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Homogeneous enzyme immunoassay modified for application to luminescence-based biosensors

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Abstract

Application of immunoassay to biosensors for use in the point-of-care setting ideally requires immunoassay without separation steps and with small volumes of both sample and reagents. The suitability of cloned enzyme donor immunoassay (CEDIA), one of a few homogeneous immunoassays available, was investigated for application to biosensors. This method is based on the bacterial enzyme β-galactosidase, which has been genetically engineered by others into two inactive fragments, enzyme donor (ED) and enzyme acceptor (EA). Association of the ED and EA fragments in the assay results in formation of active enzyme, which acts on substrate to generate a detectable signal. Sensitivity of commercially available CEDIA kits were compared, with respect to the sample and reagent volumes, using three different signal generation processes. The CEDIA kit for valproic acid and three substrates, a colorimetric (chlorophenol red-β-D-galactopyranoside), a chemiluminescent (Lumi-Gal 530), and a bioluminescent (Beta-Glo Assay System), were employed in the study. Our results indicate that the high sensitivity of the bioluminogenic substrate, D-luciferin-*O*-β-galactopyranoside, with short assay time and small volumes of sample and reagents required for the assay, simple handling, and relatively low expense, make this substrate, together with CEDIA, suitable for application to biosensors intended for drug and metabolite monitoring in the point-of-care setting.

Keywords: Homogeneous immunoassay; CEDIA; Spectrophotometry; Chemiluminescence; Bioluminescence; Substrates

There is a growing need for high-throughput analysis of small molecular substances [1]. For such analysis homogeneous rather than heterogeneous assays are preferred due to their simplicity, ease of automation, and higher throughput. A homogeneous immunoassay that employs complementation within the β-galactosidase system is known as CEDIA[1] [2,3]. CEDIA is based on the bacterial enzyme β-galactosidase, which has been genetically engineered into two inactive fragments, enzyme donor (ED) and enzyme acceptor (EA). When mixed together, the two fragments combine to form active enzyme; this process is termed "complementation." Covalent attachment of analyte to ED does not affect the ability of ED to associate with EA. Analyte present in a sample competes for binding to the limited number of antibody sites, making analyte–ED conjugate available for enzyme formation.

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[1] *Abbreviations used:* CEDIA, cloned enzyme donor immunoassay; CPRG, chlorophenol red-β-D-galactopyranoside; EA, enzyme acceptor; ED, enzyme donor; EDTA, ethylenediaminetetraacetic acid; GDP, 4-methoxy-4-(3-β-D-galactosidephenyl) spiro [1,2-dioxetane-3,2′-adamantane]; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid; R1, CEDIA reagent 1; R2, CEDIA reagent 2; TDM, therapeutic drug monitoring; VPA, valproic acid; RT, room temperature.

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The amount of active enzyme formed is directly proportional to the analyte concentration in the sample. During the assay, the level of enzyme with β-galactosidase activity can be determined either spectrophotometrically by the rate of chromogenic substrate hydrolysis or luminescently with chemi- or bioluminescent substrate. The CEDIA Kit for valproic acid (VPA), designed by Microgenics (Fremont, CA, USA) for use in automated analyzers, is a two-step procedure. For time saving, cost reduction, simplicity, and application to a ChemChip format [4], a one-step procedure introduced by Khanna et al. [5] was adapted and used in this study (Fig. 1). Based on simulation results, it is shown that one-step CEDIA has a wider analyte dynamic range than two-step CEDIA [6].

High-quality therapeutic drug monitoring (TDM) reagents, calibrators and controls for a number of drugs are available from Microgenics. This kit employs the standard two-step CEDIA procedure [3,6]. The commercial CEDIA kit for VPA contains the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) for quantification of enzyme activity. The hydrolysis of substrate is measured spectrophotometrically at 550–600 nm.

