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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 905–909
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Diastereomeric and enantiomeric high-performance liquid chromatographic separation of synthetic anisodamine

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Received 24 June 2006; received in revised form 16 August 2006; accepted 4 September 2006
Available online 17 October 2006

Abstract

In order to investigate the enantiomeric pharmacokinetics and biotransformation of synthetic anisodamine (654-2), a cholinoceptor antagonist widely used in clinic in China, it has been preparatively separated into two racemates (I and II) by using ZORBAX Eclipse XDB-C18 column. The diastereo- and/or enantioseparations of 654-2, I and II were carried out by HPLC using CHIRALPAK AD-H as chiral stationary phase (CSP) and acetonitrile–2-propanol–DEA 97:3:0.1 (v/v/v) as mobile phase. The methods were optimized by studying mobile phase modifiers, concentration of modifier and column temperature. The HPLC method for the simultaneous separation of two pairs of enantiomers of 654-2 has been validated.

Keywords: Enantioselective HPLC; Chiral stationary phases; Diastereoisomers; Anisodamine; Tropane alkaloids

1. Introduction

Anisodamine (654-1), a tropane alkaloid isolated from the Chinese solanaceous plant (*Scopolia tangutica* Maxim.) [1], is a potent cholinoceptor antagonist and has been used as a spasmolytic drug in China for decades by virtue of its weaker side effect on the central nervous system than atropine [2]. Furthermore, anisodamine was demonstrated to inhibit thrombogenesis, granulocyte and platelet aggregation and has been used in the treatment of acute microcirculatory disturbances caused by infections, such as fulminant epidemic meningitis, toxic bacillary dysentery, septic shock, severe lobar pneumonia and hemorrhagic enteritis [3,4]. The chemical structure of anisodamine is (2*S*)-3-hydroxy-2-phenyl-propionic acid (3*S*,6*S*)-6-hydroxy-8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester. Because of the increased demand in clinics and the limited amounts of anisodamine available from the natural resources, now its synthetic one (trade name 654-2), a racemic mixture of two pairs of enantiomers [5,6] shown in Fig. 1, is massively used in China.

The biological properties of this kind of alkaloids including cholinoceptor agonists [7–9] and antagonists, like most chiral drugs, depend strongly on their stereochemistry [10]. The clinical studies indicated that the spasmolysis of 654-2 was almost identical with that of 654-1, but the side effect of 654-2 was stronger than that of 654-1 [11]. Evidently, the therapeutic and side effects of 654-2 were the combination effect of four isomers. The potency differences among four isomers of 654-2 on muscarinic receptor have been observed [12]. The optimization of pharmacological properties of 654-2 warranted our evaluating the enantioselective pharmacokinetics of 654-2 and its each pair of enantiomers. Thus, there is a clear need for method able to obtain two racemates (I and II) and analytical methods by which enantioseparation of 654-2, I and II can be realized. Although certain capillary electrophoresis (CE) methods with high resolution capability and short analysis time have been established for the enantioseparation of 654-2 [5,6,13–15], no HPLC method, one of the most widely used techniques for the enantioselective analysis of chiral drugs [16], has been reported for the enantioseparation of 654-2. In this paper, we report the preparative diastereo-separation of I and II from 654-2 by the reverse phase HPLC and the direct enantioseparation of 654-2, I and II on HPLC with CHIRALPAK AD-H. Our chiral HPLC method with the possibility to transfer to preparative scale

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doi:10.1016/j.jpba.2006.09.006

