Development of a novel immunoassay for the iron regulatory peptide hepcidin

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ABSTRACT

To date there have been few published immunoassays for the important iron regulator hepcidin. This study describes a novel competitive radioimmunoassay (RIA) for the bioactive hepcidin peptide. A rabbit anti-hepcidin polyclonal antibody was produced using synthetic hepcidin radiolabelled with ^{125}I to produce a competitive RIA. Normal patient (n=47) samples were collected and assayed for hepcidin to determine a reference range. Other patient groups collected were ulcerative colitis (UC; n=40), iron deficiency anaemia (IDA; n=15), chronic kidney disease not requiring dialysis (CKD; n=45) and chronic kidney disease requiring dialysis (HCKD; n=94). Detection limit of the assay was determined as 0.6 ng/mL. Intra-assay precision was 5 ng/mL (7.2%) and 50 ng/mL (5.8%), inter-assay precision was 5 ng/mL (7.6%) and 50 ng/mL (6.7%). Analytical recovery was 98% (5 ng/mL), 94% (10 ng/mL) and 97% (50 ng/mL). The assay was linear up to 200 ng/mL. No demonstrable cross-reactivity with human insulin, glucagon I, angiotensinogen I, β-defensin 1–4, α-defensin-1 and plectasin was observed. There was significant correlation (r=0.96, P≤0.0001) between the hepcidin RIA and an established hepcidin SELDI-TOF-MS method. Analysis of the normal human samples gave a reference range of 1.1–55 ng/mL for hepcidin. Further statistical evaluation revealed a significant difference between male and female hepcidin levels. There was significant correlation between hepcidin and ferritin in the control group (r=0.6, P≤0.0001). There was also a significant difference between the normal and disease groups (P≤0.0001). Healthy volunteers (n=10) showed a diurnal increase in plasma hepcidin at 4.00 pm compared to 8.00 am. A robust and optimised immunoassay for bioactive hepcidin has been produced and the patient sample results obtained further validates the important role of hepcidin in iron regulation, and will allow further investigation of this important peptide and its role in iron homeostasis.

KEY WORDS: Ferritins.
Hepcidin.
Immunoassay.

Introduction

Hepcidin is proposed as the key regulator in the homeostasis of iron. This peptide is predominantly synthesised in the liver.[1,2] The hepcidin gene contains three exons that encode an 84aa preprohepcidin with a signal peptide. The bioactive human hepcidin is a 25aa peptide first identified in human urine and plasma, which causes hypoferremia due to inhibition of iron recycling by macrophages, iron release from hepatic stores, and iron absorption in the intestine.[3]

At the molecular level, hepcidin binds to ferroportin and induces its internalisation and lysosomal degradation.[4] Degradation products of hepcidin, hepcidin-22 and hepcidin-20, are detectable in plasma[5] and urine,[2,6] but these products show impaired ability to internalise ferroportin.[7]

Hepcidin synthesis is increased by elevated plasma iron concentration,[8,9] decreased by erythropoietic activity,[10] and pathologically increased by inflammation.[8,11] Excess hepcidin has a major role in the anaemia of inflammation[12–14] and in iron-resistant iron deficiency anaemia.[15–18] Hepcidin deficiency is the cause of iron overload in most cases of hereditary haemochromatosis[19] and contributes to iron overload in β-thalassaemia and other iron-loading anaemias.[20]

Recent studies have utilised a urinary hepcidin assay,[21] and, more recently, mass spectroscopic techniques[5,7,22–24] have been introduced, although some of these published methods did not use internal standards and were semiquantitative in nature.

To date, few immunoassays have been reported to measure hepcidin, largely due to technical difficulties in producing a suitable antibody, and also the presence of hepcidin isoforms in human samples, which could be an obvious limitation for a polyclonal-based assay, although the clinical importance of these isoforms remains inconclusive. An enzyme-linked immunosorbent assay (ELISA) for the prohormone prohepcidin is available commercially (DRG Diagnostics, Germany), but results from previous studies failed to show any correlation with ferritin, the standard measurement of iron status, in any of the patient groups studied.[25,27]

The purpose of this study is to produce a robust and optimised immunoassay for human hepcidin-25 so that further investigation of the pathophysiology of this important iron regulator can be studied in clinical samples.

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Materials and methods

Synthetic hepcidin[1,2,26] (synthesised chemically by Bachem, St. Helens, UK) was conjugated to Keyhole Limpet haemocyanin (KLH) by 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride) (EDC). Conjugation was carried out using an Imject Immunogen EDC kit with mcKLH (Thermo Fisher Scientific, Rockford, USA). Conjugation was carried out according to the manufacturer's guidelines. Briefly, 200-µL ultrapure water was added to one vial mcKLH (10 mg/mL solution). Hepcidin peptide (1 mg) was dissolved in 0.5 mL 20% acetonitrile in ultrapure water. Peptide solution was then added to the mcKLH solution. One vial of EDC was dissolved in 1 mL ultrapure water and 50 µL added to the mcKLH-peptide solution. The solution was then incubated at room temperature for 2 h. The solution was then dialysed against 0.1 mol/L phosphate-buffered saline (PBS) using a Slide-A-Lyzer dialysis membrane (Thermo Fisher Scientific).

