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Generic simple enzyme immunoassay approach to avert small molecule immobilization problems on solid phases Application to the determination of tobramycin in serum

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Abstract

Generic simple and sensitive universal enzyme immunoassay approach for the determination of small analytes has been developed to avert the problems associated with small molecule immobilization onto solid phases. The developed assay employed a heterogeneous non-competitive binding format. The assay used anti-analyte antibody coupled to polyacrylamide beads as a solid-phase and β-D-galactosidase enzyme-labeled analyte as a label. In this assay, the analyte in a sample was firstly incubated to react with an excess of the antibody-coupled beads, and then the unoccupied antibody binding sites were allowed to react with the enzyme-labeled analyte. Analyte bound to the antibody-coupled beads was separated by centrifugation, and the enzyme activity of the supernatant was measured spectrophotometrically at 420 nm, after reaction with 4-nitrophenyl-β-D-galactopyranoside as a substrate for the enzyme. The signal was directly proportional to the concentration of analyte in the sample. The optimum conditions for the developed assay were established and applied to the determination of tobramycin, as a representative example of the small analytes, in serum samples. The assay limit of detection was 10 ng mL^{-1} and the effective working range at relative standard deviation of ≤10% was 40–800 ng mL^{-1}. The assay precisions were acceptable; the relative standard deviations were 4.36–5.17 and 5.62–7.40% for intra- and inter-assay precision, respectively. Analytical recovery of tobramycin spiked in serum ranged from 95.89 ± 4.25 to 103.45 ± 4.60%. The assay results correlated well with those obtained by high-performance liquid chromatography ($r = 0.992$). The assay described herein has great practical value in determination of small analytes because it is sensitive, rapid, and easy to perform in any laboratory. Although the assay was validated for tobramycin, however, it is also anticipated that the same methodology could be used for essentially any analyte for which a selective antibody exists, and an appropriate enzyme conjugate can be made.

Keywords: Enzyme immunoassay; Non-competitive binding; Aminoglycoside antibiotics; Tobramycin; Therapeutic drug monitoring

1. Introduction

Immunoassay methods have been widely used in many important areas of pharmaceutical analysis such as diagnosis of diseases, therapeutic drug monitoring, clinical pharmacokinetic and bioequivalence studies in drug discovery and pharmaceutical industries [1,2]. The analysis in these areas usually involves measurement of very low concentrations of drugs [3–6], biomolecules of pharmaceutical interest [7], metabolites [8], and/or biomarkers which indicate disease diagnosis [9–13] and/or prognosis [14]. The importance and widespread of immunoassay methods in pharmaceutical analysis are attributed to their inherent specificity, high sensitivity, high-throughput, and applicability to the analysis of wide range of low molecular weight and macromolecular analytes in complex matrices (e.g. biological samples).

Immunoassays involving radioactive labels [15] are extremely sensitive and quite precise, however, the handling of radioactive materials and radioactive waste and high cost are inhibitory factors. Enzyme immunoassays (EIA) have become the most universal assay method in pharmaceutical analysis because of its simplicity, rapidity, sensitivity and low cost. One additional advantage of enzyme immunoassays is the possibility of utilizing the enzyme labels in the amplification of the signal, if the signal is not sufficient to give the desired assay sensitivity [16]. The use of these labels results in assay methods

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with extremely high sensitivity and low limits of detection [17]. The traditional enzyme immunoassays, e.g. microplate-based enzyme-linked immunosorbent assays (ELISA) are not practical for the analysis of small molecules (e.g. drugs, metabolites, etc.) because they adsorb very poorly or not at all to the solid-phase supports. To enhance the adsorption of small molecules to solid-phase supports, different pre-treatment approaches were used. These approaches include: irradiation of the solid-phase with ultra-violet light [18], or treatment with alcian blue [19], treatment of the target with carbodiimides [20], or conjugation with protein and subsequent immobilization of the conjugate by passive adsorption [21]. The majority of these approaches are still associated with drawbacks such as long incubation periods, multiple washing and mixing steps, high non-specific binding signals, they are labour intensive and/or expensive. Considering the drawbacks of performing ELISA for small molecules, there was a growing interest in the development of new EIA devoid of these drawbacks.

