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Improved assay method for the determination of pyronaridine in plasma and whole blood by high-performance liquid chromatography for application to clinical pharmacokinetic studies

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Abstract

An improved high-performance liquid chromatography method using a diisopropyl-C_{14} reversed-phase column (Zorbax Bonus-RP column) and a liquid–liquid extraction technique with UV detection is presented for the analysis of pyronaridine in human whole blood and plasma. Tribasic phosphate buffer (50 m*M*, pH 10.3) and diethyl ether were used for liquid–liquid extraction. The mobile phase consists of acetonitrile–0.08 *M* potassium dihydrogen phosphate buffer (13:87, v/v) with the pH 2.8 adjusted by orthophosphoric acid. Amodiaquine was found to be a suitable internal standard for the method. The quantification limit with UV detection at 275 nm was 3 ng on-column for both plasma and blood samples. The method was applied to plasma and blood specimens from a rabbit after a single intramuscular dose of pyronaridine tetraphosphate (20 mg/kg as base). From this in vivo study, evidence was found that pyronaridine is concentrated in blood cells, with a blood:plasma ratio ranging from 4.9 to 17.8. We conclude that blood is the preferred matrix for clinical pharmacokinetic studies.

Keywords: Pyronaridine

1. Introduction

Over a million deaths are caused by malaria every year, most of which are caused by *Plasmodium falciparum*. Chloroquine has been the drug of choice in the treatment of malaria. However, more and more chloroquine-resistance has developed [1]. Pyronaridine, 2-methoxy-7-chloro-10-[3′,5′-bis-(pyrolidinyl-1-methyl)-4′-hydroxyanilinol]benzo[*b*]-1,5-naphthyridines, an antimalarial drug synthesized by Zheng et al. in the early 1970s, has been used in China for over 20 years [2,3]. It has been reported to be effective against not only chloroquine-resistant but also multidrug resistant *P. falciparum* without major side effects [4,5].

Pharmacokinetic studies done in China used a spectrofluorimetric method for quantification [6]. However, this assay method is non-specific and involves laborious separation before measurement. Saleh and Loh reported a high-performance liquid chromatography (HPLC) method using ultraviolet detection [7]. However, the sensitivity of the method

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is not adequate for the analysis of pyronaridine in human plasma and blood for clinical pharmacokinetic studies. Jayaraman et al. reported a HPLC method using fluorescence detection for the analysis of pyronaridine in human plasma [8]. However, the pharmacokinetic results in human plasma were very erratic, drawing into question the validity of the method. Wages et al. reported a HPLC method using electrochemical detection for the analysis of pyronaridine in blood and urine [9]. This method involves liquid–liquid extraction and a subsequent solid-phase extraction to remove the interferences found in blood. The method uses electrochemical detection and is not very convenient for routine analysis. Despite these previous reports, there is still a need for an accurate, sensitive, specific and convenient assay method in plasma and blood for clinical pharmacokinetic studies of pyronaridine.

2. Experimental

2.1. Chemicals

Pyronaridine tetraphosphate was provided by the Tropical Disease Research (TDR) program, World Health Organization, Geneva, Switzerland. The internal standard, amodiaquine was obtained from Sigma (St. Louis, MO, USA). All solvents and all chemicals were of HPLC grade. Methanol, acetonitrile, 85% orthophosphoric acid, and diethyl ether were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Potassium dihydrogen phosphate was purchased from Aldrich (Milwaukee, WI, USA). Sodium phosphate tribasic dodecahydrate was obtained from Sigma.

