Journal of Chromatographic Science 2012;50:343–348
doi:10.1093/chromsci/bms007

Article

Method Development and Validation for the Determination of Indiquinoline Tartrate, a Novel Kappa Opioid Agonist, and its Related Substances by High-Performance Liquid Chromatography

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Received 2 February 2011; revised 16 March 2011

A stability-indicating reversed-phase high-performance liquid chromatography method has been developed and validated for the assay of indiquinoline tartrate and its related substances. The method was established by forced degradation experiments and system suitability experiments. The chromatographic separation was achieved with a Hedera ODS-3 column (5 μm, 250 mm × 4.6 mm) and the mobile phase was constituted (flow rate 1.0 mL/min) of eluant A, aqueous acetate buffer and eluant B, CH_3OH using a gradient elution. A photodiode array detector set at 254 nm was used for detection. The investigated validation elements showed that the method has acceptable specificity, accuracy, linearity, precision, robustness and high sensitivity with limit of detection and limit of quantitation. The method can be used for routine quality control analysis and stability testing of indiquinoline tartrate drug substance.

Introduction

Indiquinoline tartrate, 5-chloro-3-(1-pyrrolidin-1-ylmethyl-3, 4-dihydro-1H-isoquinoline-2-carbonyl)-indan-1-one tartrate, is a novel tetrahydroisoquinoline derivative that has high affinity and selectivity to kappa opioid receptor.

Opioid receptors are G-protein-coupled and have been pharmacologically classified into three distinct types, designated as μ, κ and δ, which are the targets responsible for pharmacological effects exhibited by opioid-type drugs (1). All three appear to be present in the central and peripheral nervous system of many organisms, including humans (2–4). As a μ-opioid receptor agonist, morphine, an alkaloid extracted from the opium poppy, *Papaver somniferum*, has been widely used in the management of pain, diarrhea and dysentery for thousands of years and is still considered to be the best analgesic available. Unfortunately, by producing euphoria, it is also one of the most frequently abused drugs (5). The use of κ selective agonists involves low abuse and milder form of dependence than the prototypic μ-opioid ligands (6).

Important aspects of HPLC method validation have been reported in many publications (7–10), and validation of analytical procedures has been discussed in the International Conference on Harmonization (ICH). As a completely synthetic compound, there are no high-performance liquid chromatography (HPLC) methods reported in literature for indiquinoline tartrate analysis. Therefore, the primary target of this work was to develop a stability-indicating HPLC method that is selective for the quantification of all possible degradation products and process impurities. The proposed method was validated as per ICH guidelines (11). The structures and names of indiquinoline tartrate and the related substances examined in this study are shown in Figure 1.

Experimental

Reagents and chemicals

Indiquinoline tartrate bulk drug was obtained from Yangze River Pharmaceutical Group (Taizhou, China), and the related substances were supplied by Department of Pharmaceutical Chemistry (China Pharmaceutical University, Nanjing). Methanol of analytical grade was obtained from Nanjing Chemical Reagent Co. Ltd. (Jiangsu, China). And all other reagents were of analytical grade.

Instrumentation and software

An Agilent 1100 series HPLC with a variable wavelength detector was used for the development and validation of the proposed method. The output signal was monitored and processed using Chemstation software (Agilent Technologies, Waldbronn, Germany).

Chromatographic conditions

The chromatographic column used in the present study was a Hedera ODS-3, 5 μm, 250 mm × 4.6 mm (Hedera Inc., China). Aqueous acetate buffer was prepared by dissolving 4.0 g ammonium acetate in 1000 mL of water with its pH adjusted to 4.0 with acetic acid, filtered through a 0.45 μm membrane filter (Millipore PVDF) and degassed in an ultrasonic bath prior to use as mobile phase A. Methanol was used as mobile phase B. Unless stated otherwise, all separations were performed at column temperature 30°C using a 1.0 mL/min flow rate and a 20 μL injection volume. The analysis was carried out under gradient conditions as follows: time (min)/A (*v*/*v*):B (*v*/*v*); $T_{0.01}$/55:45, $T_{30.0}$/15:85, $T_{31.0}$/55:45 and $T_{40.0}$/55:45. The data were acquired at 254 nm for 30 min.