In a previous study, Darwish [22] has developed a continuous-flow EIA system for determination of small analytes. The system completely resolved the adsorption problems; however, it was specifically designed for laboratories that are equipped with HPLC instrument, and it is requested to screen large number of specimens. This limited the application of this system in small pharmaceutical and clinical laboratories that are not equipped with HPLC instruments and/or requested to screen small batches of specimens (e.g. emergency rooms, urgent-care centers, physician clinics, etc.). The present study is focused on development and validation of an alternative new EIA approach for determination of small analytes. The assay is specifically designed for use in conjunction with a dedicated spectrophotometer, the common instrument in all pharmaceutical and clinical laboratories. In the study described herein, tobramycin (TOB) was used as a representative example for the small analytes. The proposed assay depended on a non-competitive binding reaction and utilized an anti-TOB monoclonal antibody coupled to polyacrylamide beads as a solid-phase, and TOB-β-galactosidase conjugate (TOB-β-GAL) as a label. In this method, an excess of the antibody-coupled beads was first incubated with TOB sample, and then separately incubated with TOB-β-GAL. After separation of the beads by centrifugation, the enzyme activity of the supernatant was measured spectrophotometrically at 420 nm, after reaction with 4-nitrophenyl-β-D-galactopyranoside as a substrate for the enzyme. The measured absorbance was directly related to the concentration of TOB in the sample.

2. Experimental

2.1. Chemicals

Tobramycin (TOB) was purchased from Eli Lilly Co. (Indianapolis, IN, USA). Monoclonal antibody against TOB was obtained from Fitzgerald Industries International Inc. (Concord, MA, USA). β-D-Galactosidase enzyme (β-GAL, EC 3.2.1.23, grade III), bovine serum albumin and Tween-20 (polyethylene sorbitan monolaurate) were purchased from Sigma Chemical Co. (St. Louis. MO, USA). *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was obtained from Pierce Chemical Co. (Rokford, IL, USA). Glutraldehyde was obtained from Merck (New York, USA). 4-Nitrophenyl-β-D-galactopyranoside (NPGP) was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN, USA). Polyacrylamide beads (Bio-Gel P) was a product of Bio-Rad Laboratories (Richmond, CA, USA), and polystyrene test tubes was a product of Walter Sarstedt (Leicester, UK). All other chemicals were of analytical grade.

2.2. Reagent solutions

Water was purified by filtration through Nanopure II water purification system (Barnsead/ThermLyne, Dubuque, IA, USA) and used for all solutions. Washing buffer; phosphate buffered saline (PBS, 137 mM NaCl, 3 mM KCl and 10 mM sodium phosphate buffer, pH 7.4) containing Tween 20 (0.05%, v/v). Assay buffer; PBS containing bovine serum albumin (0.1%, w/v). Substrate solution; PBS containing 1.5 mM of NPGP. Enzymatic reaction terminator solution; 0.1 M potassium phosphate buffer adjusted to pH 11 with NaOH.

2.3. Preparation of TOB-β-GAL conjugate and antibody-coupled beads

Tobramycin was conjugated with β-GAL enzyme using MBS reagent, and according to the method described by Darwish [22]. The unconjugated tobramycin was removed from the TOB-β-GAL conjugate by buffer exchange using a Centricon-30 filter (Amicon Inc., Beverly, MA, USA). Protein concentration of the conjugate was determined using the kit obtained from Pierce Chemical Co. (Rockford, IL, USA), and the conjugate was characterized in terms of specific activity of β-GAL. The specific activity was 242 units mg^{-1} protein; unit was defined as the amount of β-GAL enzyme that converts 1 μmol of NPGP in 1 min.

The antibody-coupled beads were prepared by activation of the polyacrylamide beads with glutraldehyde, and covalently linking the glutraldehyde-activated beads to the anti-TOB antibody [23]. The antibody-coupled beads were thoroughly washed with PBS and finally diluted into the assay buffer solution to a concentration of 50 mg mL^{-1} and stored at 4 °C.