2.2. Instrumentation

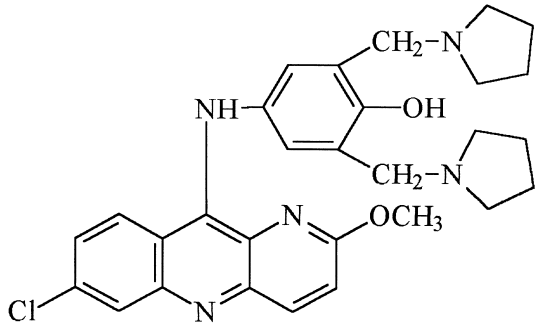
The pH was determined using a Orion Research digital ionalyzer/501 pH meter. The extraction procedure utilized a S/P vortex mixer (catalog No. S8223-1; Baxer Diagnostics, Deerfield, IL, USA) and an IEC HN-SII centrifuge (Damon/IEC division). The HPLC apparatus consisted of a Model LC-6A pumping system (Shimadzu, Kyoto, Japan), a Model SIL-6B automatic injector (Shimadzu), a Model SCL-6B system controller (Shimadzu), and a Model CR-501 integrator (Shimadzu). The chromatograph was equipped with a UV spectrophotometric detector and a Model SPD-6A UV spectrophotometric detector (Shimadzu) for peak quantification. A Zorbax Bonus-RP column (5 μm, 250×4.6 mm I.D.) was purchased from Hewlett-Packard (USA). A Platinum C_{18} column (5 μm, 250×4.6 mm I.D.) was obtained from Alltech (Deerfield, IL, USA) and a Waters Symmetry RP-8 column (5 μm, 150×4.6 mm I.D.) was purchased from Waters (Milford, MA, USA).

2.3. Standards

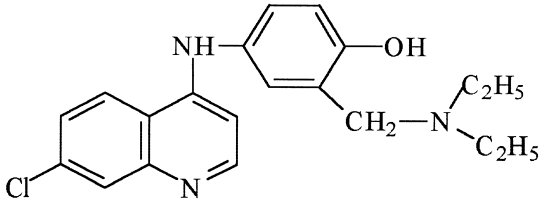
Pyronaridine standard solutions in methanol with concentrations of 28.5, 60, 110, 280, 570 and 1140 ng/ml were prepared by serial dilutions of a stock solution (1 mg/ml in methanol). The stock solution was protected from light and stored at −20°C. The internal standard stock solution (amodiaquine, 1 mg/ml) was also prepared in methanol and stored under the same conditions. Fig. 1 shows the structures of pyronaridine and amodiaquine.

2.4. Sample preparation

A human plasma or blood sample of 200 μl was spiked with 400 ng of amodiaquine in a screw-capped 100×16 mm glass test tube. Buffer solution was prepared using 500 m*M* sodium phosphate tribasic dodecahydrate with 85% orthophosphoric acid to adjust the pH value to 10.3. A 500-μl volume was added to plasma or blood, mixed briefly on a vortex mixer, and followed by the addition 3 ml of diethyl ether. The mixture was homogenized by vortex mixing for 5 min, then centrifuged for 10 min (2500 rpm). The organic layer was transferred to a new 100×13 mm glass test tube and 100 μl ethanol was added before drying in an evaporator (N-Vap, Berlin, MA, USA) under a flow of nitrogen at room temperature. The dried sample was then dissolved in 100 μl of solution containing acetonitrile–0.02 *M* KH_2PO_4 (27:73, v/v) adjusted pH to 2 using 85% orthophosphoric acid. A 50-μl aliquot was injected onto the chromatographic system.



Pyronaridine ($C_{29}H_{32}N_5O_2Cl$)



Amodiaquine ($C_{21}H_{22}$ Cl N_3O)

Fig. 1. Structures of pyronaridine and the internal standard, amodiaquine.

2.5. Chromatographic conditions

The mobile phase consists of acetonitrile–0.08 *M* potassium dihydrogen phosphate buffer (13:87, v/v). The pH value of mobile phase was adjusted to 2.8 with 85% orthophosphoric acid. The mobile phase was pumped at a flow-rate of 1.0 ml/min. Samples (50 μl) were applied with an automatic injection system onto the Zorbax Bonus-RP column (5 μm, 250×4.6 mm I.D.) with the UV detector set at a wavelength of 275 nm with aufs 0.002. Chromatography was performed at ambient temperature.

2.6. Calibration curves

Calibration curves for pyronaridine were prepared by spiking drug-free human plasma and blood samples (200 μl) with standard solution of pyronaridine in the range of 5.7 ng to 228 ng and amodiaquine 400 ng. Spiked plasma samples and blood samples were taken through the assay procedure, and calibration graphs were constructed using the peak height as a function of analyte concentration. Unweighted-squares regression analysis was used to determine the slopes, intercepts and correlation coefficient for each calibration graph.

