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Journal of Chromatography B, 843 (2006) 183–193
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Characterization and quantification of eight water-soluble constituents in tubers of *Pinellia ternata* and in tea granules from the Chinese multiherb remedy Xiaochaihu-tang

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Received 26 October 2005; accepted 31 May 2006
Available online 19 June 2006

Abstract

In traditional Chinese medicine, multiple herbs are usually used in combination to generate the joint actions of a multiherb remedy. The recent development of LC-hyphenated techniques enables efficient and rapid profiling of the chemical constituent in extracts from multiherb remedies. Xiaochaihu-tang is a seven-herb remedy that has attracted a great deal of attention for reported ability to treat liver dysfunction. Dried tubers of *Pinellia ternata* (banxia in Chinese) is one of the ingredients, but its chemical contribution to Xiaochaihu-tang remains poorly understood. In the study presented here, LC–UV–MS, LC–MS–MS, and LC–NMR were used in a complementary manner to determine the nature and content of eight water-soluble constituents of banxia and their presence in various tea granules from Xiaochaihu-tang. Among the eight chemicals identified in banxia, cytidine, adenosine, tryptophan, uridine, and adenine are reported for the first time, while tyrosine, guanosine, and phenylalanine were previously described. These chemicals are also present in all of the samples of Xiaochaihu-tang granules, and the amounts of the chemicals ingested due to a daily dose of the multiherb remedies range from 0.008 to 6.3 mg.
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Keywords: LC-hyphenated techniques; Xiaochaihu-tang; *Pinellia ternate*

1. Introduction

While screening medicinal herbs described in the traditional Chinese medicine literature for new drug leads, scientists in China and other Asian countries are also investigating the herbal remedies themselves [1]. In traditional Chinese medicine, herbs are often used in combination to achieve the joint actions of the individual herbs. This results in the chemical composition of a multiherb remedy quite complex. Before the compounds that account for specific pharmacological effects of the remedies or the compounds that are toxic can be determined, a great deal of effort is required to identify and measure the chemical constituents present in the herbal remedies. Classical strategies of phytochemistry are usually aimed at obtaining pure compounds from the crude herbal material, which are then analyzed by nuclear magnetic resonance (NMR) and mass spectrometry (MS). The sample preparation consists of extracting the raw material with organic solvents followed by isolation and purification steps of preparative chromatography and liquid handling, which is often tedious and time-consuming for study of complex multiherb remedies. Therefore, performing spectroscopic analysis of a complex mixture with reduced prior isolation and purification is expected to increase the overall efficiency.

High-performance liquid chromatography (LC) is a powerful tool for separating complex mixtures into their individual components. The coupling of conventional LC and MS has been a great advance for spectroscopic and quantitative analysis of complex mixtures [2–4]. In addition, the coupling of LC and NMR spectroscopy offers a natural and logical solution for analysis of mixture samples [5]. This hyphenated technique

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doi:10.1016/j.jchromb.2006.05.028

is increasingly used in a variety of fields, including the analysis of natural products [6–10] and the identification of drug metabolites [9–11]. Further, trapping of the individual peaks eluting from the LC column into capillary loops of an incorporated storage unit for later off-line analysis provides a method for increasing the acquisition time for the sample in the NMR detection probe. Also, the complementarity of the structural and molecular mass information derived from NMR spectroscopy and mass spectroscopy, respectively, can be enhanced if the data are collected in the same separation run or under the same chromatographic conditions.

Xiaochaihu-tang is an important herbal remedy in traditional Chinese medicine. It was first described in the *Shang Han Lun*, a treatise of febrile diseases by the physician Zhang Zhongjing, who lived from 150 to 219 A.D. during the Chinese Eastern Han Dynasty. The traditional remedy for treatment of chronic liver diseases [12–14] is a mixture of seven herbs including *Radix Bupleuri* (chaihu or Chinese thorowax root), *Radix Scutellariae* (huangqin or baical skullcap root), *Rhizoma Pinelliae* (banxia or pinellia tuber), *Radix Ginseng* (renshen or ginseng), *Radix Glycyrrhizae* (gancao or licorice root), *Rhizoma Zingiberis Recens* (shengjiang or fresh ginger), and *Fructus Jujubae* (dazhao or Chinese date). Because the constituents of the multiherb remedy have not been completely identified, its ability to treat liver dysfunction is not well understood. Additional support for a role of Xiaochaihu-tang and its active ingredients in prevention and treatment of liver disease may be provided by identification and quantification of specific herb-derived chemicals as well as by an increased understanding of their bioactivity, bioavailability, and biotransformation [15–23].