2.4. Determination of optimum concentrations of TOB-β-GAL enzyme conjugate and antibody-coupled beads

The optimal concentrations of TOB-β-GAL and antibody-coupled beads were determined by checkerboard titration. Purified TOB-β-GAL was diluted into the assay buffer at concentrations of 0.1, 0.25, 0.5, and 1 μg mL^{-1} and mixed in polystyrene test tubes with the antibody-coupled beads at concentrations of 5, 10, 15, and 20 mg mL^{-1}. The binding was then allowed to proceed by incubation at room temperature (25 ± 2 °C) for 15 min with gentle shaking to prevent the sedimentation of the beads. The tubes were centrifuged at 3000 rpm for 2 min, and 50 μL the supernatant was dispensed into a separate test tube contained 450 μL of NPGP substrate solution. The

color-developing enzymatic reaction was allowed to proceed for 10 min, and then 2.5 mL of the enzymatic reaction terminator solution was added to each tube. The absorbances were measured by spectrophotometer (UV-1601PC, Shimadzu Co., Kyoto, Japan) at 420 nm. The concentration of TOB-β-GAL that was sufficient enough to saturate all the binding sites of the antibody, and yielded a signal between 0.8 and 1.0 absorbance units, in absence of antibody-coupled beads, was considered as optimum.

2.5. Assay procedure

All procedures were carried out at room temperature (25 ± 5 °C) in triplicate. Fifty microliters of TOB sample (serum or standard solution) was dispensed into each polystyrene test tube contained 500 μL of antibody-coupled beads suspension (20 mg mL^{-1}). The tubes were incubated for 30 min with gentle shaking to prevent the sedimentation of the beads particles, and then centrifuged at 3000 rpm for 2 min. The supernatants were aspirated to waste; care being taken to avoid the disturbance of the beads pellet at the bottom of the tubes. The beads were washed three times by shaking with 1 mL of the washing buffer for about 2 min, centrifugation, and aspiration of the supernatants. Five hundred microliters of TOB-β-GAL solution (0.25 μg mL^{-1} in assay buffer) was added and the tubes were incubated for 15 min with gentle shaking. The tubes were then centrifuged for 2 min, and 50 μL of the supernatant was dispensed into a separate test tube. The enzyme activity of the supernatants was determined as described above, and the data were transformed to a four-parameter curve using Slide Write Plus software version 5.011 (Advanced Graphics Software Inc., CA, USA).

3. Results

This study describes a simple non-competitive enzyme immunoassay for determination of TOB, as a representative example for small analytes, in serum; Fig. 1 illustrates the general procedure of this assay. The assay was carried out in two main steps; the immunchemical reaction and the color developing reaction. In the first step, TOB in samples was allowed to bind to an excess of anti-TOB monoclonal antibody linked to polyacrylamide beads, as solid phase. Then, TOB-β-GAL conjugate was allowed to bind to the remaining antibody binding sites. In the second step, the enzyme activity in the supernatant, after centrifugal sedimentation of the solid-phase beads, was determined using NPGP substrate. The absorbances, measured spectrophotometrically, were directly proportional to the concentration of TOB in the sample.

3.1. Optimum assay conditions

The optimal concentration of TOB-β-GAL conjugate was considered as the concentration that was able to saturate all the binding sites of the antibody and yielded a signal between 0.8 and 1.0 absorbance unit in the absence of antibody-coupled beads. This concentration was 0.25 μg mL^{-1} when the concentration of the antibody-coupled beads was 20 mg mL^{-1} (Fig. 2). Unless otherwise stated, these concentrations were used for further experiments. The kinetics of TOB binding to the antibody was studied at different temperatures in order to determine the optimum time and incubation temperature required for the immunological reaction. As shown in Fig. 3, the reaction proceeded faster to completion as the temperature was increased