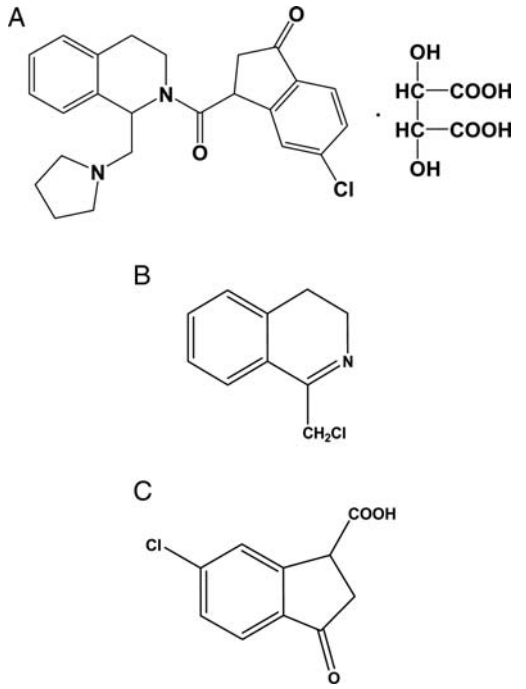

Figure 1. Structures and names of indiquinoline tartrate and the related substances: (A) 5-chloro-3-(1-(pyrrolidin-1-ylmethyl)-1,2,3,4-tetrahydroisoquinoline −2-carbonyl)-2,3-dihydroinden-1-one tartrate; Indiquinoline tartrate (molecular weight: 559.01); (B) 1-(chloromethyl)-3,4-dihydroisoquinoline; RS-2a (molecular weight: 179.65); (C) 6-chloro-3-oxo-2,3-dihydro-1H-indene-1-carboxylic acid; RS-8c (molecular weight:210.61).

Preparation of stock solution and analysis solutions

A mixture of eluant A and eluant B in the ratio of 55:45 (v/v) is used as diluent in the preparation of analysis solutions. A stock solution (1000 μg/mL) was prepared by transferring the appropriate amount of indiquinoline tartrate bulk drug into a 100 mL volumetric flask containing approximately 25 mL diluents, and the solution was sonicated for approximately 10 min or until the solid was completely dissolved. Then the volumetric flask was filled to mark with diluent. The indiquinoline tartrate stock solution was then serially diluted with diluent to provide working solutions of desired concentrations. A stock solution of impurities (RS-2a, RS-8c and enantiomer) at 100 μg/mL was also prepared in diluents. Unless stated otherwise, all the solutions were stored at 4°C.

Stress studies/specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Forced degradation of indiquinoline tartrate drug substance was carried out under acid/base hydrolytic and oxidative stress conditions. Solutions were prepared by dissolving drug substance in purified water and then treating with aqueous 1M hydrochloric acid, aqueous 1M sodium hydroxide and aqueous 10% hydrogen peroxide at 90°C for 60, 60 and 20 min, respectively. After the degradation, these solutions were diluted with diluent, filtered through a 0.45 μm membrane filter and analyzed in the proposed method.

Method Validation

Precision

The precision of the method was assessed as repeatability and intermediate precision. The repeatability was investigated by injecting six individual preparations of indiquinoline tartrate at the target concentration (100 μg/mL). The percent relative standard deviation (%RSD) of six obtained area values was calculated. The intermediate precision of the method was evaluated by a different analyst and instrument located within the same laboratory in different days. All the samples solutions were freshly prepared and analyzed daily.

Limit of detection and limit of quantificationThe limit of detection (LOD) and limit of quantification (LOQ) for indiquinoline tartrate, the enantiomer, RS-2a and RS-8c were estimated at signal-to-noise ratios of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

Linearity

Linearity test solutions for the method were prepared from a stock solution at seven concentration levels from 1 to 200% of the assay analyte concentration (1, 10, 25, 50, 100, 150 and 200 μg/mL). Each solution was injected in triplicate, and the mean peak area versus concentration data was analyzed with least squares linear regression.

