Anal. Chem. **2004,** *76,* 4208−4216

Determination of Saikosaponin Derivatives in *Radix bupleuri* and in Pharmaceuticals of the Chinese Multiherb Remedy Xiaochaihu-tang Using Liquid Chromatographic Tandem Mass Spectrometry

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Saikosaponins are bioactive oleanane saponins derived from the Chinese medicinal herb *Radix bupleuri* ("chaihu" in Chinese). An LC−MS/MS-based method has been developed for characterization and quantification of 15 saikosaponin derivatives (saikosaponin a, saikosaponin b_1, saikosaponin g, saikogenin A, saikogenin H, saikosaponin c, saikosaponin h, saikosaponin i, prosaikogenin C_2, prosaikogenin B_2, saikogenin C, saikogenin B, saikosaponin d, saikosaponin b_2, and saikogenin D) in one chromatographic run. Optimization of the ionization process was performed with electrospray and atmospheric pressure chemical ionization techniques in both positive and negative ion modes. Negative ion ESI was adopted for generation of the precursor deprotonated molecules to achieve the best ionization sensitivity for the analytes. In addition, the most abundant fragment ion was chosen for each analyte to give the best CID sensitivity. Because some of the saponin derivatives are isomeric, complete resolution for the whole analytes was achieved both chromatographically and mass spectroscopically. Furthermore, optimal internal standard was successfully discovered for determination of the analytes by making use of a combinatorial chemistry approach. Good linearity over the range ∼1.65 or 4.98 to 1200 ng/mL for the analytes was observed. The intraday accuracy and precision at nominal low, intermediate, and high concentration varied between 0.8 and 11.8% and between 80 and 116%, respectively, whereas those for interday assay were between 1.1 and 15.5% and between 86 and 119%, respectively. The lower limits of quantitation for the test compounds were ∼16.5 to 49.4 pg on-column. The new method offered higher sensitivity and greater specificity than previously reported LC methods. After the validation, the applicability of the method for determination of these chemicals present in a variety of crude chaihu roots and in different brands of the Chinese multiherb remedy Xiaochaihu-tang (or Shosaiko-to) extract granules has been demonstrated. The sensitivity and specificity of the technique will be the basis of a method for the accurate quantification of the saikosaponin derivatives in biomatrixes.

Chaihu (*Radix bupleuri*, Chinese thorowax root) is a favorite medicinal herb in traditional Chinese medicine (TCM) and is used as a key ingredient herb for many Chinese multiherb remedies. As described in the Chinese pharmacopoeia,[1] chaihu is the dried roots of *Bupleurum chinense* DC., known in commerce as Bei-chaihu, or *Bupleurum scorzonerifolium* Willd., known as Nan-chaihu, (family Umbelliferae), which are gathered in Spring or Fall. Bei-chaihu is considered to be superior to Nan-chaihu.[2] In Japan, the roots of *Bupleurum falcatum* L. (known as Mishima-saiko) have also been used as a source of chaihu ("saiko" in Japanese). Chaihu has been employed traditionally to relieve fever, to smooth the liver, and to cure drooping and ptosis. The herb contains a mixture of bioactive oleanane saponins, principally saikosaponins a (SSa), c (SSc), and d (SSd),[3−5] which are composed of a pentacyclic triterpene aglycone (saikogenin) substituted with a sugar side chain of glucose−fucose− or glucose−(rhamnose)−glucose− as monodesmosides (Scheme 1). SSa, SSc, and SSd contain an unstable allyl oxide linkage and are readily converted into diene saponins by mild acid treatment[4−6] or on heating.[7,8] The transformed saponins possess a heteroannular diene structure at C-11,13(18) or homoannular diene

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10.1021/ac0499423 CCC: $27.50
Published on Web 05/29/2004

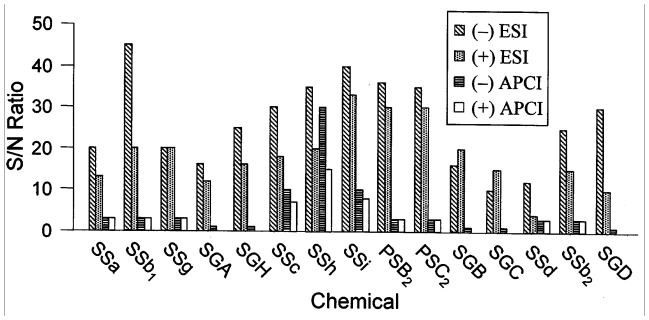

Figure 1. Comparison of different ion sources for ionization of the 15 analytes in terms of signal-to-noise (S/N) ratios. The most intense peak in each spectrum was chosen for the comparison.

structure at C-9(11),12. The ether saponins could also be transformed into the diene derivatives using gastric juice.[9] In addition, both the underivatized and the diene-transformed saikosaponins could be stripped of their sugar moieties by intestinal flora.[9,10] Saikosaponins are of interest because of their broad spectrum of biological and pharmacological activities coupled with low toxicity,[11] such as antihepatotoxic activities,[12,13] antiinflammatory activities,[14−16] immunomodulatory activities,[17,18] and anticancer activities.[19,20]

Xiaochaihu-tang is an important multiherb remedy in TCM and was first described by the famous Chinese physician Zhang Zhongjing (150 to 219 A. D. in the Chinese Eastern Han Dynasty) in his *Shang Han Lun*, a treatise on febrile diseases. The traditional remedy is a combination of seven herbs (Table 1), and chaihu is a key ingredient herb. Xiaochaihu-tang (or Shosaiko-to in Japanese) has attracted a great deal of attention for its possible healing effects on chronic hepatitis[21−23] and its beneficial effect to prevent the development of hepatocellular carcinoma in patients with cirrhosis of the liver.[24] In addition, many studies in animal models

Table 1. Herb Composition of Xiaochaihu-tang

herb	family	amt (g)	structural hierarchy
Radix bupleuri (chaihu, Chinese thorowax root)	Umbelliferae	12	prime
Radix scutellariae (huangqin, baical skullcap root)	Labiatae	9	minister
Rhizoma pinelliae (banxia, pinellia tuber)	Araceae	9	adjutant
Radix ginseng (renshen, ginseng)	Araliaceae	9	adjutant
Radix glycyrrhizae (gancao, liquorice root)	Lequminosae	6	adjutant
Rhizoma zingiberis recens (shengjiang, fresh ginger)	Zingiberaceae	9	emissary
Fructus jujubae (dazhao, Chinese date)	Rhamnaceae	12[a]	emissary

