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Macrocyclic antibiotics as chiral selectors in the design of enantioselective, potentiometric membrane electrodes for the determination of L- and D-enantiomers of methotrexate

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Abstract

Three enantioselective, potentiometric membrane electrodes (EPMEs) based on macrocyclic glycopeptide antibiotics—vancomycin and teicoplanin (modified or not with acetonitrile)—were proposed for the determination of L- and D-enantiomers of methotrexate (Mtx). The linear concentration ranges for the proposed enantioselective membrane electrodes were between 10^{-6} and 10^{-3} mol l^{-1} for L- and D- methotrexate. The slopes of the electrodes were 58.00 mV/pL-Mtx for vancomycin-based electrode; 57.60 mV/pD-Mtx for teicoplanin-based electrode and 55.40 mV/pD-Mtx for teicoplanin modified with acetonitrile-based electrode. The detection limits of the proposed electrodes were of 10^{-8} mol l^{-1} magnitude order. The surfaces of the electrodes are stable and easily renewable by polishing on alumina paper. All proposed electrodes proved to be successful for the determination of the enantiopurity of Mtx as raw material and of its pharmaceutical formulations (tablets and injections).

Keywords: Enantioselective, potentiometric membrane electrode; Macrocyclic antibiotics; Vancomycin; Teicoplanin; L(D)-Methotrexate

1. Introduction

Chirality is an important concept especially in the pharmaceutical industry and clinical analysis [1]. In many cases, only one isomer in a chiral compound is responsible for the desired activity, while the other isomer may exhibit no therapeutic value and may potentially cause adverse effects. Accordingly, the development of reliable methods for the analysis of drug, offering high efficiency and high resolution, is continuously requested by researchers working in the pharmaceutical and biomedical fields.

Discrimination of drug enantiomers and related compounds has mostly been based on chromatographic and electrophoretic techniques and more recently electrochemistry. The use of electrochemical techniques offer some advantages such as low cost, simplicity and high efficiency over chromatographic and spectrophotometric techniques, which are laborious, time consuming and requiring the preliminary treatment of the samples [2].

Macrocyclic antibiotics vancomycin (Scheme 1) and teicoplanin (Scheme 2) are produced as fermentation products of *Streptomyces orientalis* and *Actinoplanes teichomyceticus*, respectively [3]. These antibiotics are primarily active against aerobic and anaerobic Gram-positive microorganisms both in vitro and in vivo. They are known to inhibit cell wall synthesis [4].

Armstrong and co-workers introduced vancomycin and teicoplanin as powerful and efficient chiral selectors for chromatographic [5–9] and capillary electrophoretic [10–12] separations about a decade ago.

Methotrexate (Mtx, *N*-[4-[(2,4-diamino-6-pteridinyl)methyl]-benzoyl]glutamic acid (Scheme 3)) is an antifolate, that proved to be efficient in the treatment of various human neoplastic disorders including childhood leukemia [13],

