Clinica Chimica Acta 403 (2009) 56–62

Contents lists available at ScienceDirect

Clinica Chimica Acta

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

Surface plasmon resonance immunoassay analysis of pituitary hormones in urine and serum samples

Juan Treviño [a,b,*], Ana Calle [c], José Miguel Rodríguez-Frade [d], Mario Mellado [d], Laura M. Lechuga [a,b]

[a] Grupo de Nanobiosensores y Biofísica Molecular, Centro de Investigación en Nanociencia y Nanotecnología (CIN2: CSIC-ICN), ETSE, Campus UAB, Bellaterra, Barcelona, Spain
[b] Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Barcelona, Spain
[c] Instituto de Microelectrónica de Madrid (CNM-CSIC), Madrid, Spain
[d] Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología (CNB-CSIC), Madrid, Spain

ARTICLE INFO

Article history:
Received 10 October 2008
Received in revised form 28 December 2008
Accepted 20 January 2009
Available online 27 January 2009

Keywords:
SPR
Immunosensor
hTSH
hGH
hFSH
hLH

ABSTRACT

Background: Direct determination of four pituitary peptide hormones: human thyroid stimulating hormone (hTSH), growth hormone (hGH), follicle stimulating hormone (hFSH), and luteinizing hormone (hLH) has been carried out using a portable surface plasmon resonance (SPR) immunosensor.
Methods: A commercial SPR biosensor was employed. The immobilization of the hormones was optimized and monoclonal antibodies were selected in order to obtain the best sensor performance. Assay parameters as running buffer and regeneration solution composition or antibody concentration were adjusted to achieve a sensitive analyte detection.
Results: The performance of the assays was assessed in buffer solution, serum and urine, showing sensitivity in the range from 1 to 6 ng/mL. The covalent attachment of the hormones ensured the stability of the SPR signal through repeated use in up to 100 consecutive assay cycles. Mean intra- and inter-day coefficients of variation were all <7%, while batch-assay variability using different sensor surfaces was <5%.
Conclusions: Taking account both the excellent reutilization performance and the outstanding reproducibility, this SPR immunoassay method turns on a highly reliable tool for endocrine monitoring in laboratory and point-of-care (POC) settings.

1. Introduction

The pituitary gland is part of the endocrine system which regulates many physiological processes including growth, reproduction and metabolism. The anterior pituitary produces and secretes thyroid stimulating hormone (hTSH), growth hormone (hGH), follicle stimulating hormone (hFSH), luteinizing hormone (hLH), and other peptide hormones such as the adrenocorticotropic hormone (ACTH), prolactin (PRL) and endorphins. Among them, we have chosen the evaluation of the hormones hTSH, hGH, hFSH and hLH due to their high biological interest and diagnostic value. hTSH, hLH and hFSH are heterodimeric glycoprotein hormones, with molecular weights about 30 kDa, formed by an alpha chain and a beta chain [1]. The alpha chain is identical in these three two hormones as well as in the placental hormone human chorionic gonadotropin (hCG). The beta chain is unique for each hormone and is the responsible for the bioactivity and the immunological differentiation. hGH is a non-glycosylated polypeptide hormone with a molecular weight of 22 kDa [2].

hTSH regulates the endocrine function of the thyroid gland, stimulating the secretion of the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3), which are fundamental for normal metabolism. Normal hTSH values range from 0.4 to 4 μIU/mL [3,4]. Increased values are associated to hypothyroidism and pituitary tumors, while drops in its concentration reveal hyperthyroidism or hypopituitarysm. hTSH is measured as well in newborn screening for congenital hypothyroidism [3,5], and those patients with a hTSH value >20 μIU/mL are recalled for further testing.

hGH is essential for normal growth and development. Besides height growth in children, it regulates the metabolism throughout all adult life. It has a pulsatile secretion which results in widely fluctuating levels that can reach 50–100 ng/mL peaks, while basal concentration falls below 0.03 ng/mL [6,7]. Disorders in hGH secretion produce excess or deficiency problems. hGH excess, generally due to benign pituitary tumours, causes acromegaly. hGH deficiency produces different problems at various ages, but its effects are more severe in children as produces growth failure. A cut-off value of 10 ng/mL after stimulation is widely accepted for hGH deficiency diagnostic.

Gonadotropic hormones hFSH and hLH play a key role in the development and function of the reproductive system. hFSH stimulates spermatogenesis in men while it controls ovarian follicle growth

* Corresponding author. Centro de Investigación en Nanociencia y Nanotecnología (CIN2: CSIC-ICN), Edificio Q - ETSE, 3ª Planta, Campus UAB, 08193, Bellaterra, Barcelona, Spain. Tel.: +34 93 586 80 12; fax: +34 93 586 80 20.
E-mail address: juan.trevino@cin2.es (J. Treviño).

