Evaluation of Sample Preparation Options for the Simultaneous Extraction of Angiotensin and Aldosterone Prior to UHPLC-MS/MS Analysis.

Katie-Jo Teehan[1], Lee Williams[1], Alan Edgington[1], Adam Senior[1], Rhys Jones[1], Helen Lodder[1], Geoff Davies[1], Steve Jordan[1], Claire Desbrow[1], Paul Roberts[1] & Ryu Konoshita[2]

[1]Biotage GB Limited, Distribution Way, Dyffryn Business Park, Ystrad Mynach, Cardiff, CF82 7TS, UK
[2]Shimadzu Europa GmbH, Albert Hahn Strasse 6-10, Duisburg, Germany

Introduction

Traditionally the analysis of aldosterone and angiotensin (for plasma renin activity measurement) are performed separately. However, the relationship of the aldosterone-to-renin-ratio is a very useful tool to help define the cause of secondary hypertension. This poster compares sample preparation options for the simultaneous extraction of Angiotensin I, II and Aldosterone from plasma. LC-MS/MS parameters were investigated for increased sensitivity: MRM transitions, chromatography and mobile phase additives for use with positive and negative ionisation modes. Particular emphasis was placed on the sample preparation to provide high reproducible recoveries whilst minimizing matrix effects and co-extracted materials such as proteins and phospholipids.

Experimental

Reagents

Standards, ammonium acetate and ammonium fluoride were purchased from Sigma-Aldrich Company ltd. (Gillingham, UK). LC/MS grade solvents were from Honeywell Research Chemicals (Bucharest, Romania). Water (18.2 MΩ.cm) was drawn fresh daily from a Direct-Q 5 water purifier (Merck Millipore, Watford, UK). Pooled human plasma was from The Welsh Blood Service (Pontyclun, UK) and TCS Biosciences Ltd. (Buckingham, UK).

Sample Preparation

Incubation: 250 µL of plasma was incubated at 37 °C for 1 hour in the presence of 50 µL of incubation buffer. Full buffer composition was based on previously reported methods and available on request. Post incubation quenching of the reaction was performed using 10 µL of 10% (v/v) formic acid aq.

Extraction: Extractions were developed using polymer-based SPE in 96 fixed well plate formats.

EVOLUTE® EXPRESS ABN, CX and AX were used in 30 mg formats (P/N 600-0030-PX01, 601-0030-PX01 and 603-0030-PX01).

Full method optimization was performed for each sorbent chemistry, with final extraction protocols detailed in ***Table 1***.

Table 1. *Optimized Extraction Protocols.*

Step	EXPRESS ABN 30 mg	EXPRESS CX 30 mg
Condition	MeOH 1000 µL	MeOH 1000 µL
Equilibration	0.1% NH_4OH (aq) 1000 µL	0.1% Formic Acid (aq) 1000 µL
Sample load	300 µL Incubated plasma	300 µL Incubated plasma
Wash 1	0.1% NH_4OH (aq) 1000 µL	0.1% Formic Acid (aq) 1000 µL
Elution 1	400 µL 2% NH_4OH (50:50 H_2O:MeOH)	600 µL 98:2 EtOAc:IPA
Wash 2	-	MeOH 1000 µL
Elution 2	500 µL EtOAc	400 µL 2% NH_4OH (50:50 H_2O:MeOH)

Post extraction: Extracts were evaporated at 40 °C and reconstituted in 200 µL of 0.2% acetic acid (50:50 H_2O:MeOH) prior to injection.

UPLC Conditions

Instrument: Shimadzu Nexera UHPLC (Shimadzu Europa GmbH, Duisburg, Germany)
Column: ACE EXCEL C18 1.7 µm 50 x 2.1 mm + guard (ACT, UK)
Mobile phase: A, 0.2 mM NH_4F (aq); B, MeOH
Flow rate: 0.3 mL/min **Gradient:** Shown in ***Table 2***
Column temp: 30 °C **Injection volume:** 10 µL

Table 2. MRM Parameters.

Time (min)	% A	% B
0	60	40
1.5	60	40
3.5	25	75
4	20	80
4.1	60	40

Mass Spectrometry

Instrument: Shimadzu 8060 Triple Quadrupole mass spectrometer equipped with and ES interface for mass analysis (Shimadzu Europa GmbH, Duisburg, Germany). Positive or negative ions were acquired in the MRM mode (***Table 3***).
Heat Block Temp: 500° C **Interface Temp:** 400° C
DL Temp: 150° C
Nebulizing Gas: 3 L/min **Drying Gas:** 5 L/min
Heating Gas: 15 L/min **CID Gas:** 270 kPa

Table 3. MRM Parameters *(qual ions in parenthesis).*

Analyte	Transition (MRM)	Ionization Mode	Collision Energy (eV)
Angiotensin II	523.85 > 263.3	+	-21
Angiotensin I	433.0 > 110.0 (433.0 > 647.0)	+	-27 -17
Aldosterone d4	363.1 > 189.3	-	19
Aldosterone	359.1 > 189.3 (359.1 > 331.1)	-	20 16

Results

Evaporation Optimization

Peptides and steroids can exhibit non-specific binding effects to plastics during evaporation. ***Figure 1***. demonstrates the complexity of evaporation effects for this assay. Recovery (signal spiked before/after evaporation) depicts binding effects while overall signal (signal spiked before/standard) takes into account suppression effects.