Luminescence-based assays are finding increased use in chemical and medical diagnostics. A chemiluminescent assay for β-galactosidase using Lumi-Gal 530 (Lumigen, Southfield, MI, USA), wherein chemiluminescence of the product is measured with a luminometer, was evaluated. This assay exhibits advantages over the standard spectrophotometric technique. It is simple and relatively inexpensive and has a 20-fold greater sensitivity [7].

Bioluminogenic substrates with even higher sensitivity have been developed for the determination of enzymes and many different analytes. The sensitivity of CEDIA has been substantially increased by utilization of a highly sensitive substrate for β-galactosidase, based on the release of D-luciferin from 6-*O*-β-galactopyranosyl-luciferin by the action of β-galactosidase. D-Luciferin can be easily quantified in a luminometric assay [8]. The Beta-Glo Assay System (Promega, Madison, WI, USA), is based on the two coupled enzyme reactions and allows for measuring β-galactosidase activity in a homogeneous assay format.

Our goal is to develop a simple, highly sensitive, single-step homogeneous immunoassay platform for application to a luminescence-based ChemChip [4].

Materials and methods

Homogenous immunoassay with spectrophotometric detection in microplate reader

Reagents for the homogeneous immunoassay. CEDIA valproic acid II kit (Catalog No. 100013) was purchased from Microgenics. Reagent 1 (R1) of CEDIA kit contains antianalyte antibody and EA. Reagent 2 (R2) consists of analyte–ED conjugate, antiimmunoglobulin secondary antibody, and substrate CPRG. Both reagents R1 and R2, supplied in lyophilized form, were reconstituted with provided buffer solutions and stored at 4 °C.

Solutions for calibration and standard curves. CEDIA Core TDM Multi-Cal kit (Catalog No. 100007, Microgenics) contained High and Low Calibrators (143.2 μg/ml VPA and 0.1 μg/ml VPA, respectively). Solutions of different VPA concentrations were prepared by dilution of the High Calibrator with 20 mM Hepes, pH 7.5. The concentrations of VPA used in the study (from 0.1 to 144 μg/ml) covered its therapeutic range from 50 to 100 μg/ml VPA.

Spectrophotometric detection of enzyme activity during CEDIA. Optical density (O.D.) of the colorimetric product was recorded using a Microplate Reader Spectra MAX 250 with its parameters being controlled by Soft Max Pro software (Molecular Devices, Sunnyvale, CA, USA). Spectrophotometric assays were carried out in 96-well microplates (Nunc-Immuno Module; No. 120080LE P891) according to the following procedure. The CEDIA reagents (R1 and R2) and VPA samples were equilibrated to 37 °C separately in the Microplate Reader Spectra Max 250 for 8 min. At the time of experiment, the VPA samples were transferred into R1, and immediately thereafter R2 was added to a mixture of R1 and VPA. After quick mixing, the plate was placed into the Microplate Reader. O.D. was recorded at 570 nm as a function of time for nearly 10 min in 22-s intervals at 37 °C.

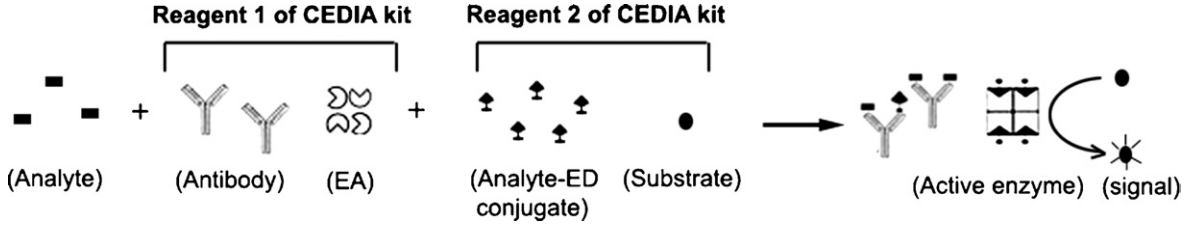

Fig. 1. One-step CEDIA assay. All of the CEDIA reactants are mixed simultaneously in the same vessel. Subsequently, color or luminescence can be measured depending on a signal generation process of substrate added.