[a] As described by Zhang Zhongjing in his treatise, the number is 12 Chinese dates.

or on cell lines have demonstrated that Xiaochaihu-tang or its ingredients possess cytoprotective effects on experimental liver injuries,[25−27] an antiinflammatory effect on chronic hepatitis,[28,29] preventive and therapeutic effects on experimental hepatic fibrosis via the inhibition of hepatic stellate cells,[30−32] and lipid peroxidation in hepatocytes.[32] The herbal remedy also exhibits anticancer properties that inhibit chemical hepatocarcinogenesis in animals[33] and suppresses the proliferation of hepatoma cells by inducing apoptosis and arrest at the G0/G1 phase.[34−36]

To understand the relationship between administration of traditional Xiaochaihu-tang and liver dysfunction, a good investigation of the bioavailability and metabolism of the active constituents presen, such as saikosaponin derivatives, is needed. Developing sensitive and reliable quantitative methods for analysis of a range of components present in Xiaochaihu-tang is a prerequisite to the drug metabolism and pharmacokinetic (DMPK) evaluation of the herbal remedy.

Several methods for the separation and quantification of saikosaponin derivatives in plant materials have been described. Although the chromatographic methods TLC densitometry,[37−40] droplet counter-current chromatography (DCCC),[41] and micellar

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electrokinetic capillary chromatography (MEKC)[42] have been reported, high-performance liquid chromatography (HPLC) on reversed-phase C_{18} columns with UV detection was the most frequently used technique for saikosaponin determination.[8,43−51] The HPLC analysis, however, is limited due to the ether saikosaponins SSa, SSc, and SSd not possessing any chromophore necessary for UV detection. The chromatographic separation of these saponins had to be traced at shorter UV wavelengths ranging from 203 to 210 nm. The specificity at shorter wavelengths was poor, and the sensitivity could also be affected in the case of using methanol−water gradients. An alternative to shorter wavelength UV detection is pre-HPLC derivatization of ether saponins into diene saponins in order to generate a characteristic chromophore that facilitates UV detection at longer wavelength.[8,45] The diene-transformed saponins possess a strong absorption at ∼250 nm. Such methods, however, are not suitable for the direct analysis of samples containing both types of the saponins. More recently, the LC−MS technique[52] was applied to analyze saikosaponins in a chaihu-containing Chinese multiherb remedy. The method was, however, only focused on analysis of SSa and SSd. The poor sensitivity and lack of specificity of the above methods demand developing a more advanced analytical procedure that can be the basis of a method that is amenable to measurement of a broad range of saikosaponins and their metabolites at low-level concentrations in plasma or other biomatrixes following oral administration of Xiaochaihu-tang.

Recent success with the use of liquid chromatography combined with tandem mass spectrometry (LC−MS/MS) for characterizing and quantifying complex plant extracts[53−57] suggests that the technique might also be effective in the comprehensive determination of multiple saikosaponin derivatives in complex mixtures. The report presented here details the establishment of an LC−MS/MS-based method that is capable of determining 15 saikosaponin derivatives in one chromatographic run and its application to analysis of the constituents present in crude drugs of chaihu and in phytopharmaceuticals of Xiaochaihu-tang or Shosaiko-to. Because some of the saikosaponins are isomeric, the compound separation was achieved both chromatographically and mass spectroscopically. On the basis of the investigation of the ESI- or APCI-generated ionization of the test compounds as well as their fragmentation, the new method provides the best sensitivity and specificity for determination of the saikosaponin derivatives so far.

EXPERIMENTAL SECTION

Chemicals. Reference standard saikosaponin a (SSa, Wako code no. 194-08411, >98.0%), saikosaponin c (SSc, Wako code no. 194-08421, >98.0%), and saikosaponin d (SSd, Wako code no. 194-08431, >98.0%) were obtained from Wako Pure Chemical (Osaka, Japan). The methods described by Shimizu et al.[58] and by Nose et al.[59] were modified for synthesis of saikosaponin b_1 (SSb_1), saikosaponin g (SSg), saikogenin A (SGA), and saikogenin H (SGH) in acidic conditions from SSa, whereas saikosaponin h (SSh), saikosaponin i (SSi), prosaikogenin B_2 (PSB_2), saikogenin B (SGB), prosaikogenin C_2 (PSC_2), and saikogenin C (SGC) were made from SSc, and saikosaponin b_2 (SSb_2) and saikogenin D (SGD) were from SSd (as depicted in Scheme 1). The reaction products were individually applied to HPLC for purification. The ^{1}H NMR data of the purified saikosaponin derivatives (>99%) were compared with the reported data,[58−62] which were consistent with the structures shown in Scheme 1. The synthesized products were also used as reference standards for analytical purposes.

SSa, SSb_1, SSg, SSc, SSh, SSi, PSB_2, PSC_2, SSd, and SSb_2 were dissolved in 50% CH_3CN at 600 μg/mL to prepare their primary stock solutions, whereas SGA, SGH, SGD, SGB, and SGC were in CH_3CN. These primary stock solutions were pooled and mixed to obtain an intermediate stock solution at 40 μg/mL for each of the 15 test compounds. The stock solutions were stored at −80 °C when not in use.

Corticosterone 21-acetate (CSA) was used as an internal standard (IS) for the quantification of 15 saikosaponin derivatives. CSA was detected neither in a crude drug of chaihu nor in phytopharmaceutical products of Xiaochaihu-tang. A primary IS stock solution was prepared using 50% MeOH as solvent. An IS spiking solution (100 ng/mL) was prepared from the primary IS solution using 50% MeOH for the dilution. The IS stock solution and the IS spiking solution were stored at −80 °C when not in use.

HPLC-grade acetonitrile (CH_3CN, 99.9%) was obtained from Merck (Darmstadt, Germany). Other organic solvents and chemical reagents used were of analytical grade and were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). HPLC water was made by distilling predeionized water twice in this laboratory.