Banxia (*Rhizoma Pinelliae*), one of the ingredient herbs of Xiaochaihu-tang, is the dried tuber of *Pinellia ternata* (Thunb.) Breit (Fam. *Araceae*) and is officially listed in the Chinese pharmacopoeia [24]. Little is known, however, about which phytochemicals present in Xiaochaihu-tang are derived from banxia. The herb is extracted in hot water for preparation of Xiaochaihu-tang, but the chemical composition of the aqueous extract has not yet been well characterized. A few earlier studies suggested that guanosine, tyrosine, and phenylalanine were contained in banxia [25–28]. In the study presented here, hyphenated techniques such as LC–UV–MS, LC–MS–MS, and LC–NMR were used to determine the structure and quantity of eight aqueous constituents present in banxia tubers and various tea granules from Xiaochaihu-tang or Shosaiko-to.

2. Materials and methods

2.1. Herb samples and reagents

Tubers of banxia came from two farms in Guang'an and Nanchong counties of Sichuan Province, China, which were in compliance with Good Agriculture Practice, and are referred to as banxia-1 and banxia-2, respectively. In addition, crude tubers of banxia of unknown source (banxia-3) were obtained from Huayu Pharmaceutical Co. (Shanghai, China). The identities of the tubers were organoleptically confirmed to be *P. ternata* by specialists from the Department of Pharmacognosy at Chengdu University of Traditional Chinese Medicine (Chengdu, Sichuan Provence, China).

For the ease of analysis, pulverized banxia (1.00 g) was extracted with 10 mL of distilled water. The mixture was vortexed for 5 min and then ultrasonicated for 5 min at ambient temperature. The supernatant was separated after centrifugation at $16,060 \times g$ for 5 min. After a further extraction of the solid residue with 10 mL of water under the same conditions, the combined aqueous extract (∼18 mL) was diluted in water to 20.0 mL and then dialyzed using Membra-cel™ dialysis membrane (molecular weight cut-off range = 8–14 kDa; Willowbrook, IL, USA) against 180 mL of water at room temperature for 12 h with gentle agitation to remove aqueous amylose and protein. For LC–UV, LC–MS, and LC–MS–MS analysis, the resulting dialysate (5 mg of banxia solid/mL) was directly used without any sample concentration. For LC–NMR analysis and HPLC isolation and purification, the dialysate (100 mL) were lyophilized to dryness and reconstituted in 2 mL of distilled water to prepare concentrated extract from banxia (250 mg of banxia solid/mL).

Samples of tea granules of Xiaochaihu-tang (X1, X2, X3, and X4) and Shosaiko-to (S1, S2, and S3) were purchased from drugstores in Shanghai (China) and Tokyo (Japan), respectively. All the manufacturers of the phytopharmaceuticals claimed that their multiherb products were composed of the ingredient herbs (including banxia) according to the recipe of Xiaochaihu-tang described by Zhang Zhongjing in *Shang Han Lun*. The powdered sample (500 mg) of each variety of Xiaochaihu-tang or Shosaiko-to granules was dissolved in 10 mL of 50% methanol in water. After centrifugation at $1175 \times g$ for 10 min, the supernatant was filtered through a 0.45-μm filter. The resulting herbal solution was stored at −70 °C until use.

HPLC-grade acetonitrile (CH_3CN, 99.9%) and methanol (MeOH, 99.9%) were purchased from Sigma–Aldrich (Darmstadt, Germany). Acetonitrile (99.9%) for LC–NMR use was the Riedel-deHaën® product (Seeize, Germany). Deuterium oxide (D_2O) of guaranteed reagent quality (99.8%) was obtained from Beijing Chemical Factory (Beijing, China). Oxymatrine (Catalog No. 784-9001, 99.6%), obtained from the National Institute for the Control of Pharmaceutical and Biological Products, was used as an internal standard for the quantification of the identified compounds derived from banxia. HPLC-grade water was made by double-distilling predeionized water.

