were eluted using 45 µL of methanol, followed by 55 µL water.

Method conditions

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.600	55	45	Initial
1.0	0.600	55	45	6
3.5	0.600	35	65	6
3.51	0.600	2	98	11
4.0	0.600	55	45	11

Table 1. Gradient table for the separation of testosterone, androstenedione, and DHEAS. Operating backpressure at the initial conditions was approximately 8500 psi.

Analyte	ESI mode	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (s)	Cone voltage (kv)	Collision energy (eV)
Testosterone (Quan)	+	289.2	97.0	0.034	40	24
Testosterone (Qual)	+	289.2	109.0	0.034	40	24
Testosterone-$^{13}C_3$	+	292.2	100.0	0.034	40	24
Androstenedione (Quan)	+	287.2	97.0	0.034	40	24
Androstenedione (Qual)	+	287.2	109.0	0.034	40	24
Androstenedione-$^{13}C_3$	+	290.2	100.0	0.034	40	24
DHEAS	–	367.2	97.0	0.250	40	30
DHEAS-$^{2}H_5$	–	372.2	98.0	0.250	40	30

Table 2. MRM parameters for testosterone, androstenedione, DHEAS, and their internal standards. The scan window for DHEAS was 1.5–2.6 minutes. The scan window for testosterone and androstenedione was 2.61–3.8 minutes. Mobile phase was directed to waste at all other times.

RESULTS

No interferences were observed at the retention time of testosterone, androstenedione, and DHEAS when the analytes themselves and eight structurally related compounds were examined (11-deoxycortisol, 21-deoxycortisol, 21-hydroxyprogesterone, 17-hydroxyprogesterone, corticosterone, cortisol, DHEA, and dihydrotestosterone). The chromatographic selectivity of the column is demonstrated through the baseline resolution of testosterone and its epimer; epitestosterone (Figure 2). In addition, separation of androstenedione, 17-hydroxyprogesterone, and DHEA from testosterone is necessary due to the detection of these analytes or their isotopes in the testosterone MRM trace at concentrations of >1 µmol/L.

No system carryover was observed from high concentration samples into subsequent blank injections. High concentration samples were at 175 nmol/L for testosterone and androstenedione, and 135 µmol/L for DHEAS. A 1:5 dilution was successfully performed on the high concentration carryover sample, providing a mean accuracy of 97%, 101%, and 96% for testosterone, androstenedione, and DHEAS respectively.

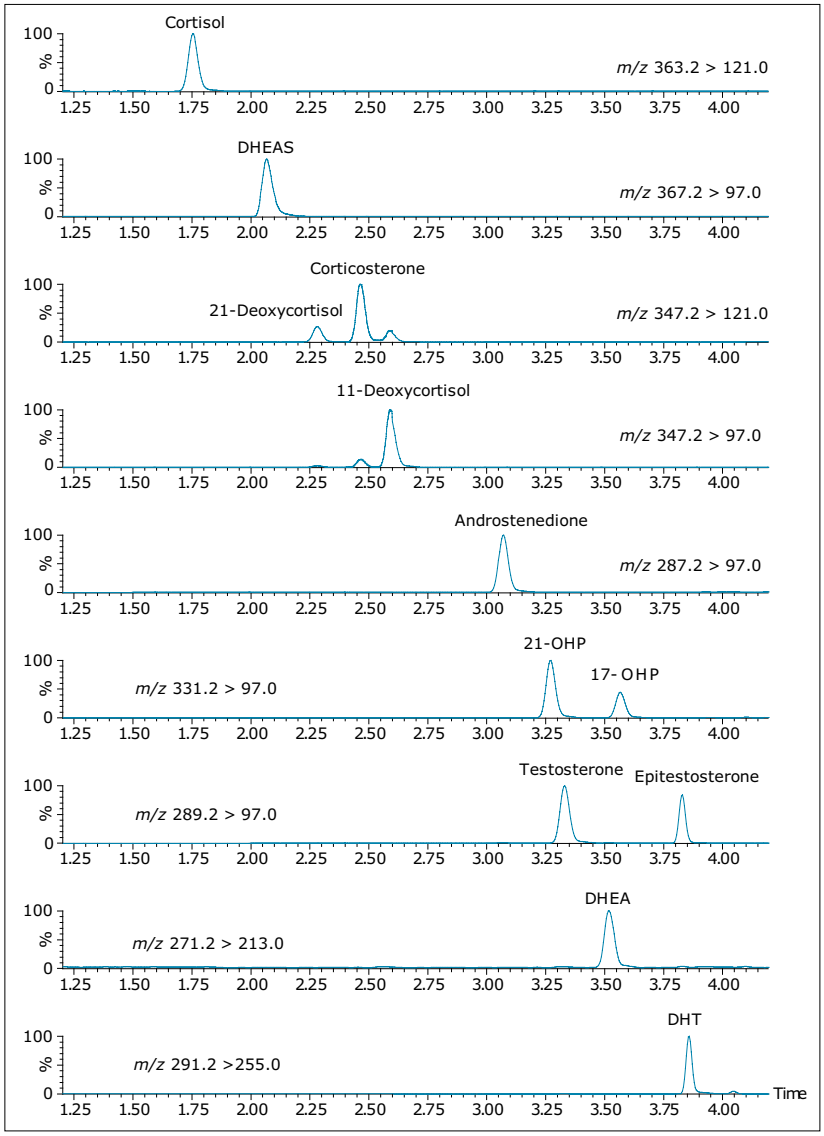

Figure 2. Chromatographic selectivity on the ACQUITY UPLC HSS T3 columns for a selection of steroid hormones, including testosterone, androstenedione, and DHEAS.

Analytical sensitivity investigations reveal that the analytical sensitivity of this method would allow precise quantification (<20% RSD) at 0.17 nmol/L for testosterone and androstenedione, and at 0.14 µmol/L for DHEAS. Signal:noise (S/N) of the lowest calibration standard was >10:1 on 10 separate occasions for all analytes.

Total precision was determined by extracting and quantifying three replicates of tri-level QC material on 2 occasions per day over 5 separate days (n=30). Repeatability was assessed by analyzing three replicates at each QC level. Low, mid, and high QC concentrations were 0.52, 5.2, and 35 nmol/L for both testosterone and androstenedione. Low, mid, and high QC concentrations were 0.41, 4.1, and 27 µmol/L for DHEAS. Total precision and repeatability using the Tecan Freedom Evo 100 Liquid Handler was ≤6.3% for all analytes (Table 3).

Compound	Total QC Precision			QC Repeatability		
	Low	Mid	High	Low	Mid	High
Testosterone	4.7%	3.3%	3.8%	3.6%	2.0%	3.4%
Androstenedione	6.3%	2.6%	4.6%	5.2%	2.3%	3.9%
DHEAS	3.3%	3.0%	3.9%	2.1%	1.8%	2.7%

Table 3. Total precision and repeatability for the analysis of testosterone, androstenedione, and DHEAS.

The method was shown to be linear over the range of 0.15–76 nmol/L for testosterone, 0.15–74 nmol/L for androstenedione, and 0.13–58 µmol/L for DHEAS, when different ratios of high and low concentration pools of the analytes were combined and analyzed. In addition, calibration lines in spiked serum were linear with coefficient of determinations (r^2) >0.998 on 10 separate occasions for all analytes.

Matrix effect investigations were performed using individual donor serum samples. The endogenous peak areas were separately quantified. Post-spiked samples were adjusted using the mean endogenous peak area to enable comparison to solvent spiked samples. The matrix factor calculated is shown in Table 4. Normalized matrix factor calculations, based on the analyte:internal standard response ratio produced similar values to peak area matrix factor.

