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Analytical performance of an immunoassay to measure proenkephalin

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ABSTRACT

Background: Endogenous opioids, enkephalins, are known to increase with acute kidney injury. Since the mature pentapeptides are unstable, we evaluated the performance of an assay that measures proenkephalin 119–159 (PENK), a stable peptide formed concomitantly with mature enkephalins.

Methods: PENK assay performance was evaluated on two microtiterplate/chemiluminescence sandwich immunoassay formats that required 18 or 1 h incubation times. PENK concentration was measured in plasma from healthy individuals to establish a reference interval and in patients with varied levels of kidney function and comorbidities to assess the association with measured glomerular filtration rate (mGFR) using iothalamate clearance.

Results: Assay performance characteristics in plasma were similar between the assay formats. Limit of quantitation was 26.0 pmol/L (CV = 20%) for the 1 h assay and 17.3 pmol/L (CV = 3%) for the 18 h assay. Measurable ranges were 26–1540 pmol/L (1 h assay) and 18–2300 pmol/L (18 h assay). PENK concentrations are stable in plasma stored ambient to 10 days, refrigerated to at least 15 days, and frozen to at least 90 days. Results were comparable in paired SST serum and EDTA plasma. Age and sex were not associated with PENK concentrations in healthy individuals (reference interval: 36–97.5 pmol/L). Plasma PENK concentration correlated with mGFR. In a multivariate model PENK concentration, age, sex and transplant status were significant predictors of mGFR, and 49% of predicted GFR values fell within 30% of the mGFR.

Conclusions: Both assay formats are accurate and precise for measuring clinically relevant PENK concentrations. The association of PENK concentration with mGFR is influenced by gender, age, and history of kidney transplantation. Future studies will determine if blood PENK can be used clinically to estimate GFR and/or detect AKI.

1. Introduction

Proenkephalin A is a precursor of the enkephalin family of endogenous opioids. It is a prohormone that is proteolytically processed to form several active pentapeptides including methionine-enkephalin and leucine-enkephalin together with several other peptide fragments (enkelytin and C-terminal extended methionine-enkephalin peptides) (Fig. 1). Enkephalins are widely secreted to act on locally expressed opioid receptors, specifically the δ opioid receptors. These opioid receptors are also widely expressed, with the highest density found in the kidney [1]. Subsequent to receptor binding the biological effects of enkephalins include nociception, anesthetics, and cardiovascular regulation [2]. These δ opioid agonists stimulate natriuresis and diuresis [3]. While several studies have demonstrated that elevated concentrations are associated with adverse outcomes, the association has in general been proportional to the change in renal function. Indeed, increased concentrations are associated with decreased renal function in several populations including sepsis [4], heart failure [5,6], cardiac surgery [7], and myocardial infarction [8].

The biologically active enkephalins are difficult to directly measure given their rapid clearance via receptor binding or degradation [9,10]. Specifically, their stability after sample collection is short (15 min half-life) making laboratory measurement of physiologic concentrations nearly impossible [11]. However, the proenkephalin A peptide (PENK, amino acids 119–159) is produced in proportional concentrations to the active peptides and is stable after collection for at least several days [12]. The previously published PENK assay utilized one polyclonal antibody and one monoclonal antibody which limited the assay specificity [12]. Thus we evaluated the analytical performance of a new PENK immunoassay that employs two highly specific monoclonal

Abbreviations: PENK, proenkephalin; CKD, chronic kidney disease; CV, coefficient of variation; mAb, monoclonal antibodies; HBT, heterophile blocking tubes

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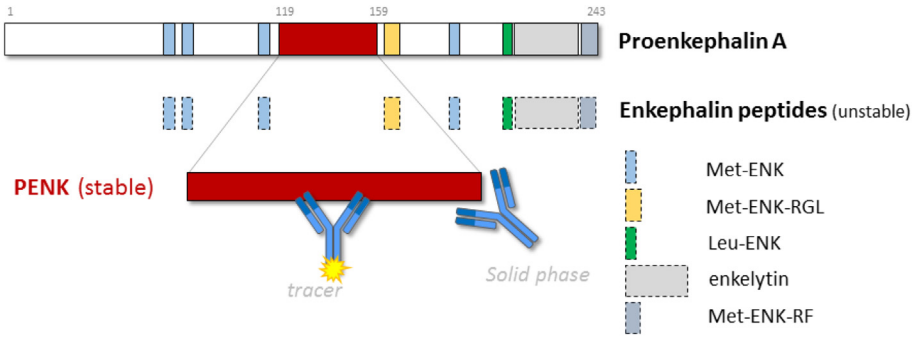

Fig. 1. Scheme showing the proenkephalin and measurement of PENK. Proteolytic cleavage of proenkephalin 1–243 results in four copies of Met-Enk, and one copy each of Leu-Enk, Met-Enk-RGL, Met-Enk-RF and enkelytin, respectively. The assay described here uses monoclonal antibodies directed against the middle portion of PENK (tracer antibody) and the C-terminus (capture antibody on solid phase), respectively.

antibodies. We also measured blood PENK concentration in a healthy reference population and a cohort of patients with and without known renal disease to establish a reference range and associations with directly measured glomerular filtration rate (mGFR). Results suggest PENK could be developed as an endogenous marker of GFR, and possibly used to detect and stage both, CKD and AKI.

