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Immunoluminometric assay for copeptin measurement in cerebrospinal fluid: Technical aspects and pilot study

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ABSTRACT

Background: Copeptin acts as surrogate marker under stress stimuli, as well as an outcome predictor based on serum or plasma concentration in patients suffering intracranial hemorrhage, aneurysmal subarachnoid hemorrhage (aSAH), and stroke. The aim of this study was to establish a method for quantification of copeptin levels in cerebrospinal fluid (CSF) and to demonstrate its clinical applicability in patients following aSAH.

Methods: This assay was validated for CSF samples using a commercial immunoluminometric assay (IMLA). For the control group (10 patients), CSF copeptin levels were determined in patients without signs of acute neurological diseases and who underwent a diagnostic lumbar puncture. The pilot cohort included calculation of copeptin levels in CSF and in serum of patients following aSAH.

Results: The control group had CSF copeptin levels lower than 0.78 pmol/L^{-1}. Among patients with aSAH, CSF copeptin values had a mean of 20.1 pmol/L^{-1} and serum copeptin concentrations had a mean of 61.39 pmol/L^{-1}.

Conclusions: This assay provides to best of our knowledge for the first time initial ranges values of CSF copeptin for patients without acute neurological disease and in patients with aSAH. Thus, it opens new doors to develop further calculations and relationships between diseases biomarker and outcome prediction.

1. Introduction

Under the stress response, the hypothalamic-pituitary axis produces stress hormones such as arginine-vasopressin (AVP) and the corticotropin-releasing factor (CRF). The AVP has several effects depending on its binding to specific receptors (V1, V2, V3), such as water retention in the kidneys or increased blood pressure mediated by vasoconstriction [1]. The AVP concentration in serum is useful as a diagnostic tool in certain endocrine disorders, sepsis or pathologies affecting osmotic and cardiovascular homeostasis [1,2]. Serious concerns exist, however, about the technical reliability of plasma AVP measurements. This is because the hormone has a relatively short plasmatic life, is rapidly cleared out of the system and is largely bound to platelets [1].

AVP is synthetized from a peptide precursor called pre-provasopressin, which is broken down into three peptides: AVP, neurophysin II, and copeptin [3]. Of particular interest, copeptin is released in equimolar quantities with vasopressin, and due to its stability and plasmatic life has emerged as a serum biomarker, and possible outcome predictor of patients affected by: intracerebral hemorrhage (ICH), ischemic stroke, aneurysmal subarachnoid hemorrhage (aSAH) and sepsis [4–8]. [5]

Previous studies have identified copeptin values only in the serum or plasma of the examined patients [4–8]. However, the determination of copeptin concentration ranges in patients' cerebrospinal fluid (CSF) has not been previously published. As such, this study aims to establish a method for estimating patient CSF copeptin levels, with the intention of investigating a more specific association with certain pathologies affecting the central nervous system. We describe here are the details of

Abbreviation list: aSAH, aneurysmal subarachnoid hemorrhage; AVP, arginine-vasopressin; CCT, cranial computer tomography; CRF, corticotropin-releasing factor; CSF, cerebrospinal fluid; CV, coefficient of variation; EVD, external ventricular drainage; HI, hemolysis index; ICH, intracerebral hemorrhage; ICP, intracranial pressure; ILMA, immunoluminometric assay; ISO, International Organization for Standardization; LD, lumbar drainage; LOQ, limit of quantitation; QC, quality control; RE, relative error; RFB, Referenz Institut für Bioanalytik; RLU, Relative Light Units; SD, standard deviation

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this methodology and demonstrate its first range of values in patients following aSAH.

2. Material and methods

2.1. Chemicals and biochemicals

The proposed assay was validated for CSF samples using the LIA CT-proAVP commercial immunoluminometric assay (ILMA) from Thermo Scientific B.R.A.H.M.S. (Hennigsdorf, Germany) [9]. This assay uses test tubes coated with anti-CT-pro-AVP monoclonal antibodies from mouse. The tracer used was a luminescence-labeled anti-CT-proAVP polyclonal antibody from sheeps. Blank CSF samples were obtained from the Referenz Institut für Bioanalytik (RFB, Bonn, Germany). The synthetic human copeptin trifluoroacetate salt with the known 39 aminoacid sequence (P01185; ASDRSNATQLDGPAGALLLRLVQLAGA-PEPFEPAQPDAY) and a degree of purity > 98% was purchased from Bachem (Bubendorf, Switzerland). This sequence corresponds to the aminoacid position 126–164. Additionally, all solutions were prepared with distilled water (Millipore, Merck, Darmstadt, Germany).