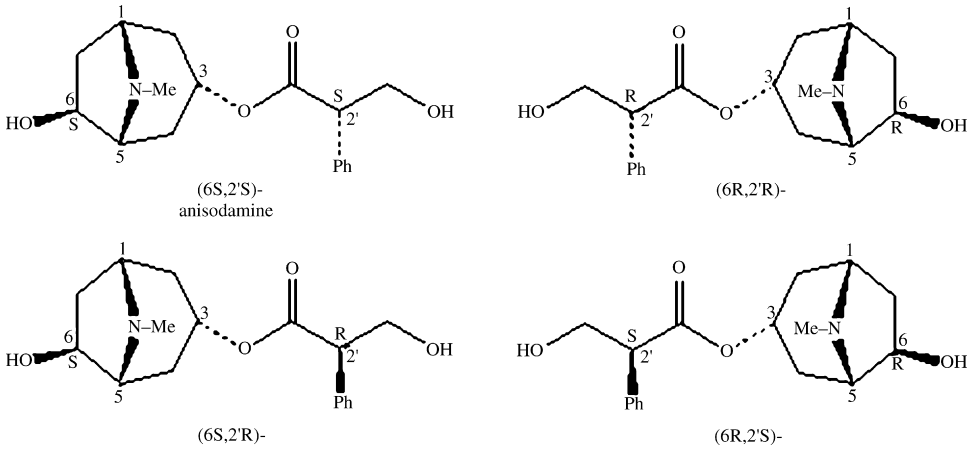

Fig. 1. Structure of four stereoisomers of 654-2.

provides an alternative for the separation of four stereoisomers of 654-2.

2. Experimental

2.1. Chemicals and materials

654-2, a mixture of (6*S*,2′*S*)-, (6*S*,2′*R*)-, (6*R*,2′*R*)- and (6*R*,2′*S*)-isomers, was provided by Shanghai No.1 Biochem & Pharm Company (Shanghai, China). HPLC grade *n*-hexane, 2-propanol and methanol were purchased from Dikma (Beijing, China), and acetonitrile from Sigma–Aldrich (St. Louis, USA). Diethylamine (DEA) was obtained from Shanghai Reagent (Shanghai, China).

2.2. Equipments

Chromatographic studies were performed on Agilent 1100 HPLCs (Agilent, Palo Alto, CA, USA), equipped with an autosampler, thermostat-column device, a variable-wavelength UV detector operating at 235 nm and a data acquisition system using the HP Chemstation software. The ^{1}H NMR spectra were measured by using Bruker AV 500 apparatus.

2.3. Chromatographic conditions

The preparative separation was achieved on ZORBAX Eclipse XDB-C18 column (250 mm × 9.4 mm i.d., packed with 5 μm diameter particles, Agilent Technologies, MN, USA) using methanol–water (53:47, v/v) containing 1.7% DEA as a mobile phase with the flow-rate 2.0 ml/min (Fig. 2). Injection volume was 30 μl.

The enantioseparation was achieved on CHIRALPAK AD-H column (250 mm × 4.6 mm i.d., particle size 5 μm) from Daicel Chemical Industries (Tokyo, Japan) using the eluents consisting of a mixture of *n*-hexane–2-propanol (92:8, v/v) or acetonitrile–2-propanol (97:3, v/v), with the addition of 0.1 vol. of DEA, with the flow-rate of 0.9 ml/min. All separations were performed at 25 °C, except those used for the study of the effect of temperature on HPLC. CHIRALPAK AD-RH column

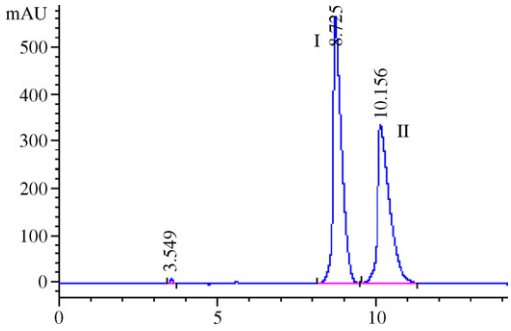

Fig. 2. Separation of diastereomers of 654-2 on ZORBAX Eclipse XDB-C18 column. Eluent: methanol–deionized water–DEA (53:47:0.017, v/v/v).

(150 mm × 4.6 mm i.d., particle size 5 μm) from Daicel was employed during method development.

2.4. Validation of the method

Detector response linearity was checked by preparing calibration sample solutions of 654-2 at concentration range of 65–1300 μg/ml using the selected mobile phase as the solvent. Linear regression curve was obtained by plotting peak area versus concentration, using the least squares method.

The method precision was assessed using three standard solutions of 654-2 at 130, 260 and 520 μg/ml. The accuracy of the method was evaluated by back-calculation. Limits of detection (LOD) were established at a signal-to-noise ratio of 3 and limits of quantification (LOQ) at a signal-to-noise ratio of 10.