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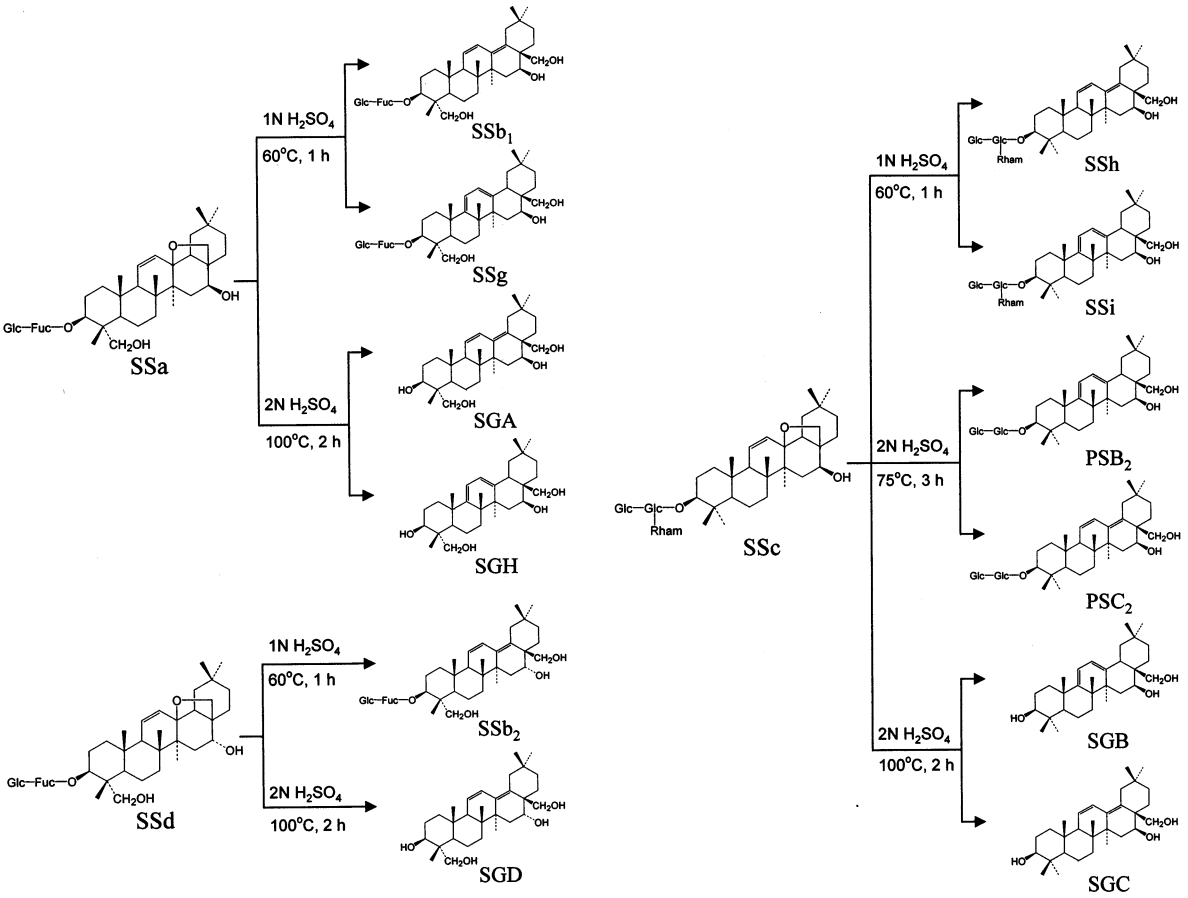
Scheme 1. Structures of the Saikosaponin Derivatives and the Chemical Conversions of the Oleanane Saponins



Liquid Chromatography−Mass Spectrometry. The liquid chromatography−mass spectrometry system consisted of an Agilent 1100 series liquid chromatograph (including a vacuum degasser, a quaternary pump, and an autosampler; Waldbronn, Germany) coupled to an Applied Biosystems/MDS SCIEX API-3000 triple-quadrupole mass spectrometer equipped with a Turboionspray ion source (Foster City, CA). The LC−MS/MS system was controlled by Analyst software. In addition, another LC−MS system consisting of an Agilent 1100 LC pump and an autosampler, and a Finnigan LCQ Deca ion-trap mass spectrometer fitted with an electrospray ionization (ESI) source or an atmospheric pressure chemical ionization (APCI) source (San Jose, CA) was used in the early phase of the method development. The LCQ Deca spectrometer was also fitted with a motorized six-pot divert valve for diverting the LC flow between the mass spectrometer and waste.

The saikosaponin derivatives were separated using a 5-μm Zorbax XDB-C_{18} column (50 mm × 2.1 mm i.d.; Chadds Ford, PA), before which a 0.2-μm filter (Upchurch Scientific, Oak Harbor, WA) was used. The LC mobile phases were CH_3CN/H_2O (10:490, v/v) for solvent A and CH_3CN/H_2O (450:50, v/v) for solvent B. The LC binary gradient program consisted of an initial 7-min linear gradient segment of increasing B from 31 to 34%, followed by an isocratic segment maintaining B at 34% from 7 to 11 min. Then the linear gradient was changed progressively by increasing B to 42% at 18 min and 100% at 24 min. Finally, B was changed back to 31% at 24.1 and maintained from 24.1 to 32 min for the analysis of the next sample. The effluent was delivered at 0.2 mL/min during the whole gradient program. The injection volume was 10 μL, and the UV absorbance of the effluent was monitored from 195 to 400 nm.

Tuning solutions of the individual analyte at the concentration of 10 μg/mL were prepared by diluting the corresponding primary stock solutions with 50% CH_3CN. The tuning solution was delivered at 5 μL/min by a syringe pump and was combined through a Peek tee union with a mixture of solvents A and B delivered at 0.2 mL/min by the LC pump. The ratio of solvents A and B varied for different test compounds according to the ratios in the postcolumn effluents containing the corresponding compounds. Instrumental parameters of the MS spectrometers were optimized to achieve the maximum ionization of the analyte molecules and the generation of the characteristic fragment ions.

Quantitative analysis of the saikosaponin derivatives in the herbal samples was performed using the triple-quadrupole LC−MS/MS system. The operating parameters of the mass spectrometer included the compound-dependent and source-dependent considerations. The optimized source-dependent parameters consisted of the flow rates of the nebulizer gas, the curtain gas, collision gas, the ionspray voltage, and the temperature of the heater gas. The compound-dependent parameters for the test compounds and IS, including the declustering potential, the focusing potential, the entrance potential, the collision energy, and

the collision cell entrance potential, were tuned for each test compound to achieve the highest instrument response. The mass spectrometer was operated at low mass resolution for both Q1 and Q3 in multiple reaction-monitoring (MRM) mode.