2. Materials and methods

2.1. Antibody generation and PENK assay

Monoclonal antibodies directed against PENK 129–144 (anti-PENK 129–144 mAb) and PENK 152–159 (anti-PENK 152–159 mAb) were generated by standard procedures [13]. Briefly, Balb/c mice were immunized and boosted with peptide-BSA conjugates (JPT Peptide Technologies GmbH; Berlin, Germany) and spleen cells were fused with SP2/0 myeloma cells to generate hybridoma cell lines. Antibodies were selected that would bind either to immobilized peptides 129–144 or 152–159, and full-length PENK 119–159, respectively and then subsequently purified using Protein G chromatography to obtain > 95% purity.

The epitope specificities of the monoclonal antibodies anti-PENK 152–159 and anti-PENK 129–144 were delineated by assessing binding to peptides related to the peptides used for immunization (Supplemental Tables 1 and 2). To map the epitope of the anti-PENK 152–159 capture antibody, different N-terminal biotinylated peptides were immobilized on microtiter plates coated with streptavidin. The binding (B) of labeled antibody on microtiter plates with the indicated peptides was analyzed and compared relative to binding with PENK 145–159, which represents the very C-terminal portion of native PENK 119–159. To map the epitope of the anti-PENK 129–144 tracer antibody, different peptides were directly immobilized on microtiter plates. The binding (B) of labeled antibody on microtiter plates with the indicated peptides was analyzed and compared relative to the binding with PENK 131–142.

The PENK assay (trade name sphingotest® penKid®) is a chemiluminescence sandwich immunoassay which uses polystyrene microtiter plates as solid phase (Sphingotec GmbH, Hennigsdorf, Germany). Purified anti-PENK 129–144 labeled with MACN-Acridinium-NHS-ester was used as the tracer antibody. The anti-PENK 152–159 antibody was coated to white polystyrene microtiter plates (Greiner Bio-One International AG, Austria) as the capture antibody. Remaining binding sites on the plate were blocked with 3% Karion, 5 g/L BSA (protease free), 6.5 mmol/L monopotassium phosphate, 3.5 mmol/L sodium dihydrogen phosphate (pH 6.5). The assay was calibrated using dilutions of synthetic PENK 119–159. The lowest calibrator did not contain PENK 119–159, but a concentration of 5.5 pmol/L was assigned to facilitate logarithmic evaluation. The calibrators were lyophilized in 20 mmol K_2PO_4, 6 mM Na_2-EDTA, 5 g/L BSA, 100 µM Leupeptin, 50 µM Amastatin, pH 8.0, and reconstituted in heat-inactivated horse serum (Gibco® Thermofisher Scientific, Boston, USA) with 0.1% ProClin950 (standard zero matrix) prior to use.

Two versions of the assay were evaluated that incorporate 1 h or 18 h sample incubations using the same set of antibodies. The 1 h assay uses microtiter strips and is best suited for measurement of fewer samples at one time while the 18 h assay uses a 96-well assay plate that is best suited for assaying larger numbers of samples. Samples/calibrators (50 µL) were pipetted into coated microtiter plates. After adding labeled anti-PENK 129–144 mAb (150 µL), the microtiter plates were incubated for either 1 h or 18 h at 22 °C without agitation. Unbound tracer was removed using washing solution (350 µL per well,5 times). Remaining chemiluminescence was measured for 1 s per well by using the Centro LB 960 microtiter plate luminescence reader (Berthold Technologies GmbH & Co. KG, Germany). The concentration of PENK was determined using a 5-point calibration curve (30.8–2496 pmol/L) for the 18 h version, and a 6-point calibration curve (0 [defined as 5.5] -1540 pmol/L) for the 1 h version. Calibrators as well as samples were run in duplicate with a required < 20% CV between the duplicates.

2.2. Patient samples

Residual EDTA plasma and serum samples were collected from routine laboratory testing for analytical validation studies. Samples used to establish reference intervals were collected from healthy donors after a minimum 12 h fast. Individuals with any major medical problems (bleeding/clotting, diabetes, heart failure or other cardiovascular events, kidney disease, malignancy, circulatory disorders) were excluded, and use of tobacco products or NSAIDs was not permitted for at least 72 h prior to sample collection.

To determine associations with mGFR, PENK was measured in plasma from patients with and without risk factors for CKD undergoing clinical GFR measurement. These studies were approved by the Mayo Clinic Institutional Review Board. In total, PENK concentrations were measured in EDTA plasma from 1191 patients. Samples were collected between December 2014–July 2015 and stored frozen at −80 °C until testing for PENK on the 18 h 96-well plate assay in December 2016. Measurement of iothalamate was performed by LC-MS/MS [14]. Indications for iothalamate clearance testing were potential kidney donation (12%), post kidney donation (4.4%), chronic kidney disease (6.9%), kidney transplant recipient (58%), and other non-kidney solid organ transplant recipient (19%).

2.3. Assay performance studies

A full analytical evaluation of the proenkephalin 119–159 (PENK) assay was performed on both, the 1 h and the 18 h assay, using similar and complementary protocols. The 18 h assay was assessed at Mayo Clinic (Rochester, MN) and the 1 h assay was assessed at Sphingotec GmbH (Hennigsdorf, Germany). Inter- and intra-assay precision was assessed by repeated measurements ($n = 20$) of pooled residual EDTA plasma containing low, mid, and high PENK on the same day (intra) or on 20 different days (inter). Acceptance criteria were CV ≤10% for intra-assay precision and CV ≤15% for inter-assay precision. Accuracy was assessed by adding known concentrations of PENK (100, 190, 265,