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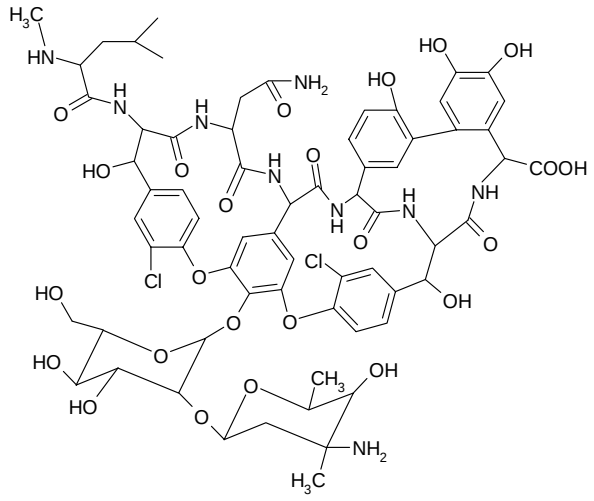
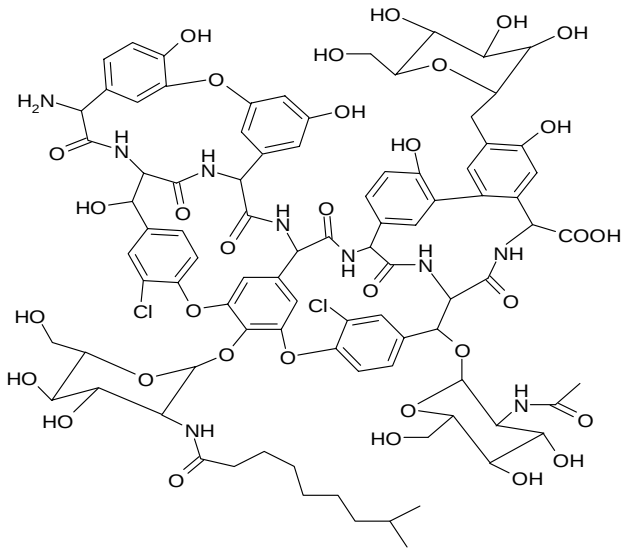


Scheme 1. Vancomycin.



Scheme 2. Teicoplanin.

head and neck cancer [14] and micrometastases of osteosarcoma [15]. Mtx-low dose therapy has also been used for the treatment of patients with rheumatoid arthritis, psoriasis [16–18] and steroid-dependent asthma [19,20]. L-Mtx acts as an inhibitor of enzyme dehydropholate reductase there by preventing cancer cells from maintaining levels of reduced folates, which supply the organism with one carbon unit in the biosynthesis of nucleic acids and some aminoacids. Like naturally occurring folates, L-Mtx is intracellularly converted into polyglutamates, which inhibit further enzymes of the folate metabolism [21–23]. Its antipode D-Mtx shows significantly different pharmacokinetic behavior. Although D-Mtx exhibits a similar inhibitory effect to dehydropholate reductase as L-Mtx, its antitumor effect is proportionally reduced [22].

Many analytical methods have been reported for the analysis of Mtx in its pharmaceutical formulations and biological fluids: high-performance liquid chromatography [24–28], capillary electrophoresis [29–31], spectrophotometry [32], fluorimetry [33] and polarography [34].

Armstrong and co-workers [10–12] previously reported the applications of vancomycin and teicoplanin as chiral selectors for capillary electrophoretic separation of methotrexate. Stefan et al. [35,36] proposed the amperometric biosensors designed using physical and chemical immobilization of glutamate oxidase and/or L-aminoacid oxidase and/or horseradish peroxidase (HRP) for the assay of L-Mtx and D-aminoacid oxidase and/or HRP for the assay of D-Mtx.

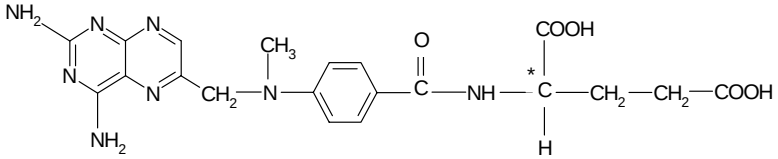
This paper reports the applications of three enantioselective, potentiometric membrane electrodes based on macrocyclic antibiotics—vancomycin and teicoplanin as chiral selectors for the determination of L- and D-enantiomers of methotrexate in pharmaceutical formulations.

2. Experimental

2.1. Reagents and materials

Graphite powder (1–2 μm, synthetic) was purchased from Aldrich (Milwaukee, WI, USA). Paraffin oil was purchased from Fluka (Buchs, Switzerland). Macrocyclic antibiotics—vancomycin and teicoplanin (as their hydrochloride salts) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The L- and D-methotrexate (L- and D-amethopterin hydrated (98% enantiopurity)) were purchased from Aldrich (Milwaukee). Phosphate buffer (pH 4.00) was obtained from Merck (Darmstadt, Germany). Deionized water from a Modulab system (Continental Water Systems, San Antonio, TX, USA) was used for all solutions preparations.