Compound	Mean matrix factor (range) based on analyte peak area	RSD
Testosterone	1.02 (0.93–1.09)	5.9%
Androstenedione	1.02 (0.94–1.07)	4.6%
DHEAS	0.93 (0.86–1.02)	6.8%

Table 4. Mean (range) matrix factor and %RSD based on peak area of testosterone, androstenedione and DHEAS.

Samples were selected (n=50) for comparison against an independently developed LC-MS/MS method for testosterone, androstenedione, and DHEAS. Comparison data was processed using Analyse-it v2.3. Altman-Bland agreement demonstrated a mean bias of 5.0%, -3.3%, and -6.3% for testosterone, androstenedione, and DHEAS, respectively.

EQA samples were analyzed for testosterone (n=30), androstenedione and DHEAS (n=50). The data obtained was compared to the mass spectrometry mean for the samples and Deming regression was performed. The correlation for testosterone, androstenedione, and DHEAS can be seen in Table 5 showing the excellent agreement with the EQA scheme MS mean. Proportional and constant bias was observed for DHEAS when all samples were analysed (0–40 µmol/L), however, the bias over the range 10.2–14.9 µmol/L was ≤6.9%. Altman-Bland agreement demonstrated a mean bias of -0.50%, 0.4%, and 5.8% for testosterone, androstenedione, and DHEAS, respectively.

Analyte	Deming equation	Proportional bias?	Constant bias?	Linear Fit (r)
Testosterone	y =1.01x–0.02	N	N	0.999
Androstenedione	y =1.03x–0.10	Y	N	0.999
DHEAS (0–40 µmol/L)	y =1.10x–0.48	Y	Y	0.996
DHEAS (0–15 µmol/L)	y =1.05x–0.09	Y	N	0.996

Table 5. Deming regression comparing the Waters LC-MS/MS method to the EQA scheme MS method for testosterone, androstenedione, and DHEAS analysis.

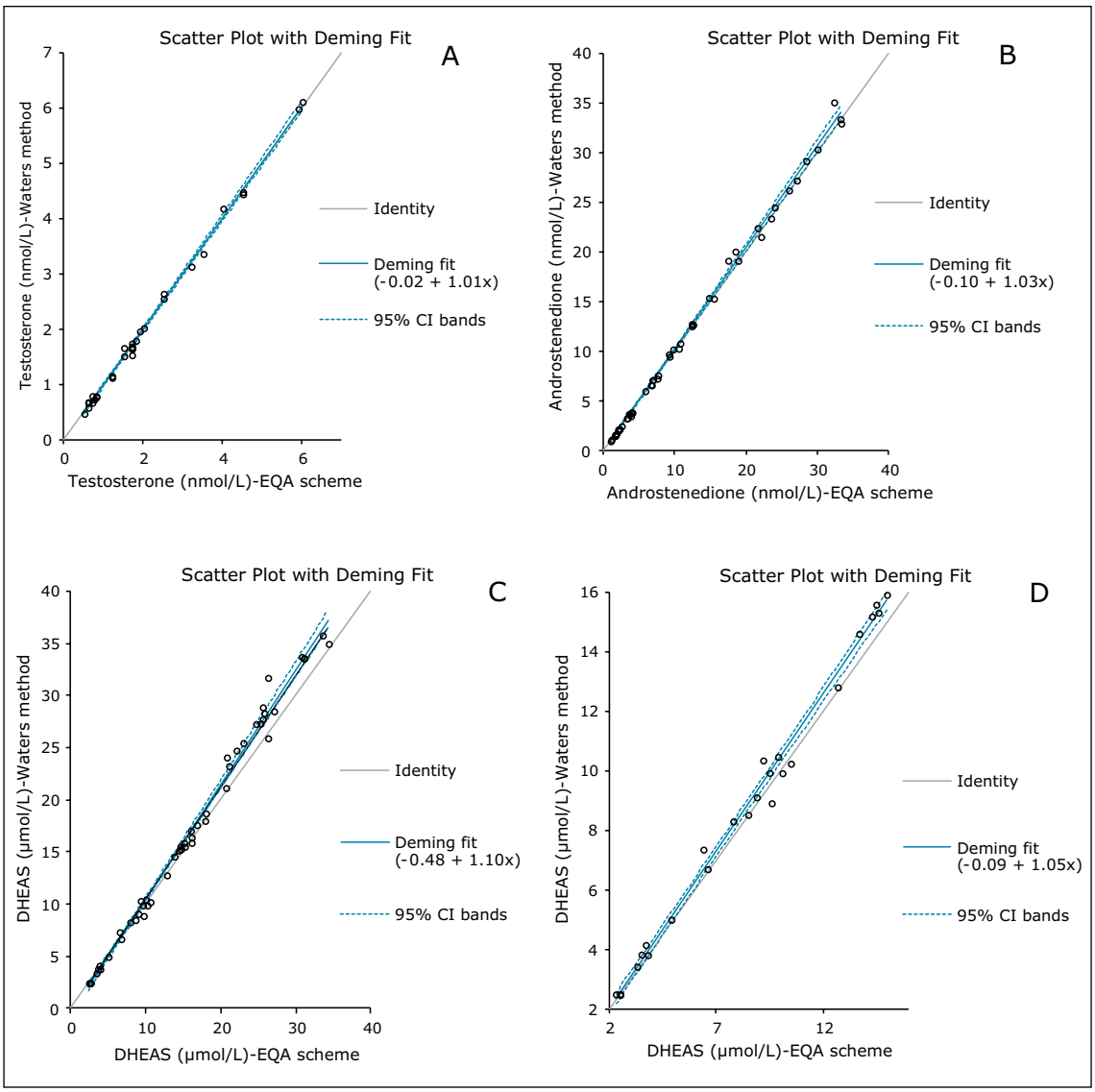

Figure 3. Deming regression comparing the Waters LC-MS/MS method to the EQA scheme MS mean for A) Testosterone, B) Androstenedione, C) DHEAS 0–40 µmol/L, and D) DHEAS 0–15 µmol/L.

CONCLUSIONS

An analytically sensitive and selective clinical research method has been developed for the analysis of testosterone, androstenedione, and DHEAS in serum.

Using only 100 µL sample volume, this method provides sufficient analytical sensitivity to analyze low physiological levels of testosterone, androstenedione, and DHEAS. Automation of the analytical method in combination with sample tracking capabilities improves laboratory workflow and reduces sample handling, which alleviates the potential for operator error.

Acknowledgement

Professor Brian Keevil and his colleagues at the Department of Clinical Biochemistry, University Hospital of South Manchester, Wythenshawe, UK, are thanked for the provision of anonymized serum samples for the analysis.

References

1. Foley D. and Calton L. A Clinical Research Method for the Analysis of Serum Testosterone and Androstenedione. Waters application note. 2015. p/n 720005274EN.

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Analysis of Plasma 17-Hydroxyprogesterone, Androstenedione, and Cortisol Using a Novel Solid-Phase Extraction (SPE) Sorbent, Oasis PRiME HLB, for UPLC-MS/MS Analysis in Clinical Research

Jonathan P. Danaceau and Erin E. Chambers
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Faster, simplified sample preparation workflow
- Elimination of at least 97% of phospholipids compared to protein precipitation
- No evaporation or reconstitution necessary
- Linear, accurate, and precise results for all analytes

WATERS SOLUTIONS

Oasis PRiME HLB µElution Plate (p/n 186008052)

ACQUITY® 96-well Sample Collection Plate, 700 µL Round well (p/n 186005837)

ACQUITY UPLC® HSS T3 Column 100Å, 1.8 µm, 2.1 x 50 mm (p/n 186003538)

ACQUITY UPLC® I-Class

Xevo® TQ-S Mass Spectrometer

KEY WORDS

Androstenedione, cortisol, 17-alpha-OH progesterone, uElution, sample preparation, SPE, steroids, matrix effects, phospholipid removal, LC-MS

INTRODUCTION

Sample preparation is an important consideration for any bioanalytical LC-MS/MS method for clinical research. Waters has developed a novel sample preparation sorbent, Oasis® PRiME HLB, which is designed to have some key advantages over traditional SPE sorbents. These include the ability to eliminate sorbent preconditioning and equilibration, allowing a more rapid workflow compared to traditional SPE products, and the ability to remove greater than 95% of phospholipids, resulting in a cleaner extracts and reducing the risk of short column lifetimes or MS source fouling.

