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A highly sensitive enzyme immunoassay for evaluation of 2′-deoxycytidine plasma level as a prognostic marker for breast cancer chemotherapy

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ABSTRACT

A highly sensitive competitive enzyme immunoassay (EIA) has been developed and validated for the determination of the plasma level of 2′-deoxycytidine (dCyd), the potential prognostic marker for breast cancer chemotherapy. This assay employed a monoclonal antibody that recognizes dCyd with a high specificity, and 5′-succinyl-dCyd (5′sdCyd) conjugate of bovine serum albumin (5′sdCyd–BSA) immobilized onto microplate wells as a solid phase. The assay involved a competitive binding reaction between dCyd, in plasma sample, and the immobilized 5′sdCyd–BSA for the binding sites of the anti-dCyd antibody. The bound antibody was quantified with horseradish peroxidase-labeled anti-immunoglobulin second antibody and 3,3′,5,5′-tetramethylbenzidine as a peroxidase substrate. The concentration of dCyd in the sample was quantified by its ability to inhibit the binding of the antibody to the immobilized 5′sdCyd–BSA and subsequently the color formation in the assay. The assay limit of detection was 8 nM and the effective working range at relative standard deviations (R.S.D.s) of ≤10% was 20–800 nM. No cross-reactivity from the structurally related nucleobases, nucleosides, and nucleotides was observed in the proposed assay. Mean analytical recovery of added dCyd was 98–100 ± 3.2–8.2%. The precision of the assay was satisfactory; R.S.D. was 3.4–4.2 and 4.3–8.9% for intra- and inter-assay precision, respectively. The proposed EIA was compared favorably with HPLC method in its ability to accurately measure dCyd spiked into plasma samples. The analytical procedure is convenient, and one can analyze 200 samples per working day, facilitating the processing of large-number batch of samples. The proposed EIA is expected to contribute in further evaluation of dCyd as a prognostic marker for breast cancer chemotherapy and elucidation of the role of dCyd in various biological and biochemical systems.

1. Introduction

Breast cancer (BC) is the main cause of mortality among women worldwide [1,2]. The high rate of mortality in BC patients is attributed to the late diagnosis of the disease, and consequently the delayed initiation of the medical treatment by surgery, radiotherapy and/or mostly chemotherapy. Most of the available chemotherapeutic agents used in the treatment of BC patients are nucleic acid anti-metabolites and/or alkylating agents, thus a disturbance of nucleic acid balance is expected. Identifying of a compound that can be used as a marker for the early diagnosis of the disease or the prognosis of the patient to therapeutic program will be clinically very valuable and ultimately reduce the rate of mortality among BC patients. 2′-Deoxycytidine (dCyd) level in plasma of BC patients was suggested as a marker for monitoring the prognosis of the BC patients treated with combined chemotherapeutic agents, cyclophosphamide, methotrexate and 5-fluorouracil [3]. For refinement of the findings about dCyd as a prognostic marker and increase the understanding about the physiology of this nucleoside, a sensitive and selective analytical method for determination of dCyd in plasma was required.

In general, high-performance liquid chromatography (HPLC) is the most commonly used technique for determination of dCyd and/or other nucleosides in biological fluids [4–12]. However, all the HPLC methods reported, so far, for determination of dCyd [10–12] suffer from major disadvantages such as inadequate sensitivity, time-consuming, and/or the inadequate accuracy of the quantitation results because of the presence of many structurally related interfering substances, which usually necessitates pretreatment of the samples. Indeed, the differences in the pretreatment of the samples and the incomplete resolution of the chromatographic peaks result in discrepancies in the results of quantification. Furthermore, the methods are not applicable for screening of large number of clinical samples. For these reasons, the development of a more convenient analytical technique for dCyd is necessary.

