Bioanalysis of Dermorphin, an Opioid Peptide in Equine Urine:

Benefit of the VIP-Heated ESI Technology for the Quantitative Analysis of Thermally Labile Molecules

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Dermorphin is an opioid hepta-peptide derived from the skin secretions of South American frogs (*Phyllomedusa sauvagei*)[1]. It is 30-to-40 times more potent an analgesic than morphine[2] and is unique because it contains D-alanine, a property that makes it highly resistant to protease mediated degradation (Figure 1). While d-amino acid peptides are sought after for their low immunogenic response and in peptidomimetic drug design, it was dermorphin's use in horse racing that catapulted this opioid peptide from obscurity into the headlines. The pain-killing properties of dermorphin were used to drive racehorse performance beyond normal physical limits often injuring the animal. As a result, dermorphin is now a Class I prohibited substance as decreed by the Association of Racing Commissioners International (RCI) Model Rules[3].

The new EVOQ™ liquid chromatography triple quadrupole mass spectrometer (LC-TQ) has several innovations that make high-sensitivity, high-flow rate quantitative analysis simpler, robust, and more routine. To deliver high-sensitivity at high-flow rates (>400uL/min) modern electrospray ionisation (ESI) probes use heated nitrogen as a nebulising gas to enhance droplet size reduction; the smaller the droplet, the higher the sensitivity. The key concept is to deliver thermal energy during the ionisation process that occurs externally at the probe tip. A good design avoids detrimental heat transfer to the liquid within the body of the electrospray probe, which results in thermal degradation of the molecule in solution before it is ionised. The VIP Heated-ESI (VIP-HESI) probe design on the new EVOQ LC-TQ uses shaped-vacuum technology as an insulating thermal barrier between the hot nitrogen gas and the LC-eluent (Figure 2). The vacuum stops conductive transfer of thermal energy to the liquid within the probe, and the heat is delivered to the tip of the probe where the ionisation occurs. This unique design efficiently ionises thermally labile molecules such as metabolites or peptides at conventional flow-rates, making it easier for automated, high-throughput analysis of real-world samples. This paper discusses the benefits of using VIP-HESI technology for the quantitative analysis of dermorphin in equine urine, using deltorphin-II as an analogue internal standard.

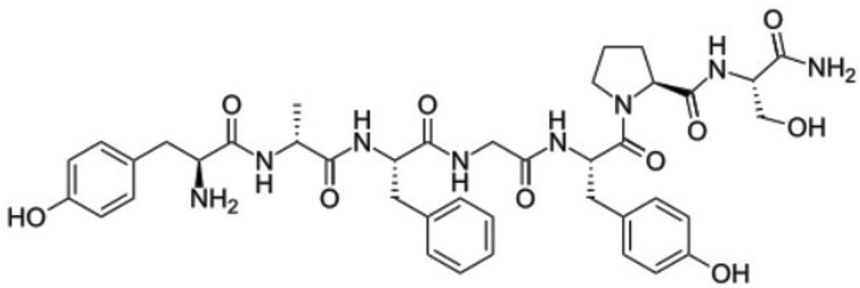

Figure 1: Chemical structure of dermorphin, a naturally occurring opioid, hepta-peptide derived from the skin secretions of South American frogs (Phyllomedusa sauvagei).

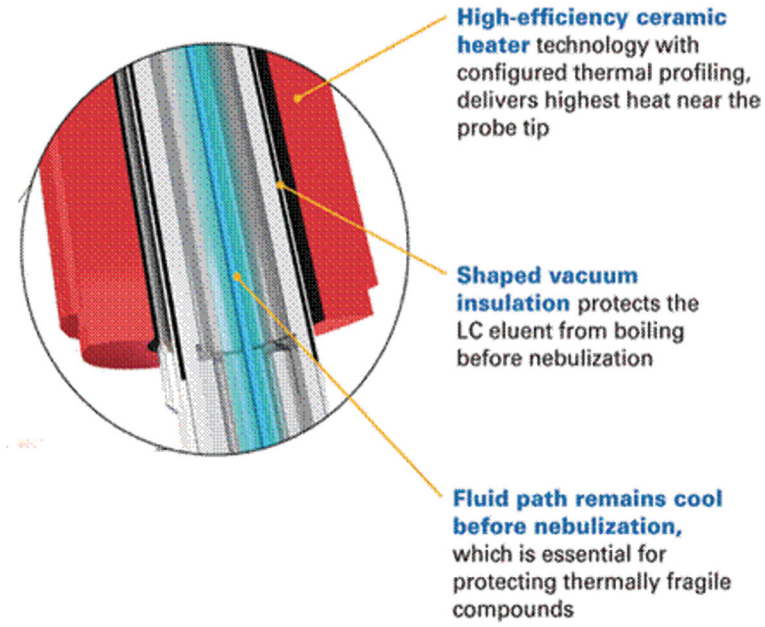

Figure 2: Cut-away of the EVOQ VIP Heated-ESI showing the novel shaped-vacuum technology that protects the LC-eluent from heat.

QC	Area Ratio	Response Factor
Low QC	0.01000	0.03335
Low QC	0.01031	0.03438
Mid QC	0.22314	0.03719
Mid QC	0.17834	0.02972
High QC	1.67945	0.03359
High QC	1.62102	0.03242

Table 1: The response factors for the QC's had an RSD of <7% RSD indicating superior sensitivity for the assay across the entire calibration range.