Quantification of the Saikosaponin Derivatives in Herbal Materials. A reference drug sample of pulverized dry roots of *Bupleurum chinense* (Chaihu-ref, Catalog No. 0992-200102) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Five other crude drugs of chaihu were also investigated. Chaihu-1 was identified as the dry roots of *B. chinense*, which was obtained from the Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine (Beijing, China). Chaihu-2 and Chaihu-3 were obtained from Shanxi Pharmaceutical Company (Taiyuan, China), which had been gathered from the wild plants (*Bupleurum* spp.) growing in Lingchuan County (Shanxi Province, China) and from stands of *B. chinense* cultivated at a local farm in compliance with good agriculture practice (GAP) in the same county, respectively. Chaihu-4 was purchased from Shanghai Lei's Pharmaceutical Co., Ltd. (Shanghai, China) with unknown habitat. Chaihu-5 was obtained from Tasly Group (Tianjin, China). The identity of these commercial crude drugs was reestablished organoleptically as *Bupleurum* spp. by the specialists from the Department of Pharmacognosy at Shanghai University of Traditional Chinese Medicine (Shanghai, China). Garbling the crude drugs of chaihu consisted of the removal of extraneous matter, such as other parts of the plant, dirt, and added adulterants and was carried out before pulverizing the crude drugs. The herbal samples were stored at −20 °C until use.

For each variety of chaihu, ∼200 mg of the pulverized crude drug was steeped at ambient temperature (∼23 °C) in 10 mL of 50% MeOH. The mixture was vortexed for 3 min, ultrasonicated for 2 min, and vortexed for 3 min again. The supernatants were then separated following centrifugation at 1175*g* for 10 min. After a second extraction with 10 mL of 50% MeOH, the combined methanol extract (∼19 mL) was filtered through a 0.45-μm nylon filter (Shanghai, China). The resultant filtants were stored at −80 °C pending LC−MS/MS analysis. Further extraction of the herbal samples under the same conditions afforded only a trace of material, indicating that the two-step extraction was enough. The formation of the diene saponins from the oleanane saikosaponins was negligible under the extracting conditions, and thus, pyridine was not added into the extracting solvent.

Seven brands of Xiaochaihu-tang phytopharmaceuticals (X-1, X-2, X-3, and X-4) and Shosaiko-to phytopharmaceuticals (S-1, S-2, and S-3) were purchased from drugstores in Shanghai (China) and Tokyo (Japan), respectively, and used for research purposes only. They were tea granules of the multiherb remedy prepared by various pharmaceutical manufactures. On the product labels, the manufacturers claimed that the herbal remedies were composed of the ingredient herbs according to the recipe of Xiaochaihu-tang described in Zhang Zhongjing's *Shang Han Lun*. The phytopharmaceuticals purchased were stored at −20 °C until use.

For LC−MS/MS analysis, each variety of Xiaochaihu-tang or Shosaiko-to granules (500 mg) was dissolved in 10 mL of 50% methanol. After centrifugation at 1175*g* for 10 min, the supernatant was filtered through the 0.45-μm nylon filter. The resultant herbal solution was kept frozen at −80 °C when not in use.

To construct standard curves for the quantification of the saikosaponin derivatives in the herbal materials investigated, calibrator pool solutions containing the 15 test compounds were prepared from the intermediate standard stock solution (40 μg/mL) by dilution with 50% MeOH to concentrations of 1200, 400, 133, 44.4, 14.8, 4.94, and 1.65 ng/mL. Ten microliters of the IS spiking solution (40 ng/mL) was added to each variety of the calibrator pool solution (100 μL). Calibration graphs were constructed using a linear regression of the test compound/IS peak area ratio (Y) to nominal concentration of the test compound (X, ng/mL) with weighting of the reciprocal concentration ($1/X$). To determine the intraday and interday accuracy and precision of the analytical method described here, 50% MeOH solutions containing the 15 test compounds at three different nominal concentrations (14.8, 44.4, and 400 ng/mL) were analyzed, and the quality control values were calculated from the regression equations (data not shown).

RESULTS AND DISCUSSION

MS and MS/MS Optimization. Using the Zorbax XDB-C_{18} column and the mobile phase of CH_3CN/H_2O provided symmetrical and sharp chromatographic peaks for the test saikosaponin derivatives. The effect of different mobile phase additives, such as formic acid, acetic acid, ammonium formate, and ammonium acetate, on the ionization in LC−MS was not further studied. To find the most sensitive ionization method for the analytes, ESI and APCI were tested with the same LC mobile phase at flow rates of 0.2 or 0.5 mL/min. The pseudomolecular ions formed were identified from the mass spectra obtained, which were achieved by applying the individual analyte to the LC−UV-MS using the C_{18} column and the mobile phase, leading to short chromatographic retention for the test compound (t_R, ∼2 to 3 min). The peak appearing in the ion chromatogram extracted from the LC−MS in full-scan mode was compared with the peak appearing in the on-line UV chromatogram (at 210 nm) in terms of their t_R. If no peak emerged at the t_R in the extracted ion chromatogram, the suspected ion could not be derived from the test compound. Since the MS spectrometer was connected behind the UV detector, there was an ∼0.1-min delay of the peak retention time in the ion chromatogram, as compared with that in the corresponding UV trace.

In positive ion ESI experiments, the saikosaponins showed a very high tendency to form alkali metal adducts. The sodiated molecule seen at m/z $[M + Na]^+$ was the base peak in the spectra of all saikosaponins, together with less abundant adduct ions $[M + K]^+$ and $[2M + Na]^+$. In addition, ion $[M + H - H_2O]^+$ was also detected for most saikosaponins, but in some cases, small peaks for ions $[M + NH_4]^+$ and $[M + H - 2H_2O]^+$ were visible in the spectra, too. The ionization profiles of the saikogenins in the positive ion ESI mode were quite different from those of the saikosaponins. Alkali metal adducts were not formed in the ESI source. Instead, the main ions derived from the saikogenins were $[M + H - H_2O]^+$ and $[M + H - 2H_2O]^+$, whereas ion $[2M + H]^+$ was also detected. The high tendency for the adduct formation substantially reduced the formation of protonated molecules so that peaks for $[M + H]^+$ were very weak or even not seen in the spectra of the saikosaponin derivatives.