This application note details the extraction and UPLC-MS/MS analysis of 17α-hydroxyprogesterone (17-OHP), androstenedione (Adione), and cortisol using Oasis PRiME HLB. Measurement of these compounds by immunoassay can be prone to cross reactivity with antibodies of chemically similar compounds. Immunoassays must also be done individually, requiring separate samples and analyses for each compound. LC-MS/MS offers greater discrimination and selectivity and the ability to multiplex methods, allowing the simultaneous determination of multiple compounds. The use of Oasis PRiME HLB resulted in consistent and highly reproducible recoveries of all compounds with minimal matrix effects. Phospholipids were almost completely eliminated compared to protein precipitation. Finally, the use of Waters® patented µElution format allowed for the concentration of the sample on the SPE column, eliminating the need to evaporate and reconstitute the sample. This resulted in a method that was linear, accurate and precise for all analytes, with limits of quantification of 50 pg/mL for androstendione and 17-OHP.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC HSS T3 Column, 100Å, 1.8 µm, 2.1 x 50 mm
Column temp.:	40 °C
Sample temp.:	10 °C
Mobile phase A (MPA):	Water with 0.1% formic acid
Mobile phase B (MPB):	ACN with 0.1% formic acid
Purge solution:	25:25:50 ACN:MeOH:Water
Wash solution:	10% ACN

The gradient ramp is shown in Table 1.

Time (min.)	Flow (mL/min.)	%A	%B
0	0.6	70	30
1.0	0.6	50	50
2.0	0.6	45	55
2.5	0.6	5	95
3.5	0.6	5	95
3.6	0.6	70	30
4.5	0.6	70	30

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the Methods section.

Mass spectrometry

MS System:	Xevo TQ-S Mass Spectrometer
Ionization mode:	ESI Positive
Capillary Voltage:	1.0 kV
Cone voltage:	Optimized for each analyte
Desolvation Gas:	1000 L/hr
Cone Gas:	150 L/hr
Desolvation Temp:	500 °C
Source Temp:	150 °C

Data were acquired and analyzed using MassLynx® Software (V4.1; SCN 876). Quantification was performed using TargetLynx.™

METHODS

All standards and stable isotope labelled internal standards were purchased from Cerilliant (Round Rock, TX). A combined stock standard (20 µg/mL cortisol; 1 µg/mL androstenedione and 17-OHP) was prepared in 25% methanol. A stock solution of 10 µg/mL cortisol-d4 and 0.5 µg/mL androstenedione-^{13}C3 and 17-OHP-d8 was prepared in methanol. A working internal standard solution of 750 ng/mL cortisol-d4 and 37.5 ng/mL androstenedione-^{13}C3 and 17-OHP-d8 was prepared in 25% methanol. Individual calibrators and quality control standards were prepared daily in 25% methanol. 25 µL of each working calibrator or QC standard was added to 475 µL of double charcoal stripped human plasma (Golden West Biological, Temecula, CA) to make calibration curves and QC samples.

SAMPLE PREPARATION

Samples were prepared as follows: 20 µL of the working internal standard solution was added to 150 µL of each calibrator or QC sample. All samples were precipitated with 300 µL of a solution of 4:1 MeOH:89 g/L $ZnSO_4$. The samples were aspirated several times to ensure full precipitation and then centrifuged at 3220 rcf for 10 minutes. 300 µL of the resulting supernatant was then added to 900 µL of 4% H_3PO_4 and aspirated to fully mix the sample. The resulting pretreated sample was then directly applied to the Oasis PRiME HLB µElution Plate in 2 aliquots. All wells of the SPE plate were subsequently washed with 2 x 200 µL aliquots of 25% methanol. The samples were then eluted with 2 x 25 µL aliquots of 90:10 ACN:MeOH and diluted with 25 µL of water. 7.5 µL was injected onto the UPLC-MS/MS system. The sample extraction procedure is summarized in Figure 1.

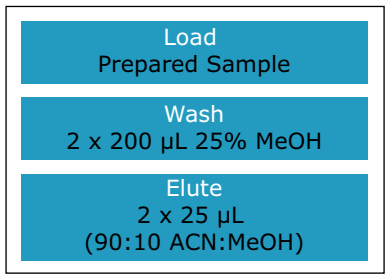

Figure 1. Oasis PRiME HLB extraction methodology for plasma corticosteroids. With no conditioning and equilibration, sample extraction is simplified to just three steps.

RESULTS AND DISCUSSION

Chromatography

Figure 2 shows the chromatography of the three steroids from an extracted calibrator. All compounds eluted within 2 minutes. The HSS T3 column offered a distinct advantage over other C_{18} columns. Even though these compounds are not polar, the enhanced retentivity of the T3 column eliminated solvent effects observed on other columns when samples were injected in high proportions of organic solvent.

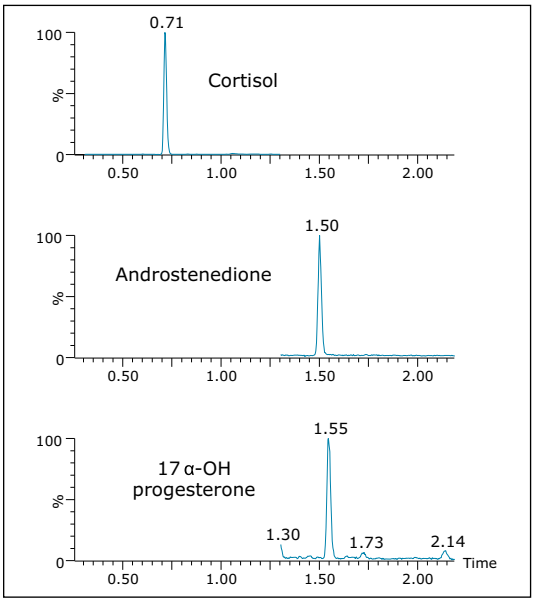

Figure 2. Chromatography of cortisol, androstendione, and 17-OHP from an extracted plasma sample on the ACQUITY UPLC HSS T3 Column, 100Å, 1.8 µm; 2.1 x 50 mm. The concentration of cortisol was 20 ng/mL. The concentration of the other compounds was 1 ng/mL.

Table 2 lists the retention time and individualized MS parameters of the steroids and their stable isotope labelled internal standards, including MRM transitions, cone voltage, and collision energy. Two MRM transitions were used for each compound, a primary (listed first) and a confirmatory transition (listed second).

Recovery and matrix effects

Extraction recovery was very consistent.
As Figure 3 shows, recovery for all compounds was 72–73% with %RSDs under 5%, demonstrating the reproducibility of Oasis PRiME.

Analyte	RT (min)	MRM transitions (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
Cotisol	0.72	336.17>121.06	42	22
		336.17>91.03	42	52
d4 Cortisol (IS)	0.71	367.17>121.12	42	22
Androstenedione	1.50	287.17>97.08	58	20
		287.17>109.04	58	26
^{13}C3 A-dione (IS)	1.50	290.17>100.07	58	20
17-OHP	1.55	331.17>97.08	58	26
		331.17>295.20	58	16
d8-17-OHP (IS)	1.53	339.23>100.07	58	26

Table 2. Mass spectral parameters for all analytes and internal standards.