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Enzyme immunoassay (EIA) is considered a more powerful alternative analytical technique [13–15]. It is remarkably quick, easily performed yielding information that would be difficult to obtain by HPLC, and also offers great sensitivity when an appropriate enzyme label is used. As well, immunoassays as they use analyte-specific antibodies do not require pretreatment for the samples and they are well suited for screening of large number of samples [16]. Omura et al. [17] has attempted the development of EIA for determination of dCyd, however the antibody employed in the assay showed high cross-reactivity with 5-methyl-2′-deoxycytidine, thus the accurate determination of dCyd was not possible. In a further study, Darwish et al. demonstrated the isolation of a monoclonal antibody that recognizes dCyd with a high specificity [18], and employed this antibody in the development of a selective EIA for determination of dCyd in plasma with a quantitation limit of 10 μM [19]. However, the normal concentrations of dCyd in plasma are ranging from 0.4 to 2.9 μM [20]. Therefore, this method was unable to determine dCyd plasma concentrations. For these reasons, the development of a new alternative more sensitive EIA for determination of dCyd was very essential. The present study describes the development and validation of a new EIA with significantly enhanced sensitivity for determination of dCyd at concentrations as low as 20 nM in plasma samples.

2. Experimental

2.1. Materials

Nucleobases, nucleosides and nucleotides were purchased from Sigma Chemical Co. (St. Louis. MO, USA), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), P-L Biochemicals, Inc. (Wis., USA), Oriental Yeast Co., Ltd. (Tokyo, Japan), Boehringer (Mannheim GmbH, Germany) or Kojin Co., Ltd. (Tokyo, Japan). Horseradish peroxidase-labeled goat anti-rat IgG (HRP–IgG), bovine serum albumin (BSA), 2,4,6-trinitrobenzene sulfonic acid, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from Sigma Chemical Co. (St. Louis. MO, USA). 3,3′,5,5′-Tetramethylbenzidine (TMB) peroxidase substrate was obtained from Kirkegaard-Perry Laboratories (Gaithersburg, MD, USA). ELISA high-binding microwell plates were a product of Corning/Costar, Inc. (Cambridge, MA, USA). Centricon-30 filter (Amicon, Inc., Beverly, MA, USA). BCA reagent for protein assay was obtained from Pierce Chemical Co. (Rockford, IL, USA). All water was purified by filtration through a Nanopure II water purification system.

2.2. Instrumentation

FLX808 microplate reader (Bio-Tek Instruments Inc., USA). FLX500 microplate washer (Bio-Tek Instruments Inc., USA). Nanopure II water purification system (Barnstead/Thermolyne, Dubuque, IA). EM-36N microtube shaker (Taitec, Japan). Biofuge Pico centrifuge (Heraeus Instruments, Germany). Model Mini/18 incubator (Genlab Ltd., UK)

2.3. Procedures

2.3.1. Antibody and coating conjugate

Anti-dCyd monoclonal antibody (RH-4) was generated by fusing SP2/0-Ag14 mouse myeloma cells with iliac lymph node cells from WKY/NCrj rats immunized with keyhole limpet hemocyanin protein conjugated with 3′-succinyl dCyd (3′sdCyd–KLH). The procedures for isolation, purification and characterization of this antibody has previously described by Darwish et al. [18]. The coating conjugate 5′-succinyl-dCyd–bovine serum albumin (5′sdCyd–BSA) was prepared by the method previously described by Darwish et al. [19] with a modification. Briefly, 5′sdCyd hapten was prepared by treating dCyd with succinic anhydride in triethylamine:dioxane (1:9, v/v) (Fig. 1). The 5′sdCyd was separated from other succinyl dCyd derivatives (3′sdCyd and 3′,5′sdCyd) and purified by HPLC, and the structure of 5′sdCyd was confirmed by mass spectroscopy and ^{1}H NMR spectroscopy [17]. EDC (75 mg) was

