Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 42 (2006) 389–394
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Potentiometric assessment of Gram-negative bacterial permeabilization of tobramycin

Amira M. El-Kosasy *

Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt

Received 10 November 2005; received in revised form 11 April 2006; accepted 12 April 2006
Available online 5 June 2006

Abstract

The construction and electrochemical response characteristics of β-cyclodextrin-based sensor for determination of the amino glycosidic antibiotic, tobramycin, is described. The membrane incorporates carboxylated poly (vinyl chloride) as matrix polymer, tetrakis-fluorophenyl borate as fixed anionic site and nitrophenyloctylether as plasticizer.

The uptake of aminoglycosidic drug was applied to determine permeability changes in the Gram-negative bacteria *Escherichia coli* as test organism and ethylenediamine tetraacetic acid as model permeabilizer.

The sensor shows linear response over concentration range of 10^{-2} to 10^{-5} M tobramycin with cationic slope of mV per concentration decade and accuracy of $99.8 \pm 2.14\%$. The proposed method was successfully applied to the determination of tobramycin uptake, by 100 μl *E. coli* suspension in presence of up to 10 μg ml^{-1} of EDTA solution, with average recovery of $99.3 \pm 2.77\%$. The obtained results were compared reasonably well with the data obtained using the compendial fluorimetric assay.

The proposed potentiometric assay serves as a convenient method in analyzing and quantifying the effects of permeabilizers on Gram-negative bacteria upon treatment with tobramycin.

Keywords: Sensor; Potentiometric determination; Tobramycin; β-cyclodextrin; Bacterial permeabilization

1. Introduction

The outer membrane (OM) of Gram-negative bacteria is unique among biological membranes in its ability to exclude external molecules [1]. This property is due to the presence of lipopolysaccharide molecules (LPS) that constitutes the outer leaflet of this membrane; the inner leaflet is composed of glycerophospholipids. Thus, there is some resistance of Gram-negative bacteria towards some antibiotics, e.g. erythromycin, novobiocin and aminoglycosides [2]. Without being directly toxic to bacteria, permeabilizers sensitize them to other external agents, enabling entry of these antibiotics [3].

By virtue of its nernestian response towards β-cyclodextrin/tetrakis-fluorophenyl borate/plasticized poly (vinyl chloride) sensor, The measurement of antibiotic uptake allows the assessment of OM permeability changes. This study, utilizes modern technique to enable versatile potentiometric assessment of bacterial permeablization.

Several techniques have been recommended to detect and/or determine bacterial permeabilization. These methods include microbiological assay via measurement of release of LPS [4]. Permeabilization is, however, not necessarily associated with LPS release as in the case of intercalating permeabilizers. Other microbiological assays use bactericidal probe antibiotic and measures the target inhibiting action such as bacterial viability [5] and protein synthesis [6]; The most famous and most applicable is the growth inhibition assay [7]. Microbiological assays offer, in this respect, an important analytical tool, since besides their flexibility and sensitivity, only nanograms, in principle, are required. Furthermore, their quantitative approaches could be correlated with the concrete chemical analysis [8].

The pioneering permeability studies were performed by using lysozym as a probe; it diffuses to exert its lytic action and allows periplasmic enzymes to be leak out and measured [9]. However, being polycationic and lacks any significant OM-permeabilizing activity, lysozyme binds to the polyanionic LPS, competes with

* Tel.: +20 27113015; fax: +20 24051106.
E-mail address: kosasy_2010@yahoo.com.

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.04.022

other external antibiotics, facilitates its own penetration and, thus, interferes with the assay.

Colorimetric methods based on using different chromogens to β-lactams, has been also recommended [10,11]. These methods are far from ideal, since many of the permeabilizers not only allow diffusion of the β-lactams probe to the periplasm but also release β-lactamase from the periplasm. Recently, fluorimetric determination of bacterial permeabilization has been reported [12]. The uptake of the fluorescent probe 1-*N* phenylnaphthylamine to the phospholipid layer of the OM, leads to prominent fluorescence that can be measured at 405 nm after excitation at 355 nm. This indirect fluorimetric assay to study OM permeabilization is a concrete method especially if permeabilizers are planned to increase the OM permeability without damaging it.