One female New Zealand white rabbit was then immunised subcutaneously with 0.5 mL immunogen (0.25 mL conjugated peptide and 0.25 mL adjuvant; Titermax, Sigma Aldrich, UK). A primary immunisation was followed by three booster immunisations before sufficient response was observed and the immunisation schedule terminated.

A test assay for determining antibody titre was produced using ^{125}I-labelled synthetic hepcidin, which was prepared by the Bolton-Hunter method and purified by reverse-phase high-performance liquid chromatography (HPLC) using a C^{18} Vydac column (Supelco, Sigma Aldrich, UK) with a 15–45% gradient of acetonitrile/water (0.05% TFA).

Rabbit immune serum was tested to determine antibody titre and affinity for the hepcidin peptide by titration of the primary antiserum diluted in assay buffer (0.01 mol/L PBS 0.5% bovine serum albumin [BSA] and 0.01% sodium azide), compared against the antiserum diluted in a known amount of hepcidin peptide (10 ng/mL hepcidin in assay buffer) (Fig. 1).

To establish the binding activity of the rabbit anti-hepcidin antibody for native endogenous hepcidin, immunohistochemistry was performed on paraffin wax-embedded healthy human liver sections. The antibody showed strong cytoplasmic staining in hepatocytes (Fig. 2). The specificity of the antibody was also confirmed by Western blot analysis of the precipitated proteins, where a single band <6 kDa was detected (Fig. 3). Briefly, the anti-hepcidin rabbit polyclonal antibody was affinity purified using protein A columns (GE Healthcare, Uppsala, Sweden).

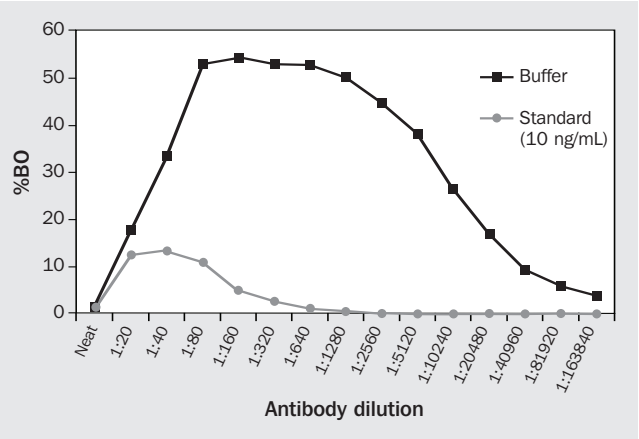

Fig. 1. Antibody titration curve using rabbit anti-hepcidin serum in the presence of a known amount of target antigen (synthetic hepcidin-25). Buffer curve: antiserum diluted in assay buffer. Standard curve: antiserum diluted in assay buffer with 10 ng/mL hepcidin-25. Labelled hepcidin ^{125}I was used for the test assay.

Immunohistochemistry was then carried out on healthy human liver tissue (supplied by the histopathology department, Hammersmith Hospital, London). Liver tissue sections were deparaffinised (x2) in xylene for 5 min and rehydrated in descending grades of ethanol, (100%, 95%, 70%) for 2 min each. Antigen retrieval was accomplished by immersing and heating slides in 10 mmol/L citrate buffer. Slides were then incubated with blocking solution, (3% FBS, 1% BSA, 0.05% Tween in Tris-buffered saline [TBS]) for 30 min, followed by a 4°C overnight incubation with purified rabbit anti-hepcidin primary antibody (1 in 800 dilution).