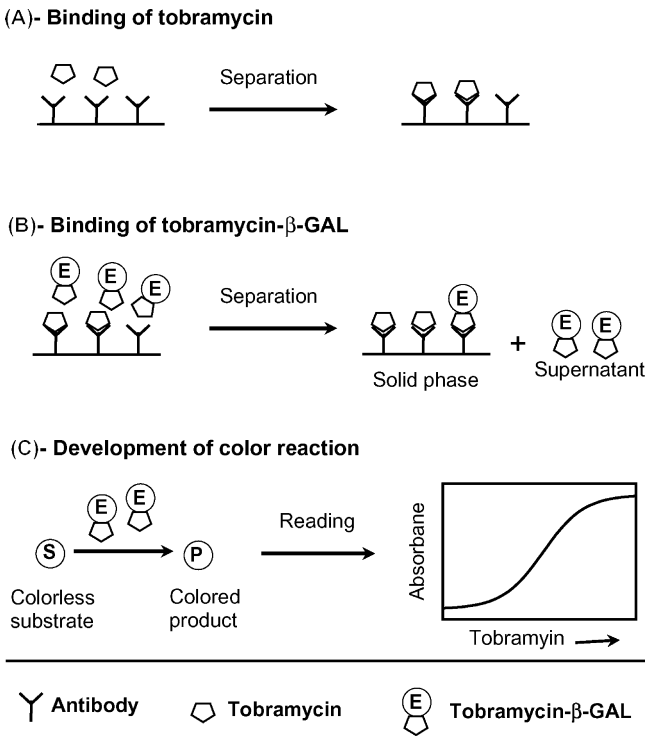

Fig. 1. Schematic diagram of the non-competitive EIA for determination of TOB in serum. (A) The serum sample-containing TOB was allowed to bind to an excess of anti-TOB antibody-coupled beads, and then the serum was removed by washing. (B) The beads were subsequently incubated with TOB-β-GAL, and then the antibody beads were separated by centrifugation. (C) The enzyme activity of the supernatant was determined by chromogenic substrate, and the color development was directly proportional to the concentration of TOB in the original sample.

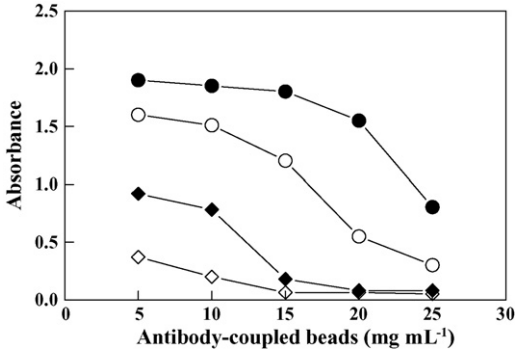

Fig. 2. Checkerboard titration of TOB-β-GAL conjugate versus anti-TOB antibody-coupled beads. The conjugate at concentrations of 0.1 μg mL^{-1} (◊), 0.25 μg mL^{-1} (♦), 0.5 μg mL^{-1} (○), and 1 μg mL^{-1} (●) were allowed to bind the antibody-coupled beads at the indicated concentrations. The signals were generated as described in Section 2.

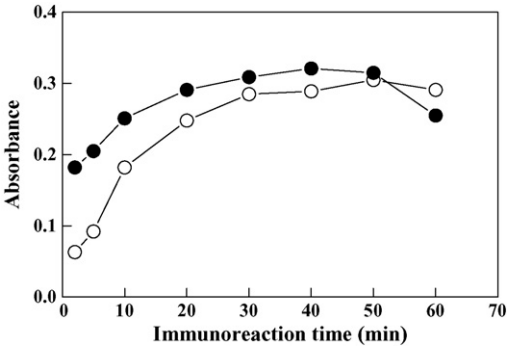

Fig. 3. Immunoreaction kinetics for binding of TOB to antibody-coupled beads. An assay buffer solution containing 50 ng mL^{-1} TOB was incubated with 20 mg mL^{-1} antibody beads suspension at 25 °C (◯) and 37 °C (●) for the indicated periods of time, and then processed as described in Section 2.

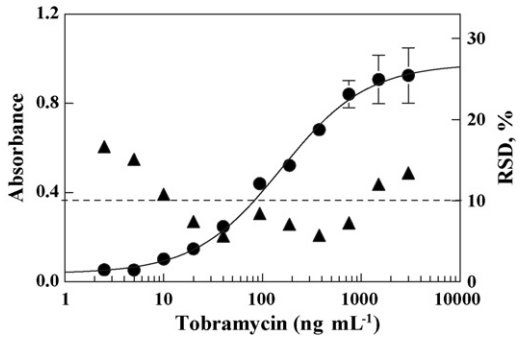

Fig. 5. Calibration curve (●) and precision profile (▲) of the non-competitive EIA for determination of TOB. Various concentrations of TOB were prepared in the assay buffer, and allowed to bind to an excess of anti-TOB antibody-coupled beads. After washing, the beads were subsequently incubated with TOB-β-GAL. The excess unbound reagent was removed by centrifugation, and the enzyme activity of the supernatant was determined by chromogenic substrate as described in Section 2. The values plotted were the mean of three determinations ± S.D.

from 25 to 37 °C. There was no significant difference between the results of the reaction at 37 °C for 30 min and that of at room temperature (25 ± 2 °C) for 40 min. For convenience, further experiments were carried out at room temperature to avoid using an additional temperature control instrument. In order to determine the color-developing time, the enzyme reaction kinetic was studied using 25, 50 or 75 μL supernatant. The results (Fig. 4) showed that 25, 50 or 75 μL could be incubated with the substrate solution for 20, 10 or 5 min, respectively. For reading precision consideration, the enzyme color reaction for the subsequent experiments was carried out using 50 μL of the supernatant and 10 min for incubation with the substrate.