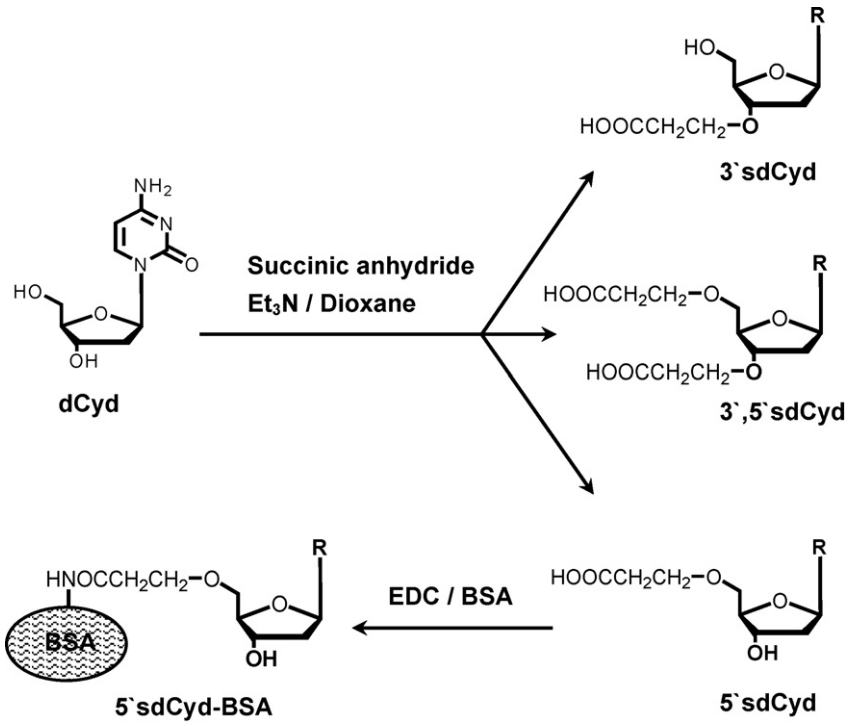

Fig. 1. Preparation of succinyl derivatives of dCyd and 5′sdCyd–BSA conjugate.

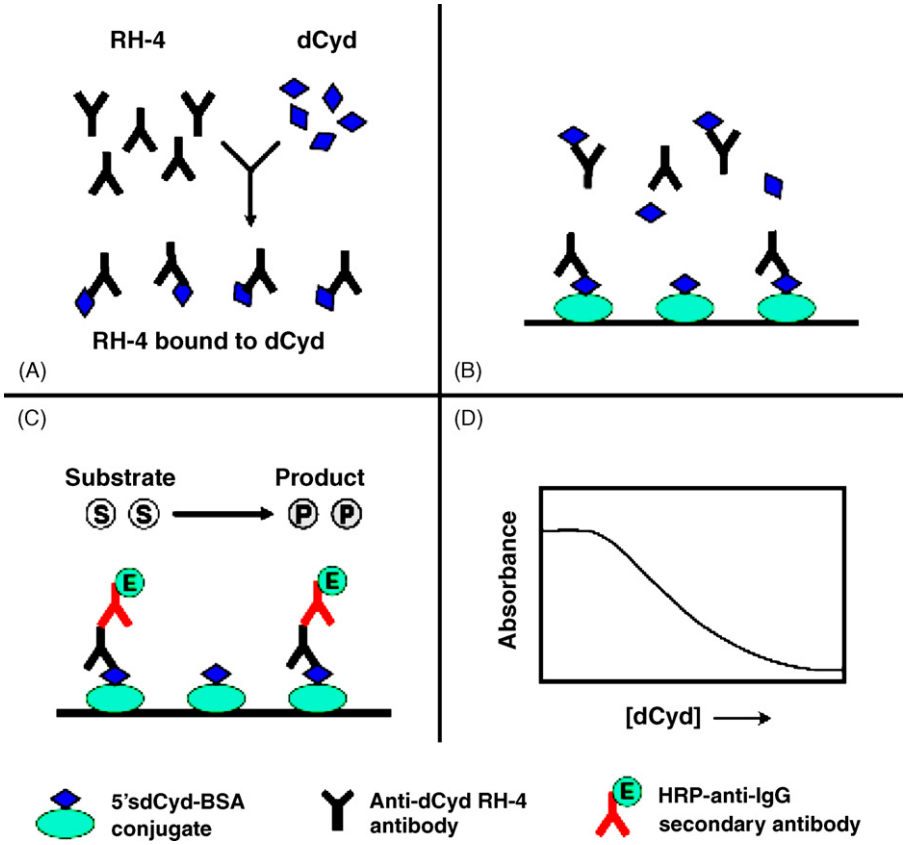

Fig. 2. Schematic diagram of the competitive EIA for dCyd. (A) Sample containing dCyd is premixed with RH-4 anti-dCyd antibody. (B) The mixture solution containing the dCyd and RH-4 antibody is dispensed into microwell plates that have been coated with 5′sdCyd–BSA conjugate. The soluble dCyd competes with the immobilized 5′sdCyd–BSA conjugate for the RH-4 antibody binding sites. (C) After a wash step to remove any antibody bound to the soluble dCyd, an enzyme-labeled secondary antibody (HRP–IgG) is added. A second wash step removes unbound secondary antibody, and a signal is generated by the addition of TMB as a colorimetric substrate. (D) Signals are correlated with the dCyd concentrations for generating the calibration curve for determination of dCyd.