3. Results and discussion

3.1. Preparative separation of diastereomers from 654-2

The preparative separation of diastereomers from 654-2 was carried out by using ZORBAX Eclipse XDB-C18 column. The capacity factor k'_1 of I, separation factor α and resolution R_s were 1.46, 1.27, 2.56, respectively, which were sufficient for the preparative separation. Two racemates (57.1 mg I and 46.4 mg II) eluted successively were collected from 654-2 (125 mg). I

Table 1
^{1}H NMR spectra of I and II

Racemates	^{1}H NMR data (CD_3OD)
I (6*S*,2′*S* and 6*R*,2′*R*)	δ 1.37–2.38 (m, 6H, 2,4,7-H), 2.47 (s, 3H, CH_3N), 2.63 (s, 2H, 2OH), 2.85 (s, 1H, 1-H), 3.22 (m, 1H, 5-H), 3.76–4.20 (m, 4H, 6-H & $CHCH_2$), 5.02 (t, *J* = 5.3 Hz, 1H, 3-H), 7.26–7.38 (m, 5H, ArH)
II (6*S*,2′*R* and 6*R*,2′*S*)	δ 1.24–2.21 (m, 6H, 2,4,7-H), 2.49 (s, 3H, CH_3N), 2.59 (s, 2H, 2 OH), 3.00 (s, 1H, 1-H), 3.13 (m, 1H, 5-H), 3.76–4.20 (m, 3H, $CHCH_2$), 4.36 (dd, *J* = 2.5, 7.3 Hz, 1H, 6-H), 5.03 (t, *J* = 5.3 Hz, 1H, 3-H), 7.26–7.38 (m, 5H, ArH)

is the pair of enantiomers 6*S*,2′*S*- and 6*R*,2′*R*-isomers, and II is another enantiomeric pair (6*S*,2′*R*- and 6*R*,2′*S*-isomers). ^{1}H NMR spectra of I and II are listed in Table 1.

3.2. Diastereo- and enantioselective separation of 654-2

The polysaccharide derivatives have been extensively used for enantioseparation of a wide variety of chiral compounds. On the basis of our own experience on enantioseparation of tropane derivatives [17], AD chiral stationary phase (CSP) consisting of silica gel coated with amylose tris(3,5-dimethylphenyl carbamate) was selected in this study to resolve the structurally related compounds from 654-2. Firstly, the normal phase conditions with CHIRALPAK AD-H column and the reversed phase conditions with CHIRALPAK AD-RH column were examined, respectively, to develop a chromatographic method for chiral separation of the investigated compounds. No satisfactory separation was found on CHIRALPAK AD-RH column using the mobile phases consisting of 20 mM borate buffer (pH 9.0):acetonitrile (60:40, 80:20, 90:10, respectively, v/v). The enantioseparation of the racemate II (k'_1 = 8.16, α = 1.20) was observed on CHIRALPAK AD-H using the mobile phase system *n*-hexane–2-propanol–DEA (92:8:0.1, v/v/v). In the subsequent investigation, in order to achieve the diastereo- and enantioseparations of four stereoisomers of 654-2 in a single run, CHIRALPAK AD-H was used and several parameters affecting the resolution, such as concentration and nature of the organic modifier, temperature were taken into account during the optimization of the method.

The mobile phase may not only influence the retention time, but also considerably affect selectivity. This is particularly the case for the polysaccharide-based phases. The direct resolution of four racemic tropane derivatives on CHIRALPAK AD-H column using 'normal-phase' eluent and the chromatographic advantages of operating this column under the polar organic elution system have been demonstrated [17,18]. Both elution systems mentioned above were evaluated to select the better mobile phase system for the simultaneous separation of two pairs of enantiomers. Four stereoisomers of 654-2 were not separated completely in the conventional 'normal-phase' using different mixtures of *n*-hexane–2-propanol (from 90:10 to 97:3, v/v) containing 0.1% DEA. The diastereo- and/or enantioseparations of 654-2, I and II were achieved using the polar organic eluent consisting of acetonitrile–2-propanol–DEA (97:3:0.1, v/v/v) (Fig. 3). Baseline separation of the four stereoisomers was obtained within 15 min under these conditions. The separation parameters are given in Table 2. This indicated that a better match between the polar nature of the analyte (log *P* = 0.54, from ChemDraw Ultra 7.0) with the presence of phenyl group on the molecule and the specific retention properties of AD CSP is

