

Full Length Research Paper

Simultaneous determination of 6 active components in traditional Chinese medicine “KANGXIN” tablets by RP-HPLC–DAD

Gang Liu¹, Hui Wang^{1*}, Yi Luo¹, Benhong Zhou¹ and Xianming Hu²

¹Department of Pharmacy, Renmin Hospital, Wuhan University, Wuhan 430060, PR China.

²College of Pharmacy, Wuhan University, Wuhan 430072, PR China.

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A HPLC method was developed for simultaneous quantitative determination of the 6 compounds namely aloin, puerarin, genistein, daidzin, genistin and daidzein in “KANGXIN” tablets. The separation was achieved on a C₁₈ column, a diode array detector at 260 nm and a mobile phase composed of acetonitrile and water under the gradient elution. Good linear relationships were obtained. The average recoveries were in the range of 95.83 – 102.57%. The validated method can be utilized as a suitable method for the quality control of “KANGXIN” tablets.

Key words: Column liquid chromatography, active components, “KANGXIN” tablet, quality control.

INTRODUCTION

“KANGXIN” tablet (KXT) studied in the paper was produced by Wuhan Jianmin Pharmacy of Traditional Chinese Medicine Co., Ltd of China (country medicine accurate character G20050145), which was a traditional medicine, including *Radix Puerariae*, the extraction of *soybean*, *Aloe* and *Fructus Lycii*. Meanwhile, *R. Puerariae*, *Aloe* and the extraction of *soybean* were the main constituents in the drug.

The main clinic application of the KXT was used for the treatment of replenishing vital essence to tonify kidney, regulating vital energy and nourishing blood, regulating endocrine and estrin lever. It was an effective adjunctive therapy for Menoxenia, osteoporosis and angiocardopathy. One or a few active constituents in a traditional Chinese medicine (TCM) can not reflect its overall efficacy. The combined action of multiple constituents is considered to be crucial for the therapeutic effect of a TCM (Xue and Roy, 2003). Concerned with the function and application of this medicine, and conformed to the TCM theory, the amount of some active ingredients should be quantized for the quality control of the production procedure and clinic application due to their effects

on the symptom. According to the literature, the compounds in KXT such as puerarin, daidzein, daidzin, genistin, genistein and aloin were reported to possess arrays of biological activities (Ho et al., 2002; Beppu et al., 2003; Wamer et al., 2003; Jun et al., 2005; Chen et al., 2006; Sepehr et al., 2007; Ward and Fonseca, 2007).

Until now TLC, HPLC and GC methods have been used in drug quality control. Because aloin and the five isoflavones were the active ingredients related to the function of KXT, the monarch herb or the minister herb should be given a content limit in quality control according to the regulations of the State Food and Drug Administration of China. Even though there were many reports on content determination of some active substances mentioned above (Wang et al., 2004; Klejdus et al., 2005; Chang et al., 2008), aloin and the five isoflavones determined in one time have not been studied to the present. Furthermore, because it was difficult to separate the five isoflavones due to their similar chemical structures (Figure 1), the study was very useful in detecting more active compounds at the same time by HPLC. While simultaneous determination of active ingredients was also done in the quality control study, which could give a comprehensive control of the medicine.

In the current work, we successfully developed a

*Corresponding author. E-mail: liugang.wh@gmail.com.