In the negative ion ESI mode experiments, the peak due to the deprotonated molecule $[M - H]^-$ was detected in all spectra,

Table 2. Ions of Saikosaponin Derivatives Detected in LC−MS Spectra in Negative ESI Mode

	ion[a]			
chemical	$[M - H]^-$	$[2M - H]^-$	$[M + Cl]^-$	$[M + HCOO]^-$
SSa	779 (100)	1559 (20)	815 (7)	825 (10)
SSb_1	779 (100)	1559 (30)	815 (8)	825 (10)
SSg	779 (100)	1559 (15)	815 (25)	825 (5)
SGA	471 (60)			517 (100)
SGH	471 (100)			517 (30)
SSc	925 (100)	1853 (12)	961 (12)	971 (15)
SSh	925 (100)	1853 (8)	961 (10)	971 (14)
SSi	925 (100)	1853 (7)	961 (26)	971 (22)
PSB_2	779 (100)	1559 (50)		825 (80)
PSC_2	779 (100)	1559 (30)		825 (65)
SGB	455 (80)			501 (100)
SGC	455 (100)			501 (50)
SSd	779 (100)	1559 (50)	815 (10)	825 (18)
SSb_2	779 (100)	1559 (15)	815 (7)	825 (15)
SGD	471 (10)			517 (100)

[a] *m*/*z* with relative abundance (%) in parentheses.

which was the most intense peak for the analytes (except the three saikogenins SGA, SGB, and SGD). Meanwhile, small peaks for $[2M - H]^-$, $[M + Cl]^-$, and $[M + HCOO]^-$ were also seen for the test saikosaponins. As shown in Table 2, formate adduct $[M + HCOO]^-$ peak was abundant for the saikogenins studied, especially for SGA, SGB, and SGD, which was the base peak.

The phenomenon of abundant adduct formation also occurred in the positive ion APCI mode. The main adduct for the saikosaponins was sodium adduct $[M + Na]^+$, but in a few cases, the ion $[M + H - H_2O]^+$ was also detected. Both the ions $[M + H - H_2O]^+$ and $[M + H - 2H_2O]^+$ were abundant in the spectra of the saikogenins. In negative ion APCI experiments, the chlorine adduct $[M + Cl]^-$ was the main peak for all saikosaponins (except SSb_1), and the deprotonated molecule $[M - H]^-$ was also detected. The saikogenins seemed to be very poorly ionized in the APCI source.

On the basis of the above MS experimental results (Figure 1), negative ion ESI was adopted for generation of the precursor deprotonated molecule $[M - H]^-$ in MS/MS experiments of this work, which gave the highest signal-to-noise ratio for most of the analytes. Formate adduct $[M + HCOO]^-$ could be a relevant precursor ion for SGA, SGB, and SGD in the future. The CID spectra from the deprotonated saikosaponins exhibited a fragmentation pattern corresponding to successive loss of glycosidic units, which allows a straightforward interpretation of the spectra. In two-stage tandem MS experiments, the most abundant ions were due to loss of the terminal rhamnose from SSc, SSh, and SSi or the glucose from the other saikosaponins. The negative ion MS/MS spectra showed a clear difference between the heteroannular and the homoannular saikogenins, in which the characteristic fragment ions of the deprotonated molecules included the ions $[M - H - H_2O]^-$, $[M - H - CH_3OH]^-$, or $[M - H - CH_2O - H_2O]^-$. After the optimization of the conditions for the individual transition, the fragment ions seen at *m*/*z* $[M - H - CH_2O - H_2O]^-$ were most abundant for SGA and SGC. In contract, those at *m*/*z* $[M - H - H_2O]^-$ were most abundant for SGH and SGB. The main fragment ion for SGD was ion $[M - H - CH_3OH]^-$, together with the less abundant ion $[M - H - H_2O]^-$ and the poorly formed ion $[M - H - CH_2O - H_2O]^-$.

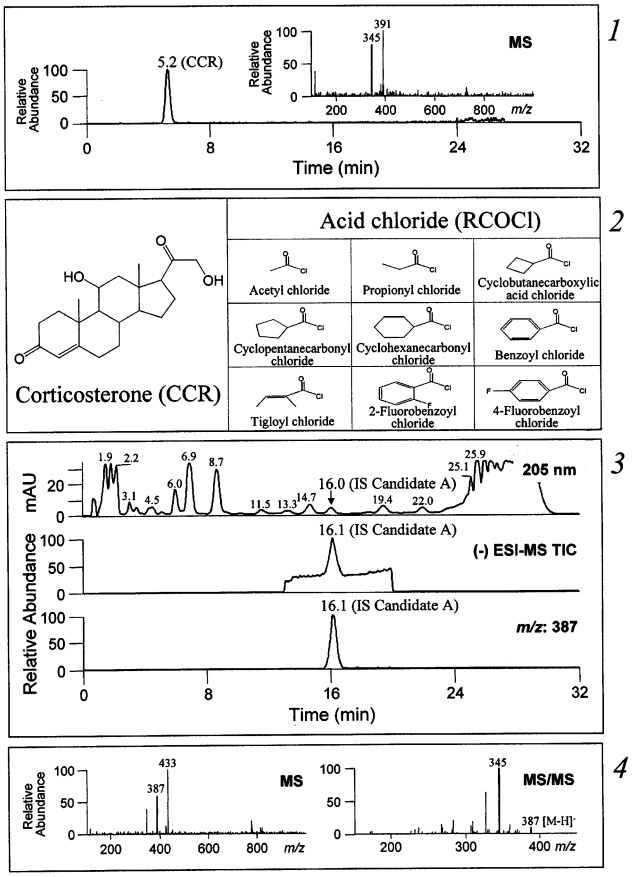


Figure 2. Discovery of an optimal internal standard for the mixture of the 15 test saikosaponin derivatives using a combinatorial chemistry approach. For panel 1, corticosterone (CCR) was tested using the LC−MS/MS method developed for the mixture of the 15 analytes. For panel 2, CCR was used as a starting material and reacted with nine acid chlorides. For panel 3, LC−UV−ESI-MS was used to trap and detect IS candidate(s) from the reaction mixture. For panel 4, the (−) ESI-MS and MS/MS spectra were obtained for IS candidate A.