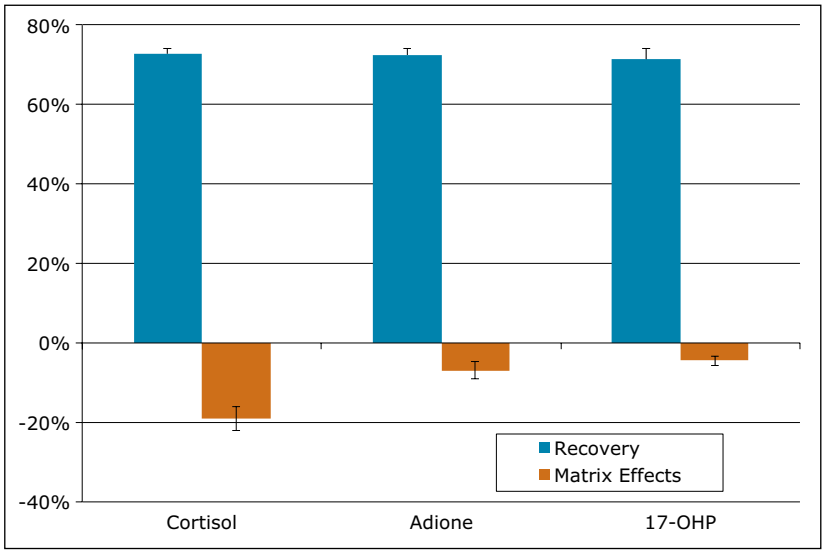

Figure 3. Recovery and matrix effects of cortisol, androstendione, and 17-OHP after extraction using the Oasis PRiME HLB µElution Plate. %RSDs for extraction recovery were less than 5% for all compounds. Matrix effects were -19% for cortisol and less than 10% for the other two steroids.

Matrix effects were low for all compounds. The matrix effect for cortisol was -19%, indicating minor ion suppression and was minimal for the other compounds. The average matrix effect for all compounds was -10.1%. Once again, the low standard deviations (3.1% or less) demonstrate the consistency of extraction and cleanup seen with Oasis PRiME. All recovery and matrix effect data are summarized in Table 3.

	Recovery			Matrix effects	
	Mean	S.D.	%RSD	Mean	S.D.
Cortisol	72.7%	3.1%	4.2%	-19.0%	3.1%
Adione	72.5%	1.9%	2.7%	-6.9%	2.2%
17-OHP	71.5%	1.9%	2.6%	-4.5%	1.3%
			Mean	**-10.1%**	

Table 3. Recovery and Matrix effects (N=4 for all tests).

Quantitative results

Calibration and quality control samples ranged prepared as previously described in the materials and method section. Calibration ranges were from 1–500 ng/mL for cortisol and from 0.05–25 ng/mL for the remaining compounds, mirroring the expected concentrations of these compounds in plasma. Quality control samples were prepared at low, medium, and high concentrations as appropriate for the calibration ranges.

All compounds had linear responses over the entire calibration range with R^2 values of 0.99 or greater with 1/x weighting. Table 4 summarizes the data from the calibration curves. Lower limits of quantification (LLOQ) were 1.0 ng/mL for cortisol and 0.05 ng/mL for androstenedione and 17-OHP. In each case, all FDA recommendations for accuracy, precision and analytical sensitivity were met for validated methods.[1]

	R^2	Mean % dev.
Cortisol	0.996	8.39
Adione	0.989	8.03
17-OHP	0.993	9.72

Table 4. Calibration Curve Summary. Linear fits with 1/x weighting were used for all compounds.

Quality control samples were accurate and precise, with all results within 10% of expected values and %CVs no greater than 7% (N=6). This data can be seen in Table 5. The excellent accuracy and precision demonstrate the consistency and robustness of this sorbent.

	Accuracy					
	Androstenedione			17α-OH progesterone		
QC Level (ng/mL)	Mean	S.D.	%CV	Mean	S.D.	%CV
0.15	94.3%	5.4%	5.7%	93.7%	6.1%	6.5%
1.5	95.0%	3.4%	3.6%	92.3%	4.7%	5.6%
15	95.4%	5.3%	**5.5%**	**93.7%**	6.1%	6.5%
Mean	**94.9%**			**92.6%**		

	Accuracy		
	Cortisol		
QC Level (ng/mL)	Mean	S.D.	%CV
3	92.3%	4.9%	5.4%
30	94.8%	2.9%	3.0%
300	94.9%	5.7%	6.0%
Mean	94.0%		

Table 5. Quality control results from extracted plasma samples (N=6 for each compound at all three levels).

Phospholipid removal

One of the key features of Oasis PRiME HLB over other reversed-phase SPE sorbents or liquid-liquid extraction is its enhanced ability to remove phospholipids, which can be major contributors to matrix effects,[2,3] accumulate on analytical columns, and may contribute to fouling of MS sources, necessitating frequent cleaning. To assess the removal of phospholipids, we analyzed the extracts for several of the most common phospholipids and compared that data to samples that had been subject to protein precipitation only. Figure 4 compares chromatograms of identical plasma samples that were ether extracted as described above, or subject only to the protein precipitation step detailed. The total area of phospholipids in the Oasis PRiME HLB extracted samples was 3% that of precipitated samples. The true removal is likely even greater than 97% as there was no sample concentration for the precipitated samples. Taking the concentration factor into account (approximately 6X) would result in a removal of greater than 99%.

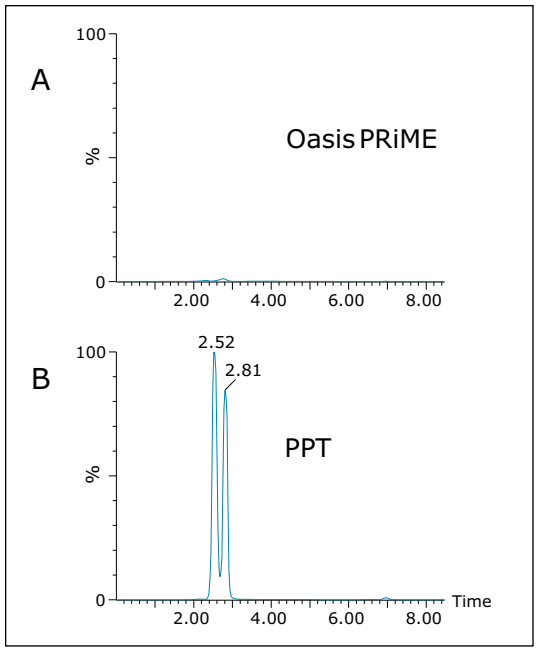

Figure 4. Removal of phospholipids compared to protein precipitaion. A. Summed chromatogram of phospholipids from a plasma sample prepared using Oasis PRiME HLB. B. Summed chromatogram of phospholipids from an identical plasma sample prepared by protein precipitation. Chromatographic scales are linked.

CONCLUSIONS

This application note details the extraction of cortisol, androstendione and 17-OHP from plasma samples using a novel SPE sorbent, Oasis PRiME HLB, in a µElution format for clinical research. The unique nature of this sorbent enabled the elimination of conditioning and equilibration steps, simplifying the extraction procedure and speeding up the sample preparation workflow. In addition, residual phospholipids were nearly eliminated compared to protein precipitation. The µElution format enabled the direct injection of extracts without evaporation or reconstitution.

Recoveries were very consistent for all compounds, and matrix effects were less than -20% for cortisol and under -10% for the other compounds. Linearity, accuracy, precision and analytical sensitivities were excellent for all compounds. All accuracies were within 8% of target concentrations and all %CVs were less than 7% for QC samples demonstrating the high reproducibility of this sorbet.