2.7. Recovery, intra- and inter-day precision

The recoveries of pyronaridine and the internal standard, amodiaquine from human plasma and blood were determined by comparing the peak heights obtained from the direct injection of stock standard solutions of compounds with those found by extraction from spiked human plasma and blood samples ($n=6$ for each concentration of pyronaridine used). Intra-day precision and accuracy were evaluated by conducting repeated analysis of spiked human plasma samples and blood samples at three different concentration levels: 57, 570 and 1140 ng/ml ($n=6$ for each concentration of pyronaridine). The inter-day precision was assessed by analyzing spiked human plasma samples at the same three concentrations on different occasions ($n=6$ for each concentration of pyronaridine). The relative standard deviation (RSD) of each concentration was calculated to determine the precision of the method.

2.8. Storage stability

The stability of pyronaridine in human plasma was investigated. Spiked samples were prepared with drug-free plasma at two concentration levels, 110 and 1140 ng/ml. Spiked human plasma samples were stored frozen at −20°C (Model ULT 2540-7-A12 freezer) and −70°C (Model ULT 2586-5-A14 freezer) (Revco Scientific, Asheville, NC, USA). Both concentrations stored at −20 and −70°C were thawed and analyzed on weeks 0, 1, 4, 8 and 12. A standard calibration curve was freshly prepared on the day of the analysis.

3. In vivo rabbit study

In order to prove the suitability of our newly developed pyronaridine HPLC assay method in

blood and plasma specimens for pharmacokinetic studies, we performed a pilot study in the rabbit. A New Zealand White rabbit weighing 4.5 kg was injected intramuscularly with pyronaridine 20 mg/kg dissolved in sterile water (0.5 ml). Plasma and blood samples were withdrawn at time 0 (predose), 1, 2, 4, 8, 12, 24, 48, 72, 96, 144 h to establish the concentration profile over time for pyronaridine. A 4-ml volume of blood was taken at each sampling time. A 2-ml volume of blood was stored for HPLC analysis and the other 2 ml of blood was centrifuged (3000 *g*; 10 min) immediately after collection. The plasma supernatant was stored at −70°C and the blood samples were stored at 4°C until analysis by HPLC. The protocol was approved by the University Animal Care and Use Committee. The maintenance and care of the laboratory animal complied with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA).

4. Results and discussion

Several columns were investigated at the outset of this project to identify a suitable column and mobile phase to optimize chromatography. The Platinum C_{18} column (Alltech; 5 μm, 250×4.6 mm I.D.) had peak splitting under the conditions tested and the Symmetry RP-8 column (Waters; 5 μm, 150×4.6 mm I.D.) gave broad, tailing peaks for pyronaridine under a variety of HPLC conditions. The HP Zorbax-Bonus RP column was found to give sharp, symmetrical peaks and good selectivity using a mobile phase containing 0.08 *M* potassium dihydrogen phosphate buffer–acetonitrile (87:13, v/v) adjusted pH to 2.8 with 85% orthophosphoric acid. As pyronaridine is a basic compound and easily ionized, the mobile phase has to be buffered for rapid equilibration and to avoid broad and asymmetrical peaks due to slow kinetic process. Melander and Horvath [10] suggested that maintaining a high concentration of buffer will speed up the protonic equilibration. Therefore, in order to improve the peak shape and eliminate tailing, a higher concentration of potassium dihydrogen phosphate buffer (0.08 *M*) and a lower pH (2.8) were needed. In Saleh and Loh's [7] study, in addition to the pH and buffer concentration of the mobile phase, triethylamine was also added to avoid surface silanol adsorption in order to improve the peak shape of pyronaridine. However, basic mobile phase modifiers such as triethylamine are not required to obtain a symmetrical peak shape for pyronaridine using the HP Zorbax-Bonus RP column. Typical chromatograms for pyronaridine and amodiaquine in human plasma and blood are shown in Figs. 2 and 3, respectively. The retention time for pyronaridine is 9.3 min and for amodiaquine is 11.8 min.

