# Using electrokinetic injection to increase throughput and improve sensitivity in the detection of basic neuropeptides by CESI-MS

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## INTRODUCTION

There are several important basic neuropeptides which include Vasoactive intestinal peptide (VIP), Pituitary adenylate cyclase-activating polypeptide (PACAP) and Parathyroid Hormone (PTH). These peptides are generally very basic (with isoelectric points > 10) and are difficult to analyze by LC-MS methods as they bind to auto-sampler components and columns resulting in very poor chromatographic results. CESI is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process in a single device. CESI-MS operates at low nL/min flowrates offering several advantages including increased ionization efficiency and a reduction in ion suppression. In this work we describe the use of the electro-kinetic injection technique to improve sensitivity in a CESI-MS method for the analysis of these intact peptides.

## MATERIALS AND METHODS

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

**Sample Preparation:**
Standard stocks were prepared by dissolving the solid peptides into water to make 0.1 mg/mL concentration standards. These stocks were serially diluted into different solvents (see results).

**CESI-MS method:** Samples were injected by pressure using transient isotachophoresis (tITP, 5 psi, 99s) or injected by electrokinetic supercharging (EKS, 99 s, 10 kV) onto a neutrally coated capillary. The capillary was thermostatically controlled using recirculating liquid coolant at 25ºC. For this analysis, a SCIEX QTRAP® 6500 system was fitted with the NanoSpray® III source. Gas 1 and 2 were not used and the temperature was set low (50 ºC) as ionization at these very low flow rates occurs by simply applying the ion-spray voltage. The curtain gas was set low (5 psi) 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 and last periods (1 minute each) the ionspray voltage was set to zero.

The CE separation was dependent on the injection used and conditions are shown in Table 2 and 3. Both methods used the same BGE which was 1% formic acid : 20% Methanol: 79% water).

| | | | | | |
|---|---|---|---|---|---|
| Curtain gas | 5psi | | 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 | 1600 V | | VIP | 666 | 663 | 24 |
| DP | 80 V | | VIP | 666 | 771.5 | 27 |
| EP | 10 V | | | | | |
| CXP | 13 V | | Resolution | Q1 Unit | Q3 Unit | |
| Heater | 50°C | | | | | |

**Table 1.** MRM conditions used for both LC-MS and CESI-MS methods

| Action | Time | Pressure (psi) | Direction | Voltage (kV) | Solution |
|---|---|---|---|---|---|
| Rinse | 2.5 min | 100 | Forward | 0 | 0.1 Molar HCl |
| Rinse | 3 min | 100 | Forward | 0 | BGE |
| Rinse | 0.75 min | 75 | Reverse | 0 | 10% Acetic Acid |
| EK plug | 60s | 0.5 | Forward | 0 | 250mM Ammonium acetate |
| EK plug | 10s | 0.5 | Forward | 0 | Water |
| EK injection | 99s | *0* | Forward | 10 | Sample Vial |
| Separation | *25 min* | 5 | Forward and reverse ramp 2 minutes | 25 | BGE |
| Separation | *2* | 100 | Forward and reverse | 10 | BGE |
| Voltage Ramp down | 2 | 10 | Forward | 1 | BGE |

**Table 2.** CE method used for EKS injections.

| Action | Time | Pressure (psi) | Direction | Voltage (kV) | Solution |
|---|---|---|---|---|---|
| Rinse | 2.5 min | 100 | Forward | 0 | 0.1 Molar HCl |
| Rinse | 3 min | 100 | Forward | 0 | BGE |
| Rinse | 0.75 min | 75 | Forward | 0 | 10% Acetic Acid |
| tITP injection | 99s | *5* | Forward | 10 | Sample Vial |
| Separation | *25 min* | 5 | Forward and reverse ramp 2 minutes | 25 | BGE |
| Separation | *2 min* | 100 | Forward and reverse | 10 | BGE |
| Voltage Ramp down | 2 min | 10 | Forward | 1 | BGE |

