analytical chemistry

Article

Cite This: Anal. Chem. 2019, 91, 9323−9329

pubs.acs.org/ac

Considerably Lower Levels of Hypocretin-1 in Cerebrospinal Fluid Is Revealed by a Novel Mass Spectrometry Method Compared with Standard Radioimmunoassay

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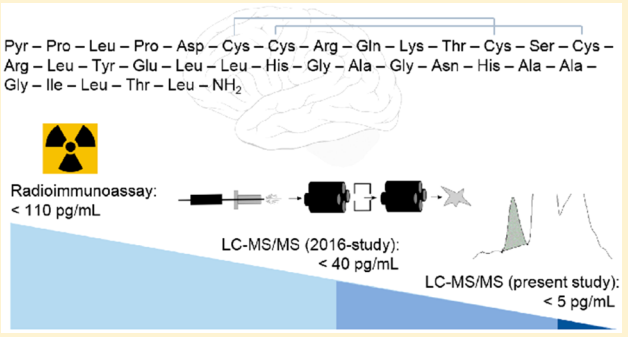
ABSTRACT: Low levels of hypocretin-1 (Hcrt1) in cerebrospinal fluid (CSF) are associated with narcolepsy type 1 (NT1). Although immunoassays are prone to antibody batch differences, detection methods and variation between laboratories, the standard method for Hcrt1 measurement is a radioimmunoassay (RIA). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is an antibody- and radioactive free alternative for precise measurement of Hcrt1. We developed an LC-MS/MS method for measurement of Hcrt1 in CSF with automated sample preparation by solid-phase extraction (SPE). The LC-MS/MS method was compared with the RIA method for Hcrt1 detection. CSF samples from healthy subjects and NT1 patients was obtained by lumbar puncture. NT1 patients were diagnosed according to the minimal criteria by the International Classification of Sleep Disorders (ICSD). The LC-MS/MS method showed linearity across the range of calibrators and had a limit of detection (LOD) of 2.5 pg/mL and a limit of quantitation (LOQ) of 3.6 pg/mL. Comparison of the LC-MS/MS method with RIA revealed a 19 times lower level in healthy controls and 22 times lower level in NT1 patients with the LC-MS/MS method than with RIA. Bland-Altman analysis demonstrated agreement between the methods. These results question what is detected by RIA and strongly suggest that the physiological concentrations of the peptide are much lower than previously believed. LC-MS/MS proves to be an alternative for detection of Hcrt1 for diagnosis of narcolepsy.



The neuropeptides hypocretin-1 and -2 (Hcrt1 and -2, also known as orexin A and -B)[1,2] are produced by specific neurons in the lateral hypothalamus. While hypocretin-2 has a very short half-life[3,4] and unknown functions, Hcrt1 is believed to have a fundamental role in regulation of wakefulness.[5,6] The hypocretins can be measured in the cerebrospinal fluid (CSF) and measurement of Hcrt1 in CSF is clinically relevant as reduced levels of Hcrt1 are observed in narcolepsy type 1 (NT1),[7−9] a chronic neurological disorder characterized mainly by excessive daytime sleepiness, cataplexy and disturbed nocturnal sleep.[10−12] Narcolepsy affects functioning in daily life and usually necessitates lifelong stimulant medication. Strong evidence supports an autoimmune genesis, and almost 100% of subjects with NT1 carry the HLA-DQB1*06:02 allele.[13,14] This theory is supported by the evident increase of narcoleptic cases in children following the Pandemrix vaccination for swine flu (H1N1).[15−17] Loss of Hcrt1 is thought to be caused by cytotoxic T-cells that specifically destroy the hypocretin producing neurons.[14,18]

The prevalence of NT1 is about 0.02 to 0.06%.[17,19,20] The typical age of onset is in childhood or adolescence, but the diagnosis of usually made several years, even more than 14 years later.[14] These facts actualize the need for more precise diagnostic tools to more quickly and easily establish a definite diagnosis and exclude potential differential diagnoses.

Received: June 14, 2019
Accepted: June 19, 2019
Published: June 19, 2019

DOI: 10.1021/acs.analchem.9b02710
Anal. Chem. 2019, 91, 9323−9329

Hcrt1 is a small peptide, and the availability of good epitopes for antibodies to bind to is therefore limited. The gold standard for diagnostic measures of Hcrt1 in CSF is today a competitive radioimmunoassay (RIA). Immunoassays like RIA are known to be highly influenced by interassay variation due to antibody batch differences, detection methods, and lab to lab variability. Indeed, different CSF levels of Hcrt1 have been reported in healthy subjects.[9,21−23]

In the clinical laboratory, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is often used for detection of small molecules, and increasingly also for detection of peptides and proteins in different biological samples. So far, just one study using LC-MS/MS with multiple reaction monitoring (MRM) on samples from human CSF has been reported,[24] reflecting on the difficulty of obtaining sensitive Hcrt1 measurements in CSF. Looking for alternative and nonradioactive methods for precise measurements of Hcrt1 in CSF, we used LC-MS/MS after automated (robot) sample preparation by solid-phase extraction (SPE) and revealed much lower levels of Hcrt1 than previously reported.