Saleh and Loh [7] carried out recovery studies on pyronaridine from human plasma buffered at pH values ranging from 1 to 13, with various extraction solvents. They tested diethyl ether, hexane, chloroform, dichloromethane and ethyl acetate and found the highest recovery was obtained at pH 9–11 using diethyl ether. We found optimal recovery by adding 500 μl tribasic phosphate buffer (pH 10.3) to 200 μl plasma or blood samples to adjust the pH in the range of 9 to 11, followed by liquid–liquid extraction with 3 ml diethyl ether. Relatively high recoveries were achieved at all concentrations that were studied with a range of 81–105% for plasma and 73–101% for blood (Table 1). These recoveries are higher than those reported by Wages et al. [9], where recoveries of pyronaridine from whole blood averaged only 60% within the range of concentration studied.

Amodiaquine was found to be a suitable internal standard, owing to its similar structure to pyronaridine, and good recovery from plasma (88.7%) and from blood (85.4%). In addition to excellent recovery, amodiaquine possesses another favorable characteristic for an internal standard by having a longer retention time than pyronaridine. Thus, it is less likely to interfere with the identification of more water-soluble metabolites of pyronaridine in future pharmacokinetic studies.

The detector response was linear over the concentration range from 28.5 to 1140 ng/ml. Linear regression analysis performed for calibration curves yielded correlation coefficients of 0.9994 for pyronaridine from plasma and 0.9997 for pyronaridine from blood. Good linearity with a negligible intercept was found for pyronaridine for plasma ($y=0.0023x-0.0115$) and for blood ($y=0.0027x-0.0283$. The quantification limit of

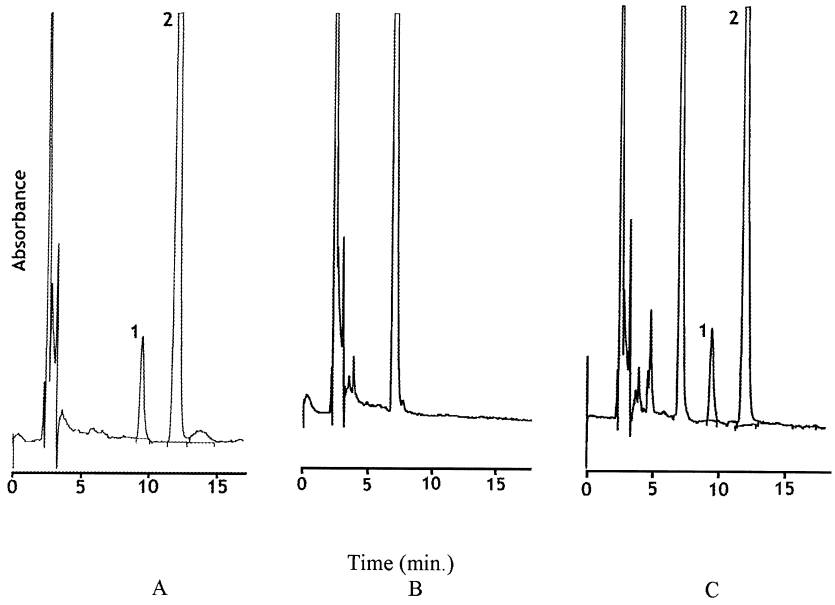


Fig. 2. Typical chromatograms of (A) standard of pyronaridine (1) (5.7 ng on-column) and amodiaquine (2) (200 ng on-column), (B) blank plasma, (C) plasma sample spiked with pyronaridine (1) (5.7 ng on-column) and amodiaquine (2) (200 ng on-column).