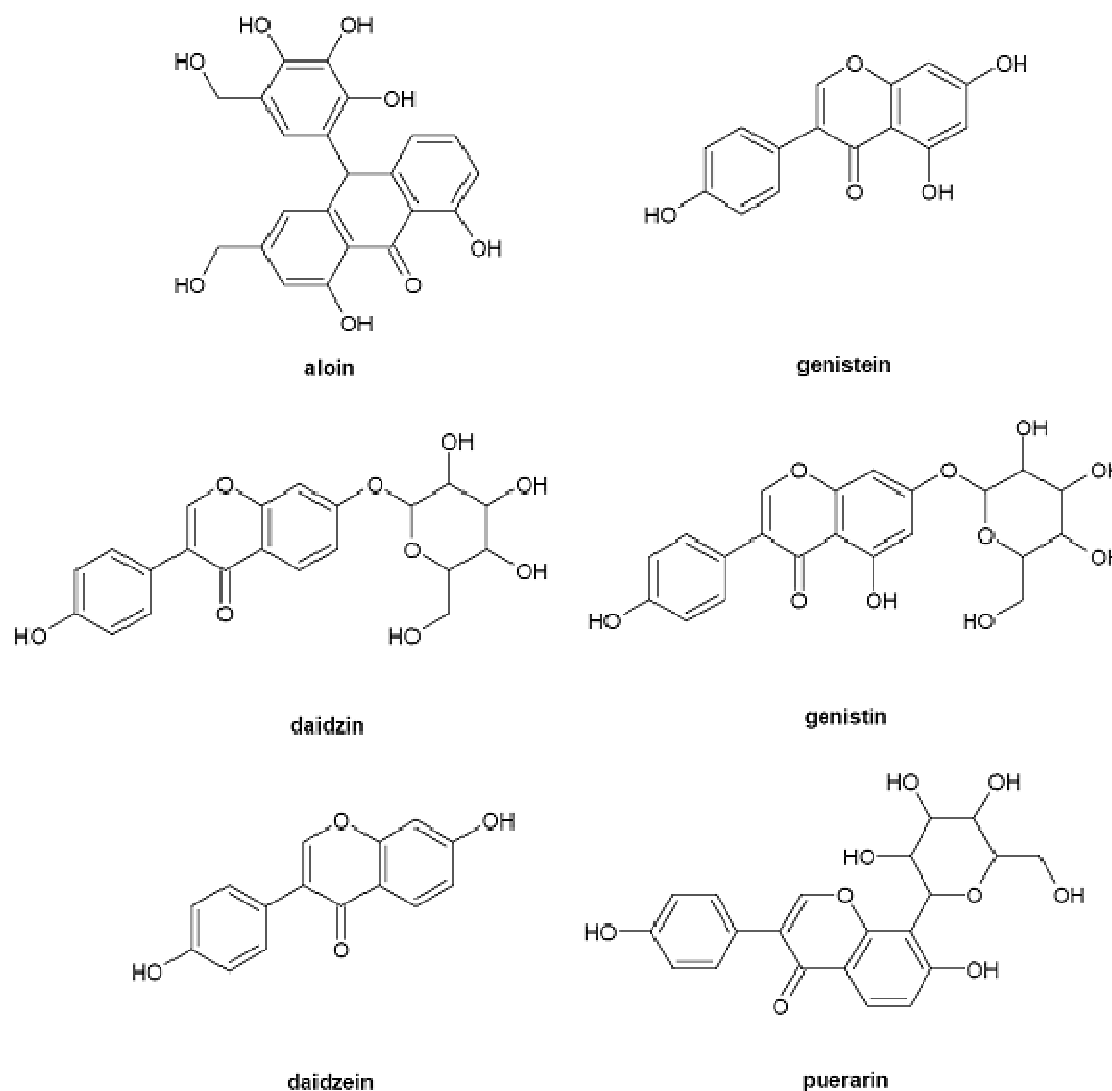


Figure 1. The structures of 6 constituents in KXT.

modified HPLC analytical method for simultaneous quantitative determination of aloin and the five isoflavones in KXT. The method was simple so as to reduce the duration of the analysis and suitable for the routine determination of aloin and five isoflavones in the drug. Furthermore, we investigated the sample preparation methods, peak confirmation, gradient elution program regarding the complexity of KXT.

MATERIALS AND METHODS

Chemicals and reagents

KXT were supplied by Wuhan Jianmin Pharmaceutical Co., Ltd. (Hubei, China). Standard substances of puerarin, daidzein, genistin, genistein and aloin were all provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and daidzin was provided from Sigma (St. Louis, USA). Methanol

and acetonitrile (HPLC-grade) were purchased from Burdick and Jackson (MI, USA). Other reagents were all of analytical grade. HPLC-grade water was produced by Milli-Q system (Milford, MA, USA).