0009-8981/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2009.01.015

and estrogens production in females. Normal hFSH levels vary from 2 to 10 mIU/mL. Concentration increases in females just before ovulation to 5–20 mIU/mL and reaches up to 100 mIU/mL in post menopause. Determination of hFSH concentration is used for the diagnosis of reproduction and development disorders as infertility and premature or delayed puberty [8]. hFSH tests are used as well to check ovarian reserve and menopausal status. In men, hLH stimulates testosterone production while in females it regulates androgen and progesterone production and activates ovulation. Normal hLH values range from 2 to 10 mIU/mL, with a marked increase to 20–100 mIU/mL in females previous to ovulation and 20–70 mIU/mL in post menopause. Abnormally increased hLH levels are related with primary gonadal dysfunction, polycystic ovary syndrome, post menopause and pituitary adenoma in females. High hLH levels are present in men with hypogonadism, and primary testicular failure [9]. hFSH and hLH are simultaneously determinate to check ovulation, for the diagnosis of reproductive disorders and for monitoring endocrine therapy.

Different immunoassay methods as radioimmunoassays (RIA), immunometric assays (IMA) or enzyme-linked immunosorbent assays (ELISA) have been developed for the determination of those hormones [3,4,6–9]. Immunoassay techniques have been extensively used for fast diagnostic and monitoring in point-of-care settings because of its simplicity, sensitivity and specificity [10]. Routine measurement for the determination of these hormones is generally carried out using automated high throughput systems based mostly in immunometric assays with chemiluminiscent labels (ICMA). These assays reach very low limits of detection, in the range of pM–fM, but they show heterogeneous results and sensitivities, mainly due to the heterogeneity of the hormones and the reference preparations, and are affected by matrix effects [3,4,6–9]. Furthermore, the complexity of the equipment make impossible to use them near the patient, enlarging the testing time due to logistics of sample transport and results management. It would be a benefit for the treatment of the diseases associated to these hormones having a diagnostic method that can produce immediate results in point-of-care (POC) settings. Biosensors can achieve similar analytical characteristics to immunoassays but they offer some additional advantages as a rapid response, portability, simple use and automation [11,12]. Surface Plasmon Resonance (SPR) biosensors [13] allow the real-time monitoring of biomolecular interactions as increases in refractive index caused by mass changes at the sensor surface. SPR devices have shown the necessary performance and in addition they provide a real-time analysis avoiding labelling steps. However, the employment of optical biosensors for real clinical applications is hampered due to the matrix effects produced by the non-specific binding, onto the sensor surface, of the components of biological fluids. Applications of SPR biosensors that measure directly clinical samples are still unusual. Some methods have developed using serum [14,15], plasma [16], saliva [17] and urine [18,19], but generally such analyses require sample pre-treatment, the employment of extremely dilute samples and the use of signal amplification methods.

The aim of the present study is to develop an SPR immunosensor capable to detect these pituitary hormones, at physiological concentration levels, directly in urine and serum, with minimal sample treatment and handling. To our knowledge, this is the first example of a biosensor method for the analytical determination of hGH and hTSH and the first SPR biosensor method for the analytical determination of hFSH and hLH. The simple operation and fast response turns the SPR device in a useful equipment for clinical laboratories and POC settings.

2. Materials and methods

2.1. Reagents

Recombinant hGH was obtained from Pfizer (Spain). hFSH (NIDDK-hFSH-I-SIAFP-2, AFP-7220C, 14,296 IU/mg in terms of WHO 2nd IRP-HMG), hLH (NIDDK-hLH-I-SIAFP-2, AFP-4395A, 8444 IU/mg in terms of WHO 1st IRP 68/40) and hTSH (NIDDK-hTSH-SIAFP-B-2, AFP-3951A, 7.54 IU/mg in terms of highly purified hTSH) were obtained from Dr. Parlow, National Hormone & Peptide Program (NHPP), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Torrance, CA (US). Human serum was purchased from Sigma-Aldrich (Steinheim, Germany). Urine was obtained from healthy laboratory volunteers and was used without any further treatment. Monoclonal antibodies (mAb) were obtained and characterized at the National Centre for Biotechnology (CNB-CSIC, Madrid, Spain). Ammonium sulphate purified mAbs were employed. Three mAbs were chosen as candidates to carry the hTSH assay: hTSH-1, hTSH-2 and hTSH-3. mAbs hFSH-1, hFSH-2, hFSH-9 and hFSH-14 were chosen for the hFSH assay and mAbs hLH-6 and hLH-7 for the hLH assay. mAb-hGH-12 was selected for the hGH assay using previous data [20].