added to a solution of 5′sdCyd (5 mg mL^{-1}) in 12.5 mM phosphate buffer (PB) at pH 5. The pH of the reaction solution was maintained at 5–5.5 for 2 min, then a solution of BSA (5 mg mL^{-1}) in 50 mM PB (pH 7.2) was added. The pH of the reaction mixture was rapidly adjusted to 6.4 and maintained constant for 90 min. The reaction was allowed to proceed for overnight in dark at 4 °C. The uncoupled 5′sdCyd was removed from the 5′sdCyd–BSA conjugate by buffer exchange using a Centricon-30 filter. Protein concentration of the conjugate was determined using BCA reagent and the extent of substitution of free amino groups on the BSA was determined by estimation of free amino groups on unreacted BSA and on BSA subjected to the conjugation procedure described by Habeeb [21]. The extent of conjugation was 45.6% of the total amino group residues.

2.3.2. Determination of the optimum concentrations of antibody and coating conjugate

The optimum 5′sdCyd–BSA concentration required for coating onto the microwell plates and the best working concentration of the anti-dCyd antibody (RH-4) were determined by checkerboard titration. Different concentrations (0.05–2 μg mL^{-1}) of 5′sdCyd–BSA in 50 μL of phosphate buffered saline (PBS; 137 mM NaCl, 3 mM KCl, and 10 mM sodium phosphate, pH 7.4) were coated onto microwell plates in duplicate rows for 2 h at 37 °C with gentle agitation by microtube shaker. After incubation, the plates were washed with 0.05% Tween 20 in PBS (PBS-T) using microplate washer. The wells were blocked with 3% BSA in PBS by incubation at 37°C for 1 h. Fifty microliters of different concentrations (0.5–4 μg mL^{-1}) of RH-4 anti-dCyd antibody solution (in PBS) was added in columns across the plates. After 1 h incubation at 37 °C, the plates were washed with PBS-T, and 50 μL of HRP–IgG (1/5000 in PBS) was added to each well. After similar incubation and washing step, 50 μL of TMB substrate solution was added and the reaction was allowed to proceed for different times at 37 °C for color development. The absorbance of each well was measured in a dual wavelength mode (450–650 nm) using a microplate reader. Concentrations of 5′sdCyd–BSA conjugate and RH-4 antibody yielded 0.8–1.2 absorbance units were considered as reference binding conditions for further testing.

2.3.3. Preparation of plasma samples

Plasma samples were centrifuged at 3500 rpm at 4 °C for 10 min and the supernatants were collected. The supernatants were 10-folds diluted in PBS and used directly for the analysis by EIA. For analysis by HPLC, supernatants were treated with 2 M perchloric acid for precipitation of proteins, and the solutions were centrifuged at 15,000 rpm at 4 °C for 30 min. The supernatants were neutralized with 2 M KOH and the neutralized solution was centrifuged at 3500 rpm at 4 °C for 5 min. The supernatants were used for analysis by HPLC [22].

2.3.4. Competitive EIA procedures and data analysis

Samples of dCyd (50 μL) were 10-folds diluted in PBS. The diluted samples were mixed with equal volumes of RH-4 anti-dCyd antibody (1 μg mL^{-1}). Aliquot (50 μL) of the mixture was added to each well of the microwell plate that had been previously coated with 0.5 μg mL^{-1} of 5′sdCy–BSA conjugate and blocked with 3%

BSA. After 1 h incubation at 37 °C, the plates were washed with PBS-T, and 50 μL of HRP–IgG (1/5000 in PBS) was added to each well. After 1 h incubation, the plates were washed with PBS-T and the amount of the bound HRP–IgG was quantified using TMB microwell substrate as described above. The data were acquisitioned by KC Junior software (Bio-Tek Instruments Inc., USA), and transformed to a four-parameter curve using Slide Write software, version 5.011 (Advanced Graphics Software, Inc., USA). Values for IC_{50} were those that gave the best fit to the following equation:

$$A = A_0 - \frac{(A_0 - A_1)\,[\mathrm{dCyd}]}{\mathrm{IC50} + [\mathrm{dCyd}]} \quad (1)$$

where A is the signal at a definite known concentration of dCyd, A_0 is the signal in the absence of dCyd, A_1 is the signal at the saturating concentration of dCyd, and IC_{50} is the dCyd concentration that produces a 50% inhibition of the signal. The concentrations of dCyd in the samples were then obtained by interpolation on the standard curve.