EXPERIMENTAL SECTION

Healthy Control Subjects. Twenty-two subjects referred to the Neurological Department at Stavanger University Hospital, underwent lumbar puncture as part of the neurological examination for various symptoms. None of the control subjects suffered from excessive daytime sleepiness or cataplexy. The investigations including CSF analyses, revealed no inflammatory, infectious or malignant diseases of the central nervous system or other somatic disease, and the patients were thus included in the study as "healthy control subjects". Median age was 43 years, range 27−29 years.

Patients with Narcolepsy. The CSF of nine patients diagnosed with NT1 according to the minimal ICSD criteria were included. All patients had previously been diagnosed and low CSF Hcrt1 levels were measured using RIA in the Rinnekoti Research Centre (Hcrt1/Orexin A RIA kit, Phoenix Pharmaceuticals, Burlingame, CA). Six out of seven available multiple sleep latency tests (MSLT) showed a sleep latency of 5 min or less and at least two sudden-onset rapid eye movement (REM) periods. The MSLT of one patient showed mean sleep latency of 13.3 min and one sudden onset of REM. All were HLA-DQB1*06:02 positive. Median age was 26.5 years (range 14−42 years).

Sample Collection. CSF was obtained by lumbar puncture. CSF of the 22 healthy control subjects was collected between 09:15 and 14:15. Samples were immediately transferred to cooled glass vials and kept on ice before centrifugation at 2500*g* for 10 min at 4 °C and stored at −80 °C. CSF of patients with NT1 was collected in the morning.

Reagents. Synthetic Hcrt1 was diluted to a 4400 ng/mL stock solution in 25% acetonitrile (ACN) and 1% formic acid (FA) and used as calibrator/standard (Phoenix Pharmaceuticals and Peptide Institute, Osaka, Japan). For optimizing MS, a Hcrt1 solution of 440 ng/mL in 25% ACN and 1% FA was used.

Synthetic Hcrt1 with ^{13}C and ^{15}N stabile isotope modification on two leucine amino acids was used for internal standard (ISTD) calibration; Glp-P-L(U13C6,15N)PDCCR-QKTCSCR-L(U13C6,15N)YELLHGAGNHAAGILTL-NH2 (CPC Scientific, Sunnyvale, CA). This internal standard was chemically similar to Hcrt1, both in terms of disulfide bridges and modifications, but had a mass shift of 14 Da compared to Hcrt1. Hcrt1 ($^{13}C_{12}$, $^{15}N_2$) internal standard stock was made by dissolving 0.1 mg in 10 mL (25% ACN, 1% FA). The stock of the ISTD was aliquoted into 1 mL aliquots and stored at −80 °C. Working solutions of Hcrt1 ($^{13}C_{12}$, $^{15}N_2$) ISTD was made by diluting the stock 1:5000 in 4% acetic acid (AcOH), giving a concentration corresponding to 2000 pg/mL. As 100 μL of the ISTD was added to 600 μL sample, a total of 333 pg/mL of ISTD was added to each sample before processing.

Calibrators, Quality Control Samples, And Blank. To make an artificial CSF solution with approximate protein concentration as in physiological CSF, 1% newborn calf serum (PN S0125, LN 1316B, Biochrom AG, Berlin, Germany) was added to phosphate buffered saline (PBS) (PN 1890535/28372, Thermo Fisher Scientific, Waltham, MA) and used as blank samples and for making up calibrators. Hcrt1 solution for calibration standards in the artificial CSF was prepared from a primary stock solution (101 ng/mL (25% ACN, 1% FA)), aliquoted (1 mL) and stored at −80 °C. Quality control samples of high and low concentrations was prepared by diluting the Hcrt1 primary stock solution into samples of pooled CSF, aliquoted and stored at −80 °C.

Sample Preparation for Mass Spectrometry. Automated sample preparation was done by a MiniPrep 75 pipetting robot (Tecan, Männendorf, Switzerland). Hcrt1 was extracted from CSF by solid phase extraction (SPE) with 33 μm Strata-X reversed phase polymer particles in 96-well format (Phenomenex, Værløse, Denmark). The adsorbent was conditioned with 300 μL of a mixture of 80% v/v methanol with 10% v/v acetic acid ("80−10"), followed by rinsing twice with 600 μL of water. Then 600 μL of CSF sample was applied, followed by rinsing twice with 600 μL water, drying by vacuum, and finally elution with 300 μL of the "80−10" mixture into a 96-well polypropylene microplate (Nunc). Samples were spun dry in a vacuum centrifuge (miVac (Genevac Ltd., Ipswich, UK)) at 80 °C for 60 min, followed by dissolving the extract in 50 μL of the "80−10" mixture.

