Enzyme-Linked Immunosorbent Assay for Determination of Cyclosporin A in the Whole Blood

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Abstract—A test-system based on enzyme-linked immunosorbent assay (ELISA) for quantitative determination of cyclosporin A (CSA) in human whole blood has been developed. The detection limit of the method was 25 ng/ml, the linearity of the method in the concentration range of 60–1400 ng/ml varied from 94 to 105%, the variation coefficient did not exceed 8%. The novel method exhibited good correlation with radio-immunoassay and polarization fluoroimmunoassay methods; the linear regression coefficients were 0.965 and 0.984, respectively. The developed test system is stable for at least 9 months when stored at 4°C and can be used in clinical practice.

Keywords: cyclosporin A, enzyme-linked immunosorbent assay (ELISA).

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INTRODUCTION

Methods of enzyme-linked immunosorbent assay (ELISA) are widely used in medical diagnostics including clinical laboratories for therapeutic monitoring of hormones, drug substances, diagnostics of viruses (HIV, hepatitis) and bacteria and also in biochemical and microbiological studies.

Therapeutic drug monitoring is usually performed to control the level of particular drug substance in blood: it is important to be sure that a dosage chosen for a particular patient is sufficient to achieve the therapeutically effective concentration that does not exhibit toxic effects.

Cyclosporin A (CSA) possesses potent immunosuppressive properties [1, 2]. CSA therapy of patients with transplanted organs resulted in a significant (more than 3-fold) increase in their survival rate after transplantation. CSA is intensively metabolized in the body by the hepatic cytochrome P450 monoxygenase system. At present, about ten CSA metabolites have been isolated from blood and internal organs and characterized. These metabolites exhibit high nephro- and hepatotoxicity. Individual variations in CSA excretion from the body and high toxicity of its metabolites require constant monitoring of CSA content in patients [3, 4].

Thus, there is a clear need for development of rapid and accurate methods for determination of blood CSA concentrations. Frequent analyses also demand additional requirements such as simplicity, reliability, reproducibility, and low cost. At present, various (both physicochemical and immunochemical) methods are used for determination of CSA concentrations [5-9]. Although the HPLC method is considered as a golden standard for most practical analyses it is widely used polarization fluoroimmunoassay (PFIA) (which requires special equipment developed by the test-system supplier, Abbott, USA) and radioimmunoassay (RIA) methods. Recent developments include combination of HPLC with tandem mass spectrometry (HPLC-MS/MS), which can simultaneously determine not only blood CSA but also other immunosuppressants [10, 11]. However, all existing methods for determination of CSA concentrations have certain drawbacks (e.g. long duration of analysis, high costs of equipment, used of radioisotope labels). In addition, high costs of imported test-systems available at the market stimulate the development of cheaper Russian analogues.

The enzyme-linked immunosorbent assay may represent good alternative for determination of blood CSA concentrations; ELISA is often used in clinical practice due to relative simplicity, reliability, sensitiv-
ity, reasonable low cost of equipment, which does not require highly qualified staff.

The aim of this study was to develop an ELISA method for CSA determination in human whole blood and to compare results obtained using this method with other existing methods.

MATERIALS AND METHODS

A conjugate of cyclosporin C (CSC) with bovine serum albumin (CSC-BSA) was prepared using the method described in [12].

Monoclonal antibodies to CSA (MAb) were kindly presented by the Russian Scientific Center of Molecular Diagnostics and Therapy (Russia). MAb cross-reactivity to CSA metabolite did not exceed 10%. Lyophilization of an aqueous solution of anti-CSA MAb with addition of 1% D(+)-trehalose was performed using a Super-modulo 12 freeze-dryer (Edwards, England) as described in [13].

The following buffers were used in this study: 0.1 M Na-carbonate buffer, pH 9.5, (CB), 0.1 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.04% Tween-20 (Tris-T).

Calibration probes containing known amounts of CSA were prepared in Tris-T buffer with addition of 20% ethanol or CSA-free blood serum from the stock CSA solution in ethanol (5 mg/ml).

