Clinica Chimica Acta 413 (2012) 483–489

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Analysis of molecular forms of urine Retinol-Binding Protein in Fanconi Syndrome and design of an accurate immunoassay

Keith A. Burling [a], Pedro R. Cutillas [b], David Church [c], Marta Lapsley [d], Anthony G.W. Norden [c,*]

[a] Core Biochemical Assay Laboratory, NIHR Cambridge Biomedical Research Centre, Addenbrooke's Hospital, Cambridge CB2 2QQ, United Kingdom
[b] Analytical Signalling Group, Centre for Cell Signalling, Barts Cancer Institute, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, United Kingdom
[c] Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge University Teaching Hospitals, Box 232, Hills Road, Cambridge CB2 2QR, United Kingdom
[d] South-West Thames Institute for Renal Research, Wrythe Lane, Carshalton, Surrey SM5 1AA, United Kingdom

ARTICLE INFO

Article history:
Received 10 October 2011
Received in revised form 28 October 2011
Accepted 8 November 2011
Available online 19 November 2011

Keywords:
'Top-down' protein mass spectrometry
Retinol-Binding Protein
Retinol-Binding Protein 4
Tubular proteinuria
Renal Fanconi Syndrome
Dissociation-enhanced lanthanide fluorescence immunoassay

ABSTRACT

Background: Retinol-Binding Protein in urine (uRBP), a biomarker for the proximal renal tubular disease of congenital and acquired Fanconi Syndrome (FS) occurs in multiple forms. However these have not had quantitative mass spectrometric (MS) analysis, nor is there a validated assay for defined molecular species of uRBP with linearity on sample dilution.
Methods: A 'Top-down' MS approach identified distinct forms of uRBP differing by only one amino acid. Based on this, we designed a dual-monoclonal antibody-based fluorescence immunoassay calibrated with intact plasma RBP4.
Results: LC–MS showed that uRBP in FS (one Dent disease urine) comprised intact plasma RBP4 and C-terminal-truncated RBP4, desL-RBP4 and desLL-RBP4 in molar ratio 2:2:1. DELFIA® assay calibrated with plasma RBP4, formulated with two monoclonal antibodies (HyTest, Finland), mAb48 for capture and biotinylated-mAb42 for detection, provided good sensitivity (1 μg/L), working range > 500 μg/L and good linearity on sample dilution. The three predominant forms of uRBP were equipotent over the assay working range. uRBP reference range was <3 μg/mmol creatinine and FS patients had concentrations of 1000–5000 μg/mmol creatinine.
Conclusions: Using 'Top-down' MS analysis of uRBP we devised an accurate, linear, fluorescence immunoassay with defined RBP molecular targets optimal for uRBP measurement. Discrimination of elevated uRBP from the upper limit of normal was some 10-fold greater than previous assays.

1. Introduction

Urine Retinol-Binding Protein is an important biomarker for proximal renal tubular disease [1]. Disease of the proximal tubule causes 'tubular proteinuria' due to a failure to reabsorb predominantly smaller proteins, including RBP (21 kDa) which are readily filtered by the renal glomerulus [2–4]. Measurement of urine RBP appears to be the optimal biomarker of tubular proteinuria, the most constant feature of the renal Fanconi Syndrome [5]. Marked tubular proteinuria with a very high level of urine RBP, is found in hereditary forms of the renal Fanconi Syndrome, including Dent disease, Cystinosis and Lowe syndrome [5]. In addition, measurements of lower levels of urine RBP are a sensitive indicator of genetic carrier status of these hereditary disorders, of idiopathic Fanconi Syndromes and also of proximal tubular damage due to environmental toxins [6–8]. Such toxins are cadmium and uranium, as well as nephrotoxic antiviral drugs, particularly some used for HIV-treatment [1,9,10].

Intact RBP in plasma, termed RBP4 is 181 amino acids in length and circulates predominantly as a complex bound to another plasma protein, transthyretin. Approximately 14% of RBP is in the free form and is readily filtered by the glomerulus [11]. Plasma RBP4 has received recent interest as a marker of insulin resistance [12] and there have been several studies defining heterogeneity of RBP in plasma [13–17].