**Table 3.** CE method used for tITP injection method

| Time (min) | % A | % B |
|---|---|---|
| 1.0 | 95 | 5 |
| 10.0 | 50 | 50 |
| 12.0 | 5 | 95 |
| 13.0 | 5 | 95 |
| 13.1 | 95 | 5 |
| 16.0 | 95 | 5 |
| | | |

**Table 4.** LC-MS gradient conditions used for analyses

**LC-MS method:** Samples were injected onto an AERIS PEPTDIE 100 x 2.1mm 1.7 µM XB-C18 COLUMN which has been developed for peptide quantitation and was held at 40ºC for all injections. Mobile phase A was water containing 0.1% formic and mobile phase B was acetonitrile containing 0.1% formic acid. For the LC-MS analysis the IonDrive™ Turbo V source was used as separations were run at 300 µL/min. The temperature of the MS source was 550 ºC, curtain gas 30 psi, gas 1 was 50 psi, gas 2 was set at 60 psi and the ionspray voltage was 4500V. The other MRM conditions were the same as used for the CESI-MS analyses. The LC system used was a Nexera system from Shimadzu.

## RESULTS

Before the EKS technique was compared with tITP in CESI-MS several factors were tested. When using EKS both the salt content and the level of organic solvent has an effect on the peak height observed. In Figure 1 and 2 both of these factors were investigated for both PACAP-38 and VIP. It was found that when the acid concentration was too high the sensitivity dropped dramatically so for this technique it is recommended that low levels of acetic acid <0.1% are used for the sample solvent. Also for reconstitution of samples an organic solvent is often required and for these two peptides acetonitrile was found to be the best (Figure 2). The level of this organic in the sample also had an effect on the response and a level of 50 -75% was found to the best. If 100% organic was used then the conductivity of the sample is low and the peak height of an injection drops.

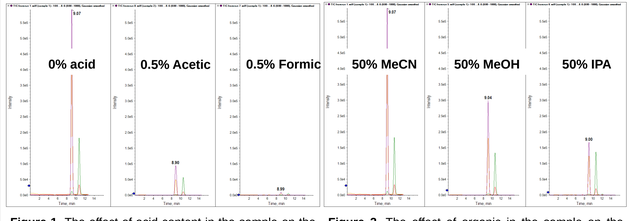


**Figure 1.** The effect of acid content in the sample on the sensitivity. In this figure both PACAP-38 (first peak) and VIP (second peak) are shown.

**Figure 2.** The effect of organic in the sample on the sensitivity. In this figure both PACAP-38 (first peak) and VIP (second peak) are shown.

Using a sample solvent of 50% acetonitrile containing 0.05% acetic acid a standard of PACAP-38 was compared with an injection of the same concentration of PACAP-38 in mobile phase A containing 5% acetonitrile (Figure 3) and also with a sample of PACAP-38 in 50% acetonitrile containing 100 mM ammonium acetate used for isotachophoresis (Figure 4).

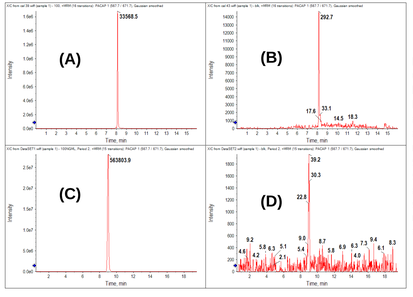


**Figure 3.** A carryover comparison of CESI-MS vs LC-MS all peaks are labelled with signal to noise for PACAP-38. (A) 10 ng/mL standard analysed by LC-MS. (B) solvent blank analysed by LC-MS after the 10 ng/mL standard. (C) 10 ng/mL standard analysed by CESI-MS. (D) solvent blank analysed by CESI-MS after the 10 ng/mL standard. Any signal due to PACAP-38 was later removed by dipping the capillary into acetonitrile.