3. Results and discussions

This study describes the development of a new highly sensitive EIA for determination of dCyd in plasma samples; Fig. 2 describes the feature of this EIA. In this assay, microwell plates were coated with 5′sdCyd–BSA conjugate. A monoclonal antibody (RH-4) that recognizes dCyd with high specificity was mixed with sample containing dCyd and the mixture was incubated with 5′sdCyd–BSA immobilized onto the microwells. During this incubation, dCyd competed with 5′sdCyd–BSA for binding to the available limited binding sites on the RH-4 antibody molecule. After removal of unbound reagents, the amount of RH-4 antibody bound to the microwells was determined using HRP–IgG and TMB as a chromogenic substrate. The concentration of dCyd in the sample was quantified by its ability to inhibit the binding of RH-4 antibody to the immobilized 5′sdCyd–BSA, and the color development was inversely proportional to the concentration of dCyd in the original sample solution.

3.1. Strategy for the assay development

The main objective behind this study was the employment of the available anti-dCyd antibody in the development of a new EIA with adequate sensitivity for the determination of normal range of plasma dCyd concentrations (0.4–2.9 μM) [19]. In the development of an immunoassay for any particular analyte, the affinity of the antibody to its target antigen is the most important factor in determining the ultimate sensitivity of the assay [23]. As well, the sensitivity of the competitive binding-based immunoassays could be significantly improved when the affinity of the antibody for the soluble free competitor is higher than the affinity to the immobilized competitor analogue [24]. In a previous study, Darwish et al. [17] determined the affinity of RH-4 anti-dCyd antibody for dCyd and its succinyl-dCyd derivatives: 3′sdCyd, 5′sdCyd, and 3′,5′,sdCyd. It was found that RH-4 shows the lowest affinity (lowest IC_{50}) for 5′sdCyd. Therefore, it is anticipated that the use of 5′sdCyd as a competitor for dCyd in the assay would ultimately provide the highest sensitivity. Based on this basis, 5′sdCyd was selected for conjugating to the BSA, and the produced conjugate (5′sdCyd–BSA) was employed as a solid-phase antigen in the present EIA. In addition to the affinity of the antibody, the experimental conditions and assay configuration would also affect the assay performance. The optimization of the assay conditions and evaluation of the assay performance are described in the following paragraphs.

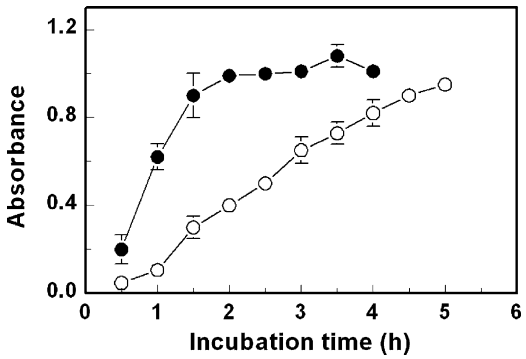

Fig. 3. Effect of temperature and incubation time on the immobilization of 5′sdCyd–BSA onto the microwells. Incubations were carried out at 37°C (●) and room temperature (25 ± 5°C) (○).

3.2. Optimization of assay variables

3.2.1. Choice of conjugate and antibody concentrations

To determine the optimum concentration of 5′sdCyd–BSA required for immobilization onto the microwell plates and the concentration of RH-4 antibody for competitive binding reaction, checkboard titration [25] of 5′sdCyd–BSA and RH-4 was carried out. The conjugate and antibody concentrations that gave 0.8–1.2 absorbance unit (after 10 min for color development) were considered as a reference optimum binding conditions. The optimum concentrations of 5′sdCyd–BSA was found to be 0.5 μg mL^{-1} and the concentrations of RH-4 was 1 μg mL^{-1}. Therefore, these concentrations were used in all further testing.