For Research Use Only. Not for Use in Diagnostic Procedures.

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[APPLICATION NOTEBOOK]

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Improving the Detection of Thyroglobulin in Human Plasma for Clinical Research by Combining SISCAPA Enrichment and Microflow LC-MS

Jay S. Johnson,[1] Morteza Razavi,[2] James Murphy,[1] Selena Larkin,[2] and Paul Rainville[1]
[1]Waters Corporation, Milford, MA, USA; [2]SISCAPA Assay Technologies, Washington, DC, USA

APPLICATION BENEFITS

- ionKey/MS™ configured for dual-pump trapping is well suited to analyze SISCAPA eluents
- Sub 1 ng/mL quantitation level of thyroglobulin is achieved using 10x less plasma than the comparable standard flow method
- Accuracy is highly correlated with the values obtained from the standard flow method but offers higher levels of precision LC over 4 replicates
- Dual-pump trapping significantly reduces cycle times to under 7 minutes allowing a similar number of samples to be run in the same time frame as the best in literature standard flow method
- Microflow is a viable and attractive solution for clinical research

WATERS SOLUTIONS

ACQUITY UPLC® M-Class System

ionKey/MS System

Xevo® TQ-S

iKey™ Separation Device

MassLynx® Software

TargetLynx™ Application Manager

KEY WORDS

Thyroglobulin, SISCAPA, UPLC, MRM, TQ-S, ionKey/MS, dual-pump trapping, health sciences, high throughput

INTRODUCTION

Current research immunoassays for Tg may be subject to high false negative rates in a significant portion of the sample population due to the presence of endogenous anti-Tg autoantibodies (Tg-AAbs) that block the binding epitope resulting in the reporting of a negative result in the immunoassay. The prevalence of these negative results has lead researchers to look for alternative analytical approaches that can improve the quality of the result.

Stable isotope standards and capture by anti-peptide antibodies (SISCAPA) enrichment for Tg combined with standard flow LC-MS has been implemented as an alternative approach in clinical research labs. The high analytical selectivity and specificity of the capture step using anti-peptide antibodies specific for a proteolytic peptide unique to Tg greatly enhances the detection and quantitiation of Tg down to levels of approximately 1 ng/mL or 1.52 amol/µL. However, standard flow LC-MS requires 200–400 µL of plasma to reach these relevant LOQ levels, a very large volume of sample.

Microflow LC-MS, exemplified by the ionKey/MS System, operating at 10's of µL/min offers substantial analytical sensitivity benefits over standard flow using less starting plasma in sample-limited applications.[1] Accordingly, we investigate here if the ionKey/MS System operating in a dual-pump trapping configuration can provide reductions in LLOQ levels for Tg, using less plasma while maintaining the requisite accuracy, precision, and throughput exemplified by published standard flow LC-MS assays. The dual-pump trapping configuration was explicitly chosen due to the ability of the set-up to handle relatively large injection volumes compared to iKey column volume, reduce carryover coming from the sample loop and trap column, and decrease cycle time by affording load ahead capability on the trap column and independent washing and equilibration of the trap column and analytical iKey.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC M-Class
Analytical column:	iKey Peptide BEH C_{18} Separation Device, 130Å, 1.7 µm, 150 µm x 50 mm (p/n 186006764)
Trap column:	Prototype Symmetry C_{18} Microfluidic Trap Column, 100Å, 5 µm, 300 µm x 50 mm (p/n TBD)
iKey temp.:	45 °C
Sample temp.:	12 °C
Injection volume:	20 µL partial loop in a 22.8 µL loop
Flow rate:	3 µL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
WNW:	0.1% formic acid in water
SNW:	2% formic acid in 25/25/25/25 water/acetonitrile/isopropanol/methanol
Gradient:	9.9% B to 27.5% B in 2.2 min
Trap loading:	99.5% A at 50 µL/min for 0.8 min
Total cycle time:	6.75 min injection to injection

MS conditions

MS system:	Xevo TQ-S operating in MRM Mode with Unit Mass Resolution
Ionization mode:	ESI Positive
Capillary voltage:	Optimized through infusion of analyte of interest
Source temp.:	100 °C
Cone gas flow:	50 L/Hr
Nano gas flow:	Off
Collision energy:	Optimized through infusion of analyte of interest, see Table 1
Cone voltage:	Optimized through infusion of analyte of interest, see Table 1

Data management

Chromatography software:	MassLynx v4.1
Quantification software:	TargetLynx

Sample preparation

The generic SISCAPA enrichment workflow coupled with ionKey/MS is detailed in Figure 1. The sample preparation detailed in this application note was performed by SISCAPA Assay Technologies following their recommended procedures.

1. Plasma sample is digested using trypsin
   Any potential auto Tg antibodies are digested along with the target, Tg, to their corresponding peptides
2. A highly selective and specific antibody against a proteotypic peptide unique to Tg with the amino acid sequence FSPDDSAGASALLR (FSP) is conjugated to a magnetic bead support
3. A stable isotope standard (SIS) of the FSP peptide and the bead-conjugated antibody is added to the plasma digest
4. The FSP peptide and SIS are selectively enriched by the anti-peptide antibody bead complex in an automated fashion in the 96-well plate format
5. The beads are then washed to remove unbound matrix material and the bound peptides are released using acid elution
6. The resulting eluent is subjected to microflow LC-MS using the conditions described below

Peptide	Precursor	Product	CE	Cone voltage
FSP.light	708.8	768.5	27	30
FSP.heavy	703.8	758.5	27	30
FSP.light	708.8	697.4	27	30
FSP.heavy	703.8	687.4	27	30
FSP.light	**708.8**	**591.8**	**21**	**30**
FSP.heavy	**703.8**	**586.8**	**21**	**30**

Table 1. Optimized MRM transition parameters for the heavy and light versions of FSP. The qualifier MRMs are shown in bold. These parameters were optimized thru infusion using the onboard fluidics of the Xevo TQ-S and an infusion iKey.

Instrumental set-up

In attempts to decrease cycle time and allow more samples to be run on the ionKey/MS System, the configuration chosen was a dual-pump trapping configuration as shown in Figure 2. In dual-pump trapping a dedicated binary solvent manager plumbed with larger I.D. transfer lines handles the loading of the trap column. A second binary solvent manager is dedicated for gradient elution of the analyte of interest off of the trap column to the analytical iKey. Due to the fact that the loading pump is plumbed with larger I.D. transfer lines, this loading step can occur at a faster flow rate without reaching the pressure limit of the system. The optimized loading flow rate in this method was found to be 50 µL/min, however, flow rates of up to 70 µL/min are possible. Furthermore as we employ two dedicated pumps, the loading of the trap column by the loading pump can be overlapped with the equilibration of the analytical iKey by the gradient pump, effectively cancelling out the sample loading time from the total cycle time, resulting in considerable analytical time savings. After the set loading time, the valve is switched to the elution configuration as seen in Figure 2 and 3, and the gradient pump forms a gradient that back flushes the analyte off the trap column to the analytical iKey. During this elution step the loading pump is in line with the sample loop and can be used to flush the loop at a high flow rate with any mixture of mobile phase which should help manage carryover.

The dual-pump trapping configuration also allows heart cutting type experiments in which the trap is decoupled from the analytical iKey just after the last analyte of interest elutes by switching the trapping valve back into the loading configuration. Decoupling is beneficial from an analytical column and MS optics cleanliness standpoint as any later eluting matrix components such as proteins and phospholipids will be directed to waste.