LC-MS/MS Analysis. The analysis was done on an Acquity UPLC coupled with a Xevo TQ-S tandem quadrupole mass spectrometer (Waters, Milford, MA). Ten microliters of sample were injected onto a 2.1 mm i.d. and 50 mm long Kinetex C18 reversed phase column with 2.6 μm superficially porous (100 Å) particles (Phenomenex). The mobile phase was delivered at a flow rate of 600 μL/min and was a mixture of 0.2% formic acid and methanol, using gradient elution with 15−50−90% methanol at times of 0−0.6−1 min, respectively. The column temperature was 40 °C and the samples were kept at 10 °C. Positive electrospray (ESI+) with 2.8 kV on the capillary and 50 V on the cone produced a Hcrt1 precursor ion with four charges. MRM[25] was used for detection, with the transitions 891.2 > 1138.4 and 891.2 > 1110.9 for hypocretin-1 and 894.7 > 1143 for the internal standard, all using 25 eV for collision-induced dissociation (CID).

RIA Analysis of Hrct1 Concentrations. To compare with the LC-MS/MS method, Hcrt1 in CSF were also analyzed by RIA using standard kits (Phoenix Pharmaceuticals) according to the manufacturer's protocol. All samples, including the quality control sample provided by the manufacturer, were analyzed using the same kit lot number. External reference sample material was not applied. CSF samples, 100 μL, were measured in duplicates and amount of the radioactive ^{125}I probe was counted on a 5-well Riastar gamma counter (Packard, IL). For one of the 22 subjects there were not

DOI: 10.1021/acs.analchem.9b02710
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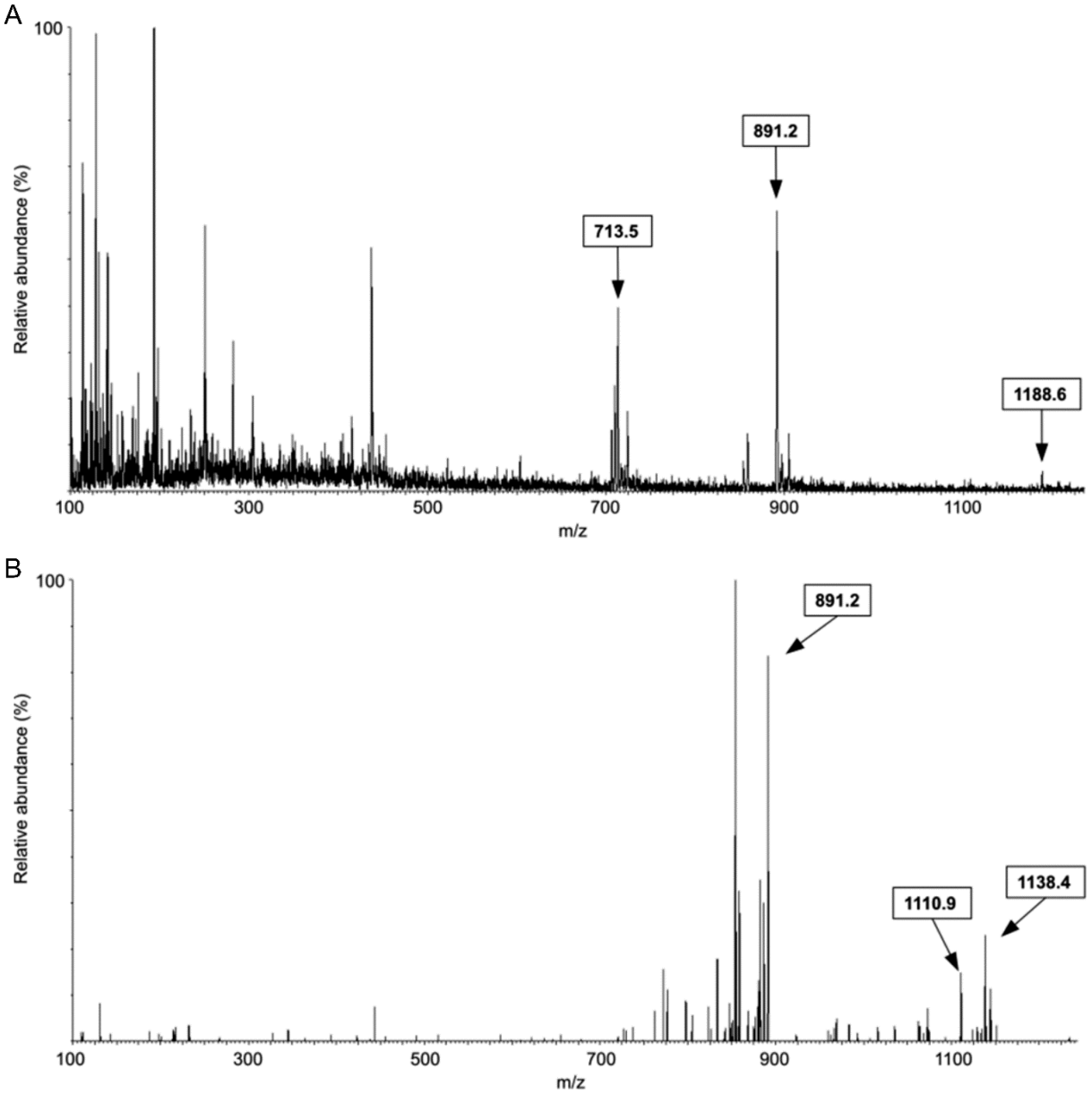