Instrumentation and analytical conditions

The HPLC system (Agilent Technologies, CA, USA) was equipped with the ChemStation software (Agilent Technologies) and composed of a quaternary pump, an online vacuum degasser, an autosampler, a thermostated compartment and a diode array detector (DAD). All separations were carried out on a Kromasil C₁₈ column (250 × 4.6 mm, 5.0 μm) from Hanbang Science and Technology (Jiangsu, China). The mobile phase was composed of double-distilled water and acetonitrile, and the elution was performed under a gradient, acetonitrile remaining 16% within 0 - 5 min, 16 - 35% within 5 - 30 min, 35 - 16% within 30 - 33 min and 16% at the last 7 min. The flow-rate was 1.0 mL·min⁻¹, the column temperature maintained at 25°C, the effluent was monitored at 260

nm, and injection volume was 10 μ l. The peak identification was based on the retention time and the DAD spectrum against the standard presented in the chromatogram.

Preparation of standard solutions

Standard stock solutions were prepared by directly dissolving the standard substances of the six compounds in methanol, respectively. Working standard solutions containing the 6 compounds were prepared and diluted with methanol to appropriate concentrations for the establishment of calibration curves. The standard stock solutions and working solutions were all put in dark brown calibrated flasks and stored at 4 °C (Klejduš et al., 2005).

Preparation of sample solution

The powder of KXT (0.5 g) was extracted with 50 ml methanol for 30 min in an ultrasonic bath, and then the extraction was performed twice using the fresh methanol. The extracted solution was collected together and filtered. The filtrate was concentrated and dried to produce residue which was then dissolved in methanol by ultrasonication and transferred to a 10 ml volumetric flask. This solution was diluted to a proper consistency and then filtered through a syringe filter (0.45 μ m) before the injection for analysis.

Method validation

The method validation was according to Qian et al. (2009) and Klejduš et al. (2005). The calibration curves were constructed by plotting the peak area versus the concentration of each analyte. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10 respectively. Intra- and inter-day variations were chosen to determine the precision of the developed assay and expressed by relative standard deviation (RSD). The recovery was used to evaluate the accuracy of the method. Three replicates were performed for the test and the recovery was calculated according to the following formula: $\text{recovery (\%)} = (\text{total amount after spiking original amount in sample}) / \text{spiked amount} \times 100\%$ (Zhang et al., 2009). To confirm the stability, the same real sample was analyzed within 24 h and at room temperature. The RSD value was calculated as a measurement of stability and method repeatability.

Application

The developed method was applied to analyse 10 battery pharmaceutical drugs. The samples of the products were extracted and analysed as previously described.

RESULTS

Chromatographic separation

The typical chromatographic profiles of the blank, standard solution and the real sample solution are shown in Figure 2. Under the present chromatographic condition, the six compounds were well separated in chromatogram. The retention times of puerarin, daidzein, genistein, aloin, daidzin and genistin were 6.817, 11.902, 15.555, 18.061, 21.103 and 25.067 min respectively, and all the peaks were sharp and of obvious baseline separation.

The overall time of analyses (approximately 30 min) was more acceptable for routine analyses than Klejduš et al. (2005).

Linearity, range and limits of detection

In order to obtain the calibration curves, chromatographic peak areas were plotted against the corresponding concentrations of the standard solutions. The regression equations were calculated by six concentration levels on the consecutive 5 days. The details of the regression curves and limits of detection were presented in Table 1, and the results showed that the linearity of each compound was good ($r > 0.999$).