3.2. Analytical performance

3.2.1. Calibration curve and sensitivity

The calibration curve of TOB using the proposed non-competitive EIA is shown in Fig. 5. The data showed good correlation coefficient ($r = 0.998$) on the four-parameter curve fit. The limit of detection of the proposed non-competitive EIA, calculated as the lowest TOB concentration significantly different from zero concentration at 95% confidence limit, i.e. mean of zero ± 4.65 S.D. [24] was 10 ng mL^{-1}, based on the basis of 5 replicate measurements of the reagent blank. The supernatant solutions that have been used for generating the calibration curve were stored overnight at 4 °C, and then were used for generating a new curve. The two curves were completely superimposed (not shown data) indicating the stability of the enzyme conjugate. This allowed the removing of the supernatants and storing them for delayed measurements of the enzyme activity. This increases the convenience of the analysis, and allows the easy handling of large batches of specimens.

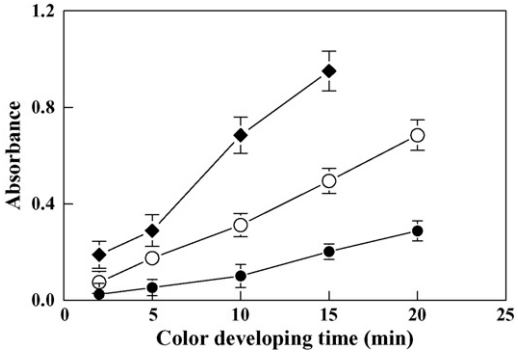

Fig. 4. Time-course of the color development reaction in the non-competitive EIA for TOB. The non-competitive assay was performed using 50 ng mL^{-1} TOB. After centrifugation of the TOB-bound antibody beads: 25 μL (●), 50 μL (◯), and 75 μL (♦) of the supernatant was incubated with 450 μL of the substrate solution for the indicated periods, and then processed as described in Section 2. Values were the mean of three determinations ± S.D.

3.2.2. Serum matrix effect and specificity

Studying the effect of serum matrix was necessary in the development of the proposed method, since the assay was designed for quantitation and therapeutic monitoring of TOB in serum. Quality control TOB-free serum sample was serially diluted into the assay buffer solution and each dilution was spiked with 200 ng mL^{-1} of TOB standard. The spiked samples were then analyzed by the proposed assay to investigate the feasibility of the assay. The measured concentrations increased with the increase in the serum dilution, and then leveled off when the serum dilution was 20-fold. Therefore, serum samples should be diluted, in assay buffer, at least 20-fold in order to reduce possible false-negative analytical results (Fig. 6). It is worth to mention that the high sensitivity of the assay (limit of detection was 10 ng mL^{-1}) allowed the dilution of a clinical specimen up to 100-fold as the TOB concentration would remain in the clinical therapeutic range (5–10 μg mL^{-1}).

Although, the assay utilized a monoclonal antibody specific to TOB, it was necessary to investigate the effect of the experimental assay conditions on this specificity. The specificity study was performed using various aminoglycoside antibiotics (kanamycin, gentamicin, amikacin, and streptomycin) at concentrations considerably higher than that may be present in patient serum [25]. No cross reactivity was detected with any of the tested compounds indicating the good specificity of the method under the experimental conditions.

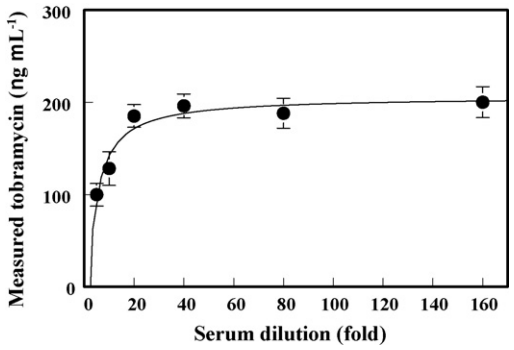

Fig. 6. Effect of serum matrix on the performance of the non-competitive EIA for TOB. TOB-free serum sample was serially diluted in the assay buffer solution, and each of the diluted samples was spiked with 200 ng mL^{-1} TOB. The spiked samples were analyzed by the EIA as described in Section 2. The plotted values were the mean of three determinations ± S.D.