2.2. Detection and molecular analysis of the major constituents in aqueous extracts of banxia

The aqueous extracts of banxia were analyzed on a LC–MS system consisting of an Agilent 1100 series liquid chromatograph (including a vacuum degaser, a binary pump, an autosampler, a thermostatted column compartment, and DAD detector; Waldbronn, Germany) coupled to a Thermo Finnigan TSQ Quantum AM triple-stage quadrupole mass spectrometer interfaced via an atmospheric pressure chemical ionization (APCI) or an electrospray ionization (ESI) source. The software packages Chemstation (Agilent) and Xcalibur (Finnigan) were used for controlling the system as well as for data acquisition and processing.

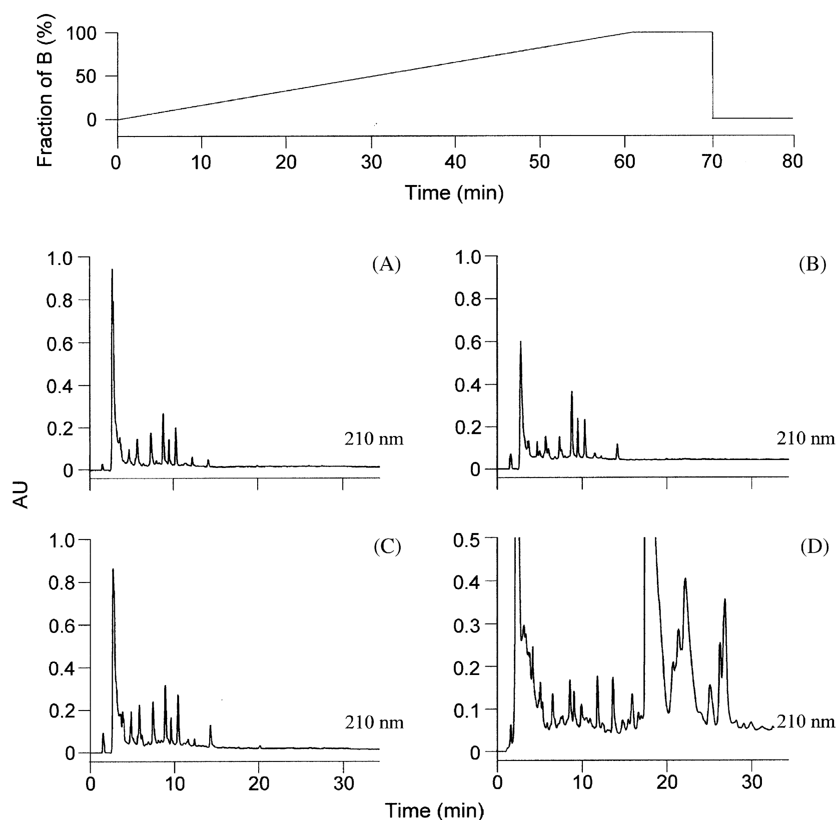

Fig. 1. Chromatographic separation of the aqueous extracts of crude banxia and a typical Xiaochaihu-tang extract. Traces A, B, C, and D are for extracts of banxia-1, -2, -3, and S1 (see Table 4), respectively. Because most of the peaks were eluting within the first 35 min, the chromatograms are the displays of the up to 35 min.

LC separations of the aqueous extracts from banxia for detecting the major constituents present in the aqueous extract and scouting their chromatographic retentions were achieved on a 5-μm Kromasil® 100-5C18 column (150 mm × 4.6 mm i.d.; Eka Chemicals, Bohus, Sweden) maintained at 30 °C. The mobile phase consisted of CH_3CN/H_2O (10:490, v/v) for solvent A and CH_3CN/H_2O (450:50, v/v) for solvent B and the gradient program is shown in Fig. 1 (the upper panel). The eluent flow, at 0.5 mL/min, was monitored for UV absorption at 210 nm.