Linearity test solutions of the enantiomer, RS-2a and RS-8c were prepared by diluting the impurity stock solutions (2.4) to the required concentrations. The test solutions of RS-2a and RS-8c were prepared at five concentration levels from 0.1 to 5.0% of the target concentration for indiquinoline tartrate (0.1, 0.5, 1.0, 2.5 and 5.0 μg/mL). For the enantiomer, linearity was checked at five different concentration levels ranging from 0.1 to 20 μg/mL. The slope and y-intercept of the calibration curve were calculated.

Accuracy

The accuracy of the method was evaluated in triplicate at three concentration levels (50, 100 and 150 μg/mL), and the percentage recoveries were calculated.

Recovery experiments were conducted to determine the accuracy of the proposed method for the quantification of all four impurities (the enantiomer, RS-2a and RS-8c) in the drug substance. The study was carried out in triplicate at 5.0, 10 and 15% of the target concentration (100 μg/mL) for the enantiomer and at 0.5, 1 and 1.5% of the target concentration (100 μg/mL) for RS-2a and RS-8c. The percentage of recoveries for the enantiomer, RS-2a and RS-8c were calculated.

Robustness

To evaluate the robustness of the proposed method, the influence of small and premeditated alterations of analytical parameters on the quantification of the related substances and selectivity was studied. The flow rate of the mobile phase was 1.0 mL/min. To study the effect of the flow rate on the resolution, the flow rate was changed by 0.2 units (0.8 and 1.2 mL/min). The effect of pH on the resolution of the impurities was studied by varying the pH by ±0.2 units in eluant A (pH 3.8 and 4.2). The

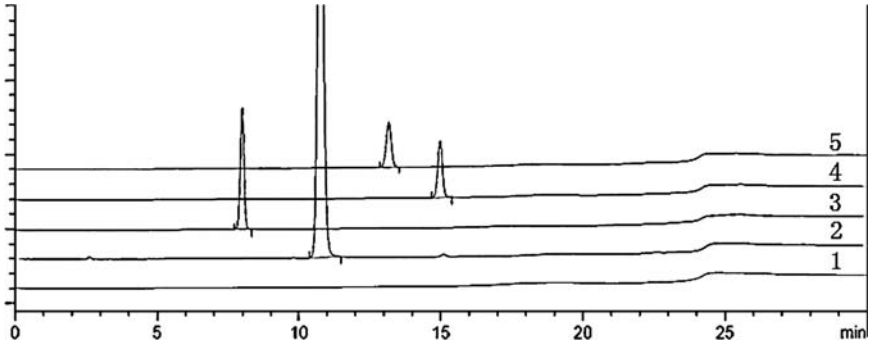

Figure 2. HPLC chromatograms of m.p. (1), indiquinoline tartrate (2), RS-8c (3), enantiomer (4) and RS-2a (5).

effect of the column temperature on the resolution was studied at 25 and 35°C instead of 30°C. In these varied conditions, the components of the mobile phase remained constant, as outlined in the "Chromatographic conditions" section.

Stability of sample solutions

The solution stability of indiquinoline tartrate in the assay method was carried out by leaving the sample solutions in tightly capped volumetric flasks at room temperature, 4°C and 25°C for 48 h. The same sample solutions were assayed at 8 h intervals over the study period. The prepared mobile phase remained constant during the study period. The %RSD of the indiquinoline tartrate assay was calculated.

Results and Discussion

Method development and optimization

The primary objective of the chromatographic method was to appropriately make the retention time of indiquinoline and separate the enantiomer, RS-2a and RS-8c from the analyte peak. Different stationary phases and various mobile phases with buffers, such as phosphate and acetate with different pH values (3–6), and organic modifiers, in the mobile phase, including acetonitrile and methanol were optimized.

A potassium dihydrogen orthophosphate buffer with a pH value of 6.0 and methanol (50:50, *v*/*v*) at a flow rate of 1.0 mL/min was chosen for the initial trial with a 250 × 4.6 mm ID column and 5 µm particle size C18 stationary phase. Considering the high concentration of the phosphate and its damage to the column, volatile mobile phases were investigated and satisfactory results were obtained when ammonium acetate buffer was used with a pH value of 6.0 and methanol (50:50, *v*/*v*) at a flow rate of 1.0 mL/min.