Precision and stability

The instrument precision was examined by the performance of the intra-day and inter-day assays by six replicated injections of the mixture standard solutions at medium concentration. The intra-day precision was performed with the interval of 4 h in the same day, and the inter-day precision was performed over 3 days. The precision results showed that the RSD values of peak area ranged from 0.86 to 1.87% both for the intra-day and inter-day precision. The same real sample was analyzed for the stability within 24 h and at the room temperature. The results demonstrated that the analytes were stable in the conditions, and RSD values of peak area of puerarin, daidzein, genistein, aloin, daidzin and genistin were 1.82, 1.41, 1.35, 1.93, 1.74 and 1.98%, respectively.

Recovery test

In order to evaluate the accuracy of the proposed methods, a recovery test was performed by determining the amount of reference substance quantitatively added to the sample of KXT before extraction. Known concentrations of accurately determined amounts of the 6 standard substances were used to spike the KXT crude mixture, and then extracted and analyzed as described in the above paragraph. The quantity of each compound was subsequently calculated according to the corresponding calibration curves. The recoveries of the six bioactive compounds were within the range of 95.83 – 102.57% with an RSD of between 1.12 and 1.94% ($n = 3$). These values indicated that the method was reliable.

Application

To simultaneously determine the six active components in Chinese medicine KXT of different batches, the HPLC method was developed, and the specialization of the method was confirmed by comparing the retention times and on-line UV spectra with those of standards. The