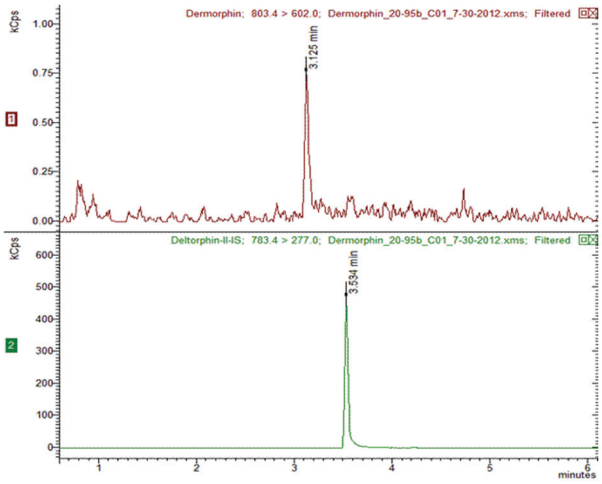

Figure 3. Shows the LLOQ for dermorphin (0.1ng/mL) in equine urine.

Experimental

Chromatography (Advance HPLC)

- Column: ACE C18, 3µ, 2.1x100mm (ACE, MAC-MOD Analytical Inc., PA)
- Injection volume: 40µL
- Flow rate: 0.4mL/min
- Mobile phase A: Water with 0.2% Formic acid
- Mobile phase A: Acetonitrile with 0.2% Formic acid
- Gradient conditions:
  - 0.00min 95% A
  - 0.20min 95% A
  - 8.00min 5% A
  - 8.50min 5% A
  - 8.51min 95% A
  - 12.50min 95% A

Mass Spectrometry (EVOQ Elite)

- VIP Heated-ESI Temp: 350°C
- Heated gas: 70 units
- Nebuliser gas: 70 units
- Cone gas temp: 250°C
- Cone gas: 10 units
- Spray voltage: 4000v
- Active exhaust: On
- Dermorphin ($C_{40}H_{50}N_8O_{10}$) transitions
  - m/z 803→ 202 (CE:32v, quantifier ion)
  - m/z 803→602 (CE:20v)
  - m/z803→325 (CE:40v)
  - m/z803→352 (CE:35v)
- Deltorphin-II (IS) transitions
  - m/z 783→227→602 (CE:40v)
  - m/z 783→119→ 202 (CE:65v)
  - m/z 783→325 (CE:45v)

Dermorphin standards and extracts were provided courtesy of Equine Analytical Chemistry Laboratory, UC Davis, Davis, CA.

Results and Discussion

Despite the extensive cleanup afforded by solid-phase extraction, separation of dermorphin and deltorphin-II required the use of shallow 13min gradient at a flow rate of 400uL/min. The separation was necessary because of the nature of matrix; equine urine is a viscid, foamy, solution containing excessive levels of salts (carbonates and phosphates) and mucus (glycoproteins, lipids, immunoglobulin)[5]. The lack of an isotope labelled internal standard makes quantitative analysis even more challenging, however ensuring adequate separation from the ion suppression causing matrix components can be largely avoided through good chromatography. Accurate quantification of dermorphin in equine urine between the range of 0.1-1000ng/mL in equine urine was easily achieved on the EVOQ Elite. Figure 3 shows the Signal to Noise ratio (S/N) of the Lower Limit of Quantitation (LLOQ) of 0.1ng/mL dermorphin in equine urine showing the high-sensitivity performance of the EVOQ Elite with the VIP Heated-ESI probe.

The calibration curve (Figure 4) had a response factor relative standard deviation (RSD) of 12% RSD. The response factors for the QC's had a RSD of 7% RSD and are shown in Table 1.

The analysis of thermally labile compounds such as peptides is an important requirement for LC-MS/MS analysis and the innovative design of the VIP Heated-ESI on the EVOQ LC-TQ provides new capabilities to quantify thermally labile molecules.

Acknowledgements

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References

1. Broccardo, M. et al. Br. J. Pharmac. 1981, 73, 625-631.
2. Kreil, G. J. Biol. Chem. 1985. 269 (15): 10967–70.
3. Bogdanich W (19 June 2012). "Turning to Frogs for Illegal Aid in Horse Races". New York Times.
4. Shaped-vacuum technology is a trademark of the Concept Group, NJ, USA.
5. Wong, A. et al. Drug Testing and Analysis. 2005. Vol 4, No. 5. 355-361.
6. Brunt, C. (30 Aug, 2012). Test Confirm Use of Dermorphin. Albuquerque Journal.

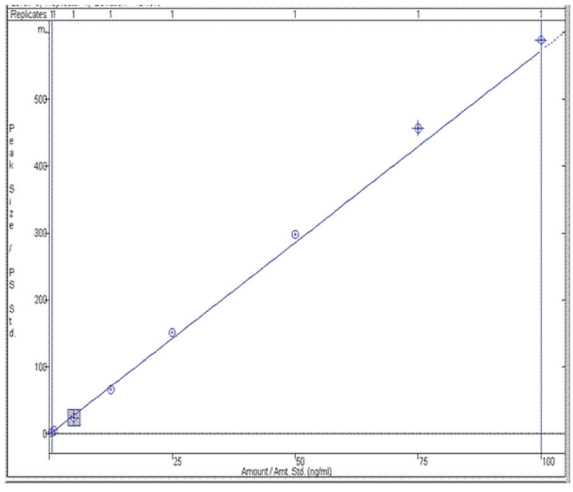

Figure 4. Calibration curve in the range 0.1-100ng/mL of dermorphin in equine urine.