The solution of vancomycin (2×10^{-3} mol l^{-1}) was prepared in phosphate buffer (pH 4.00). The solution of teicoplanin (2×10^{-3} mol l^{-1}) was prepared using pH 6.00 phosphate buffer. The solution of teicoplanin (2×10^{-3} mol l^{-1}) containing acetonitrile was prepared



Scheme 3. Methotrexate.

using pH 6.00 phosphate buffer containing 40% (v/v) of acetonitrile. All standard and diluted solutions were buffered with phosphate buffer (pH 4.30) using the ratio buffer:distilled water 1:1 (v/v).

Methotrexate® tablets (2.5 mg Mtx per tablet) were supplied by David Bull Laboratories, Warwick, UK. Methotrexate LPF® sodium injections were supplied by Immunex Corporation, Seattle, WA, USA.

2.2. Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in connection with PGSTAT 20 and Eco Chemie (Utrech, the Netherlands) Software version 4.9 were used for all potentiometrical (zero current) measurements. A Ag/AgCl (0.1 mol l^{-1} KCl) electrode served as reference electrode in the cell.

2.3. Electrode design

The paraffin oil and graphite powder were mixed in a ratio of 1:4 (w/w) followed by the addition of solution of vancomycin, teicoplanin or teicoplanin modified with acetonitrile (100 μl of chiral selector solution to 150 mg of carbon paste). A certain quantity of carbon paste free from chiral selector was prepared and placed in a plastic pipette peak, leaving 3–4 mm empty in the top to be filled with the carbon paste that contains the chiral selector. The diameters of the potentiometric, enantioselective membrane electrodes were 3 mm. Electric contacts were obtained by inserting an Ag wires into the carbon paste. The internal solution of all electrodes was 0.1 mol l^{-1} KCl.

Before each use the surface of the electrode was refreshed with a new portion of carbon paste, containing the chiral selector and then polished with alumina paper (polishing strips 30144-001 Orrin). The carbon paste prevents the leaching of the antibiotic from the membrane into the solution.

2.4. Recommended procedures

2.4.1. Direct potentiometer

The potentiometric (zero current) technique was used for potential determination of each standard solution of L- and D-methotrexate (10^{-10} to 10^{-3} mol l^{-1}). The electrodes were placed into stirred standard solutions and graphs of E (mV) versus pL-Mtx (pL−Mtx = −log[L−Mtx]) and E (mV) versus pD-Mtx (pD−Mtx = −log[D−Mtx]) were plotted. The unknown concentrations of L- and D-methotrexate were determined from the calibration graphs.

2.4.2. Content uniformity assay of Methotrexate® tablets and Methotrexate LPF® injections

Each of the 10 tablets (2.5 mg Mtx per tablet) was placed into 100 ml calibrated flask, dissolved and diluted to the mark using a phosphate buffer (pH 4.30):deionized water solution in a ratio of 1:1 (v/v). Each of 10 aliquots (50 μl) of the Mtx injection (25 mg Mtx ml^{-1} of injection) was poured into 50 ml calibrated flask and then diluted to the mark with phosphate buffer (pH 4.30):deionized water (1:1) solution. Direct potentiometer was used to determine the unknown concentrations of L- and D-methotrexate in pharmaceutical formulations.