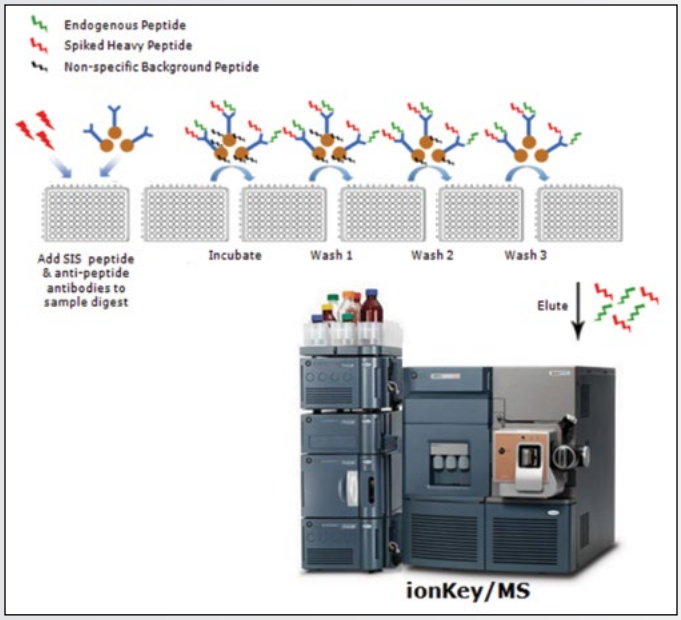
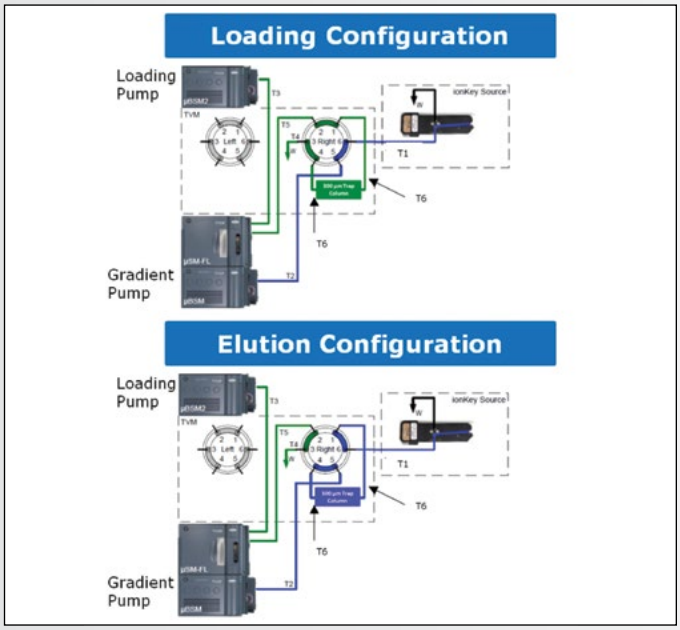


Figure 1. Analytical workflow employed combining SISCAPA enrichment and microflow LC-MS using the ionKey/MS System for the sensitive detection of Tg.

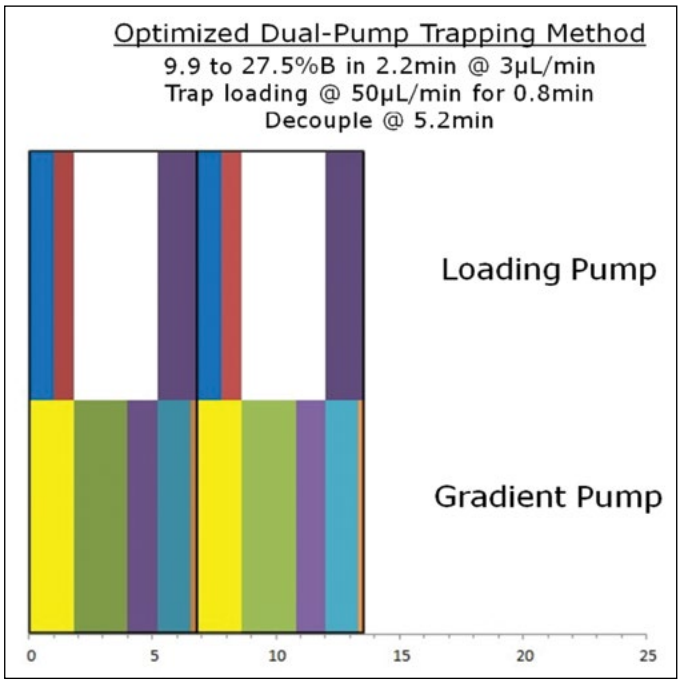


#	Length	ID	Fittings	Part #	Order #
T1	40"	40 µm	V-F	430004188	700010399
T2	30"	25 µm	M-V	430003619	700009872
T3	30"	40 µm	M-V	430003620	700009873
T4	31"	.01"	PEEK	430004090	700009892
T5	26"	40 µm	V-V	430003658	700009881
T6	6"	40 µm	V-V	430003649	700009878

Figure 2. The dual-pump trapping set-up contains a dedicated binary solvent manager for fast sample loading onto the loop and trap column and a dedicated gradient elution pump. In this set-up line T3 has a 40 µm I.D. which affords a loading flow rate of 50 µL/min. The gradient transfer line, T2, remains a 25 µm I.D.

RESULTS AND DISCUSSION

The analytical sensitivity of the ionKey/MS System in the dual-pump trapping configuration, using the parameters defined previously, was first evaluated using synthetic standards of the light and heavy FSP peptides. A 6 point calibration curve was created comprising a concentration range of 2,000 amol/µL down to 0.64 amol/µL utilizing a 1 in 5 dilution with 3% acetonitrile in 0.1% formic acid. Each calibration point was run in triplicate and we observed an excellent linear response and reproducibility over the calibration range as detailed in Figure 4 with the 13 amol level having a coefficient of variation (CV) of approximately 16%. This data reinforces the ability of the platform and method described to analyze synthetic standards of FSP.



Load pump events	Time (min)	Gradient pump events	Time (min)
Trap equilibration/sample loading onto loop	0.95	iKey equilibration	1.75
Trap loading	0.8		
Wash loop	3.4	Gradient	2.2
Wash trap	1.6	Wash iKey	1.2
		Delay volume	1.35
		Housekeeping	0.25
Cycle time	6.75	Cycle Time	6.75

Figure 3. Schedule of events in the optimized dual-pump trapping workflow.

amol on column	Avg light area	CV	Avg heavy area	CV
40000	1343852.3	4.9%	1181870.0	4.4%
8000	267515.0	1.1%	234463.0	0.8%
1600	43533.7	4.8%	39211.7	1.5%
320	8312.3	2.0%	7147.7	2.6%
64	1681.0	4.9%	1614.0	1.3%
12.8	246.0	16.3%	254.0	16.4%

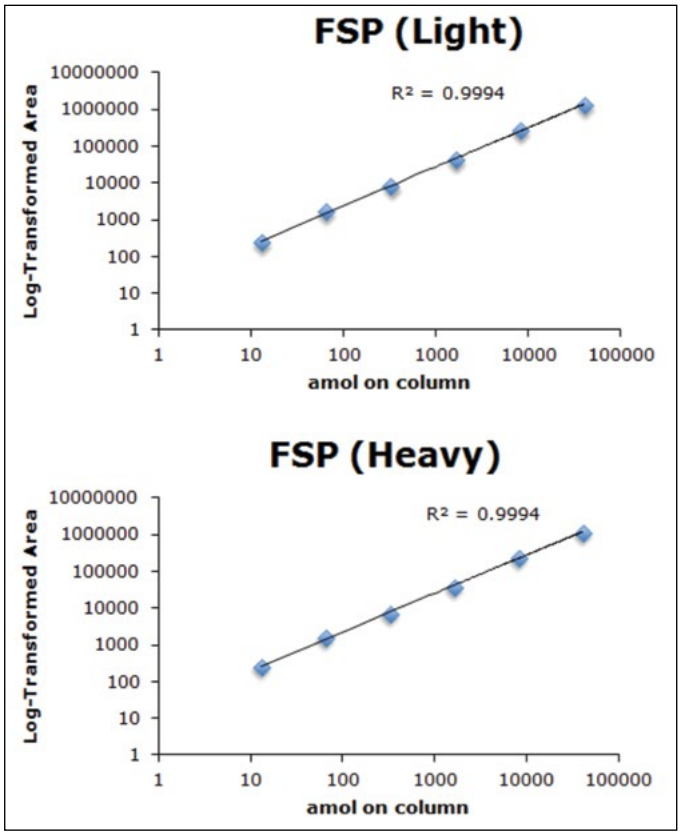


Figure 4. Calibration curve expressed in amol on column for the analysis of the synthetic standards of both the heavy and light versions of FSP. We observed an excellent linear response and reproducibility when working with these standard variants of the peptide.