Sophisticated high-pressure liquid chromatography accompanied by mass spectrometry has been recommended for structure/function studies and permeabilization assessment [13,14]. No electrochemical method has been yet reported in the literature for the assessment of bacterial permeabilization, therefore, it is worth developing a simple potentiometric sensor for its fast determination via drug uptake.

With *Escherichia coli* as test organism, tobramycin as antibiotic and ethylenediamine tetraacetic acid as model permeabilizer, quantitative and highly reproducible antibiotic uptake levels were obtained potentiometrically, using β-cyclodextrin ion selective electrode. Tobramycin molecule consists of amino sugars linked glycosidically. Additionally, it contains a highly substituted 1,3-diaminocyclohexane central ring. Thus, it is strongly basic and exists as polycations at physiologic pH and as sulfates in pharmaceutical formulations [15]. Its broad spectrum of activity against many common pathogens of both Gram-positive and Gram-negative types was reported [16]. The mechanism of uptake of aminoglycosides across the outer membrane of *E. coli* was reevaluated [17]. Beside the general advantages of electrochemical devices such as low cost, small size, low detection limits and easy automation, the ion-selective electrodes-based potentiometric procedures allow more selectivity, short experimental time and high sensitivity [18].

2. Experimental

2.1. Equipment

Potentiometric measurements at $25 \pm 1\,^{\circ}C$ were made with an Orion digital ion-analyzer model 720A using membrane sensor in conjunction with an Orion 90-20 Ag/AgCl double junction reference electrode containing 10% (w/v) potassium nitrate solution in the outer compartment. Monitoring of pH was made with an Orion 91-20 combination glass electrode.

2.2. Reagents

All chemicals were of analytical reagent grade and bidistilled deionized water was used throughout.

Chemicals for the preparation of electrode membrane were, β-cyclodextrin (β-CD) (Sigma), carboxylated poly (vinylchloride) (PVC-COOH) (Sigma); tetarkis [3,4-bis (trifluoromethyl) phenyl] borate (TFPB) (Fluka); tetrahydrofuran (THF) (Fluka) and nitrophenyloctyl ether (NPOE) (Prolabo).

Pure samples of tobramycin sulfate were obtained from Sigma Chemical Co., Germany, potency as is $648/\mu g\,mg^{-1}$ according to the official USP 23 assay [19] with purity of $99.92 \pm 0.560\%$ according to the compendial spectrophotometric method [20].

Standard solutions, 10^{-2} to 10^{-6} M tobramycin dissolved in HEPES buffer were freshly prepared. Ethylenediaminetetraacetic acid disodium dihydrate (EDTA), $1\,mg\,ml^{-1}$ aqueous solution (Merck, Darmstadt, Germany) bacterial suspension, *E. coli* 0157 was grown in Lauri Berttani broth (per litre, 10 g of Difco tryptone, 5 g of Difco yeast extract on 5 g of NaCl, pH 7.0) at 37 °C with shaking (200 rpm). Cultures with an optical density value of $0.5 + 0.02$ were centrifuged for 10 min at 1000 rpm and room temperature, and the cells were suspended in half volume of 10^5 mM HEPES buffer, pH 7.0. HEPES is *N*-[2-hydoxyethylpiprazine]-*N*-[2-ethanesulfonic acid], pH range 6.8–8.2, obtained from Sigma.

2.3. Procedure

2.3.1. Electrode fabrication and calibration

In glass Petri dish (5 cm diameter), mix 0.04 g β-CD with 0.01 g of TFPB and 0.4 g NPOE. Add 0.18 g PVC-COOH previously dissolved in 5 ml THF and thoroughly mix. Cover the Petri dish with filter paper and allow it to stand overnight to obtain solvent evaporation at room temperature; master membrane with a thickness of 0.1 mm was obtained. Assemble the electrode using punched circular membrane (0.8 mm diameter) as previously described [21]. Condition the membrane electrode by soaking in 10^{-2} M aqueous tobramycin solution for 1 h and store it in water when not in use. The membrane sensors were calibrated by immersing the fabricated electrode in 10^{-6} to 10^{-2} M tobramycin solutions and allowed to equilibrate, with constant stirring, in conjunction with an Orion reference electrode. The electrode potential was recorded as a function of logarithmic concentration of tobramycin drug. The calibration plot obtained was used for subsequent measurements of bacterial samples containing the aminoglycosidic drug with EDTA.