3. Results and discussion

3.1. Electrodes response

The response characteristics exhibited by the macrocyclic antibiotics-based carbon paste electrodes towards the enantiomers of methotrexate are summarized in Table 1. From Table 1 one can see that vancomycin-based electrode showed linear and near-Nernstian response towards L-methotrexate with lower detection limit, while teicoplanin-based electrodes exhibited near-Nernstian electrode function for D-methotrexate. The modification of teicoplanin with acetonitrile allows lowering the detection limit of the electrode. The linear range of electrode function of the proposed electrodes is situated in the higher concentrations region than the one of amperometric biosensors, previously described by Stefan et al. [35,36] for the assay of methotrexate, which minimizes the dilution of the sample to be analyzed. The values of the correlation coefficients obtained for the calibration plots are 0.9997, 0.9994 and 0.9995 for vancomycin, teicoplanin and teicoplanin-modified with acetonitrile-based electrodes, respectively.

The response times of all three electrodes are higher than 1 min for concentrations of methotrexate between 10^{-10} and 10^{-7} mol l^{-1} and less than 1 min for concentrations between 10^{-6} and 10^{-3} mol l^{-1}. The proposed electrodes exhibited highly stable and reproducible characteristics over a month test period (R.S.D. < 0.1%).

3.2. Effect of pH on the response of the electrodes

The effect of pH on the response of the proposed electrodes was checked by recording the emf of the cell, which contain 10^{-5} mol l^{-1} L-methotrexate at different pH values (pH 1–10) for vancomycin-based electrode and 10^{-5} mol l^{-1} D-methotrexate at different pH values (pH 1–10) for teicoplanin-based electrodes. These solutions were prepared by adding very small volumes of HCl and/or NaOH solution (10^{-1} or 1 mol l^{-1} of each) to L- and D-methotrexate solutions, respectively. The plots of E (mV) versus pH (shown in Figs. 1 and 2) indicate, that the response of the electrodes does not depend on the pH from 2.25 to 6.17 for vancomycin-based electrode, from 1.06 to 4.07 for teicoplanin-based electrode and from 3.05 to 6.97 for teicoplanin, modified by acetonitrile-based electrode.

Table 1
Response characteristics of enantioselective, potentiometric membrane electrodes designed for the assay of L- and D-Mtx

Chiral selector used for the design of the electrode	Enantiomer of methotrexate	Slope (mV/pMtx)	Intercept, E^0 (mV)	Linear concentration range (mol l^{-1})	Detection limit (mol l^{-1})
Vancomycin	L	58.00	416.50	10^{-6} to 10^{-3}	6.50×10^{-8}
Teicoplanin	D	57.60	407.20	10^{-6} to 10^{-3}	8.50×10^{-8}
Teicoplanin, modified with acetonitrile	D	55.40	439.30	10^{-6} to 10^{-3}	1.10×10^{-8}

All values are average of 10 determinations.

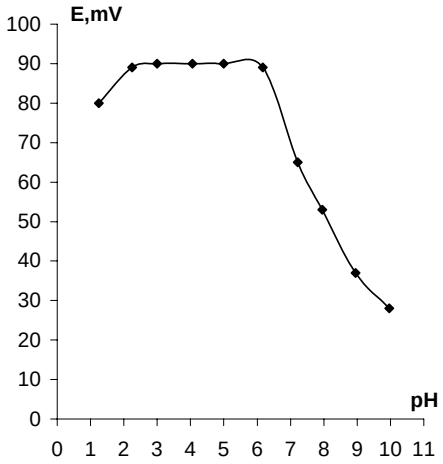


Fig. 1. Effect of pH on the response of the vancomycin-based enantioselective, potentiometric membrane electrode designed for the assay of L-methotrexate (10^{-5} mol l^{-1} L-methotrexate solution).

3.3. Selectivity of the electrodes

The selectivity of the electrodes was investigated using the mixed solution method. The concentrations of L-Mtx and D-Mtx were 10^{-5} and 10^{-4} mol l^{-1} in case of vancomycin-based electrode and 10^{-5} mol l^{-1} D-Mtx and 10^{-4} mol l^{-1} L-Mtx in case of teicoplanin-based electrodes, respectively. The values of the potentiometric selectivity coefficients are shown in Table 2. It is evident from Table 2, that the modification of teicoplanin by acetonitrile improves the selectivity of the corresponding electrode towards D-methotrexate. These results prove, that all three electrodes can be used for methotrexate enantioanalysis.