Next, to demonstrate that the platform is compatible with SISCAPA eluates and can actually detect endogenous Tg in human plasma, we performed a plasma titration experiment where varying amounts of pooled human plasma were digested followed by SISCAPA enrichment of the FSP peptide. Accordingly, it is expected that the PAR value, or the ratio of the endogenous light FSP to the heavy SIS FSP added after digestion at a consistent concentration, should increase linearly when plotted against pooled human plasma amount. The results shown in Figure 5 show the expected linear response was achieved for human plasma amounts down to 40 µL, with no observed backpressure fluctuations in the analytical iKey. Accordingly, it can be concluded that the platform is compatible, robust, and analytically sensitive for endogenous FSP in human plasma enriched using the SISCAPA workflow. Furthermore, as a positive control, the experiment was replicated on an Agilent 1290/6490 QqQ instrument operating in the standard flow regime and utilizing the recommended method parameters for the instrument. A high linear correlation of $R^2 = 0.998$ as seen in Figure 6 was achieved between the two platforms confirming the accuracy of the PAR values as measured on the ionKey platform. Additional evidence of the agreement between the PAR values obtained on the standard flow and ionKey/MS can be visualized in the Bland-Altman plot seen in Figure 7. Agreement between all measurements is within the 95% confidence interval. Furthermore, the ionKey/MS System showed better precision across 4 replicates than the standard flow system. This suggests microflow offers tangible improvements in the precision of measurement of FSP while maintaining the accuracy expected of the conventional standard flow approach.

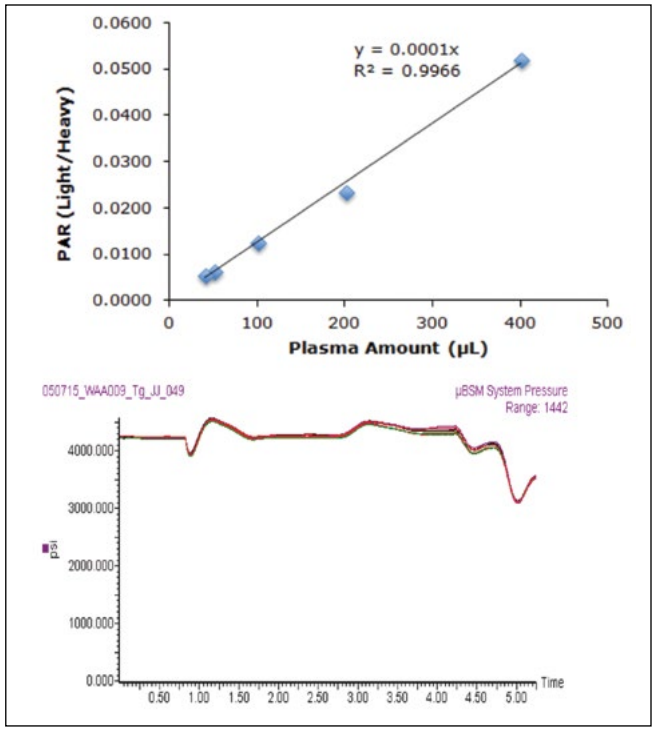
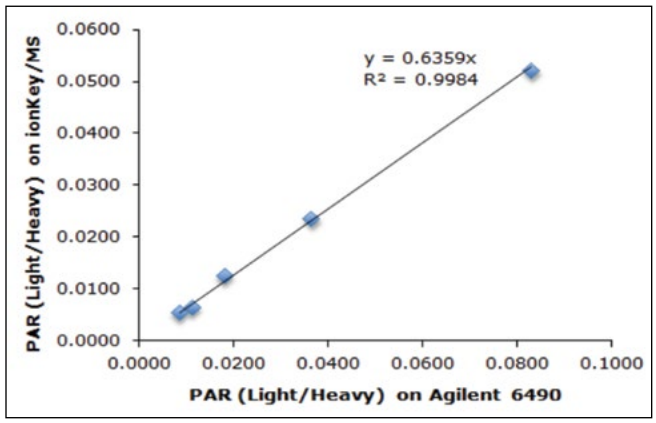


Figure 5. A linear response was achieved for pooled human plasma amounts down to 40 µL with no observed backpressure fluctuations demonstrating the ionKey/MS platform as described is compatible, robust, and sensitive for SISCAPA eluates.



Plasma (µL)	ionKey/MS CV	Agilent 6490 CV
400	6.8%	17.2%
200	6.5%	9.7%
100	9.6%	12.5%
50	13.9%	17.7%
40	6.0%	36.2%

Figure 6. The experiment above was replicated on a standard flow Agilent system as a positive control and an excellent correlation was obtained. The high correlation and better precision across 4 replicates proves that microflow offers tangible benefits in the analysis of Tg over the conventional standard flow approach.

To further study the analytical sensitivity of the platform in terms of LLOD and LLOQ, a reverse curve was generated by titrating the heavy FSP peptide from 5000 amol/µL down to 0.75 amol/µL and spiking synthetic light peptide at a constant level in human plasma. The LLOD, defined in this work as the point below which the CV is consistently above 30% for FSP, was determined to be 15 amol on column. The LLOQ, defined in this work as the point below which the CV is consistently greater than 20%, was 45 amol column. Representative chromatograms for the LLOD and LLOQ levels along with the reverse curve can be seen in Figure 8. The LLOQ of 45 amol is slightly higher than that estimated in the synthetic standard FSP work as one would expect due to the influence of the matrix.

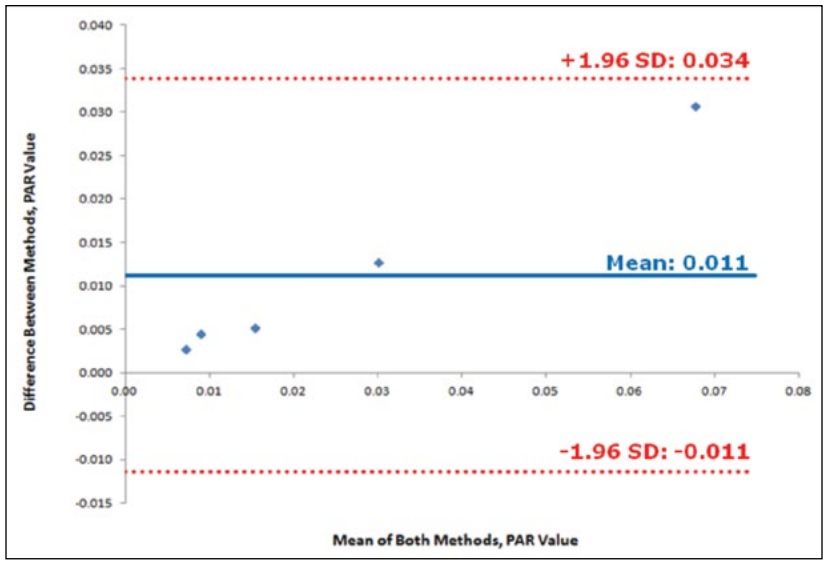


Figure 7. Bland-Altman Plot showing all differences between the standard flow Agilent method and the microflow method measurements of the FSP PAR value lie within the upper and lower confidence intervals. Agreement is therefore expected for 95% of the samples.