Figure 1. Raw data from MS scan of Hcrt1. (A) LC-MS spectra. Peak of hypocretin-1 with $[M+3H]^{3+}$, $[M+4H]^{4+}$, and $[M+5H]^{5+}$ charges are marked with m/z values 1188.6, 713.5, and 891.2 respectively. (B) MS/MS spectra of the 4-charged form of Hcrt1 (m/z 891.2) showing selected peaks for MRM (m/z 1110.9 and 1138.4).

enough CSF and therefore 21 samples were analyzed with the RIA method.

Statistics and Data Analysis. Results describing the characteristics of the LC-MS/MS method are given as mean ± SD. For comparison between the LC-MS/MS method and RIA for the control and narcolepsy group, not all data followed the normal distribution and therefore nonparametric statistics were used and values are given as median ± interquartile ranges. MassLynx 4.1 with TargetLynx (Waters) was used for peak detection, integration, calibration, and quantification. Statistical analysis was performed using RStudio 1.1.383 with R version 3.3.3.

Ethics. Patients gave written consent to participate in the study, and all samples were used in agreement with approval from Norwegian Regional Ethics Committee (REC West 2010/1455 and 2011/878), and the study was carried out in compliance with the Helsinki Declaration.

RESULTS AND DISCUSSION

The present LC-MS/MS method had a sufficient sensitivity for measurement of Hcrt1 in the majority of CSF samples. However, we found surprisingly lower levels of Hcrt1 in CSF from healthy subjects, than reported by other methods/assays (RIA, FIA, ELISA),[21,26,27] and also lower than the only other published study using a LC-MS/MS approach.[24] Recovery investigations indicated an acceptable accuracy of our assay. This was further supported by the LC-MS/MS analysis of the control sample in the RIA kit, which was found to be within the given expected range.

LC-MS/MS Method. Hcrt1 was detected as an intact peptide, and the optimization of MS signal intensity was done by infusion of 10 μL/min of 440 ng/mL of Hcrt1 into a mobile phase flow consisting of 200 μL/min of 80% methanol and 20% formic acid (0.2%). The full scan mass spectrum for Hcrt1 revealed a strong signal for the $[M+4H]^{4+}$ peptide at m/z

DOI: 10.1021/acs.analchem.9b02710
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891.2 (z = 4) and weaker signals for $[M+5H]^{5+}$ and $[M+3H]^{3+}$ at m/z 713.5 and 1188.6, respectively (Figure 1A). The full scan fragment ion mass spectrum of the $[M+4H]^{4+}$ precursor revealed strong signals at m/z 1138.4 and 1110.9, respectively (Figure 1B). A similar experiment was performed for Hrct1 ($^{13}C_{12}$, $^{15}N_2$) internal standard, resulting in detection of $[M+4H]^{4+}$ precursor ion at m/z 894.7 and a strong fragment ion at m/z 1143.0. Highest detector response was obtained with 2.8 kV capillary voltage, 50 V cone voltage, 25 eV collision energy, and an increased collision cell pressure (1.48×10^{-2} Torr) by using 0.6 mL/min of argon collision gas. We established our MS detection method by using these MS tune settings and MRM transitions.

Solubility Conditions for Solid Phase Extraction. Because of low Hcrt1 concentrations in CSF, we tried to use SPE for sample enrichment, prior to the LC-MS/MS analysis. Preliminary studies with cation exchange were not successful, so we used SPE with reversed phase retention mechanism instead. A strong eluent is needed, and we therefore investigated the solubility of Hcrt1 at low concentration (1 ng/mL) in different solvents. While Hcrt1 was soluble in 25% ACN at high concentration, we observed losses with this solvent at lower concentrations. Initial experiments demonstrated that solvents capable of hydrogen bonding, such as alcohols, worked better than solvents not capable of hydrogen bonding (i.e., ACN). The solvents ethanol (EtOH), methanol (MeOH), and ethylene glycol (EG) where therefore tested for optimizing Hcrt1 solubility. Results are shown in Figure 2. At high amounts of solvent (80%), EtOH and MeOH demonstrated good solubility for Hcrt1. EG, a solvent offering even more hydrogen bonds than EtOH or MeOH, demonstrated lower solubility for Hcrt1, with best solubility at 30%.

