Development of a high-resolution mass spectrometric immunoassay (MSIA) for human hepcidin

Handley SA [1,2], Couchman L [1], Sharp P [2], Niederkofler EE [3], Moniz CF [1]
[1] Toxicology Unit, Clinical Biochemistry, King's College Hospital, London, SE5 9RS;
[2] Diabetes and Nutritional Sciences Division, King's College London, London, SE1 9NH; [3] ThermoFisher Scientific, Tempe, AZ, USA

Introduction

- Hepcidin is a 25-amino acid polypeptide (MW 2789) that is considered to be the key regulator of systemic iron homeostasis.
- Measurement of hepcidin may aid the differential diagnosis of iron deficient anaemia, or anaemia of chronic disease, and may also help guide the treatment of anaemia with iron supplementation, erythropoietin or with novel therapies.
- Truncated isoforms of hepcidin-25; hepcidin-20 (MW 2191), -22 (MW 2436), and -24 (MW 2674) have been identified but their role has not yet been defined.
- MSIA involves the immunoenrichment of an analyte on a monolithic microcolumn which is activated with an anti-protein antibody fixed in a Disposal Automated Research Tips (D.A.R.T). The analyte is then eluted, and analysed by LC-MS(MS).
- A number of methods are available for the measurement of plasma hepcidin-25; however, there are concerns over the and poor agreement between methods has been reported
- MSIA has the required selectivity and sensitivity to make it suitable for the measurement of hepcidin-25.
- The aim of this work was to investigate the viability of MSIA for the quantification of hepcidin-25, and its isoforms.

Method

- DART'S were pre-bound with anti-hepcidin-25 antibody.
- Calibration solutions containing hepcidin-25 and isoforms over the range; 1–100 µg/L were prepared in phosphate buffered saline (PBS) containing 10 g/L (v/v) bovine serum albumin.
- Sample (200 µL) was diluted with internal standard solution [hepcidin-25-$^{13}C_{18}$,$^{15}N_3$ (48 µg/L), in HBS-EP buffer, 500 µL], and captured as per **Table 1**.
- Captured analytes were eluted from the DART'S using 33 % (v/v) acetonitrile (aq) containing 0.4 % aqueous trifluoroacetic acid.
- Samples were analysed using a Thermo Scientific™ Transcend™ II system with a Thermo Scientific™ Q-Exactive™ high-resolution mass-spectrometer.
- The following step-wise gradient was used: Starting condition 15 % B, ramped to 100 % B over 5 minutes, held for 1 minute then returned to initial conditions for 3 minutes.
- LC-MS parameters are given in **Table 2**. MS detection was carried out in positive ionisation mode using heated electrospray ionisation [spray voltage 4.5 kV; temperatures: vaporiser 200 ºC; capillary 320 ºC; auxiliary, sheath, and sweep gases 5, 50 and 0 (arbitrary units) respectively, S-lens voltage 100 V]. The C-trap capacity was set at 3 x 10^6 charges. Maximum injection time was 250 ms.
- The peak areas for the six most abundant isotopes of the +3, +4, and +5 charge states (example total ion chromatogram in **Fig. 1**) for each analyte were summed to create an extracted ion chromatogram (**Fig. 2**, mass extraction window: ± 5 parts per million).

Table 1. *Immunocapture procedure.*

Step	Solution	Solution volume (µL)	Number of cycles	Cycle volume (µL)
1 - Wash	Water	200	20	150
2 - Capture	Sample	700	500	250
3 – Wash	PBS	200	20	150
4 – Wash	Water	200	20	150
5 - Elute	Eluent	100	100	75

Table 2. *LC-MS parameters.*

Parameter	Conditions
Column:	ACE C18 (100 x 2.1 mm); 60 ºC
Eluents:	A: 0.1 % (v/v) aqueous formic acid B: 0.1% (v/v) formic acid in acetonitrile Flow-rate 0.25 mL/min
Injection volume:	100 µL
Detection:	Full-scan (resolution 140,000, *m/z* 400–1,000)

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Figure 1. *Total ion chromatogram of hepcidin-25.*