2.3.2. Tobramycin uptake assay

For standard measurements, each measurement is accompanied by control one. Each measurement was performed at least three times. The electromotive force values of different laboratory prepared mixtures contain 100 μl of bacterial suspension in HEPES buffer; pH 7, 100 ml 10^{-2} M tobramycin solution in HEPES buffer, pH 7, and EDTA aliquots equivalent to 0.2–1 mg, were obtained using the proposed calibrated sensor. The concentration of tobramycin was obtained from the corresponding calibration plot.

It was ascertained that in absence of bacterial suspension, EDTA alone-without bacterial suspension has no effect on the response of the proposed sensor towards tobramycin.

Potentiometric readings of tobramycin in presence of bacterial suspension and EDTA is subtracted by the corresponding

values of tobramycin in presence of bacterial suspension alone (in absence of EDTA), to quantify the effect of EDTA.

Alternatively, the results are expressed as antibiotic uptake factor. The uptake factor was calculated as a ratio of mVs of the bacterial suspension and of the buffer, each value was background corrected (i.e. subtracted by the value in absence of tobramycin).

3. Discussion

Measurement of the permeability alterations of the Gram-negative bacterial OM serves many purposes in the study and development of antimicrobial agents [22]. The molecular basis of the integrity of the OM lies in its LPS. Polyanionic LPS molecules are linked electrostatically by divalent cations Ca^{2+} and Mg^{2+} forming a stable "Tiled roof" on the surface of OM [23]. The OM disorganizing and permeabilizing action of EDTA, that chelates Ca^{2+} and Mg^{2+} is well known. In agreement with previous reports on the permeabilizing activity of EDTA towards Gram-negative bacteria, EDTA sensitizes *E. coli* to a number of antibiotics e.g. erythromycin, rifampin, novobiocin, cloxacillin and aminoglycosides, by a factor ranged from 5 to 500 [24].

In this work, the measurement of the permeability alterations of the polyanionic LPS membrane of *E. coli* to the poly cationic amino-glycosidic drug, tobramycin, via manufacturing of ion-selective electrode are the area of interest. The advantages they offer over other microbiological and other chemical analytical procedures are: low cost, simplicity, suitability for testing samples without prior treatment, low detection limits and enhanced selectivity.

3.1. Sensor characteristics

Plasticized PVC-COOH membrane with anionic lipophillic additive (TFPB) and ionophore (β-CD) was prepared and electrochemically evaluated as prospective sensor for the aminoglycosidic antibiotic – tobramycin – according to IUPAC standards [25]. Table 1 shows that the investigated sensor displays ideal performance characteristics. Typical calibration graph for the proposed sensor is obtained.

At 25 °C, the sensor display linear response for negative logarithmic concentrations over the range 10^{-2} to 10^{-5} M tobramycin with nernestian slope of 56 mV/concentration decade ($n = 3$).

Repeated calibrations of the sensor over a period of 3 weeks show potential and slope stabilities within ± 1 mV decade^{-1}. The mean accuracy of the results obtained is 99.8 ± 2.14% (Table 1). Repeatability was assured by immersing the sensor, alternatively, into 10^{-3} and 10^{-4} M drug solutions, where The R.S.D. was 2.74%.

3.2. Effect of β-cyclodextrin

Cyclodextrins are organized media that from inclusion complexes in aqueous solution and in solid state with organic molecules. Because their chemical structure provides well-defined inclusion cavities with a specific receptor function they are able to modify significantly some physicochemical properties of active molecules [26]. β-Cyclodextrins were previously applied as sensor ionophores to different potentiometric techniques [27].

The present work evaluates the possibility of using β-cyclodextrin as ionophore in the preparation of tobramycin-selective electrode in which carboxylated PVC was used as polymeric matrix for immobilization of the primary hydroxyl groups at the C-6 positions of the β-cyclodextrin molecules and thus attain high stability of the complex between it and the cationic tobramycin molecule. This stability reflects on both sensitivity and selectivity of the proposed sensor, as shown in Tables 1 and 2.