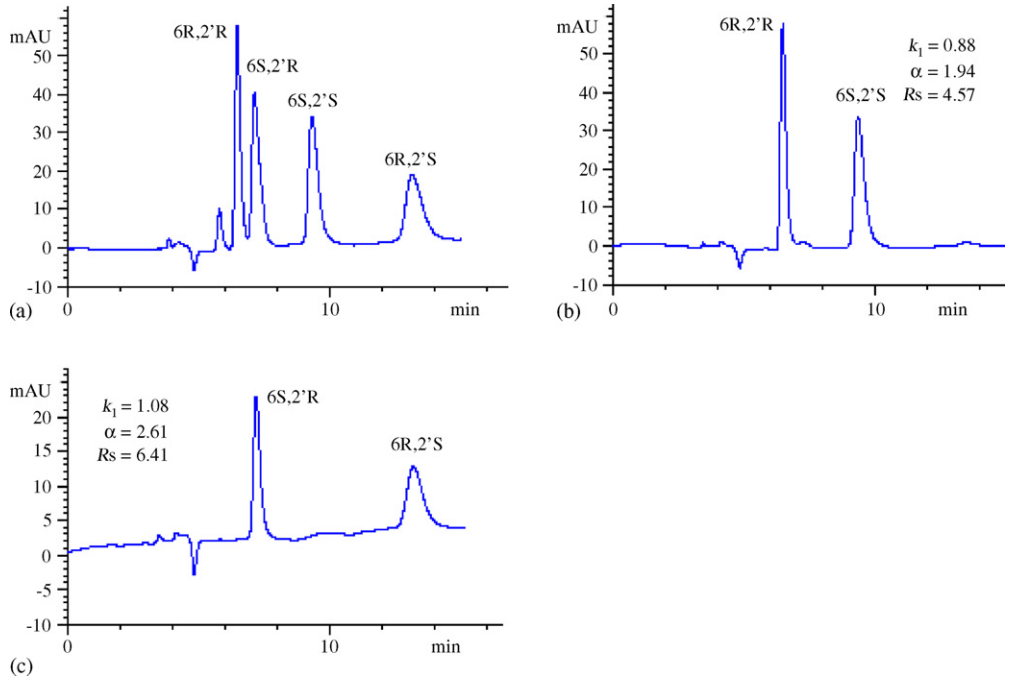

Fig. 3. Separation of 654-2 (a), I (b) and II (c) on CHIRALPAK AD-H column, acetonitrile–2-propanol–DEA 97:3:0.1 (v/v/v), 0.9 ml/min, 25 °C; UV detection at 235 nm.

Table 2
Separation parameters corresponding to the resolution of four stereoisomers of 654-2

Stereoisomers	t (min)	k	α	R_s	N
(6*R*,2′*R*)	6.46	0.87			4522
(6*S*,2′*R*)	7.13	1.07	1.23	1.52	3742
(6*S*,2′*S*)	9.32	1.70	1.59	3.67	3026
(6*R*,2′*S*)	13.15	2.81	1.65	4.28	2214

The conditions were identical to those in Fig. 3.

Table 3
The calibration functions and r^2 values

Stereoisomers	Slope	y-Intercept	Correlation coefficient (r^2)
(6*R*,2′*R*)	4.4976	−3.5978	0.9999
(6*S*,2′*R*)	4.5351	−6.3096	0.9998
(6*S*,2′*S*)	4.6156	−11.0269	0.9996
(6*R*,2′*S*)	4.5337	−7.8342	0.9998

Number of data points: $n = 6$.

obtained with the polar organic eluent than under the normal-phase eluent conditions.

To clarify the stereochemistry of the four isomers, we isolated them and evaluated their circular dichroism (CD) spectra (data not shown). Their configuration was determined by comparison of their CD spectra and HPLC with those of authentic sample and with data in ref. [19]. The four isolated isomers represent two pairs of enantiomer (6*R*,2′*R*)/(6*S*,2′*S*) and (6*R*,2′*S*)/(6*S*,2′*R*). Therefore, our investigation using CHIRALPAK AD-H column concerned not only diastereoseparation but also enantioseparation.

The separation of enantiomers on amylose-based CSP was due to the formation of solute–CSP complexes through inclusion of the enantiomers into the chiral cavities in the higher order structures of CSP [20,21]. The solute–CSP interaction was achieved between certain groups of solutes and the polar carbamate group on CSP. In the investigated compounds, hydroxyl, carbonyl, aromatic group exist. We assumed that the mentioned interactions include: (1) hydrogen bonding between hydroxyl groups on the solute and the carbamate carbonyl group on CSP; (2) hydrogen bonding between the carbonyl group on the solute and the carbamate NH group on CSP; (3) dipole–dipole interaction between the carbonyl group on the solute and that on CSP. We assumed also that aromatic group in the solute could provide additional stabilizing effect to the solute–CSP complex by insertion into the chiral cavity on CSP. The discrimination of one enantiomer from the other in CSP HPLC is due to the differences in the strength of over-mentioned interactions of two enantiomers and in their steric fit in the chiral cavities on CSP.