Calibration and control levels

Before using the commercial CSF for calibration and controls, the baseline Relative Light Units (RLU) of the blank CSF were estimated in ten independent runs. Then, the RLU of the distilled water were also determined ten times, due to the fact, that copeptin powder was initially dissolved in water. For the calibration curve and quality control (QC) samples, a stock solution of copeptin in water (1 mg ml^{-1}) was prepared. From this stock solution, a working standard solution (1240 pmol/L^{-1}) was prepared by dilution with the blank CSF. Then, a calibration curve in CSF consisting of six standards was prepared from the working standard solution at copeptin concentrations of 0.78, 1.55, 3.1, 6.2, 12.4 pmol/L^{-1}. Two levels of QC samples (1.24 and 9.69 pmol/L^{-1}) in CSF were prepared separately in the same fashion. The linearity of the assay was assessed by analyzing the calibration curve (0.78–12.4 pmol L^{-1}) in CSF using a weighted (1/y2) linear regression of the RLU of copeptin versus the nominal concentrations of the calibration standards.

2.2. Method validation

The immunoassay was validated by determining the lower detection limit, linearity, intra- and inter-assay variability as well as stability [10]. The intra-assay variability of the immunoassay was confirmed by mounting two CSF samples containing copeptin at two concentrations (1.24 and 9.69 pmol/L^{-1}), and then measuring each sample ten times within the same assay and calculating the intra-assay coefficient of variation (CV) for each sample (%CV = [SD/mean]*100). The inter-assay variability of this assay was determined by analyzing the two CSF samples in five consecutive assay runs, and then calculating the inter-assay CV. The accuracy was expressed as the relative error (RE) according to the equation:

$$RE\,(\%) = 100\% \times (\text{measured concentration} - \text{nominal concentration}) / \text{nominal concentration}$$

The acceptance criteria of the data included a precision (CV) within 20% and accuracy (RE) within ± 20% of the nominal values [11]. The stability of the analyte was tested at 4 °C and at room temperature for seven consecutive days. Therefore, five QC samples (QC level 1 and 2) were stored under the described conditions and subsequently measured.

2.3. Disruptive factors

The hemolysis index (HI) of CSF samples was determined to identify any possible contamination with peripheral blood The HI shows a nearly linear correlation with free hemoglobin concentrations, and it is subdivided into six categories according to the identified amount of free hemoglobin (Table 1). The Dimension Vista analyzer (Siemens, Erlangen, Germany) was used to measure HI, with a spectral extinction at 405 nm and 700 nm.

Table 1
Hemolysis index (HI) classification into six categories.

Hemolysis index (HI)	
HI	Hemoglobin range [mg/dl]
1	≤ 25
2	25 < H = 50
3	50 < H = 200
4	200 < H = 300
5	300 < H = 500
6	> 500

2.4. Copeptin in CSF of control samples

CSF copeptin concentrations were determined in patients (n = 10) who underwent a diagnostic lumbar puncture (normal pressure hydrocephalus) at the Mannheim University Hospital. Patients suffering of acute stroke (ischemic or hemorrhagic), heart failure, aSAH or sepsis were excluded from the control group. The values obtained from this group served as control samples, as no reference values exist for healthy populations in CSF measurements.

2.5. Copeptin in CSF clinical samples (patients with aSAH)

From June 2017 to October 2017, clinical data were prospectively collected from patients admitted to the Mannheim University Hospital neurosurgical department that met the following inclusion criteria: 1) Age within 18–75 years old, 2) aSAH within 24–48 of ictus, 3) presence of aSAH on initial CCT scan, 4) placement of an external ventricular drainage (EVD) or lumbar drainage (LD) for intracranial pressure (ICP) and/or hydrocephalus management. Additionally, corresponding venous blood samples were collected alongside the CSF from this group to investigate a possible relationship between both serum and CSF measurements.