Table 1
Analytical features of the potentiometric determination of tobramycin, solution using the investigated sensor

Parameters	Value
Slope (mV decade^{-1})	56
Slope standard deviation	1.74
Response time (min)	0.5
Working pH range	5.5–7.5
Concentration range (M)	10^{-2} to 10^{-5}
Linearity (%)	99.0
Detection limit (M)	1×10^{-6}
Intercept	389.3
Intercept standard deviation	0.96
Stability (days)	21
Temperature (°C)	25 ± 2
Accuracy, average recovery (%)[a] $n = 3$	99.8
Inter-day R.S.D[b] (intermediate precision, $n = 10$)	2.14
Intra-day R.S.D. (repeatability, $n = 10$)	2.74
Correlation coefficient	0.998
Robustness[c] (mean ± standard error, $n = 3$)	98.83 ± 0.046
Ruggedness[d] (mean ± standard error, $n = 3$)	99.16 ± 0.013

[a] Series of four standard solutions of tobramycin with concentrations between 10^{-2} and 10^{-5} M were prepared by triplicate.
[b] Relative standard deviation, using 10^{-3} M tobramycin.
[c] Using nitrophenylphenylether instead of NPOE and 10^{-3} M tobramycin.
[d] Comparing results with those obtained using Jenway potentiometer model 714, and using 10^{-3} M tobramycin.

Table 2
Selectivity coefficients ($K^{pot.}$ drug, interferent) for the proposed sensor

Interferent[a]	($K^{pot.}$ Drug, interferent)[b]
Tobramycin	1
Amikacin	8.5×10^{-2}
Gentamycin	3.3×10^{-2}
Kanamycin	2.1×10^{-2}
HEPES buffer	2.4×10^{-4}
EDTA	2.6×10^{-4}
NaCl	3.7×10^{-4}
Erythromycin	2.2×10^{-4}
Penicillin sodium	1.9×10^{-4}
Ciprofloxacin	1.7×10^{-4}
Glycine	1.5×10^{-4}

[a] 10^{-3} M aqueous solutions were used.
[b] Average of three measurements, the calculated selectivity coefficients have no units.

3.3. Effect of lipophilic additive

It has been recommended that the addition of fixed ionic sites for ionophore-based ion selective electrode is beneficial [28]. It reduces the interference by other lipophilic counter ions and creates permselectivity.

In the present work, it was found that the anionic additive TFPB with its large molecular size (larger than other tetraphenyl borate derivatives) led to the best performance. It reduced the response time, lowered the electrical membrane resistance and gave rise to improved selectivity.

3.4. Effect of PVC-COOH and plasticizer

Similar results were obtained on using PVC, polyurethane and PVC-COOH as polymer matrices. It is noteworthy to mention that the life span of the membrane containing PVC-COOH is the longest (3 weeks in comparison to 2 weeks in case of other matrices).

The potentiometric response of the investigated sensor was not influenced by the polarity of the membrane medium, which was in turn defined by the dielectric constant of the plasticizer. Different sensors with different plasticizers having dielectric constants over the range 4–24; namely dibutylsebathate, dioctylpthalate and NPOE, were prepared and tested. The results were more or less the same.

3.5. Soaking and response time

Freshly prepared electrodes must be soaked in 10^{-2} M tobramycin solution to activate the surface of the membrane. Depending on the diffusion and equilibration at the interface, complexation of tobramycin and membrane sensor constituents occurs with the result of selective sensor to the studied drug. The presoak times were 1 h.

Nevertheless, continuous soaking of the electrodes in drug solution, affects negatively their response to the analyte. This may be attributed to the leaching of the ionophore.

The response time was nearly instantaneously in relatively concentrated solutions, while in dilute solutions, about 30 s were necessary to reach stable potential readings.

3.6. Effect of permeabilizer and interferents (selectivity of the electrode)

Potentiometric selectivity coefficients ($\log K^{\text{pot.}}_{\text{drug,interferent}}$) of the proposed sensor were evaluated using the separate solution method [25], with 10^{-3} M concentration level of both drug and interferents which include; HEPES buffer, EDTA, aminoglycosides other than tobramycin and some related substances. The applied equation is:

$$\log K^{\text{pot.}}_{\text{drug,interferent}} = \frac{E_i - E_t}{S} - \left(\frac{1}{Z-1}\right)\log[\text{tobramycin}]$$

where E_t is the potential measured of 10^{-3} M tobramycin solution, E_i the potential measured of 10^{-3} M interferent solution, S the slope of the proposed sensor and Z is the charge number of the interferents.