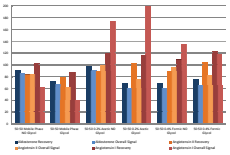
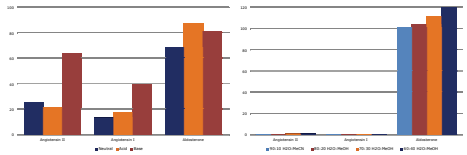


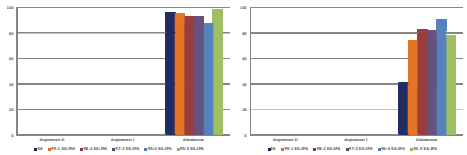
Figure 1. *Evaporative investigation.*

SPE development was carried out using ABN sorbent plates. ***Figure 2.*** Illustrates the effect of pH on the extraction. Due to differing polarities between aldosterone and angiotensin we were able to investigate analyte elution into dedicated fractions. ***Figure 3.*** demonstrates aldosterone elution with EtOAc after washing with various H_2O/MeOH ratios. Similar results were observed using CX (data not shown).



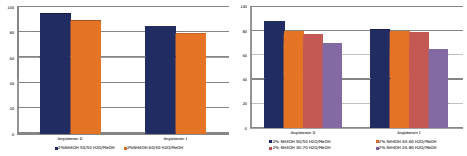
Figures 2 and 3. *pH and wash investigation using ABN.*

Various water immiscible organic solvents were investigated for the elution of Aldosterone. EtOAc performed best for ABN. However 2% IPA was required when using CX as demonstrated in ***Figures 4 and 5.***



Figures 4 and 5. *Aldosterone elution optimization using ABN and CX.*

Due to higher polarity of angiotensin, elution required pH modified H_2O/MeOH combinations. ***Figures 6 and 7.*** demonstrate elution solvent performance for ABN and CX respectively.



Figures 6 and 7. *Angiotensin elution optimization using ABN and CX.*

These solubility differences allowed fractionation of angiotensin and aldosterone in different orders as well as the ability to combine fractions. ***Figure 8.*** compares performance between these methods.

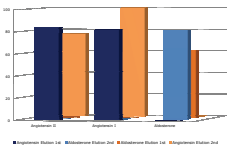
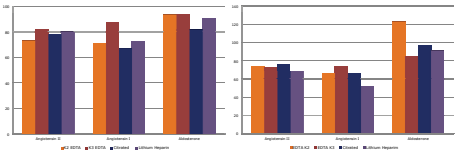


Figure 8. *Various elution order performance using ABN.*

Figures 9 and 10. demonstrates ABN and CX performance with plasma containing various anti-coagulants.



Figures 9 and 10. *ABN and CX using various anti-coagulants.*

Figure 11. demonstrates final optimized extraction recovery performance for the various SPE protocols.

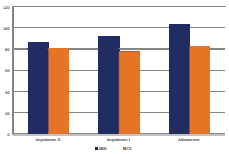


Figure 11. *Recovery profiles for optimized protocols.*

Figure 12. compares extract cleanliness in terms of efficiency of phospholipid removal. Both optimized elution fractions for EVOLUTE® EXPRESS CX and EVOLUTE® EXPRESS ABN were compared to a protein precipitated sample.

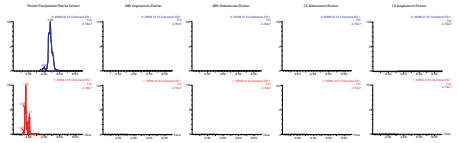


Figure 12. *Phospholipid removal using optimized extraction protocols.*

Calibration curves were constructed in human plasma from 10-10000 pg/mL. Good linearity and coefficients of determination, r^2 were obtained for EVOLUTE® EXPRESS ABN and CX protocols both delivering LOQs below the required levels.

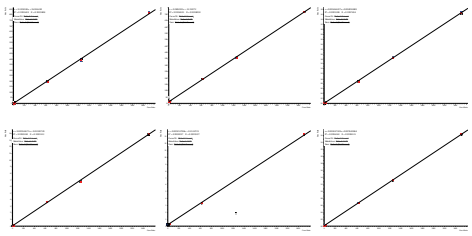


Figure 13. *Calibration curve performance (Angiotensin II, Angiotensin I, Aldosterone) for ABN (top) and CX (bottom).*

Conclusions

- This poster presents multiple optimized solid phase extraction approaches to low level analysis of Aldosterone and Angiotensin.
- Good recoveries, LOQs and linearity were obtained while demonstrating removal of matrix interferences such as phospholipids.
- The optimized protocol using EVOLUTE® EXPRESS ABN provided very subtle enhancements in performance over the CX.

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