Different stationary phases were compared to obtain suitable retention time of indiquinoline and its resolution with the enantiomer. The retention time was too short when C8 was used. The Agilent C18 column (5 µm, 250 × 4.6 mm) and Hedera ODS-2 column (5 µm, 250 × 4.6 mm) both showed weaker absorbability than the Hedera ODS-3 column (5 µm, 250 × 4.6 mm). Additionally, the sensitivity of indiquinoline was lower when the Phenomenex C18 column (5 µm, 250 × 4.6 mm) was used. Therefore, the Hedera ODS-3 column (5 µm, 250 × 4.6 mm) was selected for further method development.

Table I
System Suitability Results

Compound	Resolution(Rs)	Symmetry factor	No. of theoretical plates
RS-8c	–	1.08	17327
Indiquinoline	8.60	0.82	13831
RS-2a	7.12	1.09	24272
Enantiomer	5.78	1.01	41604

To improve the resolution between the impurities and analyte, methanol was replaced with acetonitrile in the mobile phase and injected the impurity-spiked solution. The resolution between the impurities and analyte was slightly decreased because acetonitrile has stronger eluting power, which makes the retention time shorter.

The effect of the buffer pH was also studied under the previously mentioned conditions. At pH 6.0, the resolution between indiquinoline and the enantiomer was poor and the number of theoretical plates of indiquinoline was fewer. At pH 2.5, the retention time was shorter and the resolution between indiquinoline and the enantiomer was poorer than than at pH 4.0. In contrast, a pH value of 4.0 was chosen, at which a suitable retention time of indiquinoline and good resolution between the impurities and analyte were obtained.

The retention time of the enantiomer was 19.7 min under the previously discussed conditions. To optimize the total run time, a gradient method was selected using ammonium acetate buffer with a pH value of 4.0 as mobile phase A and methanol as mobile phase B, under gradient conditions as follows: time (min)/A (*v*/*v*):B (*v*/*v*); T 0.01 /55:45, T 30.0 /15:85, T 31.0 /55:45 and T 40.0 /55:45.

Using the optimized conditions, stress studies and method validation were carried out as described in the following. The system suitability results are given in Figure 2 and Table I and the developed LC method was determined to be specific for indiquinoline tartrate, the enantiomer, RS-2a and RS-8c.

Stress studies/specificity

HPLC chromatographs of acid, base and oxidative degradation are shown in Figure 4. Significant degradation of the drug substance was detected. Peak purity test results (Figure 1) derived from the photodiode array detector confirmed that the indiquinoline tartrate peak and the degraded peaks were homogeneous and pure in all of the analyzed stress samples.

Using the proposed method, indiquinoline tartrate, the enantiomer, RS-2a and RS-8c were well separated, with a resolution of greater than 5 and typical retention times for RS-8c, indiquinoline tartrate, RS-2a and the enantiomer of approximately 8.0, 10.7, 13.1 and 15.0 min, respectively. The system suitability results are given in Table I and the proposed method was determined to be specific for indiquinoline tartrate and the three related substances, RS-2a, RS-8c and the enantiomer.

Method Validation

Precision

The %RSD of indiquinoline tartrate during the proposed method precision study was not more than 0.1% and the %RSD of the results obtained in the intermediate precision study was not more than 1.0% (Table II).

Table II
Regression and Precise Data

Parameter	Indiquinoline	Enantiomer	RS-2a	RS-8c
LOD(μg/ml)	0.02	0.02	0.02	0.01
LOD(μg/ml)	0.1	0.05	0.05	0.02
Regression equation				
Slope(m)	46.8414	44.5182	36.6545	74.2878
Intercept(C)	18.8001	−3.6455	−0.5196	−1.1085
Correlation coefficient	0.9998	0.9998	0.9999	0.9998
Repeatability(%RSD)[a]	0.07	/	/	/
Intermediate Precision(%RSD)[a]	0.75	/	/	/
Accuracy at 100% for drug substance	99.83 ± 0.48	99.45 ± 0.39	101.52 ± 1.63	99.45 ± 1.40

Linearity range was ranging 0.1% to 5%, 0.1% to 5% and 0.1% to 20% with respect to 100 μg/ml indiquinoline tartrate for RS-2a, RS-8c and the enantiomer, respectively; Linearity range was 1% to 200% with respect to 100 μg/ml indiquinoline tartrate for the drug substance analysis.
[a] Six determinations using 100 μg/ml for indiquinoline tartrate.