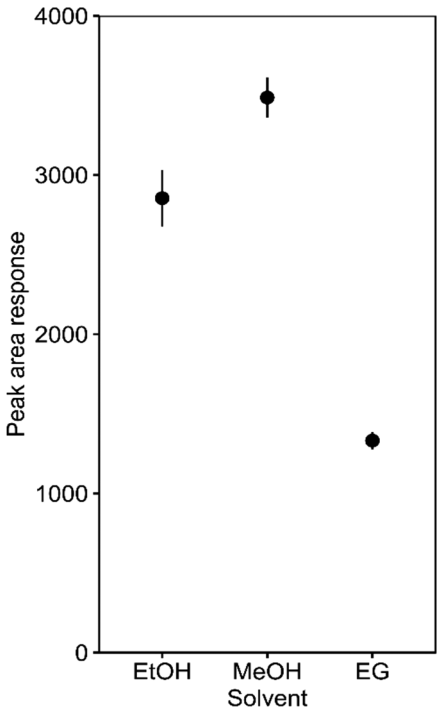

Figure 2. Solubility parameters for solid phase extraction (SPE). Results from experiments to find optimal conditions for up concentration and purification of Hcrt1 (1 ng/mL final concentration) are shown as peak area response for the solvents 80% ethanol (EtOH), 80% methanol (MeOH), and 30% ethylene glycol (EG), all with added 10% acetic acid.

Hcrt1 Peak Detection, Column Properties, Interference. High throughput analysis (3.1 min/sample) by using a short column of high efficiency, and therefore included ultraperformance liquid chromatography (UPLC) and fused core (solid core) type of columns for the LC separation. More than five different columns were evaluated. We selected a 50 mm long and 2.1 mm i.d. Kinetex C18 column with 2.6 um particle size, because this column provided sufficient selectivity for separation of Hcrt1 from interference (Figure 3).

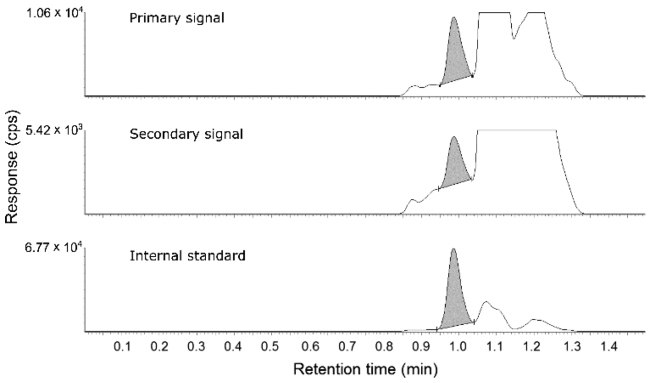

Figure 3. MRM chromatograms from the hypocretin-1 in a human CSF sample. Primary signal (m/z 891.2 > 1138.4), secondary signal (m/z 891.2 > 1110.9), and internal standard signal (m/z 894.7 > 1143). Analytical peak of hypocretin-1 is marked in gray. The response represents the area of the peak generated by count per second (cps) for each fragment ion.

Interference of eluted peaks and their association to the analytical peak of Hcrt1 was detected by inspection of MRM chromatograms. For calibrators diluted in ACSF the analytical peak of Hrct1 was in clear distance from other eluted peaks, resulting in low background. For CSF samples we observed an interfering peak close to the analytical peak. By careful selection of columns and evaluation of column properties we were able to separate the analytical peak (marked with gray) from the interfering peak observed to elute directly after Hcrt1, as shown in Figure 3. Other columns that were tested, even some with higher chromatographic efficiency, did not exhibit the selectivity required to remove this interference, and hence resulted in erroneous measurement of Hcrt1 in CSF. We were not able to identify the interference.