Sections were then incubated for 1 h with secondary anti-

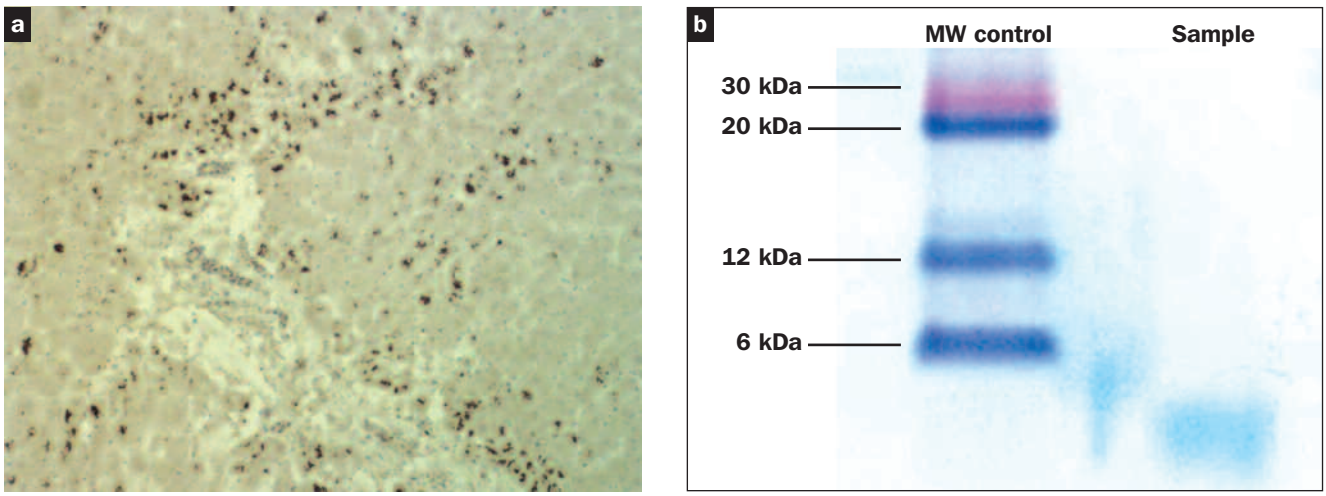

Fig. 2. Specificity of rabbit anti-hepcidin antibody against native hepcidin. a) Immunohistochemical staining of healthy human liver tissue (HRP-DAB staining, original magnification x40). b) Western blot analysis of serum proteins using rabbit anti-hepcidin antibody demonstrating the presence of a protein band at <6 kDa.

rabbit antibody conjugated with horseradish peroxidase (HRP; Sigma Aldrich). The reaction was visualised by the addition of 3,3′diaminobenzidine (DAB) substrate (Sigma Aldrich) for 5 min, and the reaction was stopped by washing with laboratory-grade water. Sections were counterstained with haematoxylin (Sigma Aldrich, UK) for 1 min. Slides were then dehydrated in 70%, 95% and 100% ethanol (2 min each), cleared in xylene for 5 min and then mounted in DPX (Sigma Aldrich).

Western blot analysis was carried out by using 10-mL normal human plasma, which was first centrifuged and filtered through a 0.22 mm filter (Millipore, MA). After addition of 10-mL DTT, plasma was again filtered through a 30 kDa filter (Millipore) and the filtrate precipitated using 25% TCA. The precipitated proteins were resuspended in loading buffer and subjected to SDS electrophoresis on a 4–16% NuPAGE Novex Bis/Tris gel (Invitrogen, UK). Western blot was performed using an XCell II blot module (Invitrogen) and a 0.1 mm pore size nitrocellulose membrane (Sigma Aldrich). Non-specific sites were blocked with 5% BSA in PBS and the membrane probed with purified rabbit anti-hepcidin antibody (1 in 2000 dilution in blocking buffer), followed by incubation with a secondary anti-rabbit antibody conjugated with HRP (Sigma Aldrich). Specific signals were detected with 3,3′, 5,5′ tetramethyl-benzidine liquid substrate (Sigma Aldrich).

For the hepcidin radioimmunoassay (RIA), a standard curve covering the analytical range (1.25–160 ng/mL) was produced using synthetic hepcidin (Bachem) (Fig. 2). A secondary antibody precipitation reagent was used to separate bound from non-bound label (sheep anti-rabbit IgG [Scipac, Kent, UK] with normal pre-immune rabbit serum and 4% polyethylene glycol [PEG] in assay buffer). A competitive RIA was produced using 100-µL appropriate standard/sample (in duplicate), 100-µL assay buffer, 100-µL rabbit anti-hepcidin antiserum and 100-µL ^{125}I-labelled hepcidin.

The assay was then incubated for 16–24 hours at 4°C. After overnight incubation, 500-µL separation reagent was added and further incubated for 30–40 min at 4°C. Triton (0.01%, 1 mL) was added and the assay centrifuged for 20 min at 3000 rpm. Tubes were decanted and the pellet collected and counted in a gamma counter for 1 min. Assay parameters were calculated using an appropriate computer package (RiaCalc Software, Perkin Elmer, UK).