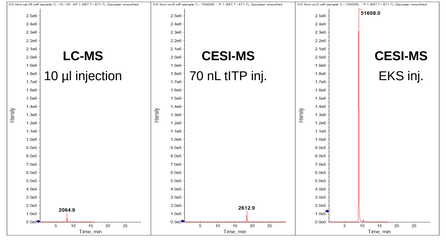


**Figure 4.** Comparison of a LC-MS 10 µL injection of a 10 ng/mL standard with EKS and a tITP injection by CESI-MS all peaks are labelled with signal to noise for PACAP-38.

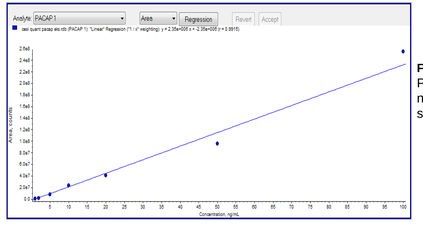


**Figure 5.** Calibration Line for PACAP-38 range was 1 – 100 ng/mL. In this example no internal standard has been used.

When EKS injection was compared with the standard LC-MS approach (Figure 3) the first noticeable difference was that the EKS injection was more sensitive than the LC-MS analysis also when a blank was injected, after the 10 ng/mL standard, the carryover with CESI-MS was only 0.0069% compared 0.87% with LC-MS which used the standard auto-sampler wash cycle. The small amount of carry over seen in CESI-MS was later removed by added a dip step into acetonitrile (10s) after the EK injection and was attributed to PACAP-38 on the outside of the capillary.

When the EKS injection was compared with the tITP injection the tITP injection gave a similar response to the LC-MS analyses (Figure 4) but was slower than the EKS approach. The EKS approach was further shown to give a linear response over the range tested (Figure 5) even though no internal standard was used and when it was tested on an SPE extract (Figure 6) the response was equally high even though the current SPE recovery was only in the range of 10-20% and so still needs more development. When compared to the tITP previously tested1 the EKS injection was 10 times more sensitive with a signal to noise of 1708.

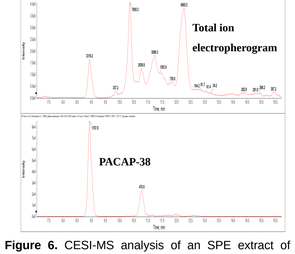


**Figure 6.** CESI-MS analysis of an SPE extract of a 1 ng/ml PACAP-38 spike into human plasma. The initial SPE protocol was based on the method previously developed[3] except the reconstitution solvent was 50% acetonitrile containing 0.05% acetic acid.

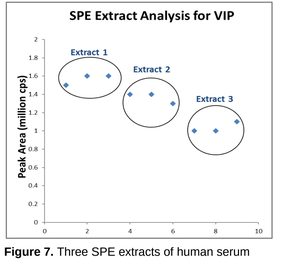


**Figure 7.** Three SPE extracts of human serum samples spiked with 1 ng/mL of VIP, injected separately by EKS. This shows reproducibility by area even without the use of internal standards.

## CONCLUSIONS

- CESI is not hampered by hydrophobic absorptive losses and carryover as in conventional LC workflows.
- High resolution, high sensitivity results were achieved using CESI-MS without sacrificing run time and with zero-carryover.
- CESI-MS was able to separate & detect PACAP-38 and VIP in serum samples reproducibly.
- Initial tests show new EKS method is 10x more sensitive on biological extracts compared to a previous tITP injection approach.
- Electrokinetic supercharging technique further improves detection limits of basic peptides by a factor of 20 fold compared to traditional LC-MS approach.

## REFERENCES

1. Lock, S. 'The application of Capillary Electrospray Ionization to the detection of Neuropeptides'. Conference Proceedings ASMS 2016, San Antonio, June 5-9, 2016.

## TRADEMARKS/LICENSING

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