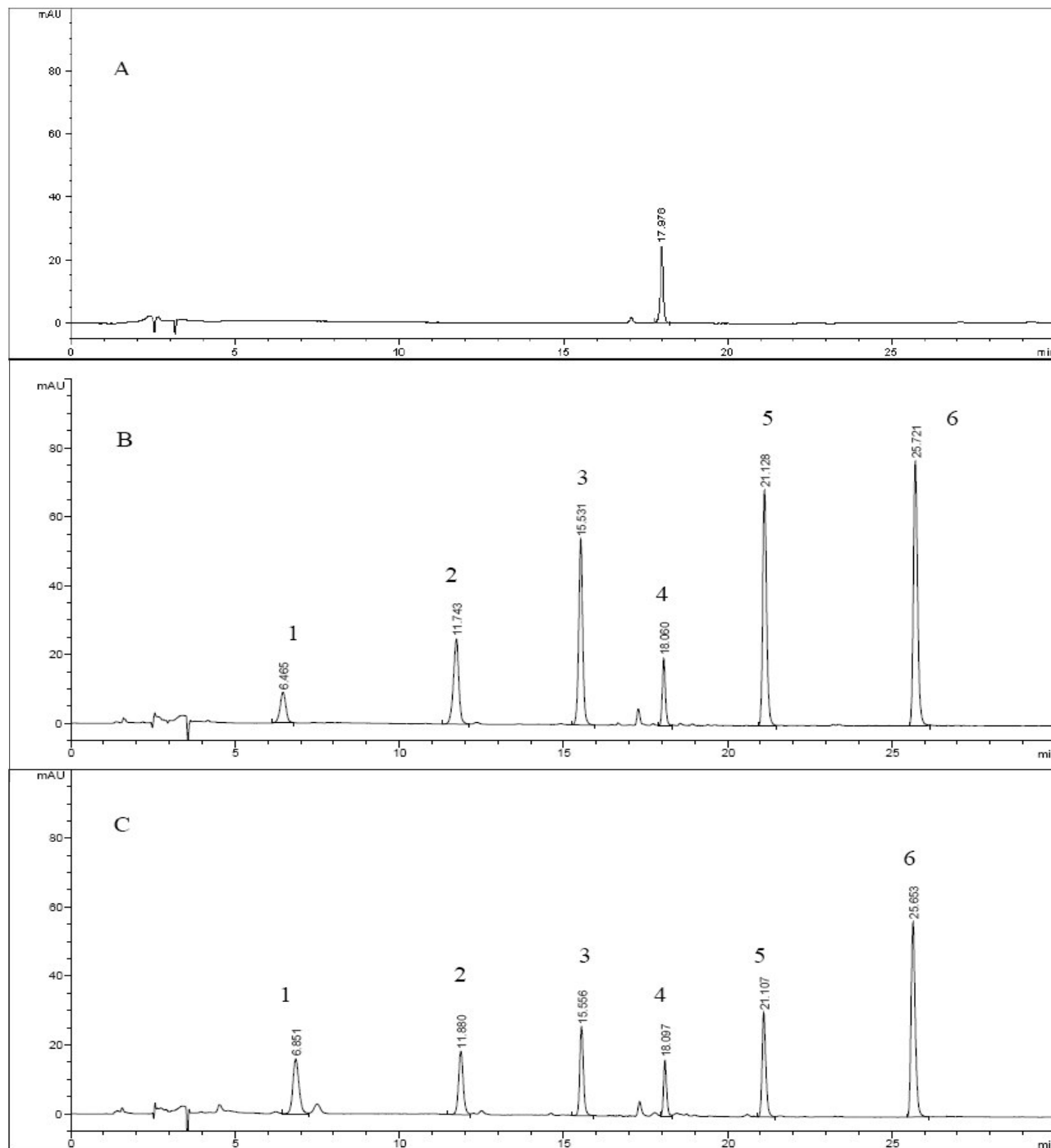


Figure 2. The superimposed typical HPLC chromatograms of the blank (A). Standard solution (B) and the real sample solution (C) at 260 nm. The peaks marked were 1 = puerarin, 2 = daidzein, 3 = genistein, 4 = aloin, 5 = daidzin, 6 = genistin, respectively.

amounts of the six compounds of the samples were determined, and the results showed that they were of no significant difference among ten batches of products (Table 2). This study indicated that the proposed method was suitable for the simultaneous determination of the six compounds in KXT.

DISCUSSION

The five isoflavones with similar basic structure were commonly observed to co-exist in herbs which made it difficult to separate them from each other so that the HPLC method was selected for determination. At the

Table 1. Linear relationship between peak area and concentration.

Components	Regression equation ($y = ax+b$)	r	Linear range (mg/ml)	LOD (mg/ml)	LOQ (mg/ml)	Retention time (min)
Puerarin	$y=3.552x+4.9812$	0.9991	1.63-32.6	0.24	0.92	6.817
Daidzein	$y=3.755x+8.7058$	0.9991	3.96-79.20	0.68	1.39	11.902
Genistein	$y=5.069x+4.8321$	0.9997	4.50-90.00	0.15	0.31	15.555
Aloin	$y=3.636x+0.1908$	0.9993	1.76-35.20	0.14	0.74	18.061
Genistin	$y=3.879x-2.8506$	0.9996	8.80-176.00	0.30	0.60	25.067
Daidzin	$y=3.638x-1.6120$	0.9994	7.92-158.40	0.19	0.38	31.103

y: peak area of components; x: concentration of components; r: correlation coefficient.

Table 2. Determination of the active components in KXY by HPLC method.