Discovery of An Optimal Internal Standard. Since its chemical structure is close to those of the analytes, corticosterone (CCR) was first examined for IS discovery. Under the MS/MS conditions (in the negative ion ESI mode) developed for the quantification of the saikosaponin derivatives, CCR exhibited abundant generation of the deprotonated molecule ($[M - H]^-$, *m*/*z* 345) (Figure 2/panel 1) and efficient production of its characteristic fragment ion (*m*/*z* 327) (data not shown). The chromatographic retention of CCR (t_R, 5.2 min), however, was too short and out of the retention time range of the 15 analytes (t_R, ~5.8 to 27.7 min, see Figure 3).

Instead of the traditional way of serially testing compounds for IS discovery by trial and error, that is, giving up CCR and testing another compound, CCR was retained as an IS lead for further optimization. With a combinatorial chemistry approach, about 100 CCR derivatives were rapidly synthesized as a mixture in the same reaction vessel. In brief, CCR was used as starting material and was dissolved in dry dichloromethane at 0 °C. A mixture of nine acid chlorides (0.6 mmol for each, Figure 2, panel 2) and triethylamine (12 mmol) were added to the CH_2Cl_2 solution of CCR (0.15 mmol). The reaction mixture was stirred until the disappearance of CCR (checked by TLC) and then mixed with

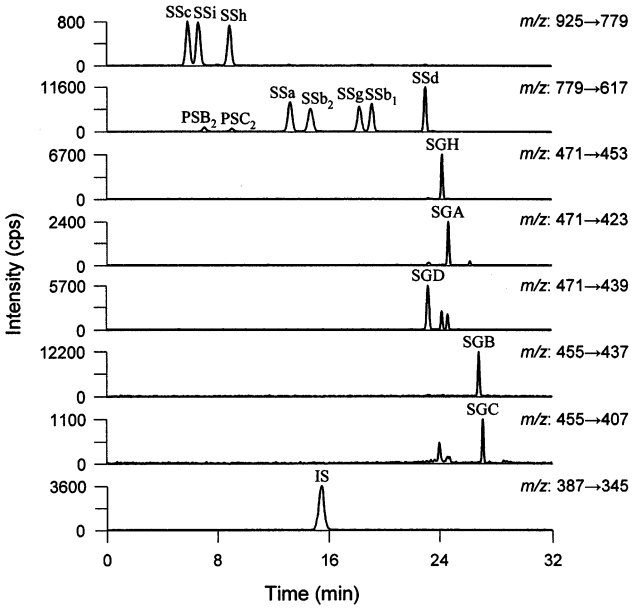


Figure 3. Extracted ion chromatograms of the mixture of the saikosaponin derivatives SSa, SSb_1, SSg, SGA, SGH, SSc, SSh, SSi, PSB_2, PSC_2, SGB, SGC, SSd, SSb_2, SGD, and IS (CSA) from the LC−MS/MS experiment (MRM mode) with negative ESI. The ion transitions used for extraction are marked for each chromatogram ($[M - H]^- \rightarrow$ fragment ion).

10 mL of H_2O. The resultant mixture was extracted three times with 10 mL of EtOAc. After centrifugation, the combined EtOAc extract was treated in turn with 1 N HCl, H_2O, brine, and $MgSO_4$ and then concentrated by evaporation under reduced pressure to yield an oily mixture of esters. The resultant reaction mixture was then applied to LC−MS/MS using the chromatographic and ionizing conditions developed for the mixture of the 15 test saikosaponin derivatives. To trap the IS candidates, the divert valve on the LCQ_{DECA} MS spectrometer was set to introduce the eluent flow during 13−20 min to the ion source with the other eluent flows to waste. As depicted in Figure 2, panel 3, there was one compound detected at t_R 16.1 min, the chromatographic peak that was labeled "IS candidate A". The IS candidate A (ISC-A) showed an abundant $[M - H]^-$ at m/z 387 and an abundant $[M + HCOO]^-$ at m/z 433 in the ESI-MS spectrum (Figure 2, panel 4), the ionization pattern of which was similar to those of the analytes (see Table 2). The difference of the molecular weight between ISC-A and CCR is 42 mass units, suggesting that ISC-A might be an acetylated product of CCR. The CID spectrum of the deprotonated molecule, obtained in the full scan MS/MS mode, exhibited a characteristic fragment ion at m/z 345. To confirm the chemical structure of ISC-A, CCR was reacted with acetyl chloride. Fortunately, ISC-A was also synthesized from the reaction and purified by HPLC. On the basis of ^1H NMR analysis (data not shown), ISC-A was identified as corticosterone 21-acetate (CSA). CSA was found by LC−MS/MS in neither test herbal samples of this study nor blank plasma, urine, or tissue homogenate samples of this laboratory, suggesting it is amenable to being used as IS for the analysis.

Development and Validation of Quantification Method. The optimal conditions were applied for the TurboIonSpray ionizations (an ESI) in negative ion mode and ion transitions. The fragment ions chosen for quantification purposes were most abundant for the corresponding analytes and gave the best sensitivity. Because some of the saikosaponins are isomeric, showing the same MS/MS fragmentation pattern, compound resolution cannot be achieved spectroscopically only. An advanced liquid chromatographic separation system was needed to adequately resolve the isomers between SSa and SSb_2 (m/z 779 → 617), between SSg and SSb_1 (m/z 779 → 617), and between SSc and SSi (m/z 925 → 779). As depicted in Figure 3, complete resolution was successfully achieved both chromatographically and spectroscopically for the 15 analytes in a 32-min chromatographic run. Due to the LINAC collision cell technology of API-3000, varying the dwell times for the ion transitions and grouping the analytes in chromatographic time windows for MRM analysis did not exhibit evidently enhanced sensitivity of the detection method. Matrix-induced interference resulting in suppression of ionization of test compounds in LC−MS has been well-recognized.[63−65] Under the LC−MS/MS conditions developed in this work, however, no obvious interference was observed in the analysis of the complex herbal extract samples.