Because alcohol content in the mobile phase is responsible for retention as well as chiral discrimination, 2-propanol concentration in the polar organic eluent should be important for the separation of the four stereoisomers of 654-2. A general decrease in retention factor (k) of the enantiomers was observed when 2-propanol content rises from 2 to 5% (v/v) (Fig. 4(a)). This indicated that the higher the content of alcohols in the eluent, the higher the elution strength of the mobile phase will be, which reduces retention times. Fig. 4(b) represents the variation of the stereoselectivity of each two adjacent peaks in four peaks versus 2-propanol percentage in the mobile phase. No general trend is observable and the selected mobile phase composition acetonitrile–2-propanol–DEA (97:3:0.1, v/v/v) mixture is a compromise between resolution and analysis time.

In order to elucidate the temperature effect on the chiral resolution of stereoisomers of 654-2 by CHIRALPAK AD-H, variable temperature studies were carried out between 15 and 30 °C. With decreasing temperature, the enantioseparation factor α improved (1.89 at 30 °C, 2.02 at 15 °C for I, and 2.50 at 30 °C, 3.25 at 15 °C for II) and the column efficiency reduced due to a slower kinetics of the absorption–desorption process, which are consistent with the observation of Cirilli et al. [22]. Considering better resolution, higher column efficiency, and convenience, the enantioseparation at room temperature was selected for the analytical studies.

3.3. Validation results of the chiral separation method

Linear relationships of peak area versus concentration were obtained for all four stereoisomers, with the correlation coefficient (r^2) consistently greater than 0.9996. The calibration functions with r^2 are presented in Table 3. LOD (S/N = 3) and LOQ (S/N = 10) were determined to be 1.2 and 3.6 μg/ml for (6*R*,2′*S*)-isomer, 0.7 and 1.4 μg/ml for other three isomers. The higher LOD and LOQ of (6*R*,2′*S*)-isomer than those of three others should be due to its lower number of theoretical plates.

The results of intra- and inter-day repeatability, expressed as R.S.D.s, were found ($n = 3$) to range between 0.30–2.64 and

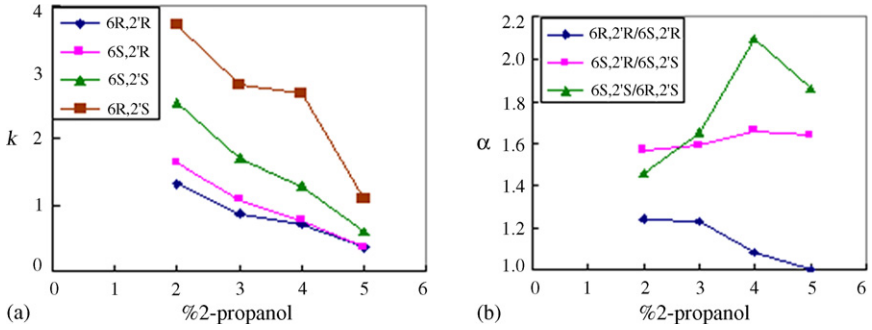

Fig. 4. Influence of 2-propanol fraction volume in the polar organic eluent upon (a) retention factor (b) selectivity on CHIRALPAK AD-H.

Table 4
Intra- and inter-day precision and accuracy data for the enantiomers of 654-2 ($n = 3$)

Concentration (μg/ml)			
(6*R*,2′*R*)/(6*S*,2′*R*)/(6*S*,2′*S*)/(6*R*,2′*S*)	33.8/31.6/33.6/29.0	67.6/63.2/67.2/58.0	135.2/126.4/134.4/116.0
Intra-day			
Precision (R.S.D.%)	2.64/1.76/0.68/1.48	1.53/1.52/2.35/2.37	1.07/0.89/0.30/0.51
Accuracy (%)	100/103/102/101	97/97/99/97	97/97/97/97
Inter-day			
Precision (R.S.D.%)	1.67/2.00/0.82/3.72	1.63/0.73/0.57/1.04	2.53/2.23/1.86/1.44
Accuracy (%)	100/100/103/105	99/98/99/99	99/99/98/99

0.57–3.72%, respectively. Precision and accuracy values are shown in Table 4.