LOD and LOQ

The limits of detection and quantification of indiquinoline tartrate, RS-2a, RS-8c and the enantiomer for a 20 μL injection volume are given in Table II.

Linearity

The linear calibration plot of indiquinoline tartrate for the proposed method was obtained over the tested calibration range (1–200 μg/mL) and the obtained correlation coefficient was greater than 0.999. The results revealed an excellent correlation between the peak area and analyte concentration. The slope and *y*-intercept of the calibration curve were 46.8414 and 18.8001, respectively.

The linear calibration plots were determined over the calibration ranges (0.1 to 5%, 0.1 to 5% and 0.1 to 20%) for RS-2a, RS-8c and the enantiomer, and a correlation coefficient of greater than 0.999 was obtained. These results showed an excellent correlation between the peak areas and concentrations of the enantiomer, RS-2a and RS-8c (Table II, data not shown).

Accuracy

The percentage recovery of indiquinoline tartrate in the drug substance ranged from 99.07 to 100.57%. The percentage recoveries of the enantiomer, RS-2a and RS-8c in the drug substance ranged from 98.93 to 100.01%, 98.92 to 103.94% and 97.08 to 101.08%, respectively. The HPLC chromatograms of spiked samples at the 10% level of all three related substances in the indiquinoline tartrate drug substance sample are shown in Figure 3.

Robustness

In two of the deliberately varied chromatographic conditions carried out as described in the "Robustness" section (flow rate

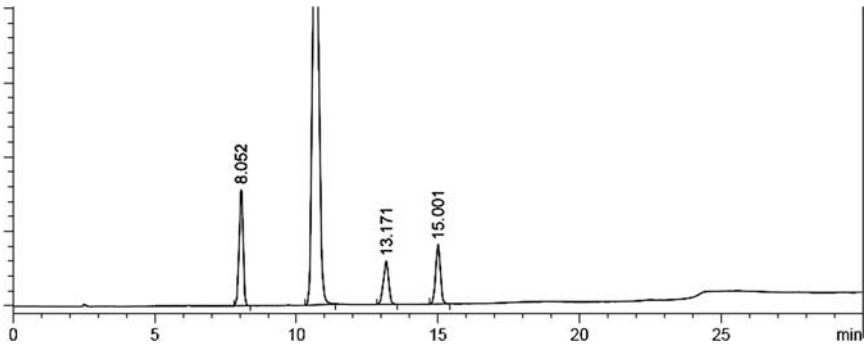

Figure 3. HPLC chromatogram of system suitability.

Table III
Solution Stability Results of Indiquinoline Tartrate for 48h

	0h	8h	16h	24h	32h	40h	48h	Average	%RSD
4°C	4662.43	4647.67	4639.33	4631.20	4658.41	4640.21	4647.08	4646.62	0.24
25°C	4606.98	4630.23	4638.60	4630.05	4624.69	4623.52	4619.98	4624.86	0.21