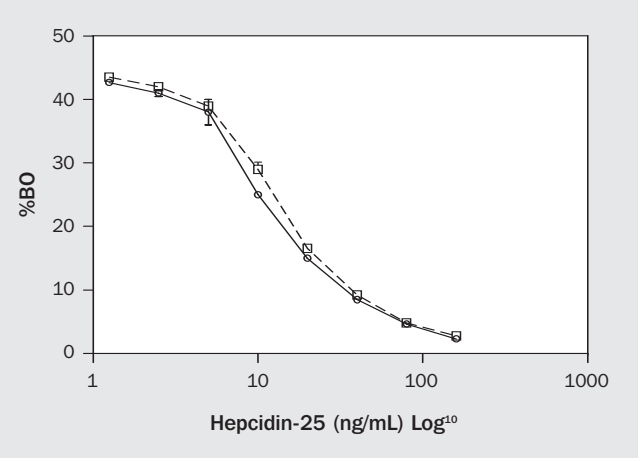

Fig. 3. Calibration data represent three calibration curves over three different days, (mean data with SEM errors plotted). Dashed line represents curve in serum-free assay buffer. Solid line represents curve. Diluted (1 in 10) in pooled human plasma from iron-deficient patients (n=3).

Ferritin, iron, unsaturated iron-binding capacity (UIBC) and C-reactive protein (CRP) were measured using the Abbott Architect *ic*8000 system, (Abbott Diagnostics, Ireland).

Patient samples

This study was approved by Ealing Hospitals and Hammersmith Hospital NHS Trust local research ethics committee. Informed consent was obtained in accordance with the Declaration of Helsinki.

To establish whether or not the hepcidin immunoassay developed provided important biological results, samples from patients with probable low hepcidin levels (iron deficiency anaemia and ulcerative colitis) were compared to healthy controls, as well as to patients with probable high hepcidin levels (chronic renal failure).

Forty-seven sample controls were recruited for the healthy control group (HC). Blood (3–4 mL) was taken by

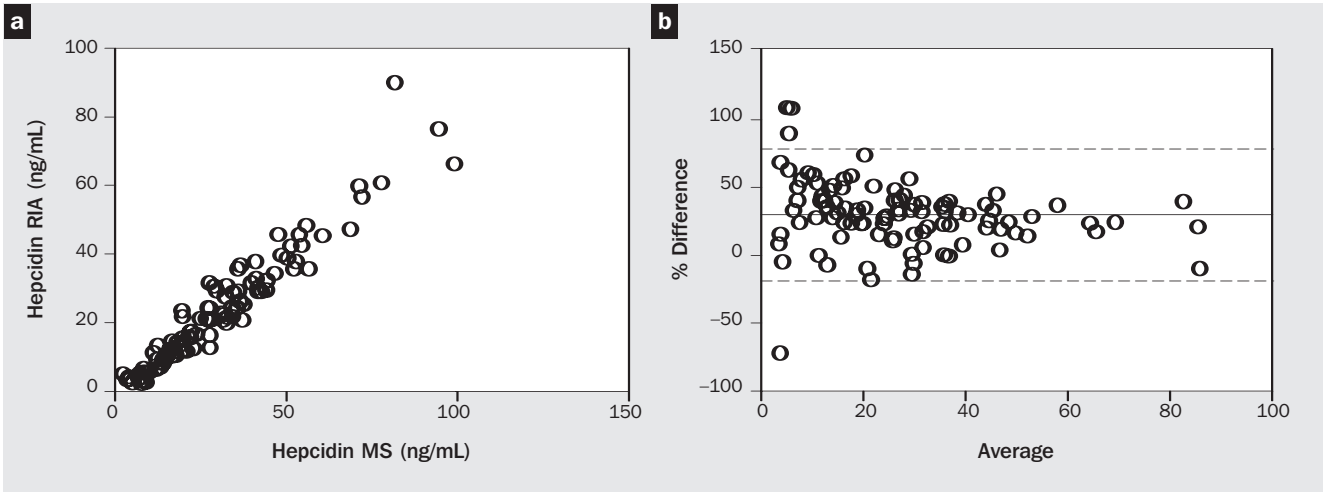

Fig. 4. Method comparison data (n=99). a) Pearson rank correlation (r=0.92, P<0.001). b) Bland-Altman (%difference between methods *vs*. average of methods). Solid line represents mean bias, dotted lines represent 95% confidence interval levels.

venepuncture into one serum separator tube (SST) and one lithium heparin tube. Both samples were centrifuged for 5 min at 2000 rpm and the serum/plasma frozen at –20°C. Samples were assayed initially to determine any partitioning effect between serum and plasma.

Patient plasma samples for the ulcerative colitis (UC; $n=40$) and iron deficiency anaemia (IDA; $n=15$) groups were collected over a six-month period through the out-patient clinic of the gastroenterology department, Ealing Hospital, London, and frozen at –20°C.

In the UC group, the condition was diagnosed on clinical features supported by endoscopy, histological and/or radiological findings. None of the patients in this group were on immune modulators or anti-tumour necrosis factor therapy. The anaemia seen in the UC group was defined as haemoglobin (Hb) <12 g/dL in females and <13 g/dL in males. Iron deficiency anaemia was considered to be present if ferritin <15 µg/L and iron <10 µmol/L.