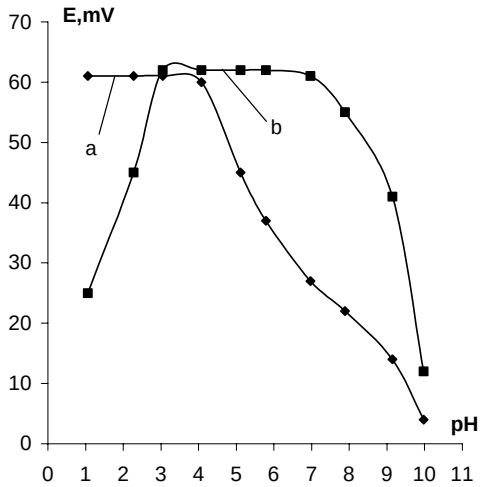


Fig. 2. Effect of pH on the response of the teicoplanin-based enantioselective, potentiometric membrane electrodes designed for the assay of D-methotrexate (10^{-5} mol l^{-1} D-methotrexate solution). Curve (a) shows the effect of pH on the response of teicoplanin-based electrode; curve (b) shows the effect of pH on the response of teicoplanin modified with acetonitrile-based electrode.

3.4. Analytical applications

The proposed enantioselective, potentiometric membrane electrodes proved to be useful for the determination of enantiopurity of L-Mtx raw materials and for performing the content uniformity test of Mtx tablets and Mtx injections due to recovery values obtained for the pure L- and D-enantiomer of Mtx: higher than 99.96% (R.S.D. $< 0.10\%$, $n = 10$) and higher than 99.95% (R.S.D. $< 0.10\%$, $n = 10$), respectively for all three proposed electrodes. The assays of L- and D-Mtx were conducted using different ratios between L- and D-Mtx and D- and L-Mtx, respectively. The good recovery values obtained for the determination of L-enantiomer in the presence of D-enantiomer and for D-enantiomer in the presence of L-enantiomer (Tables 3 and 4) demonstrated the suitability of the proposed enantioselective, potentiometric membrane electrodes for testing the enantiopurity of Mtx raw material.

The results obtained for the content uniformity tests of Mtx tablets and Mtx injections are shown in Tables 5 and 6, respectively. The uniformity content tests show that the pharmaceutical formulations contain L-methotrexate as the main component and only a small amount of D-methotrexate. The recovery values for the L-methotrexate—an active component of Mtx tablets and Mtx injections—are within the limits requested by the USP XXV [37]: recoveries—90–100% with the R.S.D. values less than 6%. The proposed enantioselective, potentiometric membrane electrodes allow to

Table 2
Selectivity coefficients (K_{sel}^{pot}) for the enantioselective, potentiometric membrane electrodes

Chiral selector used for the design of the electrode	Interfering enantiomer	K_{sel}^{pot}
Vancomycin	D	5.40×10^{-3}
Teicoplanin	L	6.20×10^{-3}
Teicoplanin, modified with acetonitrile	L	2.10×10^{-3}

All values are average of 10 determinations.

Table 3
Determination of L-Mtx in the presence of D-Mtx

Chiral selector used for the design of the electrode	Recovery L-Mtx, (%), L:D				
	2:1	1:1	1:2	1:4	1:9
Vancomycin	100.00 ± 0.01	100.04 ± 0.02	100.02 ± 0.03	99.99 ± 0.02	100.00 ± 0.01

All values are average of 10 determinations.

Table 4
Determination of D-Mtx in the presence of L-Mtx

Chiral selector used for the design of the electrode	Recovery D-Mtx, (%), D:L				
	2:1	1:1	1:2	1:4	1:9
Teicoplanin	100.00 ± 0.01	99.92 ± 0.02	99.92 ± 0.02	99.93 ± 0.02	99.94 ± 0.01
Teicoplanin, modified with acetonitrile	99.93 ± 0.02	99.95 ± 0.03	100.00 ± 0.01	99.94 ± 0.02	100.00 ± 0.01

All values are average of 10 determinations.