On the basis of the results of the LC–UV detection of the major constituents, the gradient program was modified for subsequent LC–MS and LC–MS–MS analyses, which consisted of an initial 12-min linear gradient segment of increasing B from 0 to 12%, followed by an isocratic segment maintaining B at 12% from 12 to 19 min. Then solvent was changed back to 0% B at 19.1 min and then maintained at 0% B from 19.1 to 25 min for the analysis of the next sample. The eluent flow (0.5 mL/min) was first introduced into the UV detector (set at 200–400 nm) and then combined through a Peek T-union with 0.5 mL/min CH_3CN, delivered by an Agilent 1100 LC isopump, before being directed to APCI source without splitting. The tandem TSQ Quantum mass spectrometer was operated in both positive and negative ion polarity modes in separate analytical run. The parameters of the mass spectrometer including the discharge current, the vaporizer temperature, the sheath gas pressure, the auxiliary gas pressure, the capillary temperature, the tube lens offset, and the lens O offset were set at the generally recommended values by the instrument manufacturer for molecular analysis of the herbal analytes.

The MS spectrometer acquisition time for each chromatographic run was divided into three segments (i.e., 0–4, 4–16, and 16–25 min). During the 4–16 min period, the divert valve was set to send the eluent flow to the mass spectrometer with the other eluent flow to the waste. The major banxia components were analyzed in the second segment. In addition, the protonated molecules, as well as the other ions of the analytes generated in the APCI source, were dissociated in the Q2 collision cell with 10-, 20-, 30-, or 40-eV collision energy to produce fragment ions. The LC–MS–MS experiments were also operated in full scan mode.

Accurate mass measurement of $[M+H]^+$ ions for the major constituents of banxia was achieved in ESI mode using the enhanced mass-resolution capacity of the triple quadruple mass spectrometer. With the high resolution calibration using the polytyrosine mixture containing alanine and leucine and the accurate mass calibration using methanol clusters accomplished, an internal mass-locking procedure was employed for accurate mass measurement. The final accurate mass determination was generated by averaging spectra across each LC peak of interest.

2.3. LC–NMR for characterization of the constituents in aqueous extract of banxia

The chromatographic method used for LC–NMR experiments was the same as the preceding LC method for LC–MS

except that D_2O and LC–NMR CH_3CN were used to prepare the mobile phase to minimize the intensity of the solvent signals in the NMR spectra. LC separation was carried out using a Varian chromatography system (including a Prostar 230 solvent delivery module, a Prostar 330 PDA-UVdetector, and a Valco air actuator for two position valves with a 100-μL sample loop; Palo Alto, CA, USA) under the control of Prostar chromatography workstation and Varian Cascade software. The eluent flow was monitored by absorption at 235 nm. The chromatographic peaks of interest were first captured in the 130-μL capillary loops (0.14 mm i.d.) on a Varian LC–NMR Analyte Collector. The saved peaks were later pumped into a flow cell (active volume: 60 μL; 3 mm o.d.) for ^{1}H NMR measurements. A volume of 100 μL of the concentrated aqueous banxia extract was applied to the LC–NMR system for multi-component analysis. Before applying the real samples, the delay times both from the UV detector to the analyte collector and from the analyte collector to the microflow probe were calibrated using the method described in the Varian LC–NMR Accessory Installation Manual.

^{1}H NMR data were acquired with a Varian Inova-600 MHz spectrometer equipped with a ^{1}H{^{13}C} pulsed field gradient LC–NMR probe with the 60-μL flow cell. The ^{2}H resonance of the D_2O was used for a field-frequency lock. Prior to acquisition, all samples were tuned and shimmed until lineshapes were within specification and optimized. The water suppression enhanced through T_1 effect (WET) was used to suppress CH_3CN, its ^{13}C satellites, and the residual water peaks. In order to obtain a better looking data, WET used a composite shape pulse of comp_44g. Free induction decays were collected with 16K data points, a spectra width of 8000 Hz, a 4.4 μs 90 pulse, a 2 s acquisition time and a 1 s pulse decay. Prior to Fourier transformation, an exponential apodization function was applied to the free induction decay corresponding to a line broadening of 0.25 Hz. The NMR data were recorded at 23 °C and processed using Varian VNMR Version 6.1C.