The chronic kidney disease patients not requiring dialysis (CKD; $n=37$) and stable haemodialysis patients (HCKD; $n=94$) were recruited from the West London Renal Service, Hammersmith Hospital NHS Trust. Samples were collected over a three-month period and frozen at –20°C. The CKD group had varying degrees of renal impairment (estimated glomerular filtration rate [eGFR] 13–98 mL/min), and none were receiving recombinant erythropoietin (rEPO) or intravenous (iv) iron therapy, whereas those in the HCKD group were all treated with rEPO and iv iron intended to maintain Hb >11 g/dL and ferritin >400 ng/mL.

Demographic patient data are shown in Table 1. Ten healthy volunteers (male) were recruited for the diurnal study. Blood (3–4 mL) was taken by venepuncture into one SST tube with a gel separator and one lithium heparin tube. Samples were taken over a 24-h period at 8.00 am, noon, 4.00 pm and 8.00 am the following day. All samples were centrifuged for 5 min at 2000 rpm and the serum/plasma frozen at –20°C.

Statistical analysis

Study sample size required for an alpha at 0.05 and a power of 80% was calculated as 15 cases per group (anticipated difference: 13.7 ng/mL, anticipated standard deviation [SD]: 16.3 ng/mL) to test the null hypothesis that any difference observed between the sample groups is significantly different. Variables were expressed as mean (or median) with range as indicated, depending on their normality. Quantitative variables were compared using unpaired *t*-test, one-way ANOVA with Dunnets multiple comparison test, and two-way ANOVA repeated measurements for test of difference. Pearson's rank correlation was used to calculate the correlation between variables. Bland-Altman analysis was used for method comparison data. $P<0.05$ was considered significant. All statistical analyses were carried out using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, California USA [www.graphpad.com]).

Assay performance

The minimum detection limit of the hepcidin RIA was calculated from the mean+3SD of 20 replicates of the BO (maximum binding in a competitive immunoassay in the absence of antigen; only labelled peptide is bound, resulting in the maximum detection for the assay). The minimum detection limit of the assay was found to be 0.6 ng/mL. This was repeated in three separate assays.

Analytical specificity was assessed by measuring cross-reactivity with proteins that share some structural homology with hepcidin. Human insulin (200 ng/mL), glucagon I (200 ng/mL), angiotensinogen I (200 ng/mL), β-defensins 1–4 (200 ng/mL), α-defensin 1 (200 ng/mL) and plectasin (200 ng/mL) demonstrated no observable cross-reactivity (<0.1%). There was 100% cross-reactivity with synthetic hepcidin (200 ng/mL). Human insulin, glucagon I, angiotensinogen I and plectasin proteins were obtained from Bachem. The defensin proteins were obtained from the Peptide Institute, Osaka, Japan.

Intra- and inter-assay variations were evaluated using a low (5 ng/mL) and high (50 ng/mL) serum sample. These were prepared by spiking double charcoal-stripped human serum (Serotec, UK) with the synthetic hepcidin. Intra-assay precision was reported as %CV calculated from the mean of 10 results from a single assay (low: 7.2%, high: 5.8%). Inter-assay precision was reported as the %CV calculated from the mean value of the 25 results across five assays (low: 7.6%, high: 6.7%).

To assess recovery, three human samples were spiked with 100-mL of 5, 10 and 50 ng/mL concentrations of synthetic hepcidin. Each set of samples was assayed to generate three results and repeated in six assays. Accuracy was reported as the mean of percentage recovery (5 ng/mL: 98%, 10 ng/mL: 94%, 50 ng/mL: 97%).

Assay linearity was evaluated by diluting two human plasma samples (1 in 2, 1 in 4 and 1 in 8) with assay buffer. The method was found to be linear up to 200 ng/mL. No

Table 1. Demographic and biochemical parameters of disease groups and controls.

	HC	UC	IDA	CKD	HCKD
n (men/women)	47 (27/20)	40 (26/14)	15 (N/A)	37 (11/16)	94 (62/32)
Age* (years)	37 (20–62)	45 (20–88)	NA	45 (23–84)	64 (39–83)
Hb+ (g/dL)	NA	12.2 (6.5–15.8)	8.1 (6.1–11)	12.3 (9.9–16.5)	12.4 (9.2–14.2)
Iron+ (µmol/L)	16.1 (8.8–30)	6 (1–39)	5.3 (1–25)	12.3 (5–24.2)	9.5 (3–27.6)
Ferritin+ (µg/L)	74 (11.2–372)	28 (1–317)	9 (2.7–15)	521 (79–1470)	35.3 (11.2–286)
TSAT %+	27.2 (13.6–48.5)	14.3 (1.6–47.1)	7.1 (2.2–29)	19.7 (7.1–42.9)	23.6 (9–75)
Hepcidin+ (ng/mL)	13 (1.1–55.3)	4.5 (2.8–54)	4.5 (0.6–8.7)	26 (0.8–130)	58.5 (16–436)

Data presented as median (range), *mean (range).