The results obtained in Table 2 show high selectivity of the investigated electrode; it respond primarily to the analyte ion in the presence of interfering ions that have the same charge sign. The effect of diverse ions on the proposed tobramycin electrode is negligible. The selectivity observed seems to be a result of size/charge exclusion effects. Also, the strong electrostatic interactions between the negative ionic charge of TFPB and dipoles of β-CD and the polycationic tobramycin molecules are responsible for this selectivity improvement.

HEPES buffer represents the base line (zero reading) of the calibration curve. Aqueous EDTA solution has no effect on the mV readings of the calibration curve of tobramycin in the absence of bacterial suspension. On the other hand, in presence of bacterial suspension, increasing EDTA concentration, leads to increasing tobramycin uptake by *E. coli*, which in turn, decreases the tobramycin concentration level in the external measured solution, and decreases mV readings; selectively. Table 3 shows that uptake drug factor in ranged from 1.97 to 478.4 and the

Table 3
Potentiometric measurements of tobramycin uptake by 100 μl *E. coli* buffered suspension in presence of different concentrations of EDTA, using the proposed sensor

Sample[a]	mV reading [b]	mV value after back ground subtractions	Tobramycin uptake factor [c]
HEPES buffer	40.3	–	–
HEPES buffer + 100 μl bacterial suspension	42.0	–	–
10^{-2} M tobramycin (prepared in HEPES buffer)	281.2	239.2	1
10^{-2} M tobramycin + 100 μl bacterial suspension	190.7	148.7	1.97
10^{-2} M tobramycin + 100 μl bacterial suspension + 0.2 ml EDTA	140.4	98.4	2.43
10^{-2} M tobramycin + 100 μl bacterial suspension + 0.4 ml EDTA	100.3	58.3	4.12
10^{-2} M tobramycin + 100 μl bacterial suspension + 0.6 ml EDTA	70.6	28.6	8.36
10^{-2} M tobramycin + 100 μl bacterial suspension + 0.8 ml EDTA	45.6	3.6	66.44
10^{-2} M tobramycin + 100 μl bacterial suspension + 1.0 ml EDTA	42.5	0.5	478.4
10^{-2} M tobramycin + 100 μl bacterial suspension + 1.2 ml EDTA	42.5	0.5	478.4

[a] Final volume of each sample is 100 ml.
[b] Average of five measurements.
[c] Ratio of background subtracted value, in absence of EDTA to that in presence of it.

Table 4
Recoveries of tobramycin in synthetic mixtures containing 100 μl buffered *E. coli* suspension and 10 μg ml^{-1} EDTA solution using the proposed potentiometric sensor

Tobramycin samples (M)	Drug recovery ± S.D. (%)[a]	
	The proposed potentiometric method	The compendial fluorimetric method
10^{-2}	99.7 ± 0.5, F^{b} = 0.74, t^{c} = 0.89	99.8 ± 0.4
10^{-3}	99.7 ± 0.8, F^{b} = 0.60, t^{c} = 0.36	98.9 ± 1.4
10^{-4}	98.4 ± 1.0, F^{b} = 0.76, t^{c} = 0.39	98.9 ± 1.5

[a] Average of five determinations.
[b] Theoretical *F*-value = 6.39.
[c] Theoretical *t*-value = 2.78 for *p* = 5%.

maximal permeabilization, was attained by 10 μg ml^{-1} of EDTA solution.

3.7. Effect of pH

The pH dependence of the investigated sensor was examined using 10^{-4} and 10^{-5} M drug solutions. Fig. 1 shows stable responses over the pH range 5.5–7.5. monitoring of pH was performed using dilute LiOH and HCl. Below pH 5.5, the sensor response increases with the increase of the analyte acidity; at such high acidity, the membrane may extracts H^+ ions.

HEPES buffer; pH 7, was found optimum for both electrode behavior and bacterial viability.