■ ASSAY CHARACTERISTICS, LINEARITY OF STANDARD CURVE, AND LIMIT OF DETECTION

Repeatability was measured in 11 aliquots (600 μL) of a pooled CSF sample. Mean Hcrt1 concentration was 11 ± 2 pg/mL, with a CV% = 16. For RIA the repeatability was measured on a sample split in 12 aliquots (100 μL). The mean Hcrt1 level was 240 ± 35 pg/mL, with a CV% of 15. Recovery was tested at two different spiking levels, low-middle (59.4 pg/mL, N = 8) and high (142 pg/mL, N = 4), compared to unspiked portions of the same samples. At low-middle spiking level the recovery was found to be 142 ± 14% and 90 ± 15% at high spiking level. For the RIA recovery was 96 ± 28 pg/mL when tested for one spiking level (128 pg/mL, N = 4). Linearity of the method was tested by serial dilutions of a 156 pg/mL calibrator spiked into artificial CSF. Linear regression analysis of serial dilutions vs peak response of Hcrt1

DOI: 10.1021/acs.analchem.9b02710
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demonstrated linearity trough the range of calibrators, R^2 = 0.99 (Figure 4). Lower limit of detection (LOD), defined as

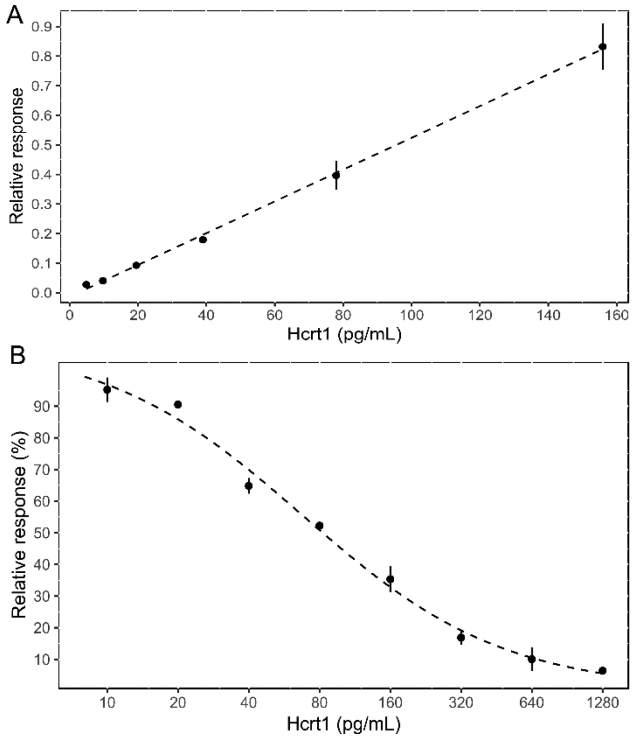

Figure 4. (A) Calibration curve for the hypocretin-1 LC-MS/MS method. Calibrators were diluted by serial dilution in artificial CSF. Final range of calibrators were 4.8−155.9 pg/mL, and linear regression demonstrated linearity throughout the analytical range. Each calibrator was measured in duplicates and shown as mean ± SD. Relative response represents area of the hypocretin-1 detected peak divided by the peak area of the hypocretin-1 ISTD. (B) Calibration curve for the RIA method. Calibrators were generated by serial dilution to a final range of 10−1280 pg/mL and measured in duplicates. Data are shown as mean ± SD. Four-parametric logistic regression was used to generate the calibration curve.

mean of the zero calibrator ±3 SDs, was 2.5 pg/mL, while the limit of quantitation (LOQ), defined as mean of the zero calibrator ±10 SDs, was 3.6 pg/mL.

Hcrt1 by LC-MS/MS. CSF levels of free Hcrt1 were measured by LC-MS/MS in samples from 22 healthy subjects, where the median level was 11 ± 3 pg/mL (median ± IQR, Figure 5a). Median Hcrt1 level measured in CSF from nine patients with NT1 was 2 ± 3 pg/mL (median ± IQR), overlapping the method LOD. Hcrt1 levels in 6 of the 10 samples were below LOD, and therefore these samples were given the value of LOD/$\sqrt{2}$ = 1.75 pg/mL (Figure 5a). There was a significant difference in Hcrt1 levels between CSF samples from the narcolepsy group compared with the healthy subjects measured by the LC-MS/MS method (p < 0.001, Mann−Whitney U test). One NT1 patient had Hcrt1 level within the range of the healthy controls. This subject had low Hcrt1 levels measured by RIA when initially diagnosed and fulfilled the minimal criteria according to the ICSD. However, the MSLT revealed normal sleep latency and only one out of five possible sleep onset REM periods.

Hcrt1 by RIA. Median Hcrt1 level measured with RIA in the same subset of healthy subjects (minus one because of limited sample) was 213 ± 90 pg/mL (median ± IQR, Figure 5b). Median level in the NT1 group was 45 ± 33 pg/mL (median ± IQR, Figure 5b), significantly lower than in the group of healthy subjects (p < 0.001, Mann−Whitney U test).

Method Comparison. A Bland-Altman plot was generated for comparison of the RIA method to the LC-MS/MS method (Figure 6). Due to differences in scale between the two methods the data from both methods was log-transformed before analysis. All values where within two SD from the mean.