2.6. Samples handling

Venous blood samples and CSF were collected in serum tubes and in sterile urine tubes, respectively (Sarstedt, Nümbrecht, Germany), centrifuged at 2800 rpm for 10 min (Heraeus, Darmstadt, Germany) at 4 °C and stored at −70 °C until further analysis.

2.7. Determination of copeptin in serum and CSF specimens

For serum samples, the validated immunoassay from Thermo Scientific B.R.A.H.M.S. was used, as previously described elsewhere [9]. The intra-laboratory CV is ≤5% and the inter-assay CV is ≤10%. The assay is linear over the concentration range of 1.96 pmol/L^{-1}–1250 pmol/L^{-1}.

For CSF samples, a six-point calibration was performed and QC levels 1 and 2 were measured in each series.

2.8. Ethics and statistics

This study has the approval of the Mannheim University Hospital Ethics committee. Data were analyzed with a commercial statistical software package (SPSS for Mac, version 22.0 2012; SPSS). The variables were expressed as medians and quartiles when appropriate. They were correlated with the x2 test, Mann-Whitney U Test, and Pearson correlations when appropriate, with a P value < 0,05 considered

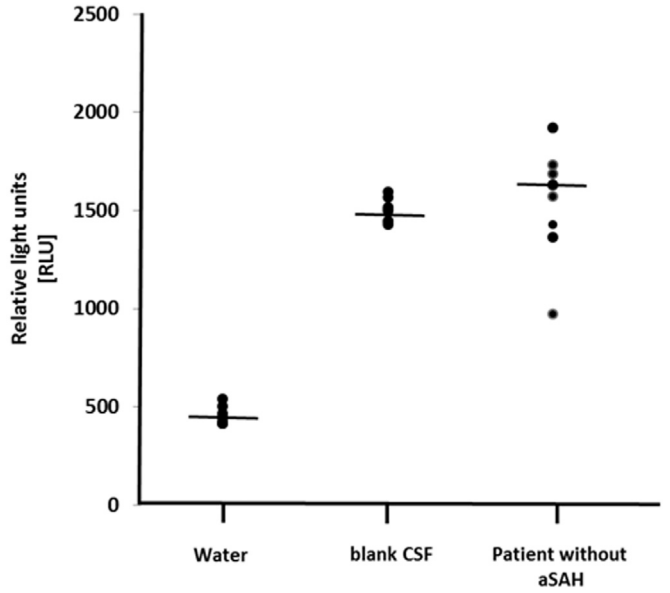

Fig. 1. Measurement of Relative Light Units [RLU] in different matrices (Water, blank CSF, and control group). Horizontal lines illustrate mean values.

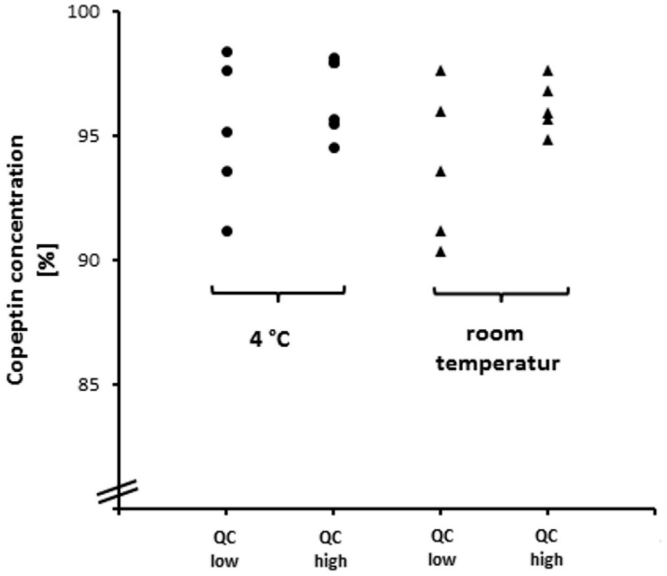

Fig. 2. The QC samples (QC level 1 (●), QC level 2 (▲)) were stored at 4 °C and at room temperature. Each QC sample was measured after seven days.

statistically significant.