3.8. Analytical applications

Utility, sensitivity and selectivity of the proposed sensor was verified by the determination of recovery % of tobramycin in presence of *E. coli* suspension and EDTA solution. Table 4 shows excellent accuracy and precision. The results obtained were compared with the data obtained using the compendial fluorimetric assay [12]. The *t*- and *F*-tests [29] revealed no significant difference between the means and variances of the two sets of the results. The good agreement between the proposed potentiometric and the compendial fluorimetric data demonstrates the applicability of the investigated procedure for assessment of Gram-negative bacterial permeabilization of tobramycin. It was found that 100 μl bacterial suspension is optimal; higher bacterial concentration leads to electrode fouling. On the other hand, lower concentrations of *E. coli* suspension leads to unobserved differences in the mV readings (small changes around the mV of the base line).

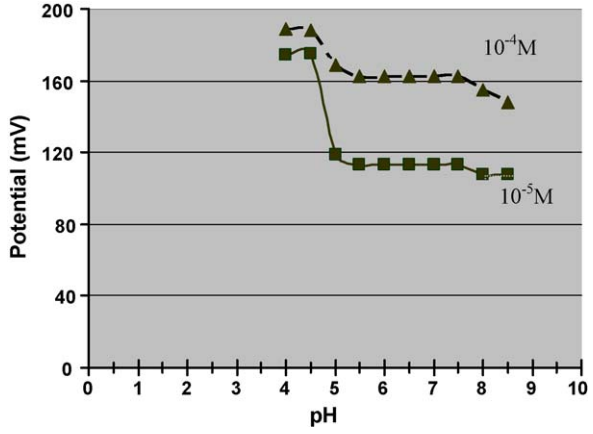

Fig. 1. Effect of pH on the proposed sensor using 10^{-4} and 10^{-5} M tobramycin.

3.9. Figures of merit of the investigated potentiometric procedure

Series of four standard solutions of tobramycin with concentrations between 10^{-2} and 10^{-5} M were prepared by triplicate and measured by following the procedure described under Section 2.3.1. Table 1 resumes the calibration parameters obtained from statistical analysis of the data.

Detection limit (LOD) was taken, under the specific experimental conditions, as the concentration of tobramycin at the point of intersection of the two extrapolations of the straight line segment and the lower curved segment of calibration curve [18]. The resultant LOD (1×10^{-6} M tobramycin) is one of the advantages of the investigated procedure. This value is better than those reported previously in literatures [6,7,10], leading to at least 10 times more sensitive determination for the Gram-negative bacterial permeabilization by the proposed cyclodextrin–based sensor.

In order to study the intra-day precision (repeatability) and inter-day precision (intermediate precision) [30], 10 replicates with a concentration of 10^{-3} M tobramycin were measured (over a 3 weeks period for the inter-day study). The intra- and inter-day relative standard deviations (R.S.D.) obtained were 2.74% and 2.14%.

The proposed cyclodextrin sensor was applied to the determination of tobramycin uptake by *E. coli* as described under Section 2.3.2. Recoveries were calculated for both intra-

Table 5
Intra-day and Inter-day determinations of tobramycin uptake factor by100 μl buffered *E. coli* suspension in presence of 10 μg ml^{-1} EDTA solution using the proposed potentiometric sensor

Tobramycin samples (M)	Recoveries (%)[a] *n* = 5	
	Intra-day determinations	Inter-day determinations
10^{-2}	100.0 ± 2.4	97.3 ± 2.9
10^{-3}	100.1 ± 2.9	98.2 ± 1.8
10^{-4}	98.8 ± 1.8	100.6 ± 2.6
10^{-5}	102.3 ± 3.5	101.1 ± 3.8

[a] Mean ± $t \times$ S.D./$n^{1/2}$ (n = 5, t = 2.78 for p = 5%) [31].

day (five replicates for each concentration measured in the same day) and inter-day (five replicates for each concentration measured over a period of 3 weeks). The obtained results with their respective confidence ranges, calculated as the: mean $\pm t \times$ S.D./$n^{1/2}$ ($n=5$, $t=2.78$ for $p=5\%$) [31], are resumed in Table 5.

4. Conclusion

We have reported here an electrochemical method for the simple assessment of Gram-negative bacterial permeabilization towards some antibiotics. With *E. coli* as test organism, tobramycin as antibiotic and EDTA as model permeabilizer, potentiometric measurements were applied using the proposed β-CD/TFPB/PVC-COOH sensor. The previously recommended methods (microbiological, chromatographic, colorimetric and fluorimelric assays) are complicated, time-consuming and non-specific. The proposed potentiometric method is far more simple, selective and sensitive compared to other reported methods. The short experimental time and the ease of electrode fabrication allows measurement of parallel samples and dilutions. Also, it allows the test bacteria to be in an identical state during potentiometric measurements; it is inherently reliable and reproducible, allowing an effective comparison of the results from different laboratories.