Quantification was performed using internal standard method. The introduction of IS for biomatrixes before sample cleanup will improve precision and accuracy for the quantitative analysis. Using CSA as IS, good correlations were achieved for all the 15 analytes relative to the compound with calibration curve regression coefficients >0.99 spanning a concentration range of ~1.65 or 4.94 to 1200 ng/mL. The precision of this analytical method was determined by calculating the relative standard deviation (RSD) of the concentrations measured on the same day ($n = 5$) and on different days ($n = 5$) for the calibration standard samples of different nominal concentrations. As shown in Table 3, the RSD never exceeded 15.5% at the concentrations examined, indicating good assay precision. Meanwhile, the accuracy ranged from 80 to 119% for the 15 analytes. The method provided a lower limit of quantification (LLOQ) of 16.5 or 49.4 pg on-column for the 15 test saikosaponin derivatives, which is quite sensitive and can be the basis of method utilized for DMPK studies of Xiaochaihu-tang or other chaihu-containing herbal remedies.

Application to Analysis of Samples of Chaihu and Xiaochaihu-tang. The quantified results obtained for six crude chaihu roots are present in Table 4. The test crude drugs except Chaihu-ref were garbled before sampling. Each sample was analyzed in duplicate. Chaihu-ref and the other chaihu samples analyzed in this work ranged from ~4.18 to 6.84 mg total saikosaponin derivatives/g of pulverized sample. The major saikosaponins detected in Chaihu-ref and the other chaihu samples were the oleanane saponins SSa, SSc, and SSd, whereas the other saikosaponins derivatives, such as the diene saponins and the hydrolyzed products, were scarcely detected (<LLOQ of our present analytical method) or not found in any sample. The levels of the individual saikosaponins SSa, SSc, and SSd present in Chaihu-ref were 2.54, 0.99, and 1.06 mg/g, respectively. A comparable content of the individual saikosaponin in samples was found for garbled Chaihu-1 to be approximately −5, i.e., ~2.13 to 3.62 mg/g for SSa, ~1.10 to 2.10 mg/g for SSc, and ~0.88 to 1.12

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Table 3. Intraday and Interday Variations of Saikosaponin Derivative Calibrations[a]

	low concn (ng/g)			intermediate concn (ng/g)			high concn (ng/g)		
chemical	FC[b]	RSD	A[c]	FC	RSD	A	FC	RSD	A
				Intraday ($n = 5$)					
SSa	15.4 ± 1.8	11.8	104	45.1 ± 2.3	5.1	102	409.0 ± 15.8	3.8	103
SSb_1	16.3 ± 0.4	2.7	110	46.1 ± 3.6	7.7	104	406.6 ± 25.6	6.3	102
SSg	13.2 ± 1.2	9.3	89	46.1 ± 3.3	7.1	104	392.5 ± 24.6	6.3	98
SGA	15.2 ± 1.5	10.2	103	40.0 ± 2.1	5.3	90	433.7 ± 13.2	3.0	108
SGH	15.2 ± 0.6	4.2	103	51.4 ± 2.2	4.4	116	431.6 ± 16.7	3.9	108
SSc	13.9 ± 1.4	9.9	94	46.3 ± 0.4	0.8	104	415.8 ± 11.3	4.6	104
SSh	14.6 ± 1.2	7.9	99	44.8 ± 3.0	6.6	101	410.5 ± 13.0	3.1	103
SSi	14.1 ± 0.2	1.7	95	45.3 ± 1.9	4.2	102	409.6 ± 4.8	5.1	102
PSB_2	14.3 ± 0.8	5.5	97	47.1 ± 2.5	5.2	106	406.0 ± 12.9	3.2	102
PSC_2	15.1 ± 0.2	1.3	102	46.1 ± 1.7	3.1	104	407.7 ± 12.9	3.2	102
SGB	15.3 ± 1.2	7.9	103	42.1 ± 2.7	6.5	95	391.8 ± 28.0	7.1	98
SGC	11.9 ± 1.2	10.1	80	36.2 ± 1.3	3.6	82	374.1 ± 37.9	10.1	94
SSd	14.9 ± 0.7	4.6	100	43.7 ± 1.0	2.3	98	409.9 ± 28.8	7.0	103
SSb_2	14.7 ± 1.2	8.3	99	48.5 ± 1.1	2.2	109	405.4 ± 15.8	4.0	101
SGD	14.9 ± 1.4	9.2	100	46.0 ± 3.0	6.5	104	438.9 ± 20.3	4.6	109
				Interday ($n = 5$)					
SSa	12.8 ± 0.4	3.2	86	42.8 ± 1.5	3.4	96	390.7 ± 20.7	5.3	98
SSb_1	13.6 ± 1.6	11.6	92	43.2 ± 4.6	10.7	97	397.5 ± 32.9	8.3	99
SSg	15.9 ± 0.9	5.5	107	43.0 ± 5.5	12.7	97	414.3 ± 49.6	12.0	104
SGA	15.3 ± 2.0	13.5	103	41.0 ± 2.3	5.7	92	415.3 ± 58.8	14.2	104
SGH	16.6 ± 0.8	4.9	112	53.0 ± 1.6	3.0	119	401.7 ± 21.2	5.3	100
SSc	13.6 ± 1.6	11.0	92	44.5 ± 2.2	5.0	100	414.2 ± 21.2	5.1	104
SSh	13.1 ± 1.1	8.3	88	45.3 ± 3.0	6.7	102	406.7 ± 10.8	2.7	102
SSi	13.3 ± 1.0	7.5	90	44.4 ± 1.1	2.5	100	404.7±4.5	1.1	101
PSB_2	13.4 ± 1.3	8.8	91	49.2 ± 1.3	2.6	111	410.4 ± 32.8	7.9	103
PSC_2	15.0 ± 0.7	4.9	101	44.8 ± 1.3	2.8	101	409.4 ± 15.5	4.8	102
SGB	15.7 ± 1.5	9.3	106	39.4 ± 3.0	7.5	89	392.1 ± 60.9	15.5	98
SGC	13.5 ± 1.5	11.1	91	38.6 ± 3.9	10.1	87	358.5 ± 44.0	12.3	90
SSd	13.1 ± 0.9	7.0	88	39.2 ± 2.9	7.3	88	418.3 ± 34.7	8.3	105
SSb_2	13.5 ± 0.4	2.7	91	48.6 ± 1.3	2.6	109	403.1 ± 15.1	3.7	101
SGD	13.4 ± 1.1	8.3	91	42.9 ± 2.4	5.6	97	415.9 ± 55.3	13.3	104

[a] Nominal low, intermediate, and high concentrations were 14.8, 44.4, and 400 ng/mL, respectively. [b] FC: found concentration in ng/mL. [c] A: accuracy in %.