3.2.3. Precision

The assay precision profile obtained from the results of calibration standard samples, assayed in triplicate, is shown in Fig. 5. From this profile, the working range of the assay (40–800 ng mL^{-1}), whereas the values of relative standard deviation (R.S.D.) were less than 10%, was derived. The R.S.D. at the detection limit of the assay (10 ng mL^{-1}) was found to be 12.02%. The intra- and inter-assay precisions were tested at three different levels (low, intermediate, and high) of TOB concentrations (40, 100, and 500 ng mL^{-1}). 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 [26], the assay gave satisfactory results; the R.S.D. was 4.36–5.17 and 5.62–7.40% for intra- and inter-assay precision, respectively (Table 1).

3.2.4. Analytical recovery

Recovery of the assay was assessed by adding various known concentrations (40, 100 and 500 ng mL^{-1}) of TOB to three different individual samples of drug-free serum, and the samples were analyzed for their TOB content, as described in Section 2. The mean analytical recovery was calculated as the ratio between the TOB concentrations found and the concentrations added, expressed as percentage. As shown in Table 2, a quantitative recovery was obtained; the recovery percentages ranged from 95.89 ± 4.25 to 103.45 ± 4.60%). This indicated the accuracy of the proposed method for determination of TOB in serum samples.

Table 1
Precision data of the proposed EIA for tobramycin

Precision	Tobramycin (ng mL^{-1})		
	40	100	500
Intra-assay (n = 8)			
Mean (ng mL^{-1})	41.00	97.74	505.422
S.D. (ng mL^{-1})	2.12	4.26	2.30
R.S.D. (%)	5.17	4.36	4.41
Inter-assay (n = 8)			
Mean (ng mL^{-1})	38.92	104.24	496.25
S.D. (ng mL^{-1})	2.88	5.86	31.20
R.S.D. (%)	7.40	5.62	6.29

Table 2
Analytical recovery of tobramycin added to serum

Serum code	Tobramycin (ng mL^{-1})		Recovery (%)
	Added	Found	
A	40	41.38 ± 0.92[a]	103.45 ± 4.60
	100	100.20 ± 2.68	100.20 ± 2.68
	500	479.45 ± 21.26	95.89 ± 4.25
B	40	39.14 ± 1.02	97.58 ± 5.10
	100	100.50 ± 3.72	100.50 ± 3.72
	500	502.10 ± 20.16	100.42 ± 4.03
C	40	40.80 ± 1.08	102.13 ± 5.40
	100	96.70 ± 4.74	96.70 ± 4.74
	500	502.60 ± 30.57	100.52 ± 6.11

[a] Values are mean of three determinations ± S.D.

Table 3
Comparison of the proposed EIA with HPLC for determination of serum samples spiked with tobramycin

Spiked tobramycin (μg mL^{-1})	Found tobramycin (μg mL^{-1})	
	EIA	HPLC
2.0	1.86 ± 0.12[a]	1.65 ± 0.04
4.0	4.28 ± 0.22	4.12 ± 0.10
6.0	5.05 ± 0.19	6.05 ± 0.13
8.0	8.26 ± 0.42	7.84 ± 0.14
10.0	9.53 ± 0.61	10.75 ± 0.26

[a] Values are mean of three determinations ± S.D.

3.2.5. Comparison with HPLC

In order to compare the present EIA with HPLC, serum samples were spiked with TOB at concentrations of 2, 4, 6, 8 and 10 μg mL^{-1}, and then analyzed by HPLC [27]. These samples were then diluted 40 times with the assay buffer to get their concentrations within the working range of the proposed EIA method, and analyzed by the proposed method. The values obtained by both methods were correlated well with each other (Table 3). The regression analysis of the results showed a good agreement between the results obtained by the two methods: HPLC = 0.146 + 1.118EIA (r = 0.992).

4. Discussion

This study described the development of new sensitive assay format for determination of small analytes in serum. Two main objectives were behind this study; the first one was the elimination of the adsorption problems encountered in the microwell plate-based assays, and the second one was the applicability of the assay in any pharmaceutical and/or clinical laboratory.