2.4. HPLC isolation and purification of P1, P7, and P8 from aqueous extract of banxia for ^{1}H NMR analysis

The concentrated extract from banxia (250 mg of banxia solid/mL) was repeatedly introduced at the sample size of 200 μL into a 5 μm Kromasil® 100-5C18 column (150 mm × 4.6 mm i.d.) maintained at 30 °C. The composition of the mobile phase delivered at 1 mL/min was the same as that described earlier in this report. A gradient elution program monitored at 210 nm was used, which consisted of an initial 9 min linear gradient segment of increasing B from 0 to 6%, followed by B going to 95% at 9.1 min and an isocratic segment maintaining B at 95% from 9.1 to 11 min. Then mobile phase was changed back to 0% B at 11.1 min and then maintained at 0% B from 11.1 to 20 min for the preparation of the next sample. Experiments were performed on an Agilent 1100 Series purification system (analytical scale) consisting of a quaternary pump with degasser, a refrigerated autosampler (set at 4 °C), a thermostatted column compartment, a diode array detector, and a refrigerated fraction collector (set at 4 °C). The system was controlled using the Agilent ChemStation and the Agilent Purification software module. The target fractions were collected in time-based mode. The pooled fractions containing P1, P7, and P8 were first reduced at 60 °C in a Savant SpeedVac concentration system (Holbrook, NY, USA) to remove the organic solvent and frozen at −70 °C, followed by further lyophilizing to dryness. The resulting residue of P1 was reconstituted in water and rechromatographed on another 5 μm Kromasil® 100-5C18 column (150 mm × 4.6 mm i.d.) at 30 °C. In isocratic pump mode, CH_3OH/H_2O (4:96, v/v) was used as mobile phase. The purified fraction was reduced to dryness using the preceding method. The chemical identity and purity were further checked by LC–UV–MS. About 0.2–0.5 mg amounts of the three compounds were prepared for conventional ^{1}H NMR analysis.

2.5. LC–MS–MS for quantification of the constituents present in a variety of banxia tubers and various tea granules from Xiaochaihu-tang

For the quantitative analysis, fast-isocratic LC was performed on a shorter 5 μm Inertsil® ODS-3 column (50 mm × 3.0 mm i.d.; GL Sciences Inc., Tokyo, Japan). The LC mobile phase was $MeOH/H_2O$ (142:358, v/v) and pumped at 0.3 mL/min. MS–MS data for the analytes were collected in positive ESI mode by selected reaction monitoring of the ion transitions. The instrumental parameters were optimized to maximize generation of the protonated molecules ($[M+H]^+$) of the test compounds and to efficiently produce the characteristic fragment ions (data not shown). The mass spectrometer was set at Q1 resolution 0.7 Da FWHM and Q3 resolution 0.7 Da FWHM.

To prepare calibration curves for the purpose of quantifying components, a set of working calibration standards containing the eight analytes (1000, 200, 40, 8.0, 1.6, and 0.32 ng/mL) was prepared by serial dilutions of the stock solution in water. Ten microliters of internal standard spiking solution (50 ng/mL) were added to each calibration standard (100 μL). Calibration graphs were constructed using a linear regression of the analyte/internal standard peak area ratio (Y) versus the nominal concentration of the analyte (X; ng/mL) with weighting by the reciprocal concentration ($1/X$). To determine the within-run and between-run accuracy and precision of the analytical method, aqueous solutions containing the eight test compounds at three different nominal concentrations (1.6, 40, and 1000 ng/mL) were analyzed, and the quality control values were calculated from the linear equations (data not shown). The real herbal extract samples were diluted in water by 10–100 times before spiking with IS and LC–MS–MS analysis.