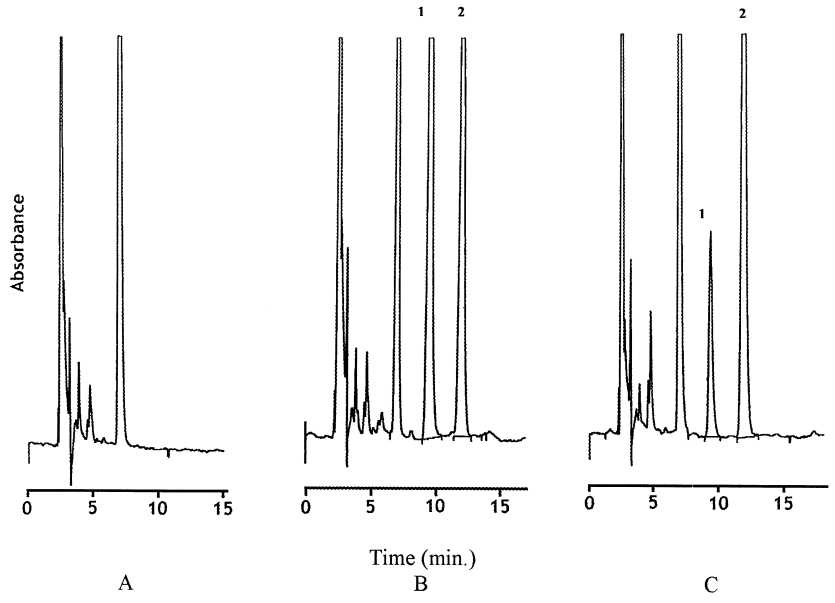


Fig. 3. Typical chromatograms of (A) blank blood, (B) blood sample spiked with pyronaridine (1) (57 ng on-column) and amodiaquine (2) (200 ng on-column), (C) blood sample spiked with pyronaridine (1) (11 ng on-column) and amodiaquine (2) (200 ng on-column).

Table 1
Recovery of pyronaridine from 200 μl of human plasma and blood (n=6)

Pyronaridine concentration added to plasma or blood (ng/ml)	Recovery from plasma (%)	Recovery from blood (%)
57	105.1	101.3
570	99.1	79.1
1140	81.0	73.1

Table 3
Inter-day precision of the HPLC method for the determination of pyronaridine in plasma (n=12)

Concentration added (ng/ml)	Concentration found in plasma (ng/ml) (mean±SD)	RSD (%)
57	66.0±0.6	10
570	581.4±34.2	6.0
1140	1162.8±62.7	5.0

pyronaridine in plasma samples was 3 ng on-column with an RSD of 15% (n=6). Compared to the study of Saleh and Loh [7] using HPLC with UV detection, the lowest amount detected was 70 ng on-column for pyronaridine in plasma, our methodology has a much higher sensitivity than the previous study due to the improvement in the peak shape for pyronaridine.

Validation of our assay method consisted of the intra- and inter-day reproducibility studies at three concentration levels: 57, 570, 1140 ng/ml (n=6). These concentrations were selected based on a previous pharmacokinetic study in malaria patients [3]. The intra-day precision for plasma and whole blood with RSD ranges from 3 to 5.9% for plasma and 2 to 3.9% for blood, respectively (Table 2) indicating a very good reproducibility of this method. As for inter-day precision, a high RSD of 10% was only observed at the low concentration of pyronaridine (57 ng/ml). At higher concentrations, the RSDs were around 5% (Table 3).

A stability study was conducted to determine the best storage temperature for plasma samples. The differences between measured concentration and real concentration for (1140 ng/ml) are shown in Table 4. For blood, Wages et al. studied the storage stability study of pyronaridine refrigerated at 4°C and frozen at −20°C. The refrigerated pyronaridine samples showed good stability up to 4 weeks, but by week 8 the concentration in those samples had decreased by 13–21%. The same trend was seen for frozen whole blood, except that a significant decrease in pyronaridine concentration (16%) was seen by week 4 [9]. Therefore, all blood specimens for the rabbit study were stored refrigerated at 4°C, and all plasma samples were stored at −70°C. The analysis

Table 4
Storage stability of pyronaridine plasma samples in −20°C and −70°C

Times (weeks)	Concentration of pyronaridine 110 ng/ml	Concentration of pyronaridine 1140 ng/ml
(−20°C)		
0	108.3±0.0	1140.0±62.7
1	119.7±5.7	1071.0±34.2
4	102.6±0.0	1003.0±51.3
8	114.0±0.0	991.8±28.5
12	125.4±5.7	1112.0±11.4
(−70°C)		
0	108.3±0.0	1140.0±62.7
1	119.7±5.7	1088.7±45.6
4	108.3±0.0	997.5±22.8
8	114.0±0.0	1077.3±28.5
12	125.4±0.0	1162.8±96.9

Table 2
Intra-day precision of the HPLC method for the determination of pyronaridine in plasma (n=6)

Concentration added (ng/ml)	Concentration measured in plasma (ng/ml) (mean±SD)	RSD (%)	Concentration found in blood (ng/ml) (mean±SD)	RSD (%)
57	60±0.6	5.9	60.0±0.0	3.9
570	604.2±34.2	5.5	541.5±11.4	2.3
1140	1202.7±28.5	3.0	1117.2±17.1	2.0

of rabbit specimens was completed within 2 weeks of collection.