3.2.2. Binding of 5′sdCyd–BSA to microplate wells

Optimum binding of the conjugate to the plate wells was attained when the incubation time was 2 h at 37 °C, and at least for 5 h at room temperature (25 ± 5 °C) (Fig. 3). For more convenience in clinical testing, it was important to check the stability of the conjugate after its coating onto the microwell plates. Plates were coated with the 5′sdCyd–BSA and stored for varying periods of time at 4 and −20 °C, and then were analyzed for the amount of immunoreactive conjugate remaining on the microwells. The results indicated that the plates could be stored for at least 4 and 8 weeks at 4 and −20 °C, respectively. This gives an advantage that the plates could be kept, after coating with the conjugate and blocking with BSA, until the assay time, and consequently reduction of 3 h from the total time.

3.3. Validation of the assay

3.3.1. Calibration curve and detection limit

The calibration curve of dCyd using the proposed EIA is shown in Fig. 4. This curve was generated by adding dCyd at concentrations from 0.1 to 10,000 nM to plasma and treating the samples as described in Section 2. The data showed good correlation coefficient (r = 0.998) on the four-parameter curve fit. The limit of detection of the proposed EIA, defined as the lowest dCyd concentration significantly different from zero concentration at 95% confidence limit (mean of zero ± 4.65 S.D.) was determined [26]. Based on the basis of 8 replicate measurements, the limit of detection was found to be 8 nM.

3.3.2. Precision profile

The assay precision profile obtained from the results of calibration standard samples, assayed in triplicate, is shown in Fig. 4. From this profile, the working range of the assay at values of relative

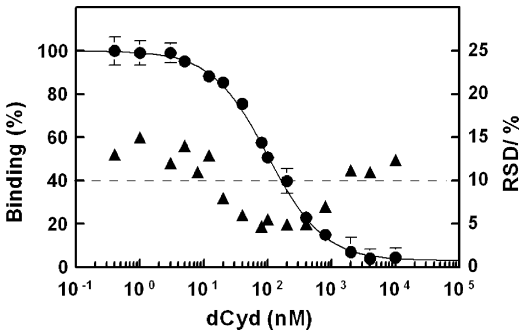

Fig. 4. Calibration curve (●) and precision profile (▲) of the proposed EIA for dCyd. Varying concentrations of standard dCyd were mixed with RH-4 anti-dCyd antibody (1 μg mL^{-1}). The reaction mixtures were further manipulated as described in Section 2. The values plotted are mean ± S.D. of three determinations.

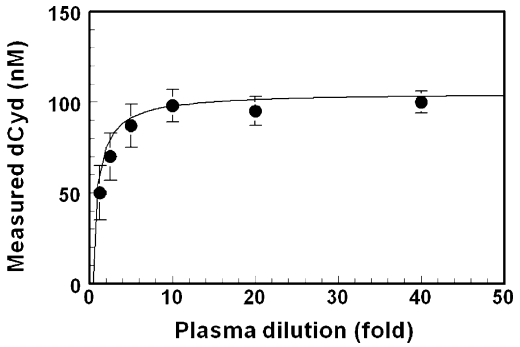

Fig. 5. Effect of the plasma matrix on the feasibility of the proposed EIA for dCyd.

standard deviation (R.S.D.) less than 10% was derived. This range was found to be 20–800 nM. The R.S.D. at the detection limit of the assay (8 nM) was found to be 10.5%. The intra- and inter-assay precision was tested at three different levels (low, intermediate, and high) of dCyd concentrations (40, 100, and 500 nM). The intra-assay precision was assessed by analyzing 8 replicates of each sample in a single run and the inter-assay precision was assessed by analyzing the same sample, as duplicates, in four separate runs. According to the recommendation of immunoassay validation [27], the assay gave satisfactory results; the R.S.D. was 3.4–4.2 and 4.3–8.9% for intra- and inter-assay precision, respectively.

In general, the precision in competitive immunoassays depends mainly upon the uniformity in the quantity of the coated reagent from well to well in a microwell plate. Any interference in this uniformity could arise from the experimental protocol and other manipulations; change in the temperature of incubation and dispensing the reagents. The good precision observed in the proposed EIA was attributed to the facts that all the incubation steps of the assay were carried out at the same temperature, and the reagents and plates were brought to the temperature of incubations before pipettings.