References

[1] I. Helander, T. Mattila-Sandholm, Int. J. Food Microbiol. 60 (2000) 153–161.
[2] H. Van Leeuwen, M. Van Der Tol, J. Van Strijp, J. Verhoef, K. Van Kessel, Clin. Exp. Immunol. 140 (2005) 65–72.
[3] M. Vaara, Microbiol. Rev. 56 (1992) 395–411.
[4] L. Leive, Biochem. Biophys. Res. Commun. 21 (1965) 290–296.
[5] M. Vaara, Drugs Exp. Clin. Res. 3 (1991) 437–444.
[6] A. Lannartsson, K. Pieters, K. Vidovic, U. Gullberg, J. Leuk. Biol. 77 (2005) 377–396.
[7] W. Ernst, S. Thoma, R. Teitelboum, C. Ko, D. Hansom, C. Clayberger, A. Krensky, J. Immunol. 165 (2000) 7102–7108.
[8] P. Tumer, in: Thomas A. McMeeken (Ed.), Microbiological Analysis and GMP System, 1st ed., 2003.
[9] H. Nikaido, M. Vaara, Microbiol. Rev. 49 (1985) 1–32.
[10] Y. Uratanc, J. Bacteriol. 149 (1982) 523–528.
[11] S. Koluskeva, J. Boyer, R. Jelinek, Nat. Biotechnol. 18 (2000) 225–227.
[12] I. Helander, T. Mattila-Sandholm, J. Appl. Microbiol. 88 (2000) 213–219.
[13] H. Tsubery, I. Ofek, S. Cohen, M. Fridkin, J. Med. Chem. 43 (2000) 3085–3092.
[14] M. Martinez, M. Flickinger, L. Higgins, T. Krick, G. Nelsestuen, Biochemistry 40 (2001) 11965–11974.
[15] B. Rosenkratz, J. Gerco, J. Hoogerheide, E. Oden, Analytical Profile of Drug Substances, vol. 9, Academic Press, New York, 1980.
[16] A. Dash, Analytical Profiles of Drug Substances, vol. 24, Academic Press, New York, 1996.
[17] J. Stephen, C. Mailaender, G. Etienne, M. Daffe, M. Niederweis, Antimicrob. Agents Chemother. 48 (2004) 4163–4170.
[18] G.G. Guilbault, Ion Select. Electr. Rev. 1 (1979) 139–143.
[19] The United States Pharmacopoeia, The National Formulary USP NF20, United States Pharmacopoeial Convention Inc., 2002.
[20] S. Sampath, D. Robenson, J. Pharm. Sci. 79 (1990) 428–431.
[21] M. Abbas, A. Abdel Fattah, E. Zahran, Anal. Sci. 20 (2004) 1137–1142.
[22] E. Begunova, O. Stepenaia, I. Tsfasman, I. Kulaev, Microbiologia 73 (2004) 320–325.
[23] M. Bos, B. Tefsen, J. Geurtsen, J. Tommassen, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 9417–9422.
[24] B. Denny, P. West, D. Panigrahi, J. Microbiol. Immunol. Infect. 36 (2003) 72–77.
[25] IUPAC, Analytical Chemistry Division, Commission on Analytical Nomenclature, Pure Appl. Chem. 67 (1995) 507–517.
[26] P. Bates, R. Kataky, D. Parker, Analyst 119 (1994) 181–192.
[27] K. Kelani, J. AOAC Int. 87 (2004) 1309–1318.
[28] S.S. Hassan, W. Mahmoud, A. Othman, Talanta 44 (1997) 1087–1096.
[29] J. Ermer, J.M. Miller, Method Validation in Pharmaceutical Analysis, 2nd ed., Wiley–VCH, Verlag GmbH and Co. KGaA, 2005.
[30] International Conference of Harmonization in Q2B: Validation of Analytical procedures Methodology, 1996. http://www.fda.gov./guidance/index.htm.
[31] J.C. Miller, J.M. Miller, Statistics and Chemometric for Analytical Chemistry, 4th ed., Ellis Horwood, New York, 2004.