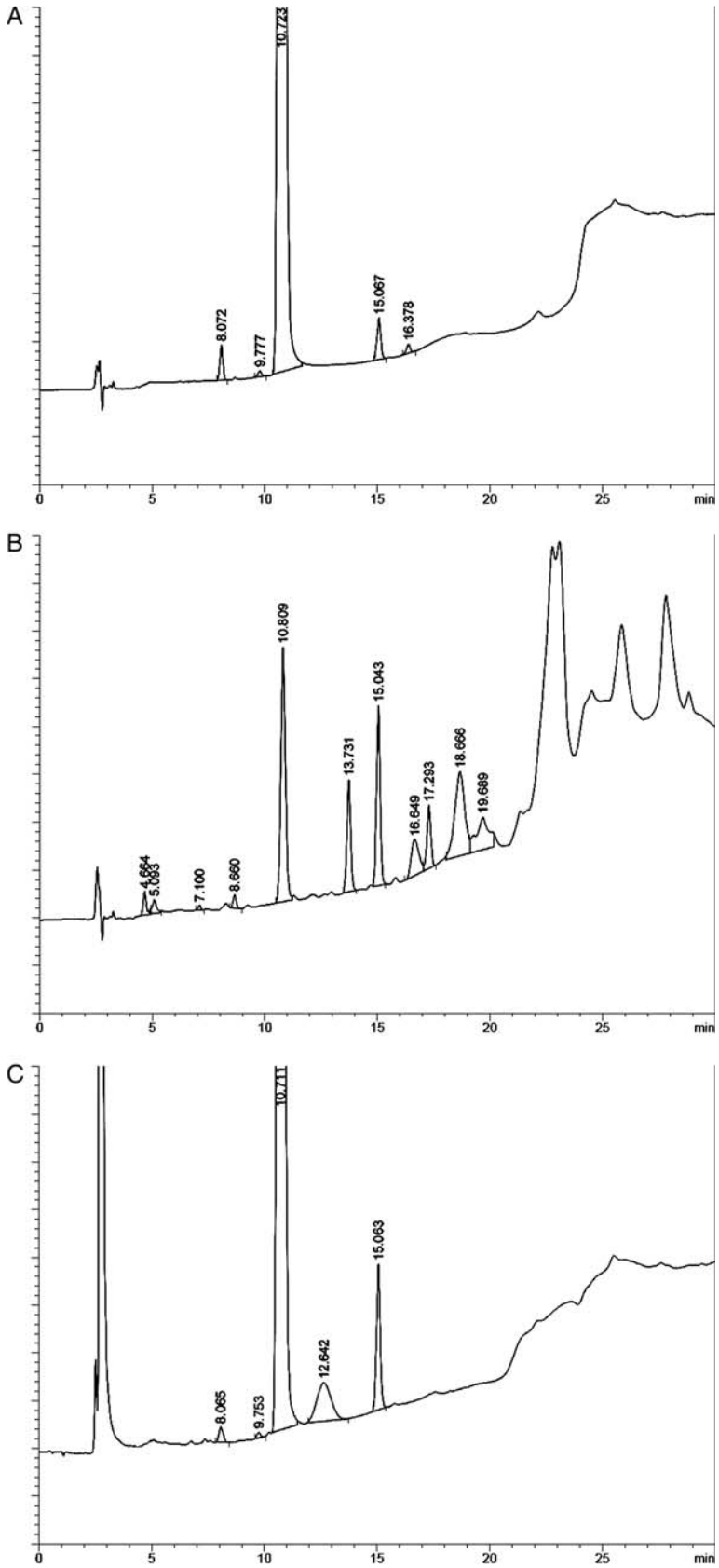

Figure 4. HPLC chromatograms of (A) acid degradation; (B) base degradation; (C) oxidative degradation.

and column temperature), the resolution between the closely eluting impurities, namely 3.61 and 4.77, was greater than 1.5. However, ± 0.2 variation of pH of eluant A has an obvious effect on the retention time of RS-2a, inducing poor resolution with indiquinoline tartrate at pH 3.8 and with the enantiomer at pH 4.2. Thus, the PH of eluant is an important factor for the robustness of the method.

Stability of standard solutions

The %RSD of assaying indiquinoline tartrate during the solution stability experiment was not more than 0.5% (Table III). No significant changes were observed in the content of the enantiomer, RS-2a and RS-8c during the solution stability experiments when performed using the proposed method. The results of the solution stability experiments confirm that the sample solutions used during the assays and related substance determinations were stable up to 48 h.

Conclusion

In the development of this method, the behavior of indiquinoline tartrate under various stress conditions was studied. All of the degradation products and process impurities were well separated from the drug substance, which demonstrates the stability-indicating power of the method. The investigated validation of the elements, namely, accuracy, linearity, precision, robustness and high sensitivity with LOD and LOQ proved the validated HPLC method to be simple, precise, rapid and reliable. The method can be used for routine quality control analysis and stability testing of indiquinoline tartrate drug substance.

Acknowledgment

The authors wish to acknowledge the support received from Prof. Yungen Xu, Department of Pharmaceutical Chemistry, China Pharmaceutical University, Nanjing, China. They also thank the colleagues of Yangze River Pharmaceutical Group for their cooperation in carrying out this work. This work was supported by three grants from the Ministry of Science and Technology of the People's Republic of China (2008BAI55B03, 2009ZX09310−004 and 2009ZX09103−096)

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