To show the applicability of the plasma and blood methods to pharmacokinetic studies, an in vivo pilot pharmacokinetic study was done with rabbit. The rabbit was given an intramuscular (i.m.) dose of pyronaridine tetraphosphate (20 mg/kg as base) and plasma and blood samples were drawn over a 7-day period. The results of the pyronaridine concentration profile are shown in Table 5. Blood pyronaridine concentrations are substantially higher than plasma concentrations. In blood, the C_{max} occurs within 1 h of i.m. administration. Pyronaridine was eliminated from blood with a half-life of 49 h. The results obtained in this pilot study are consistent with a previously published pharmacokinetic study in rabbits using a non-specific spectrofluorometric method [11]. We found evidence of concentration of pyronaridine in red blood cells. The blood:plasma ratio was approximately 10 (range 4.9–17.8). The variability in the blood:plasma ratio may explain the erratic plasma levels determined by Jayaraman et al. in a clinical pharmacological study. With high concentrations of pyronaridine in blood cells, a modest shift in the equilibrium between blood and plasma or hemolysis, could markedly alter plasma drug concentrations [8]. These results imply that blood is the preferred biological matrix for clinical pharmacokinetic studies of pyronaridine.

5. Conclusions

A sensitive and selective HPLC method using UV detection is described for the determination of pyronaridine concentrations in human blood and plasma. The extraction procedure is simple and the chromatographic is relative short allowing sufficient sample throughput to be applied to clinical pharmacokinetic studies. We believe the described method satisfies the need for an accurate, sensitive, specific and convenient assay method in plasma and blood for clinical pharmacokinetic studies of pyronaridine. The results of a pilot study in rabbits demonstrates pyronaridine concentrates in blood cells and thus blood is the preferred matrix for application to clinical pharmacokinetic studies to evaluate the kinetic properties of this promising antimalarial drug.

References

[1] A.M. Breckenridge, P.A. Winstanley, Ann. Trop. Med. Parasitol. 91 (1997) 727.
[2] X.Y. Zheng, X.Y. Gao, H.Z. Gua, C. Chem, Yao Hsueh Pao 14 (1979) 736.
[3] Z. Feng, Z.F. Wu, C.Y. Wang, N.X. Jiang, Acta Pharmacol. Sin. 8 (1987) 543.
[4] S. Looareesuwan, D.E. Kyle, C. Viravan, S. Vanijanonta, Am. J. Trop. Med. Hyg. 54 (2) (1996) 205.

Table 5
Concentration of pyronaridine in whole blood and plasma of rabbit dosed intramuscularly

Time after single i.m. dose (h)	Concentration in blood (ng/ml)	Concentration in plasma (ng/ml)	Blood:plasma ratio
1	3747.0	411.8	9.1
2	3578.0	342.8	10.4
4	2706.0	553.3	4.9
8	1110.0	62.5	17.8
12	806.9	46.7	17.3
24	438.6	41.2	10.6
48	223.0	–[b]	
72	73.7	–[b]	
96	90.6	–[b]	
120	–[a]	–[b]	
144	60.3	–[b]	

[a] Insufficient volume obtained for blood and plasma sample.
[b] Below limit of quantification.

[5] P. Ringwald, J. Bickii, L. Basco, Lancet 347 (1996) 24.
[6] Z. Feng, C.Y. Wang, Acta Pharmacol. Sin. 7 (4) (1986) 354.
[7] M.I. Saleh, H.K. Loh, Anal. Chim. Acta 282 (1993) 559.
[8] S.D. Jayaraman, S. Ismail, N.K. Nair, V. Navaratnam, J. Chromatogr. B 690 (1997) 253.
[9] S.A. Wages, L.C. Patchen, F.C. Churchill, J. Chromatogr. B 527 (1990) 115.
[10] W.R. Melander, C. Horvath, in: C. Horvath (Ed.), High Performance Liquid Chromatography, Advances and Perspectives, Vol. 2, Academic Press, New York, 1980, p. 114.
[11] Z. Feng, N.X. Jiang, C.Y. Wang, W. Zhang, Acta Pharm. Sin. 21 (11) (1986) 801.