4. Conclusion

By using the described methods, we separated successfully at the preparative level two racemates (I and II) from 654-2 and at analytical level four stereoisomers of 654-2 and the enantiomers of I and II, respectively. The method for the separation of the four stereoisomers of 654-2 has been validated. The methods can be used in our future study to the enantiomeric pharmacokinetic evaluation of 654-2, I and II, and the determination of the enantiomeric purity of individual stereoisomers of 654-2.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant No.: 30371731), Ph.D. Program Foundation of Ministry of Education of China (Grant No.: 20050266003) and Ph.D. Foundation of Shanghai Jiao Tong University School of Medicine (Grant No.: BXJ0503).

References

[1] Pharmacopoeia Committee of People's Republic of China, Pharmacopoeia of People's Republic of China, Chemical Industry Press, 2005, pp. 407–408.
[2] Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences, Natl. Med. J. Chin. 53 (1973) 269–273.
[3] R.J. Xiu, D.E. Hammerschmidt, P.A. Coppo, H.S. Jacob, J. Am. Med. Assoc. 247 (1982) 1458–1460.
[4] S. Zhang, A.M. Chang, C.F. Li, Z.J. Li, Z.J. Yin, X. Zhao, S.L. Liang, Exp. Hematol. 15 (1987) 65–71.
[5] G.R. Fan, Z.Y. Hong, M. Lin, X.P. Yin, Y.T. Wu, J. Chromatogr. B 809 (2004) 265–271.
[6] H. Wang, J.L. Gu, H.F. Hu, R.J. Dai, T.H. Ding, R.N. Fu, Anal. Chim. Acta 359 (1998) 39–46.
[7] Y.Y. Niu, L.M. Yang, H.Z. Liu, Y.Y. Cui, L. Zhu, J.M. Feng, J.H. Yao, H.Z. Chen, B.T. Fan, Z.N. Chen, Y. Lu, Bioorg. Med. Chem. Lett. 15 (2005) 4814–4818.
[8] L.M. Yang, Y.Y. Niu, Y.F. Xie, J.M. Feng, H.Z. Chen, Y. Lu, Acad. J. Shanghai Second Med. Univ. 25 (2005) 220–222.
[9] L.M. Yang, H.N. Wang, Acta Pharmacol. Sin. 33 (1998) 832–835.
[10] E.J.D. Lee, K.M. Williams, Clin. Pharmacokinet. 18 (1990) 339–345.
[11] X.Q. Chen, Y.Y. Jin, G. Tang, Xin Bian Yao Wu Xue, People's Medical Publishing House, 2003, pp. 308–309.
[12] X.Y. Niu, Z.H. Ren, L. Xie, J. Chin. Pharm. Sci. 1 (1992) 84–85.
[13] Z. Wang, A.J. Huang, Y.L. Sun, Z.P. Sun, J. High Resolut. Chromatogr. 19 (1996) 697–699.
[14] Y. Dong, Y. Sun, Z. Sun, J. High Resolut. Chromatogr. 21 (1998) 445–449.
[15] Y. Wei, J. Li, C. Zhu, A. Hao, M. Zhao, Anal. Sci. 21 (2005) 959–962.
[16] J. Haginaka, J. Pharm. Biomed. Anal. 27 (2002) 357–372.
[17] Y.F. Xie, L.M. Yang, Y. Lu, Acad. J. Shanghai Second Med. Univ. 25 (2005) 229–231.
[18] J.K. Mclninch, F. Geiser, K.B. Prickett, S.W. May, J. Chromatogr. A 828 (1998) 191–198.
[19] C.S. Zheng, J.X. Xie, Acta Pharmacol. Sin. 26 (1991) 96–102.
[20] M. Kato, T. Fukushima, N. Shimba, I. Shimada, Y. Kawakami, K. Imai, Biomed. Chromatogr. 15 (2001) 227–237.
[21] Y. Okamato, Y. Kaida, J. Chromatogr. A 666 (1994) 403–419.
[22] R. Cirilli, M.R. Del Giudice, R. Ferretti, F. La Torre, J. Chromatogr. A 923 (2001) 27–36.