Measurement of urine RBP presents unique challenges. Early work demonstrated two major forms of RBP in urine with distinct immunological properties [18] though recent studies on the heterogeneity of urine RBP are limited [19]. There has been no recent quantitative characterisation of urine RBP using mass spectrometric techniques nor do the assays described for urine RBP have defined molecular targets. Unlike plasma, measurements of RBP in urine must be reliable over an approximate 10^5-fold range since slight increases as well as

Abbreviations: *CLCN5*, Chloride Channel 5, HGNC: 2023; Des L-RBP4, RBP4 truncated by loss of one L-leucine residue from the C-terminus; DesLL-RBP4, RBP4 truncated by the loss of two L-leucine residues from the C-terminus; DELFIA®, Dissociation-Enhanced Lanthanide Fluorescent Immunoassay; LC–MS, Liquid chromatography, mass spectrometry; mAb, Monoclonal antibody; pAb, Polyclonal antibody; *OCRL1*, Oculocerebrorenal Syndrome of Lowe, HGNC: 8108; uRBP, Retinol-Binding Protein, refers to RBP4 and truncated forms of RBP found in urine; RBP4, Retinol-Binding Protein 4, intact form of RBP, circulating in plasma, 181 amino acids in length, protein product of HGNC:9922 gene; TTR, Transthyretin (Prealbumin).

* Corresponding author. Tel.: +44 1223 586820; fax: +44 1223 216862.
E-mail addresses: kab45@cam.ac.uk (K.A. Burling), p.cutillas@qmul.ac.ul (P.R. Cutillas), david.church@addenbrookes.nhs.uk (D. Church), marta.lapsley@esth.nhs.uk (M. Lapsley), agwnorden@aol.com (A.G.W. Norden).

0009-8981/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2011.11.007

very high levels may be of diagnostic and prognostic importance [5,8,20]. Assays usually require multiple dilutions to be performed to cope with the wide range of concentrations in urine. Furthermore, standardisation of the urine assay is problematic since the forms found in urine have not been fully characterised. Marked non-linearity in the assay for urine RBP has been noted and may be due to differences between assay standard(s) and samples in the forms of RBP present [21]. Poor linearity on dilution has also been noted for the assay of plasma RBP4 [22].

A 'Top-down' mass spectrometric approach was chosen to characterise urine RBP molecular species differing by only one amino acid [23]. This approach is based on the MS analysis of the intact protein ion generated by electrospray ionisation. Although their exact origin is unclear, previous work suggested that forms of RBP sequentially truncated from the C-terminus would be found in urine [13–15,24]. Inspection of the amino acid sequence in this region suggested that usual 'Bottom-up' approaches, such as initial trypsinisation, were unsuitable to quantitate forms of RBP caused by such post-translational cleavages [13,23]. Based on MS analysis, it should be possible to design a urine RBP immunoassay with defined molecular targets that is also well-standardised.

Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA®), a technique with both high sensitivity and a wide dynamic range is attractive for assaying urine RBP [25]. This assay should show linearity between assay standard and samples and use commercially-available antibodies and standards [26]. Based on results of mass spectrometric analysis, we designed a urine RBP DELFIA® assay with a capture mAb and biotinylated detection mAb and a free plasma RBP4 standard which meets these requirements.

2. Materials and methods

2.1. Patient samples

Use of patient samples was in accordance with Addenbrooke's Hospital Research and Development Committee rules. Use of samples from healthy individuals was approved by the Local Research Ethics Committee and individuals also gave their informed consent. Urine samples from Fanconi Syndrome patients were stored in liquid nitrogen for up to 8 years prior to analysis; other samples were stored for up to 6 months at −80 °C. Diagnoses of patients used for the clinical validation study and numbers of samples were as follows. Patients with inherited Fanconi Syndrome: Dent disease *CLCN5* mutation, 20, [5]; Dent disease unidentified mutation, 4; Lowe syndrome, 5; Dent disease *OCRL1* mutation, 2 [27]; Autosomal Dominant Idiopathic Fanconi Syndrome, 4 [28] and Cystinosis, 1. Patients with acquired Fanconi Syndrome: Oncogenic Osteomalacia, 18 samples on one patient obtained pre-, peri- and post-operatively over approximately 10 months; Idiopathic, 9 and secondary to HIV therapy, 1. Carriers of inherited Fanconi Syndrome: carriers of Dent disease due to *CLCN5* mutation, 8.

2.2. Chemical and reagents

Recombinant RBP4 with a 6-His tag (Cat. #3378-LC) was from R&D Systems, Abingdon, England. RBP isolated from a pool of human Fanconi urine, Cat. #P124-1, was from Scipac Ltd., Sittingbourne, England. RBP4 complexed to transthyretin (Cat #8RP7) was from Hytest, Turku, Finland.