3. Results and discussion

As an initial attempt to analyze the major constituents in crude aqueous extracts of banxia, reversed-phase LC separation, monitored by UV absorption at 210 nm, was carried out using a CH_3CN/H_2O gradient to allow analysis of highly retained non-polar constituents within the same analytical run as poorly retained polar constituents while providing good chromatographic resolution of the peaks. As shown in Fig. 1, the LC–UV traces of the extract of banxia-1 revealed eight major peaks with

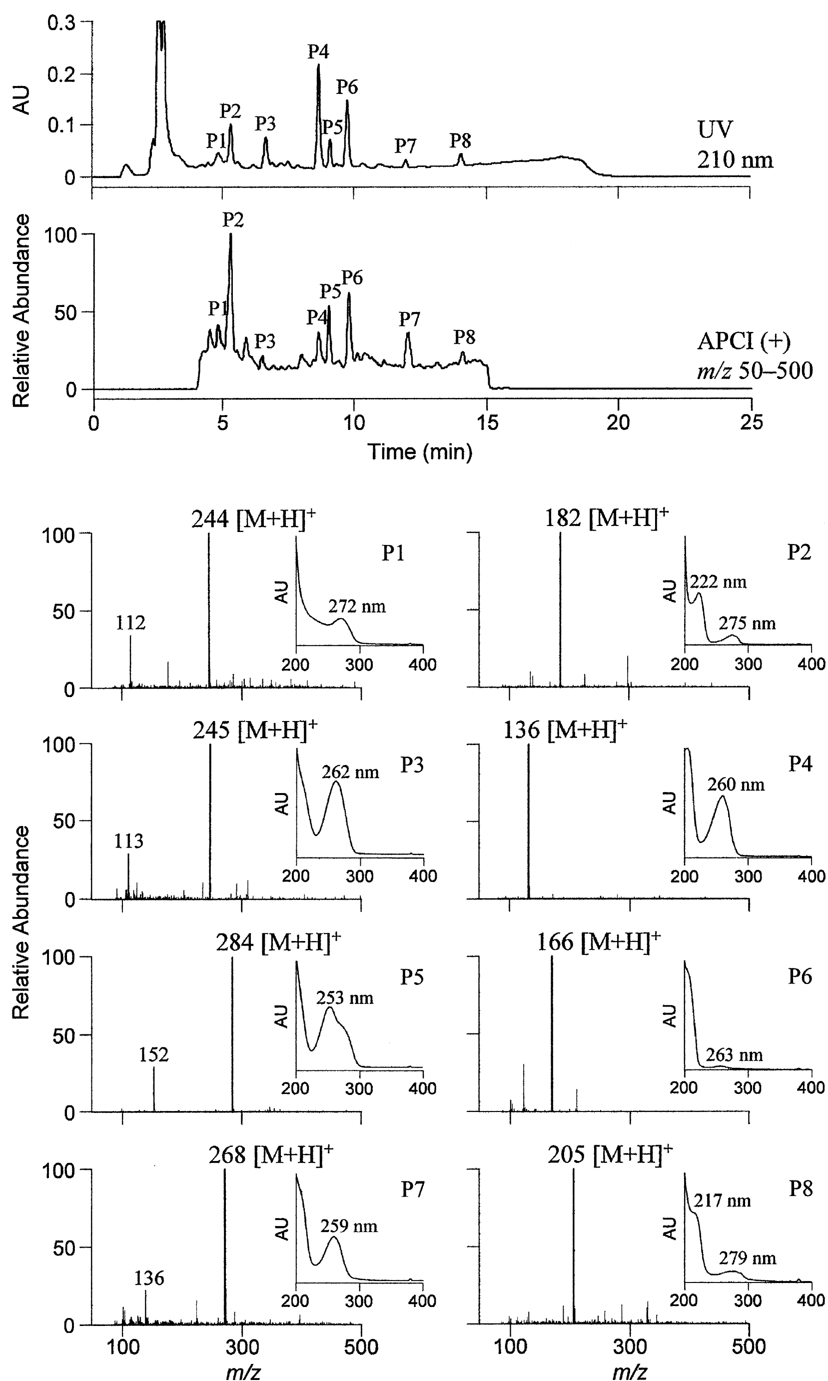

Fig. 2. LC–UV–MS-based analysis of banxia-1 extract. The mass spectra of the peaks of interest were obtained in positive APCI mode.

retention times of 4–15 min, with no peaks appearing thereafter. Similar results were obtained for the extracts of banxia-2 and banxia-3.