RIA has been the standard assay for Hcrt1 in clinical laboratories. It may suffer from similar type of interference as other immunoassays, making it necessary to establish laboratory specific reference ranges. Commutability of samples has become a matter of interest in recent years,[28] which implies interassay agreement and a strong focus on method trueness. Hence, alternative methods are needed, such as the LC-MS/MS method reported in the present work. Shifting from immunoassay to mass spectrometry does not necessarily mean an end to interference, but it may provide us with clues to the true concentration levels of hypocretin-1 in CSF. However, the discrepancy of measured concentration levels should be discussed further, in search of an explanation to why we are measuring lower levels, especially compared to RIA, but also compared to the only other LC-MS/MS method reported for determination of Hcrt1 in CSF.[24]

We applied highly specific MS/MS detection and used a LC separation column that was capable of elimination of interfering substances. We achieved a perfect match in retention time for our internal standard and used the corresponding MRM transition for the internal standard as for the analyte. We added the internal standard to the CSF directly before SPE, which strongly suggests that we are measuring the free, unmodified, unaggregated and unbound hypocretin-1 molecule. Loss during the sample preparation and instrumental analysis can occur, but was not likely in this case, as it would have been corrected for by the internal standard. Hence, if dimerization, aggregation, or other protein inclusion bodies were the cause of the discrepancy from the RIA measurements, this must have occurred in CSF before our sample preparation. We therefore believe our assay is capable of measuring the free, unmodified and active level of hypocretin-1 in CSF. An alternative explanation is the possibility of protein inclusion bodies being formed during sample storage. However, our study indicated a rather good sample stability of the spiked CSF samples, even at room temperature.

In light of our results, we hypothesize that levels of free Hcrt1 in CSF are considerably lower than previously reported by RIA. Interestingly, a recent study revealed extremely low levels of intact Hcrt1 in CSF samples measured by HPLC combined with offline RIA detection.[29] Low CSF levels were observed in healthy subjects and also in patients with NT1 as well as NT2, and the results indicate that the intact Hcrt1 peptide accounts for less than 10% of the total amount. The authors hypothesize that unidentified metabolites of Hcrt1 account for the majority of the immunoreactive signal. Previous observations by microdialysis in both human and animal studies also support our hypothesis that the active neuropeptide concentration is much lower than previously believed. Blouin et al.[30] used RIA to measure a level of 38 pg/mL when sampling from the amygdala in patients with pharmacologically resistant epilepsy. Zhou et al.[31] sampled from the arcuate nucleus of the hypothalamus in rats and measured a level of 36 pg/mL using a LC-MS/MS method.

DOI: 10.1021/acs.analchem.9b02710
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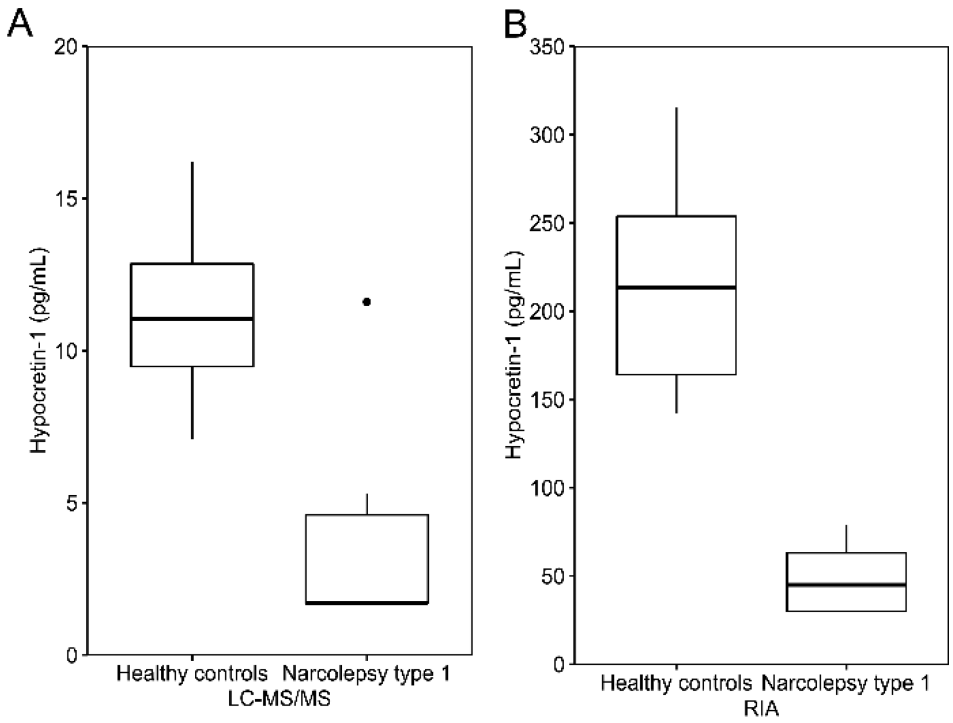

Figure 5. (A) Levels of hypocretin-1 measured by LC-MS/MS. Median Hcrt1 concentration measured in CSF from healthy subjects was 11 ± 3 pg/mL (median ± IQR) (N = 22). Median Hcrt1 measured in patients with NT1 was 2 ± 1 pg/mL (N = 9). (B) RIA measurements of Hcrt1 in healthy subjects with median concentration at 213 ± 90 pg/mL (N = 21), and in NT1 patients with median Hcrt1 concentration at 45 ± 24 pg/mL (N = 9). All samples were measured in duplicates.

