The application of Capillary Electrospray Ionization to the detection of Neuropeptides

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INTRODUCTION

Standard reverse phase liquid chromatography separates constituents based on their differential interactions with the column stationary phase and an organic mobile phase. CESI-MS is a different separation technique and is the integration of CE and ESI into one dynamic process and provides highly efficient peptide and protein separations based on their size and charge (1), therefore offering different selectivity compared to reverse phase liquid chromatography. The ionization is at the ultralow nanoflow regime (25-100 nL/min) and it uses an open capillary which eliminates some of the challenges of stationary phase based separations such as lack or retention of small polar peptides as well as over retention of larger, basic or hydrophobic peptides (Figure 1).

Vasoactive intestinal peptide (VIP) is an important peptide hormone and a neuropeptide containing 28 amino acid residues and is involved in biological functions such as stimulated contractility in the heart, vasodilation and relaxation of the smooth muscle. Pituitary adenylate cyclase-activating polypeptide also known as PACAPA is similar to VIP. One of PACAP effects is to function as a neurotransmitter and neuromodulator. Both peptides have been shown to be important neuropeptides.

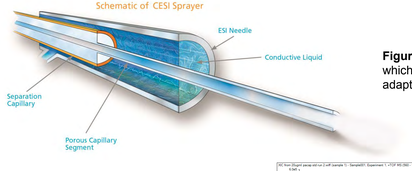

Figure 1. Schematic for CESI Sprayer which connects to MS systems via an adapter fitted to a nano spray source

His-Ser-**Asp**-Ala-Val-Phe-Thr-**Asp**-Asn-Tyr-Thr-**Arg**-Leu-**Arg**-**Lys**-Gln-Met-Ala-Val-**Lys**-**Lys**-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH2

VIP sequence, molecular weight = 3325.8

His-Ser-**Asp**-Gly-Ile-Phe-Thr-**Asp**-Ser-Tyr-Ser-**Arg**-Tyr-**Arg**-**Lys**-Gln-Met-Ala-Val-**Lys**-**Lys**-Tyr-Leu-Ala-Ala-Val-Leu-Gly-**Lys**-**Arg**-Tyr-**Lys**-Gln-Arg-Val-**Lys**-Asn-**Lys**-NH2

PACAP sequence, molecular weight = 4534.26

Figure 2: VIP and PACAP amino acid sequence with basic residue in bold and acidic residues in red.

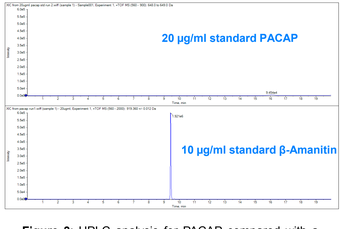

Figure 3: HPLC analysis for PACAP compared with a standard peptide (Gradient from 5 to 100% acetonitrile).

MATERIALS AND METHODS

Chemicals: All chemicals were Reagent Grade and were purchased from Sigma Aldrich including standards of VIP and PACAP-38.

Sample Preparation: Standards were prepared by initially dissolved the solid peptides into water to make 0.1mg/ml concentration standards in the vials supplied by Sigma. These concentrated standards were then serial diluted into a mixture of 45:5:50 Ethanol : Acetic Acid : 100 mM acetate. Spiked calf serum was used as a model matrix and prepared using solid phase extraction as shown in Figure 4.

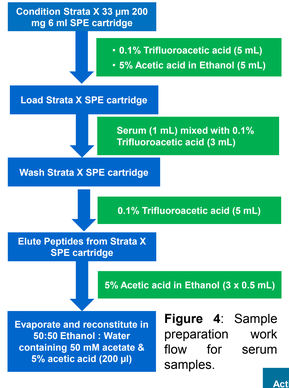

Figure 4: Sample preparation work flow for serum samples.

CESI-MS method: The sample was injected by pressure (5 psi, 30 s) onto a 30 µm ID x 91 cm bare-fused-silica capillary (including porous spray tip) housed in an OptiMS CESI cartridge. The capillary was covalently coated with Polyethylenimine (PEI) (2) and thermostatted using recirculating liquid coolant regulated at 25 ºC. For this analysis, the SCIEX QTRAP® 5500 LC-MS/MS system was fitted with the NanoSpray® III source. Gas 1 and 2 were not used and temperature set low (50 ^{0}C) as ionization at these very low flow rates occurs by simply applying the ion-spray voltage was set to 1600 V. The curtain gas was set low (5psi) and other MS conditions are shown in Table 1. The MS method was split into 3 periods to cover the CE separation, in the first period and last period (1 minute each) the ionspray voltage was set to zero, this was the only difference in the MS conditions. The CE separation used the condition shown in Table 2 with a background electrolyte (BGE) of a mixture of 5% Acetic acid : 30% Acetonitrile : 65% Water. The voltage used for the separation was in reverse polarity.