3. Results

3.1. Background signal determination

To avoid unspecific background signals, separate water and blank CSF samples were measured ten times. In this manner the maximum allowed baseline value of RLU was determined. Water showed the lowest RLU value, with a mean of 446 and blank CSF samples exhibited a mean of 1498. Based on these results, blank CSF had a maximum allowed RLU of 1500 (Fig. 1).

3.2. Assay validation

The calibration curves for determining copeptin concentrations were prepared by analyzing spiked CSF samples. The mean correlation coefficient (r) of the weighted calibration curve generated during the validation was ≥0.95. The concentrations of copeptin in QC or unknown samples were subsequently interpolated from these curves.

The limit of quantitation (LOQ) was the lowest calibration level, 0.78 pmol/L^{-1}. Ten replicates of each QC level were measured to calculate the intra-run precision. The inter-run precision was determined on the basis of different assays measured over five days. The within-batch precision and accuracy met the acceptance criteria (≤ 20%). For the between-batch precision experiments, the values ranged from 4.04% to 15.82%, and the accuracy from 90.63% to 103.84% (Table 2). We tested the stability of the analytes at 4 °C and at room temperature in different spiked QC samples for seven days. The stability of each sample is expressed as a percentage of the initial value, with stabilities between 91.2% - 98.5% for the low concentrated QC samples and between 94.6% - 98.1% for the high concentrated QC samples (Fig. 2).

3.3. Excluding confounding variables

CSF samples with an HI > 2 were excluded from further analysis; due to the possibility of contamination of the CSF with peripheral blood, which might produce false positives in our findings. One out of the ten samples had an HI of 3, and was therefore excluded from further evaluation. The remaining nine samples had an HI of 1. These samples were inserted directly without restriction into the ILMA.

3.4. Physiological and pathological ranges of copeptin in CSF (Control and following aSAH)

The control group was comprised of 10 patients (6 men and 4 women), with a mean age of 33 years. The RLU for this group had a mean of 1582 (range 969–1921 RLU; Fig. 1). The aSAH group was also comprised of 10 patients (9 women and 1 man), with a mean age at onset of 54 years (range 26–72 years). Serum copeptin values among the aSAH group had a mean of 61.39 pmol/L^{-1} (median 17.75 pmol/L^{-1}, range: 11.3–179.7 pmol/L^{-1}), and CSF copeptin values had a mean of 20.1 pmol/L^{-1} (median of 5.79 pmol/L^{-1}, range: 1–69.9 pmol/L^{-1}). The aSAH group showed no statistical correlation between serum and CSF copeptin values (p = .76). Table 3 demonstrates patients' characteristics and copeptin values.

4. Discussion

In the last years, copeptin has emerged as a promising prognostic factor in several neurological diseases, such as intracerebral hemorrhage, ischemic stroke, and aSAH [4,12,13]. Additionally, the release of

Table 2
Summary of precision and accuracy for QC samples of spiked CSF.

Concentration added (pmol/l)	Intra-assay precision (n = 10)			Inter-assay precision (n = 5)		
	Concentration found mean ± SD: (pmol/l)	Precision (%)	Accuracy (%)	Concentration found mean ± SD: (pmol/l)	Precision (%)	Accuracy (%)
1.24	1.28 ± 0.05	4.07	102.90	1.29 ± 0.05	4.04	103.84
9.69	10.18 ± 1.06	10.42	105.99	8.70 ± 1.38	15.82	90.63

Table 3
Patients' clinical characteristics and copetin values.

Patient	Age	Sex	Number of aneurysms	Aneurysm location	Aneurysm ethiology	WFNS score	Modified Fisher Scale at admission	Copeptin CSF [pmol/L^{-1}]	Copeptin serum [pmol/L^{-1}]
1	70	w	1	Anterior communicating artery	Saccular	5	4	69.96	17.77
2	64	w	1	Posterior communicating artery	Saccular	4	4	1.20	17.45
3	48	w	1	Anterior communicating artery	Saccular	5	4	30.38	12.60
4	52	w	3	Basilar trunk	Saccular	4	2	1.00	11.33
5	26	w	1	Vertebral. PICA origin	Saccular	2	4	36.75	64.11
6	46	w	1	Anterior communicating artery	Saccular	2	4	4.88	179.73
7	54	m	1	Anterior communicating artery	Saccular	4	3	46.48	119.13
8	55	w	2	Anterior communicating artery	Saccular	4	4	6.70	17.73
9	55	w	1	Vertebral = PICA origin	Saccular	2	4	2.91	13.64
10	72	w	1	Anterior communicating artery	Saccular	5	4	1.21	160.42

w: woman; m: man.