Table 4. Quantification of Saikosaponins Present in Various Crude Drugs of Chaihu[a]

chemical	Chaihu-ref	Chaihu-1	Chaihu-2	Chaihu-3	Chaihu-4	Chaihu-5
SSa	2.54 (55.3%)	2.36 (49.2%)	3.62 (52.9%)	3.34 (55.4%)	2.13 (51.0%)	2.23 (51.8%)
SSc	0.99 (21.6%)	1.52 (31.7%)	2.10 (30.7%)	1.63 (27.0%)	1.17 (28.0%)	1.10 (25.5%)
SSd	1.06 (23.1%)	0.92 (19.1%)	1.12 (16.4%)	1.06 (17.6%)	0.88 (21.0%)	0.98 (22.7%)
Total	4.59	4.80	6.84	6.03	4.18	4.31

[a] Values are the measured levels expressed in mg/g. Values in parentheses are the percentage of the chemical in the total saikosaponin measured.

mg/g for SSd (Table 4). The ratio of SSa, SSc, and SSd was around 2.6:1.4:1 (w/w/w).

The quantified results obtained for the seven brands of Xiaochaihu-tang or Shosaiko-to phytopharmaceuticals are presented in Table 5. Besides the oleanane saponins SSa and SSc, the diene saponins SSb_1, SSg, SSh, SSi, and SSb_2 were also found in the samples of the herbal extract granules, suggesting the formation of the diene saikosaponins during Xiaochaihu-tang or Shosaiko-to granule manufacturing. There is no data supporting any occurrence of further hydrolysis of these saponins during the manufacturing course. SSd was not detected in most of the granule samples, except for the Japanese Shosaiko-to samples S1 and S2, in which only a quite low amount of SSd was found. SSd seemed to be transformed into SSb_2. In the cases of SSa and SSc, the oleanane saponins coexisted with their transformed diene saponins to various extents. The heteroannular diene products SSb_1 and SSh seemed to be more abundant than their corresponding homoannular isomers SSg and SSi, respectively. The ratios of SSb_1 and SSg and that of SSh and SSi were 1.61 ± 0.10 and 1.76 ± 0.05 for Chinese granules ($n = 4$) and 1.39 ± 0.29 and 1.94 ± 0.10 for Japanese granules ($n = 3$), respectively. The major saikosaponins contained in the test Xiaochaihu-tang and Shosaiko-to samples were SSb_2 (26.9%), SSa (25.8%), SSb_1 (22.4%), SSg (14.3%), SSc (6.9%), SSh (5.4%), and SSi (2.9%). Although the daily ingested amount of the total saikosaponin for the four brands of Chinese Xiaochaihu-tang granules (20.32 ± 8.55 mg, RSD 42.1%, $n = 4$) was comparable to that of the three brands of Japanese Shosaiko-to granules (14.01± 5.21 mg, RSD 36.5%, $n = 3$), the saikosaponin composition of these phytopharmaceuticals was inconsistent, probably as a result of the discrepancy in raw materials used and during the herb extraction process. The Chinese manufacturers used a greater amount of excipients in their Xiaochaihu-tang granules than Japanese manufacturers did, resulting in a bigger size of the products for daily administration.

Table 5. Quantification of Saikosaponins Present in a Variety of Phytopharmaceuticals of Xiaochaihu-tang or Shosaiko-to[a]

chemical	X1 (60 g)[b]	X2 (60 g)	X3 (60 g)	X4 (60 g)	S1 (7.5 g)	S2 (6 g)	S3 (7.5 g)
SSa	7.2	90.8	72.9	9.6	673.7	928.2	352.0
SSb_1	66.1	75.7	204.8	95.4	238.8	214.6	132.3
SSg	42.5	50.0	123.3	55.3	183.7	186.1	77.5
SSc	TA[c]	4.6	4.0	2.1	389.0	341.6	176.0
SSh	16.5	5.4	16.5	29.1	124.2	119.0	64.3
SSi	9.3	3.1	9.7	15.9	67.1	58.3	32.9
SSd	ND[d]	ND	ND	ND	7.7	70.0	ND
SSb_2	63.8	71.9	109.4	99.7	685.9	762.5	257.1
Total	205.4	301.5	540.6	307.1	2370.1	2680.3	1092.1

[a] The values are the measured levels in μg/g granules. The amount of the saikosaponin for administration per day can be calculated by multiplying the measured level by the amount of the granules for daily ingestion. [b] The amount of the granules for ingestion per day. [c] TA, trace level detected (lower than the LLOQ). [d] ND, not detected.

Full evaluation of Xiaochaihu-tang products depends not only on the analysis of saikosaponins but also on the active principles of the other herbs. The methods for the quantification of the medicinal constituents in the other herbs of Xiaochaihu-tang have also been developed in this laboratory for determining the quality of the multiherb products and will be reported elsewhere.

CONCLUSION

In this work, a new LC−MS/MS-based methodology for characterization and quantification of a broad range of 15 saikosaponin derivatives is described. Negative ion ESI-MS/MS method was chosen for the saikosaponin derivatives. Complete resolution for these chemicals was achieved both chromatographically and spectroscopically. Making use of combinatorial chemistry approach successfully created an optimal internal standard for quantification. The glycosidic linkages can be determined from the CID multistage MS experiments using $[M - H]^-$ as the precursor ions. The applicability of the method for determination of these chemicals present in a variety of crude chaihu roots and in different brands of Xiaochaihu-tang (or Shosaiko-to) extract granules has been demonstrated. The sensitivity and specificity of the described LC−MS/MS technique will be the basis of a method for the accurate quantification of the saikosaponin derivatives in biological fluids and could be applied to the DMPK evaluation and studies of the Chinese multiherb remedy Xiaochaihu-tang.

ACKNOWLEDGMENT

This study was supported, in part, by Grant 30271596 of the National Natural Science Foundation of China, Grant 01DJ19008 of the Science and Technology Commission of Shanghai Municipality, and Grant 2003AA2Z347A of the Chinese Ministry of Science and Technology. Some experiments of this work were performed in Prof. Y-M. Yang's laboratory at this research institute or in Prof. C. Yu's laboratory at Shanghai Xuhui Central Hospital. The authors thank Dr. J. Zheng from Northeastern University for the helpful suggestions.

Received for review January 9, 2004. Accepted April 27, 2004.

AC0499423