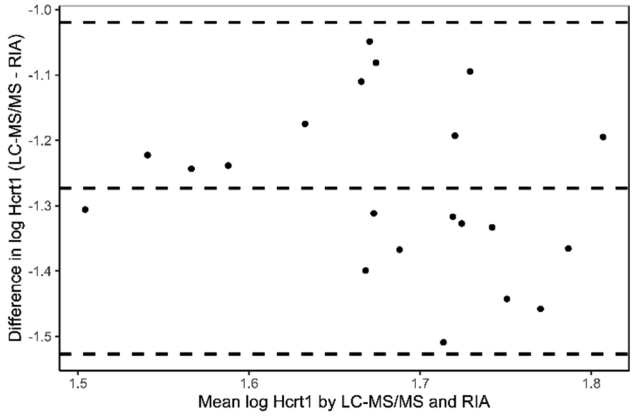

Figure 6. Estimation of agreement between the methods. Comparison of the LC-MS/MS method versus RIA was analyzed by a Bland-Altman plot of the log-transformed data. The results were within the ±1.96 (2 SD) limits (upper and lower broken lines) demonstrating acceptable agreement.

The molecular cutoff of the dialysis membranes used in these studies was 12 and 20 kDa, respectively, and will reduce the possibility of protein interactions as only small proteins/peptides will pass the microdialysis membrane. Although Hcrt1 was measured in different species using different methods and in different brain regions, it is an interesting observation that the Hcrt1 levels in brain parenchyma are lower than what is observed in CSF with the RIA method.

We also measured lower levels than another study using LC-MS/MS. An explanation for this discrepancy can be found in the purity of stock solution, or in the preparation of calibration solutions. Unlike the other LC-MS/MS assay, we did not acidify our CSF samples with phosphoric acid to break up any potential aggregates of Hcrt1 before sample preparation with SPE. We applied as much as 10% acidic acid in the elution solvent, which would likely have a similar effect. Also, the ISTD would correct for any modifications during sample preparation. Finally, it must be mentioned that Hcrt1 is a sticky and difficult molecule to measure, with the potential to be adsorbed on surfaces during the analysis. Adsorption losses in the sample preparation and instrumental analysis should, however, be corrected for by the internal standard.

A major limitation of the study is the limited number of samples used for establishing a normal reference range for Hcrt1 with our method. CSF from clinically examined controls and narcolepsy patients are difficult to obtain. A major strength of the study is the use of a very specific LC-MS/MS method, capable of detecting a Hcrt1 peak at the expected retention time, the confirmation of Hcrt1 identity by a second fragment, and the correct ratio of the two MRM channels.

One of nine patients with NT1 showed normal levels of Hrct1 using LC-MS/MS methods and had inconclusive neurophysiological result. For better interpretation of these findings, the measurement using both methods should have been repeated. This was not possible due to low amount of remaining CSF.

Measurement of the free and unmodified form of Hcrt1 by LC-MS/MS as presented here can be valuable not only as an aid in the diagnosis of NT1, but also in neurodegenerative diseases and other neurological conditions where measurement and analysis of Hcrt1 levels would be relevant but investigations so far have been limited due to the complicated nature of Hcrt1 measurements. Our results question the physiological levels of Hcrt1 in humans, both in health and disease. This can also have implications for in vitro and in vivo research on Hcrt1 function. Future improvement of this

DOI: 10.1021/acs.analchem.9b02710
Anal. Chem. 2019, 91, 9323−9329

method could aim at measurements of Hcrt1 in blood, an easier obtainable sample material.

CONCLUSION

Although the instrumentation for LC-MS/MS is expensive, we argue that the LC-MS/MS application is relevant in the routine clinical laboratory. The method is sensitive, reproducible, can be fully automated, and has low running costs. Further investigations are needed to fully answer why our method measures lower levels than immunoassays and to improve sensitivity in order to reduce the volume of CSF needed.

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Author Contributions

The manuscript was written through contributions of all authors, and all authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Sandnes, Norway, for use of their RIASTAR gamma counter.

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DOI: 10.1021/acs.analchem.9b02710
Anal. Chem. 2019, 91, 9323−9329