this peptide also appears to increase in other diseases, including electrolytes disorders, cardiac infarction and sepsis [5,14]. Up to now, copeptin levels have been identified only in patients' serum or plasma and limitation regarding sensitivity and specificity are well described [4,15]. The possibility to measure copeptin in CSF would expand laboratory diagnostics in several neurological disorders. Therefore, we have developed a method to identify the presence of copeptin in CSF and to possibly determine its local presence among patients suffering neurological diseases.

4.1. Quality assessment and reliability of copeptin in CSF

The reproducibility and standardization of a new diagnostic method relies on the use of a standard sample material. Therefore, to ensure comparable preparation across all samples, a commercial survey of CSF was used for calibration curves and quality controls. This approach provides homogenous sample material, which is then further evaluated regarding total protein content and albumin concentration, and as such meets the required criteria for metrological traceability (ISO 15193) and qualified reference materials (ISO 15194). To our knowledge, only a few studies have previously evaluated CSF for the presence of biomarkers [16,17]. For example, Schoch et al. analyzed IL-6 in CSF samples from patients after aSAH [17]. However, the methodology used was not properly adapted for evaluating CSF. This is totally opposite to our method, since every step of the technique was properly adapted for CSF samples.

4.2. Error corrections

The standardized preparation method detailed previously ensures the comparability of the sample materials. Another critical aspect is the calculation of background signals from blank samples. These samples should not contain copeptin and therefore generate baseline RLU values. Regarding the application of immune−/immunoluminomatric assays, the common presumption is that minimal measurement signals indicate analyte-free material. The manufacturer of the copeptin assay specifies a mean RLU value for blank serum of 182. However, our testing showed that even water demonstrates an average RLU of 446. This observation is likely due nonspecific binding as well as imperfect washing steps, resulting in heightened measurement signals.

As such, blank CSF samples were measured to minimize potential sources of error or falsely high signals. Using this method, the background signal RLU value of 1500 was determined. This value was comparable with RLU values of patients of the control group.

The HI measurement allowed for the assessment of potential sample contamination with peripheral blood, providing a useful tool in excluding potentially erroneous samples from our analysis.

4.3. Copeptin concentrations in the serum and CSF of aSAH patients

The median serum copeptin concentration of the study cohort was 17.75 pmol/L^{-1}, similar to values previously reported by Fung et al. for patients with aSAH [4]. CSF copeptin values in patients with aSAH had a mean of 20.1 pmol/l (median of 5.79 pmol L^{-1}, range of 1–69.9 pmol/L^{-1}). This represents the first report measuring copeptin concentrations in CSF of patients with confirmed aSAH or patients suffering any form of neurological disease. The presence of copeptin in CSF of patients with aSAH could be associated with the experimental hypothesis that vasopressin plays a role in the development of vasospasm and edema, which results after aSAH. Since copeptin is released from the same common precursor as vasopressin, its detection may provide evidence regarding this theory [18,19]. In contrast, the control group had copeptin concentrations below the lower detection limit (< 0.78 pmol/L^{-1}) of the assay used. This supports the possibility of a relationship between the presence of copeptin in CSF and neurological diseases such as aSAH.

However, in order to make a definitive statement regarding the utility of CSF copeptin concentration as a prognostic biomarker, a larger patient cohort would be necessary. Nevertheless, this work demonstrates the feasibility of CSF copeptin measurement and provides the foundation for further analyses and research in neurological diseases and their potential biomarkers. Additionally, the results suggest a possible relationship between the local presence of copeptin and acute neurological diseases.

5. Conclusions

The immunoluminometric assay is a useful tool to measure copeptin values in the cerebrospinal fluid of patients. This assay opens a new door for further analysis of possible effects and relationships between CSF copeptin levels and neurological disorders. Additionally, it represents the first attempt to demonstrate the presence of copeptin levels in the CSF of patients with aSAH and provide the first range of values in such patients.

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