Mercaptoundecanoic acid, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(-3 dimethyl-amino-propyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (Steinheim, Germany). Organic solvents used in gold chip cleansing process: tricholoroethylene, acetone and ethanol, and piranha solution components H_2SO_4 and H_2O_2 were supplied by Merck (Darmstadt, Germany). Potassium chloride, sodium chloride, disodium hydrogen phosphate and potassium dihydrogen phosphate, used for the preparation of PBST buffer (10 mM phosphate pH 7.4 with 137 mM NaCl, 2.7 mM KCl and 0.05% Tween 20), and PBST-S buffer (10 mM phosphate pH 8 with 500 mM NaCl, 2.7 mM KCl and 0.1% Tween 20), acetic acid and sodium acetate for the preparation of acetate buffers were provided by Panreac (Barcelona, Spain). Ethanolamine hydrochloride blocking agent was obtained from Acros Organics (Geel, Belgium). Tween-20 was purchased from Quantum Appligene (Heidelberg, Germany).

2.2. Instrumentation

A commercial Surface Plasmon Resonance biosensor, from Sensia SL (Spain) was employed for SPR measurements. The sensor has two flow cells with a volume of 300 nL each, allowing the measurement of two independent samples or the use of a reference channel. The device incorporates optics and upgradeable electronic modules as well as computer controlled pumps, valves and injection fluidics. All the measurements were performed by sample injection using the flow delivery system incorporated in the platform that assures the injection for analysis of precise volumes of 220 µL while maintaining a continuous flow of buffer between 10 and 40 µL/min. Further description of this system can be found elsewhere [21].

2.3. Preparation of the sensor surface. Immobilization procedure

A monomolecular layer of the ligand was achieved by the formation of a self-assembled monolayer (SAM). The different hormones were covalently attached to the sensor surface using amino coupling between carboxyl group end in the SAM and amino groups in the protein. For this purpose, SPR gold chips were cleaned using trichloroethylene, acetone and ethanol. Chips were then submerged in fresh piranha solution (H_2SO_4/H_2O_2, 3:1), rinsed, ultrasonicated in distilled water and dried with N_2. Finally, gold chip was pushed over the flow cells and the prism was adhered using matching refractive index oil. The protein covalent immobilization process was carried at a constant flow of 10 µL/min. An alkanethiol SAM was formed by adsorption of a solution 0.05 mM of mercaptoundecanoic acid on the gold surface. Then, ethanol was flowed to remove alkanethiol excess and a continuous flow of water was maintained for the following steps of the immobilization process.

For protein amino coupling, the surface was activated using a mixture of EDC 0.2 M and NHS 0.05 M. Immediately after, the appropriate hormone was immobilized via its amino groups. Then, an ethanolamine 1 M and pH 8.5 solution was injected to deactivate residual unreacted groups on the surface. Finally, potential non-covalent bound biomolecules remaining on the surface were removed by an injection of 100 mM HCl at 30 µL/min.

2.4. SPR immunoassay format

A binding inhibition immunoassay format was used for the detection of the hormones. For calibration curves, triplicate standard concentrations of the correspondent hormone in the 10^{-4} µg/mL–100 µg/mL range in PBST and blank controls were mixed (1:1) with the antibody in PBST during 10 min. Afterwards, solutions were injected sequentially over the sensor surface at 20 µL/min and SPR signal was monitored in real-time using the two flow cells of the device in parallel. Reutilization of the sensor surface was achieved by an injection of HCl 5 mM regeneration solution at a flow speed of 30 µL/min. SPR signal of each standard was expressed as the percentage of the maximum response [100 × (SPRsignal/SPRsignal,max)]. The averaged responses of the three standards measured for each concentration were plotted versus the logarithm of the hormone concentration and fitted to a four-parameter logistic equation:

$$y = \left\{D + (A - D) / \left[1 + (x/C)^B\right]\right\}$$

were x refers to hormone concentration, y is the response, A is the asymptotic maximum, corresponding to the signal in absence of analyte, B is the slope at the inflection point, C is the inflection point, equivalent to the half inhibitory concentration I_{50} and D is the asymptotic minimum, corresponding to the background signal.

2.5. Antibody selection

The candidate antibodies were tested in order to choose the ones which could provide the best assay performance. Calibration curves in PBST using different