3.3.3. Assay specificity

Although, the assay utilized a monoclonal antibody specific to dCyd, it was necessary to investigate this specificity under the specified experimental assay conditions. The specificity of the present method was determined by carrying out the competitive assay using various bases, nucleosides, and nucleotides as competitors [18] at concentrations of 0–10^6 nM. The cross-reactivity was calculated using the formula: IC_{50}(competitor)/IC_{50}(dCyd) × 100, defining IC_{50} is the concentration of the competitor that causes 50% reduction of the maximum RH-4 antibody binding to the coating conjugates (50% of the maximum color signal). It was found that the values of IC_{50} for all the competitors used [18] were higher than the maximum concentration used in the assay (1 × 10^6 nM). These data proved that none of these competitors showed any cross-reactivity with dCyd in the assay indicating the high specificity of the proposed EIA for dCyd determination.

3.3.4. Matrix effect

Since the proposed EIA was designed for quantitation of dCyd in plasma samples, it was necessary to investigate the effect of plasma matrix on the feasibility of the method. Quality control plasma sample was spiked with 100 nM of dCyd and it was serially diluted into PBS. The diluted samples were then analyzed by the proposed EIA. It was found that measured concentrations increased with the increase in the plasma dilution, and then leveled off when the plasma dilution was 10-fold (Fig. 5). Therefore, plasma samples should be diluted, in PBS at least 10-folds in order to reduce possible false analytical results. It is worth mentioning that the high sensitivity of the assay (limit of quantitation was 20 nM) allowed the dilution of a clinical specimen up to ~20-folds; the dCyd concentration would remain in the clinical normal range (0.4–2.9 μM).

3.3.5. Analytical recovery

Recovery of the assay was assessed by adding three known concentrations (40, 100, and 600 nM) of dCyd to three different individual samples of blank plasma, and the samples were analyzed for their dCyd content, as described in Section 2. The mean analytical recovery was calculated as the ratio between the dCyd concentrations found and the concentrations added, expressed as percentage. The results showed that a quantitative recovery was obtained; the recovery percentages ranged from 96 to 102 ± 3.2 to 8.2%. This indicated the accuracy of the proposed method for determination of dCyd in plasma samples, and absence of endogenous interfering substances in the samples.

3.3.6. Comparison with HPLC

In order to compare the proposed EIA with HPLC, plasma samples were spiked with dCyd at concentrations of 2, 5, 10, 20, 40, and 80 μM, and analyzed by both HPLC [22] and the proposed EIA. As the proposed EIA has higher sensitivity (~1000-folds more than the previous EIA [19]), the samples were diluted with PBS to get their concentrations within the working range of the proposed EIA method. The values obtained by both methods were correlated well with each other (Table 1). The regression analysis of the results showed a good agreement between the results obtained by the two methods:

$$\text{HPLC} = 0.807 + 0.977\,\text{EIA} \quad (r = 0.997) \tag{2}$$

Table 1
Comparison of the proposed EIA with HPLC for the determination of plasma samples spiked with dCyd.

Spiked dCyd (μM)[a]	Found dCyd (μM)[b]	
	HPLC	EIA
2	1.89 ± 0.05	1.92 ± 0.08
5	5.02 ± 0.12	4.82 ± 0.45
10	9.85 ± 0.25	9.55 ± 0.91
20	20.04 ± 1.45	17.62 ± 1.54
40	39.75 ± 1.64	39.98 ± 1.85
80	76.98 ± 3.75	78.25 ± 4.25

[a] Samples were diluted (10-folds) in PBS to get their concentration in the range of 20–800 nM, prior to the analysis by EIA.
[b] Values are mean of three determinations ± S.D.

4. Conclusions

The present study, demonstrated the development and validation of a new EIA for the determination of plasma levels of dCyd, the potential prognostic marker for breast cancer chemotherapy. The proposed assay (linear range is 20–800 nM) is superior to the previously reported EIA (linear range is 10–1000 μM) in its sensitivity (~1000-folds). Since the assay produces a colored read-out, only a colorimetric plate reader is required. The entire protocol of the present assay is very easy to perform in a 96-well plate and permits an operator to analyze a batch of 200 samples, in triplicate, and obtain the results of analysis in less than 4 h when the plates have been previously coated with 5′sdCyd–BSA and blocked with BSA. This facilitates the processing of serial samples. Unlike HPLC, the marked specificity of the proposed EIA eliminates the need for pretreatment of plasma samples by affinity chromatography or other sophisticated equipment. For these reasons, the proposed EIA is expected to contribute to further elucidation of the role of dCyd in various biological and biochemical systems.

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