NA: no data available.

significant difference was observed between the normal control plasma and serum hepcidin concentrations (n=47, P=0.87). There was no observable decrease in hepcidin concentration in plasma samples (n=5) either over the long term (four weeks) at 4°C or due to multiple (x3) freeze–thaw cycles (P=0.84).

For method comparison studies, the hepcidin RIA was compared against a published SELDI-TOF-MS method.[23] Briefly, sera were analysed on Cu^{2+}-loaded IMAC30 ProteinChips using a PBS IIc time-of-flight mass spectrometer. Sera were diluted five-fold in 8 mol/L urea, 1% CHAPS in binding buffer (0.5 mol/L NaCl, 0.1 mol/L sodium phosphate [pH 7.0]) followed by a further 10-fold dilution in binding buffer, and 100 µL applied to the chips. Following 30-min binding, the chips were washed with binding buffer, rinsed with water, dried and 2 × 1 µL 50% saturated sinapinic acid in 50% acetonitrile/0.5% TFA added. Spectra were collected over m/z 0–20,000 focused at m/z 2800 using a laser power of 165. Following mass calibration, total ion current normalisation and baseline subtraction, the hepcidin peaks were manually picked and intensities (peak heights) extracted using ProteinChip software.

Healthy serum control samples (n=99) were kindly donated by Professor K. Srai (University College London) for method comparison (Table 2). Bland-Altman analysis was used for method comparison between the RIA and mass spectrometry (MS) hepcidin methods (Fig. 3) and Pearson's rank for correlation between methods.

Results

Normal control samples (n=47) were assayed using the validated hepcidin RIA to give a primary reference range of 1.1–55 ng/mL (Fig. 4). The ferritin, iron, UIBC and CRP results were within quoted laboratory reference ranges for the normal patient group.

A significant difference (P=0.0082) was observed between male and female hepcidin results (n=27, median age: 34 years, median male: 20.1 ng/mL, range: 2.9–55.3 ng/mL and n=20, median age: 31 years, median female: 6.8 ng/mL, range: 1.1–43.3 ng/mL). Pearson's rank correlation with the HC group revealed a significant positive correlation between ferritin and hepcidin (r=0.6, P≤0.0001). This was observed in all groups (UC: r=0.5, P≤0.05, IDA: r=0.7, P≤0.05 and CKD: r=0.84, P≤0.05). In the HCKD group no relationship with ferritin was observed, due to treatment with iv iron. There was also a significant difference observed between the HC group and all the disease groups (P≤0.0001). There was no relationship between age and hepcidin in any of the patient groups studied.

Table 2. Demographic and biochemical parameters of samples used for method comparison (MC).

	MC
n	99
Iron⁺ (µmol/L)	16.2 (5.8–35)
Ferritin⁺ (µg/L)	86 (8–1039)
TSAT %⁺	26 (7–61)
Hepcidin MS⁺ (ng/mL)	27.6 (2.4–99.1)
Hepcidin RIA⁺ (ng/mL)	21.2 (2.3–90)

Data presented as median (range)

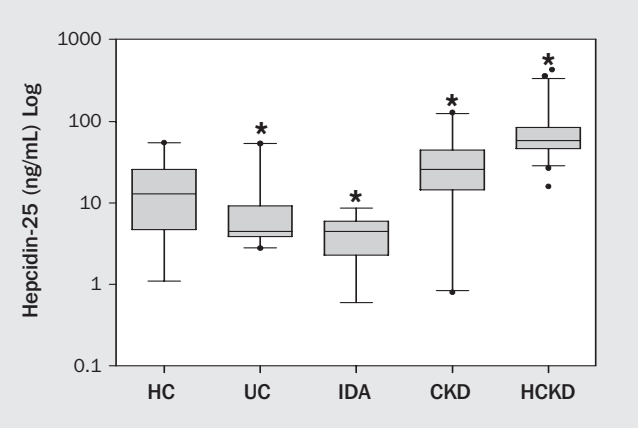

Fig. 5. Plasma hepcidin in iron disorders (box and whisker plot [2.5–97.5 percentile] with outliers). Groups include healthy controls (HC, n=47), ulcerative colitis (UC, n=40), iron deficiency anaemia (IDA, n=15), chronic kidney disease, not requiring dialysis (CKD, n=45) and chronic kidney disease, stable haemodialysis patients (HCKD, n=94). All disease groups differed significantly from HC (*P<0.0001, one-way ANOVA, Dunnett's multiple comparison test P<0.05 for all groups compared to HC).