Table 5
Determination of L-Mtx and D-Mtx in Methotrexate® tablets (2.5 mg Mtx per tablet)

Chiral selector used for the design of the electrode	Average recovery	
	L-Mtx (%)	D-Mtx (%)
Vancomycin	94.47 ± 1.59	–
Teicoplanin	–	3.02 ± 1.38
Teicoplanin, modified with acetonitrile	–	3.08 ± 1.24

All values are average of 10 determinations.

Table 6
Determination of L-Mtx and D-Mtx in Methotrexate LPF® injections (25 mg Mtx ml^{-1} injection)

Chiral selector used for the design of the electrode	Average recovery	
	L-Mtx (%)	D-Mtx (%)
Vancomycin	98.40 ± 0.04	–
Teicoplanin	–	0.87 ± 0.03
Teicoplanin, modified with acetonitrile	–	0.83 ± 0.02

All values are average of 10 determinations.

analyze Mtx in pharmaceutical formulations without additional dilution of the samples, which is needed in case of amperometric biosensors [35].

4. Conclusions

The proposed enantioselective, potentiometric membrane electrodes based on macrocyclic antibiotics—vancomycin and teicoplanin—have good features in enantioselective analysis. The simple, fast and reproducible construction of the electrodes assures the reliable response characteristics. The good enantioselectivity of the proposed electrodes allowed to perform the enantiopurity assay of L-Mtx raw material and from its pharmaceutical formulations.

The main advantage of the proposed electrodes over amperometric biosensors is working concentration range, which is situated in the higher concentration region for the enantioselective, potentiometric membrane electrodes making possible the enantioanalysis with a minimum dilution of the samples.

References

[1] T.J. Ward, K.D. Ward, in: H.Y. Aboul-Enein, I.W. Wainer (Eds.), The Impact of Stereochemistry on Drug Development and Use, Chemical Analysis Series, vol. 142, John Wiley and Sons, New York, 1997, p. 317.
[2] R.I. Stefan, J.F. van Staden, H.Y. Aboul-Enein, Electrochemical Sensors in Bioanalysis, Marcel Dekker, New York, 2001.
[3] H. Yoshikawa, K. Doi, Biosci. Biotechnol. Biochem. 62 (1998) 996.
[4] K. Kato, S. Tanaka, S. Fujii, M. Katayama, H. Kimoto, J. Biosci. Bioeng. 87 (1999) 76.
[5] D.W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill, J.-R. Chen, Anal. Chem. 66 (1994) 1473.
[6] D.W. Armstrong, Y. Zhou, J. Liquid Chromatogr. 17 (1994) 1695.
[7] D.W. Armstrong, Y. Liu, K.H. Ekborg-Ott, Chirality 7 (1995) 474.
[8] A. Berthod, Y. Liu, C. Bagwill, D.W. Armstrong, J. Chromatogr. A 731 (1996) 123.
[9] F. Hui, K.H. Ekborg-Ott, D.W. Armstrong, J. Chromatogr. A 906 (2001) 91.
[10] U.B. Nair, S.S.C. Chang, D.W. Armstrong, Y.Y. Rawjee, D.S. Eggleston, J.V. McArdle, Chirality 8 (1996) 590.
[11] K.L. Rundlett, M.P. Gasper, E.Y. Zhou, D.W. Armstrong, Chirality 8 (1996) 88.
[12] D.W. Armstrong, K.L. Rundlett, J.-R. Chen, Chirality 6 (1994) 496.
[13] S. Farber, L.K. Diamond, R.D. Mercer, R.F. Sylvester, J.A. Wolff, N. Engl. J. Med. 238 (1948) 787.
[14] M. Levitt, M.B. Mosher, R.C. DeConti, L.R. Farber, R.T. Skeel, J.C. Marsh, M.S. Mitchell, R.J. Papac, E.D. Thomas, J.R. Berthino, Cancer Res. 33 (1973) 1729.
[15] E. Frei, N. Jaffe, M.H.N. Tattersall, S. Ptiman, L. Parker, N. Engl. J. Med. 292 (1975) 846.
[16] D.I. Cargill, D.E. Furst, in: M. Yaron (Ed.), Methotrexate for Rheumatoid Arthritis, Ichlove Hospital, Tel Aviv, 1990, p. 5.
[17] P.A. Anderson, S.A. West, J.R. O'Dell, C.S. Vi, G. Claypool, B.L. Katxin, Ann. Int. Med. 103 (1985) 489.
[18] D.E. Furst, J.M. Kremer, Arthritis Rheum. 31 (1988) 305.
[19] M.F. Mullarkey, D.R. Webb, N.E. Pardee, Ann. Allergy 56 (1986) 347.
[20] M.F. Mullarkey, B.A. Blumenstein, W.P. Andrade, G.A. Bailey, I. Olason, C.E. Wetzel, N. Engl. J. Med. 318 (1988) 603.