Tobramycin (TOB) is widely used in the treatment of human and animal diseases caused by aerobic gram-negative bacillary infections [28]. The major concern in treatment with TOB is

its low therapeutic index whereas serum concentrations of less than 5 $\mu g\,mL^{-1}$ are generally ineffective, and the concentrations exceeding 10 $\mu g\,mL^{-1}$ are associated with oto- and nephrotoxicity [29]. For safe and effective treatment with TOB, routine monitoring of its serum concentrations followed by adjustment of the patient dose regimen is required [30].

Tobramycin levels in serum have been measured by microbiological assay [31], high-performance liquid chromatography (HPLC) [32–36], and enzyme immunoassay (EIA) [37,38]. Microbiological assays are semi-quantitative approach, time consuming, and subject to interferences by other antimicrobial drugs. HPLC is a very accurate method; however, the analysis requires derivatization of the sample to improve detectability, and long time for carrying out. The conventional EIA methods were not practical because it adsorbs very poorly or not at all to the solid-phase supports, in standard adsorption conditions, as well as after using various approaches to enhance its adsorption of TOB to solid-phase supports [18–20,39,40]. Therefore, an alternative assay formats that would be able to overcome these drawbacks was essential. For these reasons, the present study was dedicated to adopt TOB as an analyte in the development of the targeted analytical approach.

β-GAL enzyme was chosen for this work because it is absent from human blood, therefore avoid interferences from endogenous enzymes, particularly the proposed assay was designed to use serum samples in the analysis. Furthermore, the optimal enzyme-substrate reaction of β-GAL takes place at pH of 6–8 which is compatible with most antigen–antibody interaction systems. 4-Nitrophenyl-β-D-galactopyranoside was selected from the β-GAL substrates because it has high turnover rate for the generation of the product (λ_{max} at 420 nm), and consequently gives sensitive assay.

The solid-phase beads used in the assay allowed the employment of the simplest separation technique, which is the centrifugation, and only two washing steps were required. A final washing step was not necessary because the enzymatic activity was measured in the supernatant after separation of the solid-phase antibody. The assay was carried out in test tubes, thus avoided the problems of TOB adsorption to the microwell plates. Measurement of the enzyme activity in the supernatant was adapted to spectrophotometer, the common instrument in all clinical laboratories. The assay procedure was carried out at room temperature, thus no extra temperature control instrument was needed. For these reasons, the assay is applicable in most clinical laboratory.

Although the assay was specifically designed for use where small batches of specimens have to be screened (physician clinics, emergency rooms, urgent-care centers, etc.); however, the stability of the TOB-β-GAL conjugate in the supernatant solution allowed gathering the supernatants and storing them in large batches. This increased the convenience of the assay as well as made it applicable for large number of specimens.

The present assay depended on non-competitive binding reaction format. The use of excess reagents in this assay droved the immunochemical reaction to the formation of the immune complex, thus yielded rapid assay with high sensitivity and wide dynamic range. In the present approach, the sample's analyte (TOB) and enzyme conjugate (TOB-β-GAL) were sequentially added to the antibody, and never been in contact. This sequential addition procedure completely eliminated the non-specific antibody binding interferences that are encountered with competitive assay format. The assay was very sensitive; its sensitivity was higher than that achieved by other non-competitive immunoassay [19], and entire protocol of the assay described herein could be performed in less than one hour, compared to more than 5 h in the other method. The assay reproducibility was acceptable, however in order to obtain good precision, it was essential that a precise amount of the antibody-coupled beads should be used. This was achieved by keeping the beads in suspension using a magnetic stirrer during the pipetting process.

With attention to reagents preparation and technical manipulations, coupling of the antibody to the beads and conjugation of TOB to the enzyme could be easily performed using established procedures, and once prepared they were stable. All other reagents are inexpensive, could be easily obtained and had excellent shelf life.

5. Conclusion

The immunoassay approach described in this study has great practical value in the determination of small analytes as it eliminated the adsorption problems encountered in the microwell plate-based assays, and it is applicable in any pharmaceutical and/or clinical laboratory, as the approach adapted to a spectrophotometer, the common instrument in these laboratories. Furthermore, the approach showed high sensitivity, reproducibility, and simplicity to perform. Although, the approach was validated for tobramycin, however, it is anticipated that the same methodology could be essentially used for any analyte for which a selective antibody exists, and an appropriate enzyme conjugate can be made.

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