Patients’ whole blood samples containing CSA were supplied by the National Research Centre of Surgery (Russian Academy of Medical Sciences).

Calibration Probes And Blood Samples Preparation For Analysis

Blood samples and calibration probes prepared based on blood serum were incubated at room temperature for 30 min. Their aliquots (50 µl) were placed into microcentrifuge tubes and after addition of 150 µl of methanol into each tube they were thoroughly mixed 2–3 times for 10 min and then centrifuged (10 min. 12000 g, 20–25°C). Supernatant (100 µl) was added to Tris-T buffer (275 µl) and after mixing the resultant solutions were used for analysis.

ELISA procedure of cyclosporin A

The wells of microtiter plates (Greiner, Austria) were coated overnight at 4°C with solution of the CSC-BSA conjugate (150 µg/well) in CB with the chosen concentration. The plates were washed with Tris-T buffer (4 × 200 µl/well) and after addition of BSA solution in CB (5 mg/ml, 150 µl/well) they were incubated for 1 h at 37°C. Then plates were washed with Tris-T buffer (4 × 200 µl/well), dried and stored at 4°C.

Extracts of calibration probes of CSA or analyzed samples (50 µl) and 50 µl of MAb solution (with chosen concentration) in Tris-T buffer were sequentially added into wells with the adsorbed antigen. After incubation (1 h, 4°C) the wells were washed with Tris-T buffer (3 × 200 µl). Then 100 µl of working solution (1) of conjugate of sheep anti-mouse IgG antibody with horseradish peroxidase (Ab2-HRP) in Tris-T buffer was added to the wells and plates were incubated for 30 min at 37°C and washed as above. The color reaction was initiated by adding 100 µl of the substrate solution containing 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide into all wells (Immunotek, Russia). After 10–15 min the reaction was stopped by adding 100 µl of 0.5 M H₂SO₄ and optical density was registered at 450 nm. The measurement of the optical density of the enzymatic reaction product was performed using a multi-channel spectrophotometer for 96 well plates (Anthos 2020, Austria).

Sensitivity of this method was evaluated as mean value of optical density of CSA free solution minus 2 SD (n = 20). Statistical treatment of results was carried out using Microsoft Excel 2000. Here we report only statistically significant result (p < 0.05).

RESULTS AND DISCUSSION

For determination of whole blood CSA concentration we have chosen the scheme of indirect competitive ELISA. This method is based on competition between CSA in the analyzed sample and cyclosporin immobilized as CSC-BSA conjugate on the surface of polystyrene wells for binding to anti-CSA monoclonal antibodies (MAb). MAb bind both cyclosporin in solution and immobilized in polystyrene wells. The amount of antibodies bound with immobilized cyclosporin are detected by conjugate of Ab2-HRP. Complex formation is determined by oxidation of chromogenic substrate 3,3',5,5'-tetramethylbenzidine by HRP in the presence of hydrogen peroxide. Color intensity is inversely proportional to CSA concentration in the analyzed sample.

During the first step of the method development it was necessary to optimize concentrations of main immunoreagents. The following criteria were used: maximal difference between optical densities corresponding to the calibration probes 0 and 1400 ng/ml (at least 0.7 optical units), optical density of the calibration probe 0 ng/ml in the range 1–1.5 optical units; minimal consumption of reagents. Figure 1 shows the effect of concentrations of the immobilized CSC-BSA conjugate (from 0.25 to 0.75 µg/ml) and anti-CSA MAb in the reaction mixture (from 0.25 to 1.0 µg/ml) on the shape of the calibration curve. Although the curve characterized by the highest sensitivity corresponded to minimal concentrations of reagents (Fig. 1, curve 5) the maximal signal of optical density was less than 1. So, the following concentrations were used as the optimal ones: CSC-BSA conjugate – 0.5 µg/ml; MAb – 0.5 µg/ml, dilution of the Ab2-HRP conjugate – 1 : 6000 (Fig. 1, curve 4); they provided