Curtain gas	5 psi	Peptide	Q1 Mass	Q3 Mass	CE (V)
Gas 1	0 psi	PACAP	567.6	671.8	23
Gas 2	0 psi	PACAP	567.6	647.7	23
ISV	1600V	VIP	666	663	24
DP	80 V	VIP	666	771.5	27
EP	10 V				
CXP	13 V	Resolution	Q1 Low	Q3 Unit	
Heater	50 C				

Table 1: MS conditions

Table 2: CESI separation method

Action	Time (mins)	Pressure (psi)	Direction	Voltage (kV)	Solution	Description
Rinse	1	100	Forward	0	Water	Wash
Rinse	3	50	Forward	0	1 Molar NaCl	Condition
Rinse	0.5	100	Forward	0	Water	Wash
Rinse	2	100	Forward	0	BGE	Capillary Fill
Rinse	0.5	75	Reverse	0	10% Acetic acid	Conductive Fill
Injection	0.5	5	Forward	0	Sample Vial	Injection
Injection	0.5	0.5	Forward	0	BGE	Push
Sep.	15	1	Forward	20	BGE	Sep
Voltage	3	5	Forward	1	BGE	Ramp down

RESULTS AND DISCUSSION

Analytes are separated in CESI based on their mobility (m/z) and elute in HPLC based on hydrophobic interactions with the stationary phase of the column so CESI is a orthogonal technique to LC. Before any CESI method development was attempted, a high level standard of PACAP was first analyzed by a standard reverse phase LCMS method using a mobile phase containing 0.1% formic acid and a gradient from 2% acetonitrile to 100% acetonitrile on a small particle C18 reverse phase column. Figure 3 shows that PACAP was retained on the C18 reverse phase column unlike a marker peptide. This was due to the basic hydrophobic nature of these peptides. Due to the nature of these peptides a coated capillary was essential otherwise the peptides would bind to the silanol groups on the bare fused silica capillary. A PEI coated capillary (+ve charge) for CESI-MS achieved baseline separation of the two peptides due to their size difference with a migration difference of over 1 minute. Figure 5 shows the low level detection of VIP and PACAP in standards with both peptides easily detected at a 2 ng/ml level.

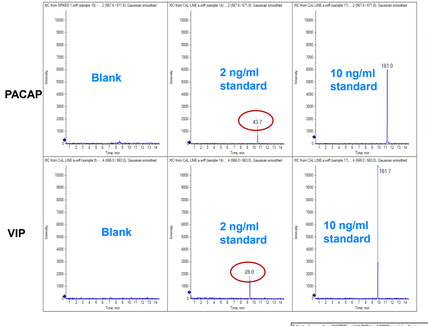

Figure 5: CESI-MS/MS electropherogram of a solvent blank, 2 ng/ml and 10 ng/ml standards of VIP AND PACAP (the peaks are labelled with signal:noise calculated in PeakView®).

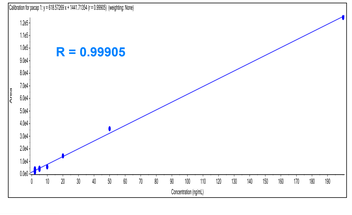

Figure 6: Calibration line for PACAP. The range for the calibration test was 1 – 200 ng/ml and this was also the range for VIP (data not shown here). This corresponded to 200 fMol/mL - 40 pMol/mL.

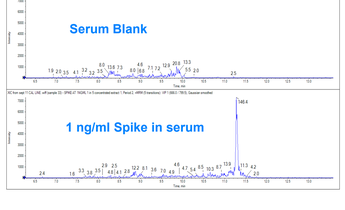

Figure 7: CESI-MS/MS electropherogram for VIP of a serum blank sample compared with a 1 ng/ml serum spiked sample (the peaks are labelled with signal:noise calculated in PeakView® software).

The linearity of response were tested for both VIP and PACAP an example of the result is shown in Figure 6. It was observed that both peptides gave a linear response over the range 1-200 ng/ml as shown by the R value= 0.99905 displayed in Figure 6 for PACAP.

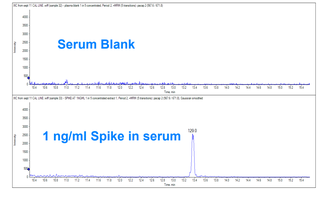

Figure 8: CESI-MS/MS electropherogram for PACAP of a serum blank sample compared with a 1 ng/ml serum spiked sample (the peaks are labelled with signal:noise calculated in Peak View® software).

Serum samples were then spiked with PACAP and VIP, extracted and reconstituted. Figure 7 shows the result for the primary MRM for VIP and it clearly shows the detection of VIP at this level in serum. For PACAP again the signal:noise obtained exceeded 100:1 (Figure 8) for the primary MRM transition. In these early tests PACAP recoveries, from the SPE protocol, were calculated to be in the range of 80 -110%.

CONCLUSIONS

In this early study CESI-MS/MS was used for detection and quantitation of PACAP & VIP in the same method as compared to conventional RPLC-MS analysis methods which are not able to.

Using 1 mL of plasma and a simple solid phase extraction protocol, a 1 ng/mL spike into serum could be detected for both PACAP and VIP with no peak observed in the serum blank. As the signal to noise currently observed is at least 10 fold higher than an expected LOD level, and the SPE extraction involved just a 1 in 5 concentration step, there is a large room to get even lower levels of detection for both peptides. This can be done by simply reducing the volume of the final extract to 50 µl or below. In addition, as this is a MS based detection method it can be further expanded to include other neuropeptides. For example, PACAP-27 or PACAP metabolites to further study the role of these peptides in biological systems.

FUTURE WORK

Recently this method has been transferred to a neutrally coated capillary which is commercially available. Work is currently underway to investigate different injections techniques to further reduce the detection limits by either the addition of more salt to the sample to improve isotachophoresis or try to use electrokinetic injection instead of a pressure injection. Further to this, additional peptides (such as oxytocin and PACAP-27) are currently being added to this method.

REFERENCES

1. Busnel, J-M., et. al., “High Capacity Capillary Electrophoresis-Electrospray Ionization Mass Spectrometry: Coupling a Porous Sheath less Interface with Transient-Isotachophoresis”, Anal. Chem. 82 9476-9483 2010.
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Document number: RUO-MKT-10-3951-A.