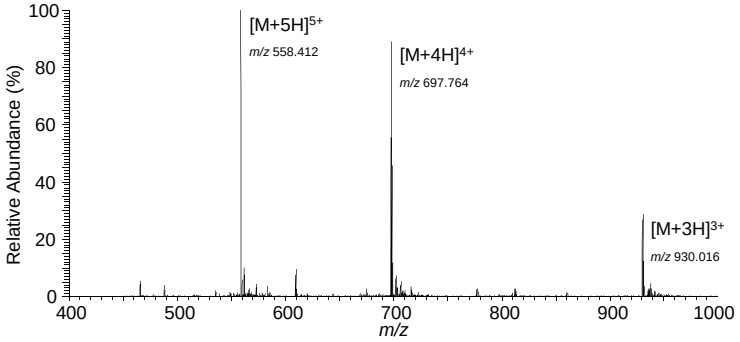


Figure 2. *Extracted ion chromatogram for (A) hepcidin-20, (B) hepcidin-22, (C) hepcidin-24, (D) hepcidin-25, and (E) hepcidin-25-$^{13}C_{18}$,$^{15}N_3$.*

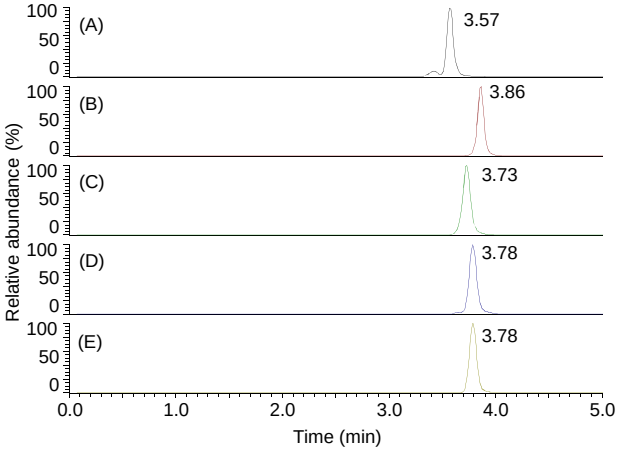
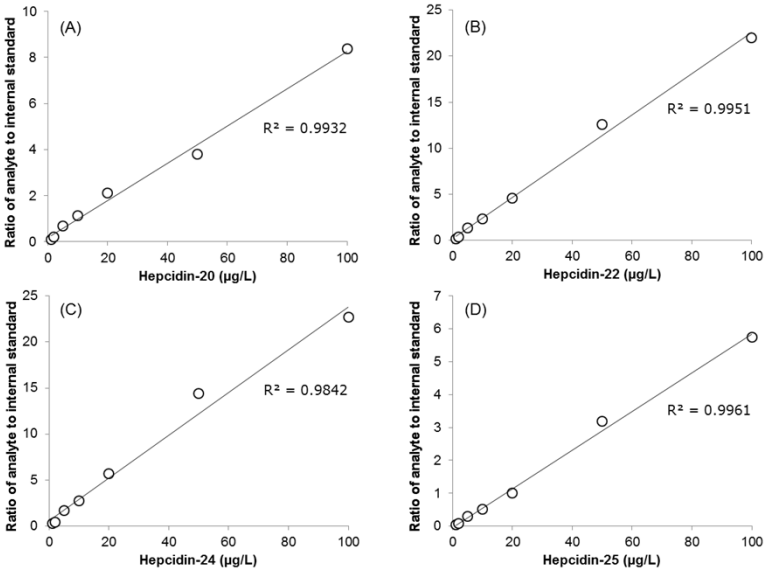


Figure 3. *Calibration curves for: (A) hepcidin-20; (B) hepcidin-22; (C) hepcidin-24; and (D) hepcidin-25.*



Results and Discussion

- All calibrations were linear over the range studied (R_2 > 0.99, **Fig. 3**), and precision (% CV, N = 3) for all analytes at 100 µg/L was < 1 %.
- Overall recovery from dH_20 (all analytes 100 µg/L) was: 56, 60, 73, 62 and 64 %, for hepcidin-20, -22, -24, -25, and the internal standard respectively.
- The proposed method is simple, easily automated, and shows the potential for MSIA in the analysis of hepcidin-25 and isoforms.
- MSIA is an ideal workflow solution for analytes which are present at low concentrations, and where interference from similar compounds is likely with conventional immunoassays.