2.3. Preparation of RBP from Fanconi Syndrome urine for mass spectrometric analysis

Urine RBP was prepared from the urine of a patient with Dent disease by a minor modification of the method of Vahlquist et al. [29]. The RBP concentration of the final preparation was determined by UV spectrophotometry at 280 nm based on the reported absorption coefficient of 1.87 for a 1 mg/mL solution of RBP isolated from Fanconi urine [30].

2.4. 'Top-down' mass spectrometric analysis

MS analysis was performed by an LC–MS system consisting of a nanoAcquity LC connected to a Q-TOF Premier (Waters Ltd., Manchester, U.K.). Separation was by reversed phase liquid chromatography (RPLC) on a column packed in-house with POROS® R10 material (Applied Biosystems, Warrington, U.K.) in capillary PEEK tubing of dimensions 0.1 × 100 mm. Mobile phase A was 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile. Gradient elution was from 1% B to 35% B in 15 min at a flow rate of 1 μL/min. Eluted polypeptides were introduced online into the Q-TOF MS and ionized in a nanoflow electrospray ion source equipped with a stainless steel emitter (Proxeon, Thermo Scientific, Hemel Hempstead, U.K.) maintained at about 3 kV. The instrument was calibrated prior to the analysis and then spectra were lock-mass corrected post-acquisition to produce mass accuracies in the region of 10 ppm or better. MassLynx™ MS 4.0 (Waters Ltd) was used for data acquisition, visualisation and deconvolution of multiply charged protein ions to reveal the mass of the analysed proteins.

2.5. DELFIA® mAb assay

Nunc® MaxiSorp® microtitre plates (PerkinElmer, Cambridge, U.K.) were coated with anti-RBP4 mAb RB48 (Hytest Ltd., Turku, Finland) using 200 μL per well of a 1:4000 dilution in 0.1 mol/L sodium bicarbonate buffer (pH 9.2) and the plate incubated overnight at 4 °C, followed by 4 washes in an automated plate washer (PerkinElmer DELFIA® Platewash Cat. #1296-026) using DELFIA® Wash Buffer (PerkinElmer, Cat. #1244-114).

The assay stock calibrant was Free Plasma RBP4, 10,000 μg/L, Cat. #8RF9, Hytest (Turku, Finland), made by adding 1 mL water to the purified protein which had been lyophilised from PBS, pH 7.4, 0.1% sodium azide. This stock calibrant was diluted 1:20 in DELFIA® Multibuffer (PerkinElmer Cat. #1244-106) to prepare a working calibrant of 500 μg/L, this was stored in aliquots at −40 °C. A fresh aliquot was used for each assay. The working calibrant was serially diluted 1 in 4 in DELFIA® Multibuffer to create a 5 point calibration curve with a range of 500 to 1.95 μg/L. DELFIA® Multibuffer was used as the zero calibrant.

Urine samples were routinely diluted 1 in 5 in DELFIA® Multibuffer before analysis. When further dilutions were required, these were in the same buffer.

Three QC samples (with low, mid and high concentrations of RBP) were prepared from pooled human urine. These samples were diluted 1 in 5 in DELFIA® Multibuffer and run at the beginning and end of each plate.

For the assay, 100 μL of RBP4 calibrant, QC sample or patient sample was pipetted into the wells in duplicate followed by an additional 100 μL of DELFIA® Multibuffer. The plate was incubated for 3 h on a DELFIA® plateshaker (Perkin Elmer Cat. #1296-001) set at 'slow' speed. The plate was then washed 6-times as before and 100 μL of biotinylated anti-RBP4 mAb added to each well (Biotinylated-RB42, Hytest, diluted 1:2000 in DELFIA® Multibuffer). The plate was incubated for 2 h on a DELFIA® plateshaker. After incubation, the plate was washed six times as before; 100 μL of streptavidin conjugated to Europium (PerkinElmer Cat.#1244-360, diluted 1 in 1000 in Multibuffer) was added to each well followed by incubation for 40 min on a DELFIA® plateshaker.

After incubation, the plate was washed six times. 200 μL of DELFIA® Enhancement Solution (Perkin Elmer Cat#1244-105) was added to each well. The plate was incubated for 5 min as before and then incubated for a further 5 min without shaking. Time-Resolved Fluorescence in each well was determined using a Victor3™ plate reader (PerkinElmer).