[21] S.P. Ackland, R.L. Schilsky, J. Clin. Oncol. 11 (1993) 2017.
[22] J. Hendel, H. Brodthagen, Eur. J. Clin. Pharmacol. 26 (1984) 103.
[23] C.J. Allegra, B.A. Chabner, J.C. Drake, R. Lotz, D. Rodbard, J. Jolivet, J. Biol. Chem. 260 (1985) 9720.
[24] F. Albertioni, B. Pettersson, O. Beck, C. Rask, P. Seideman, C. Peterson, J. Chromatogr. B 665 (1995) 163.
[25] M. Cociglio, D.H. Hillaire-Buys, C. Alric, J. Chromatogr. B 674 (1995) 101.
[26] H. Aboleneen, J. Simpson, D. Backles, J. Chromatogr. B 681 (1996) 317.
[27] A. Sparreboom, W.J. Loos, K. Nooter, G. Storer, J. Verweij, J. Chromatogr. B 735 (1999) 111.
[28] S. Sadray, S. Rezall, S. Rezakhah, J. Chromatogr. B 787 (2003) 293.
[29] F. Sczesny, G. Hempel, J. Boos, G. Blaschke, J. Chromatogr. B 718 (1998) 177.
[30] C.Y. Kuo, H.L. Wu, S.M. Wu, Anal. Chim. Acta 471 (2) (2002) 211.
[31] C.Y. Kuo, H.L. Wu, H.S. Kou, S.S. Chiou, D.C. Wu, S.M. Wu, J. Chromatogr. A 1014 (2003) 93.
[32] A. Espinosa-Mansilla, I. Duran-Meras, M.J. Rodriguez-Gomez, A. Munoz-de-la-Pena, F. Salinas, Talanta 58 (2) (2002) 255.
[33] A. Espinosa-Mansilla, I. Duran-Meras, A. Zamora-Madera, L. Pedano, C. Ferreyra, J. Pharm. Biomed. Anal. 29 (2002) 851.
[34] M.O. Kovbuz, N.M. Felitsin, Ya.S. Khim'yak, B.S. Zimenkovskii, Farm. Zhurn. (Kiev) 3 (1995) 80.
[35] R.I. Stefan, R.G. Bokretsion, J.F. van Staden, H.Y. Aboul-Enein, Talanta 60 (2003) 983.
[36] R.I. Stefan, R.G. Bokretsion, J.F. van Staden, H.Y. Aboul-Enein, Biosens. Bioelect. 19 (2003) 261.
[37] U.S. Pharmacopoeia 25-National Formulary 20, Asian Ed., 2002, p. 1111.