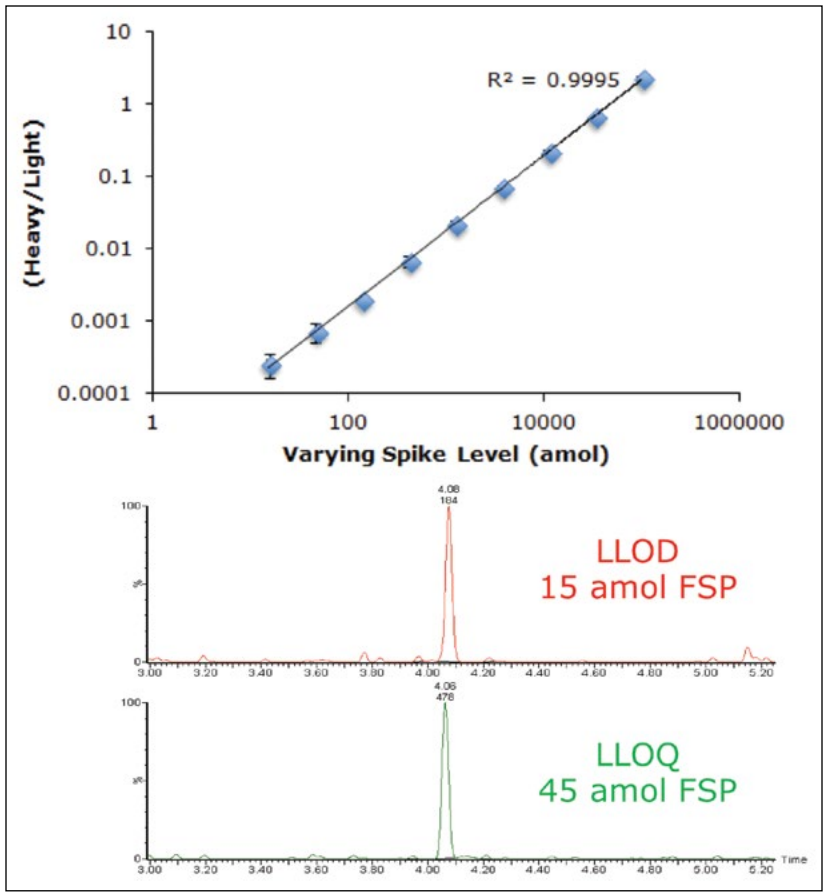


Figure 8. A reverse curve was generated to determine the approximate LLOD and LLOQ of the peptide measurement. The LLOD is 15 amol FSP on column. The LLOQ is 45 amol FSP on column.

A final curve was generated by titrating purified Tg protein in bovine plasma known to be deficient in Tg from 152 to 0.152 amol/µL followed by SISCAPA enrichment in attempts to get an estimated LLOQ value for the entire assay including the digestion step. The LLOQ of the method using only 50 µL of plasma as shown in Figure 9 is estimated to be approximately 1.18 amol/µL (0.78 ng/mL). Accordingly, the method achieves quantitation levels of Tg in bovine serum of 1.52 amol/µL (1 ng/mL) with ease.

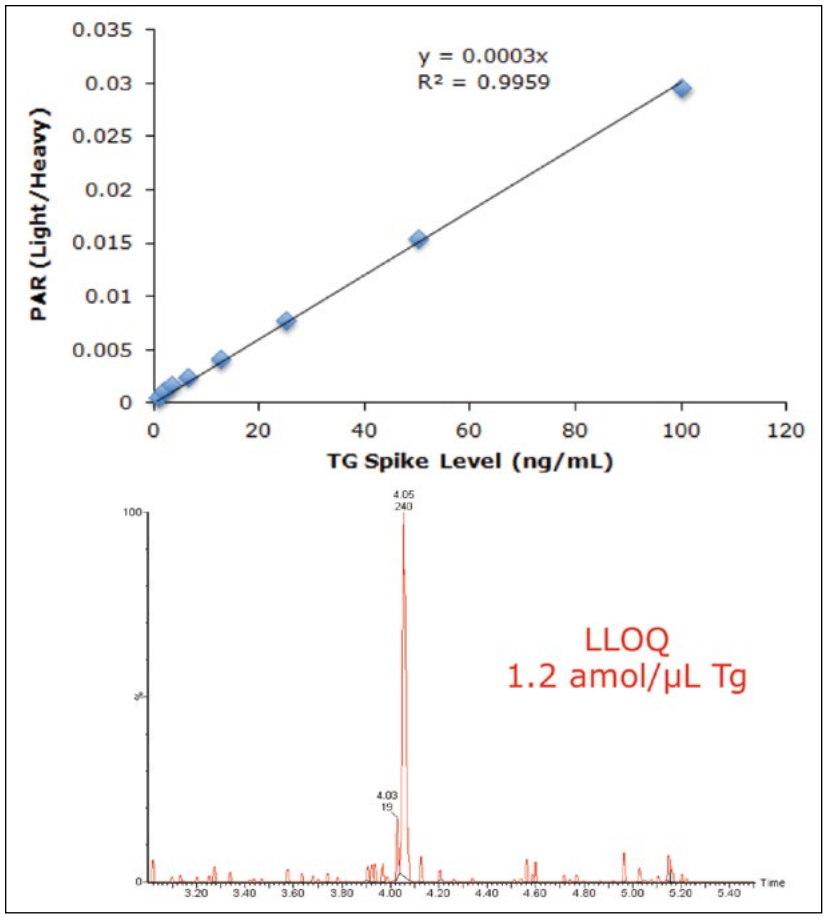


Figure 9. A curve was generated by titrating purified Tg in bovine plasma from 152 to 0.152 amol/µL. The LLOQ of the assay including the digestion step using 50 µL of sample is estimated to be 1.2 amol/µL (0.78 ng/mL).

CONCLUSIONS

Use of an optimized SISCAPA enrichment that is highly specific for a signature peptide of Tg combined with LC/MS using a vetted dual-pump trapping ionKey/MS configuration provides a sub 1.52 amol/µL (1 ng/mL) quantification limit of Tg protein with a cycle time of 6.75 min. This quantification limit is comparable with the best in literature for standard flow. However, the ionKey/MS methodology outlined above also offers a few tangible benefits to the standard flow method including; a simplified enrichment procedure, the use of ten times less starting plasma prior to enrichment, an injection volume that is two times smaller, and significantly less solvent consumption. Additionally, evidence is provided in a head-to-head comparison with standard flow in which the microflow approach offers highly correlated PAR measurements while being significantly more precise across 4 replicate measures. Furthermore, the cycle time on the microflow system is only 0.25 min longer than the standard flow method outlined in the literature allowing a similar number of samples to be run in the same time frame but with higher analytical sensitivity and lower sample volumes.

We therefore conclude that the ionKey/MS System operating in the dual-pump trapping configuration does provide acceptable LLOQ levels for Tg using significantly less plasma while maintaining the requisite accuracy, better precision, and throughput levels exemplified by standard flow LC-MS methods. Accordingly, microflow is a viable and attractive solution for clinical research.

References

1. Lame, M.E., Chambers, E.E., Improving a High Sensitivity Assay for the Quantification of Teriparatide in Human Plasma Using the ionKey/MS System, Library Number APNT134788933, PN 720004948EN.

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Waters Corporation
34 Maple Street
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