Diurnal studies revealed an observable peak level of hepcidin in the late afternoon over the 24-h period studied, which reflected similar data seen in other studies. There was a significant difference in the hepcidin diurnal data overall (P≤0.0001), with a significant difference between the 8.00 am samples and other time points (P≤0.0001) (Fig. 5).

To confirm that hepcidin responds to iron status, samples were taken from four CKD patients as they were receiving iron infusions for the first time. Hepcidin levels in these patients increased promptly from 18.1±9.6 to 59.3±18.6 ng/mL at 24 h (P=0.047) (Fig. 6).

Discussion

This study introduces a new, biologically significant immunological assay for hepcidin measurement in human sera and plasma, based on a synthetic hepcidin peptide and a rabbit anti-hepcidin polyclonal antibody. The hepcidin concentrations observed in human plasma were highly heterogeneous in all the patient groups.

The significant variation in plasma hepcidin concentrations found in the control group may therefore reflect the true biological variability of hepcidin as a sensitive regulator of iron homeostasis in humans. It has been postulated in other studies that a diurnal cycle may also be present with hepcidin, as seen with iron concentrations.[24] The present study has demonstrated the presence of diurnal variation using the hepcidin immunoassay, recording an afternoon peak and an 8.00 am trough in hepcidin concentration. It was possible to standardise sample times for the HC, CKD and HCKD groups, but impossible to guarantee standardised sample times for the patient samples in the UC and IDA groups.