Batches	Content (n = 5, mean \pm S.D., mg/g)					
	Puerarin	Daidzein	Genistein	Aloin	Daidzin	Genistin
20080611	6.79 \pm 0.09	5.38 \pm 0.07	4.61 \pm 0.08	6.64 \pm 0.10	8.49 \pm 0.11	16.05 \pm 0.17
20080623	6.85 \pm 0.10	5.46 \pm 0.11	4.28 \pm 0.09	6.29 \pm 0.13	8.27 \pm 0.13	16.02 \pm 0.18
20080708	6.47 \pm 0.08	5.29 \pm 0.07	4.19 \pm 0.09	6.08 \pm 0.08	8.19 \pm 0.12	15.87 \pm 0.20
20080714	6.59 \pm 0.12	5.31 \pm 0.14	4.56 \pm 0.10	6.55 \pm 0.13	8.45 \pm 0.14	15.96 \pm 0.20
20080725	6.82 \pm 0.07	5.42 \pm 0.14	4.78 \pm 0.11	6.87 \pm 0.15	8.72 \pm 0.16	16.37 \pm 0.24
20080729	6.55 \pm 0.13	5.09 \pm 0.10	4.33 \pm 0.12	6.92 \pm 0.13	8.82 \pm 0.16	16.41 \pm 0.25
20080805	6.91 \pm 0.14	5.57 \pm 0.16	4.81 \pm 0.09	6.95 \pm 0.14	8.93 \pm 0.14	16.49 \pm 0.26
20080810	6.71 \pm 0.09	5.33 \pm 0.15	4.52 \pm 0.08	6.37 \pm 0.09	8.32 \pm 0.09	15.62 \pm 0.09
20080821	6.73 \pm 0.13	5.52 \pm 0.15	4.61 \pm 0.09	6.75 \pm 0.15	8.83 \pm 0.15	16.39 \pm 0.29
20080826	6.78 \pm 0.12	5.43 \pm 0.14	4.57 \pm 0.09	6.47 \pm 0.08	8.42 \pm 0.09	15.46 \pm 0.10
Average	6.72	5.38	4.53	6.59	8.54	16.06
RSD (%)	2.10	2.52	4.51	4.41	3.06	2.19

same time, during the HPLC detection system establishing procedure, the sample preparation method, the effects of gradient elution condition with double-distilled water and acetonitrile on the chromatographic behavior of the detection ingredients was investigated.

In order to optimize the extraction conditions for the quantity, several variables such as extraction solvent, extraction method and extraction time were investigated (Feng et al., 2008). In the result, pure methanol was selected as optimum solvent because it gave rise to a high yield of the six compounds with a broad range of polarity, and the ultrasonic treatment was much better than the refluxing extraction. It was mentioned in the previous report (Chen et al., 2006). In order to investigate the extraction time, powdered KXT samples were extracted with pure methanol for 20, 30, 45 and 60 min, respectively. The results showed that all the six compounds can almost be completely extracted within 30 min.

Due to the complex composition of the sample solution, different mobile phases (CH₃OH-H₂O, CH₃CN-H₂O, CH₃CN-CH₃OH-H₂O) were investigated for the separation of the six compounds. Because aloin and the five isoflavones were not easily ionized in water, the pH value

was not a key factor for the chromatographic separation. Considering the running time and the total resolution of the chromatographic separation, CH₃CN-H₂O was chosen as the mobile phase for the separation. Apart from the elution conditions, the choice of detection at 260 nm provides an optimum signal to noise for quantitative analysis. In previous reports (Chen et al., 2006; Klejdus et al., 2005), it were 270 and 254 nm. The comparison between the flow rate of 0.9 and 1.0 ml min⁻¹ was performed, and the results showed that 1.0 ml min⁻¹ was much better, producing appropriate retention time and good peak shape. Additionally, it did not make any difference between the column temperature at 25 and 30°C on the chromatogram.

Since genistein, daiazin and genstin were the common active substances in *soybean* and *Radix puerariae*. The control sample was prepared using the tablet not including *soybean* and *R. puerariae*. The desired compound from KXT was identified by comparing the retention times and UV spectra with those of the authentic standard just as Luczkiewicz et al. (2004) and Maria et al. (2007). The analyte was further confirmed by spiking the actual sample with the standard. The excellent consistency between standard and sample spectra in all analytic

samples of KXT indicated that the six marker compounds were successfully separated under the proposed analytical conditions. The peak purity was confirmed by the DAD data of the peaks of aloin and five isoflavones, and no indication of impurities and chromatographic interference was found. At last a satisfactory chromatographic separation of aloin and five isoflavones was achieved.

Method validation data indicated that the present method was a reliable, repeating and accurate HPLC method for simultaneous determination of aloin and five isoflavones in KXT with optimizing extraction and analysis conditions. So the method could be used as a routine measurement to provide a safe application for good manufacture practices quality control of the medicine and other related Chinese traditional patent medicines containing these active ingredients.

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