Using the optimised hepcidin RIA, it was possible to

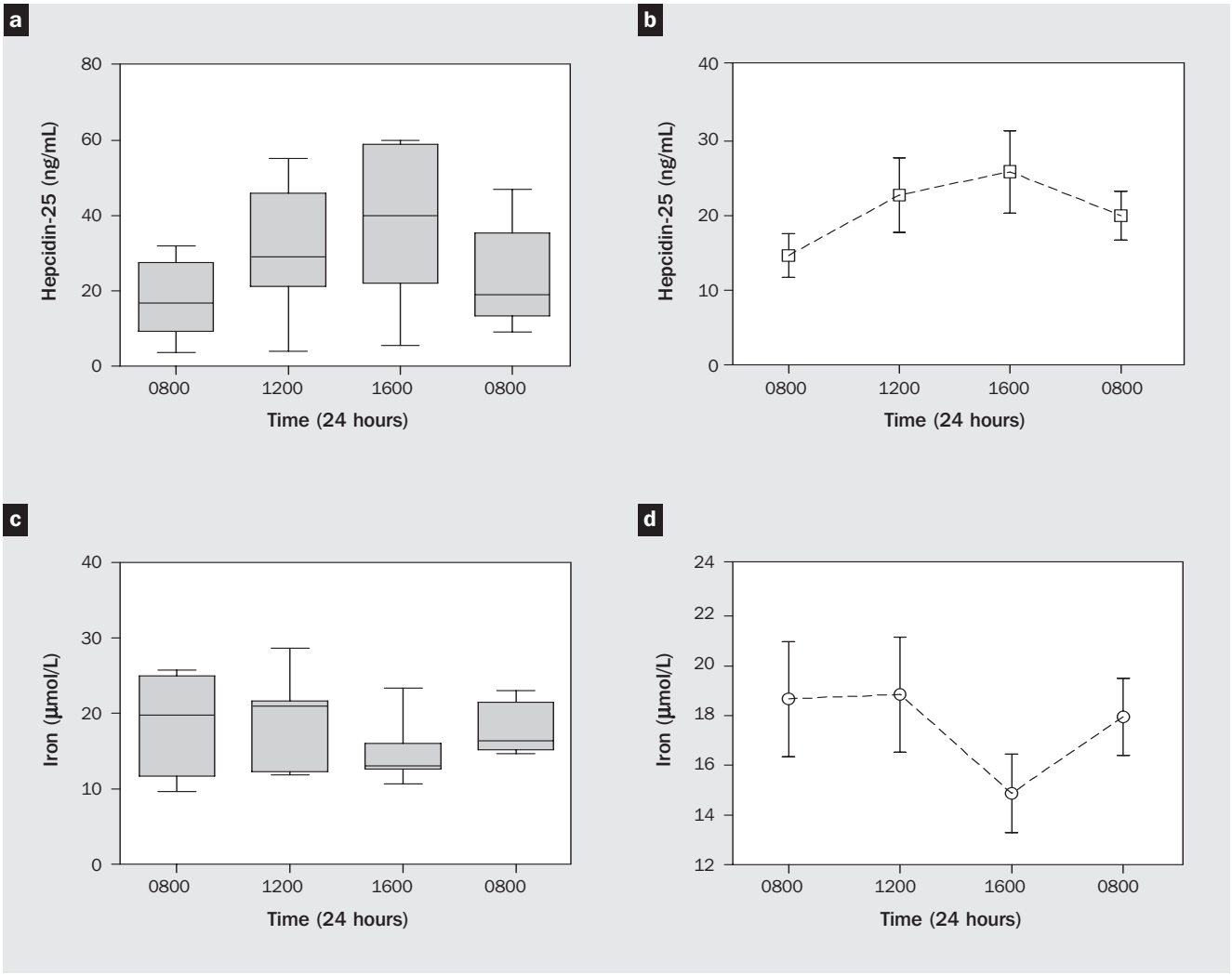

Fig. 6. Distribution of results for hepcidin and iron results of plasma samples ($n=10$) collected at four time points over a 24-h period showing the diurnal variation of hepcidin and iron. A/C) Box and whisker plot (2.5–97.5 percentile). B/D) Mean result from each time point plotted with SEM error bars. Two-way ANOVA repeated measures ($P<0.0001$ for all the diurnal data, $P<0.0001$ for 0800 sample versus other time points).

demonstrate a significant gender difference, although the higher hepcidin levels seen in males could be due to gender variation in ferritin stores. The significant correlation between hepcidin with ferritin, seen in the HC and IDA group, has been reported in other studies,[24,28] although the significant correlation of hepcidin with ferritin in the UC and CKD groups has not previously been reported and implies a high association with iron stores, which is important for the verification of the biological significance of the hepcidin assay.

Hepcidin levels in the UC and IDA groups (ferritin <15 mg/L) were inappropriately low compared to the HC group, as would be expected from the physiology of hepcidin regulation in anaemia-related conditions. Hepcidin concentrations were abnormally increased in the CKD and HCKD groups without associated inflammatory disorders, most probably due to retention caused by renal impairment. A method comparison against a published MS method demonstrated adequate concordance between the methods, although absolute hepcidin values varied between the two methods. The Bland-Altman plot shows high variability at low levels, which is probably due to a combination of differences in quantification of the internal standards used, pretreatment steps in the MS method, and detection limits for the assays. The assay has also been used to verify the significant response of hepcidin to an infused iron load in human patients, where, even in the presence of moderate renal impairment, hepcidin remains closely related to iron stores and is responsive to changes in iron status.

Most studies of hepcidin quantification have used urine as the sample and various MS techniques to estimate hepcidin concentration. Although these are sensitive techniques, they do require specialised and expensive equipment, a degree of sample pretreatment and the use of appropriate internal reference standards. Urine as a sample matrix presents a number of analytical difficulties (e.g., use of random or timed collections, correction for creatinine, and time-consuming extraction procedures to purify and concentrate the urine sample). The development of this RIA, and subsequent patient results, permit the conclusion that this assay can quantify hepcidin effectively in human serum and plasma.

In conclusion, this study reports the development of an RIA assay capable of measuring hepcidin in human serum

and plasma samples at a range of 1.25–160 ng/mL with acceptable imprecision, linearity, recovery and specificity. With this immunoassay it may be possible to simplify the measurement of hepcidin in serum or plasma samples, eliminating time-consuming pre-analytical procedures.

A well characterised and validated immunoassay, which shows good concordance with an established SELDI-TOF-MS method, has been developed for the bioactive peptide hepcidin. This will now allow further investigation of this important peptide in iron homeostasis and human physiology.

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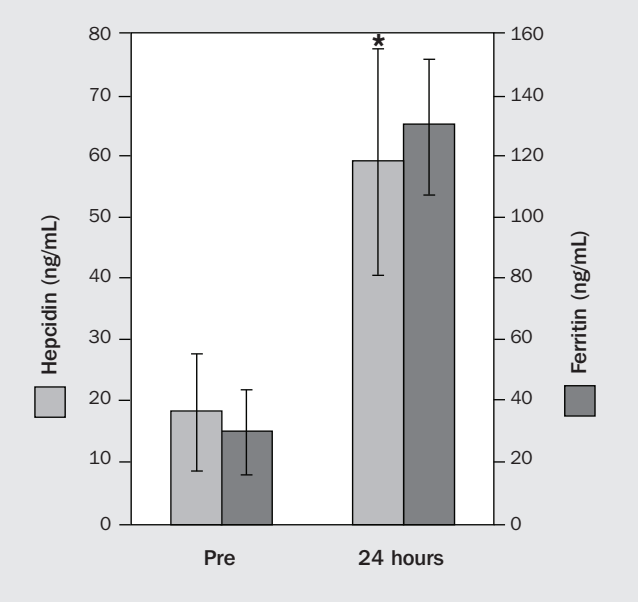

Fig. 7. Chronic kidney disease patients receiving intravenous (iv) iron for the first time (10-min iv 200 mg iron sucrose, plasma samples taken pre-iv and 24 h post-iv). Hepcidin rises promptly after infusion of iron load (*P=0.047). All samples were taken at 1500.

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