Based on these preliminary results, we performed further LC-hyphenated analysis with shorter chromatographic run time, focusing on the eight peaks denoted P1 through P8 (Fig. 2). The aqueous extract from banxia-1 was used for the following LC-hyphenated analysis. Alignment of characteristic APCI-mass spectra with UV profiles (inserts to the corresponding mass spectra) allows peak classification for the detected constituents of interest. As depicted in Fig. 2, similar UV spectra were observed for peaks P2 and P8, and peaks P3, P4, P5, and P7 also share similar UV spectra. In addition, the LC eluent from the UV detector was directed to the APCI-mass spectrometer without splitting and analyzed in both positive and negative ion polarity modes. LC–MS analysis of the banxia extract revealed that the positive APCI-mass spectra of P1, P2, P3, P4, P5, P6, P7, and P8 were dominated by ion peaks at *m/z* 244, 182, 245, 136, 284, 166, 268, and 205, respectively. The most intense ions in their negative APCI-mass spectra were observed at *m/z* 180 for P2, 243 for P3, 282 for P5, and 164 for P6 (data not shown). P1, P4, P7, and P8 seemed to be poorly ionized by the negative APCI

source. The intensities of the preceding ions changed as a function of the banxia concentration in the extract applied, indicating that these ions were derived from the constituents of the banxia extract.

The positive APCI-mass spectra of P1, P3, P5, and P7 also included another prominent peak with a mass-to-charge ratio value 132 less than that of the corresponding base peak. In general, a higher vaporizer temperature or turning on source CID led to an increase in the intensity of the characteristic "132 abstraction" ions that was proportional to the decrease in size of the corresponding most intense ions in the APCI-mass spectra. This suggested that the "132 abstraction" ions arose from the fragmentation of the most intense ions in the APCI source. Using LC–ESI-MS–MS of softer ionization technique for the analysis of the herb extract sample confirmed that the most intense ions in the positive APCI-mass spectra were the protonated molecule ions of the constituents of interest (data not shown). These data suggested that the eight constituents detected from the banxia extracts can be sorted into different compound classes: a first class including P1, P3, P5, and P7 (showing the $[M+H-132]^+$ ions in their APCI-MS spectra); and a second class including P2, P6, and P8 (showing no $[M+H-132]^+$ ions). Although P4 also shows no $[M+H-132]^+$ ions, the similarity of the UV spectra of P4 and P7, as well as the same mass to charge ratios observed for the $[M+H]^+$ ion of P4 and the $[M+H-132]^+$ ion of P7 in their APCI-MS spectra, suggested that P4 and P7 are structurally related.

To gain more structural information, LC–APCI-MS–MS experiments were performed on the analytes from the banxia extract. Peaks corresponding to the characteristic fragmentation of the protonated ions were observed at *m/z* 112 for P1, 113 for P3, 152 for P5, and 136 for P7, which shared the identical fragmentation pattern of generating $[M+H-132]^+$ ions. Because guanosine (MW = 283) has been reported in banxia [26], we inferred that the chemical identity of P5 was guanosine based on its MW and fragmentation pattern. The concomitant appearance of another fragment ion at *m/z* 135 for P5 agreed with the inferred structure, which might result from the dissociation of the fragment ion at *m/z* 152 to yield the product $[M+H-132-17]^+$.

Because P1, P3, and P7 were in the same class with P5, their characteristic fragment $[M+H-132]^+$ ions at *m/z* 112 (P1), 113 (P3), and 136 (P7) also suggested that they were ribose-containing nucleosides. For P1 and P7, the fragment ions (probably $[M+H-132-17]^+$) at *m/z* 95 and 119, respectively, also appeared in their MS–MS spectra, but such a product ion was not found for P3. The MWs of P1, P3, and P7 were the same as those of cytidine (MW = 243), uridine (MW = 244), and adenosine (MW = 267), respectively. For P4, a fragment $[M+H-17]^+$ ion at *m/z* 119 was generated by dissociation of its protonated ion at *m/z* 136. The protonated molecule of P4 at *m/z* 136 exhibited the identical mass-to-charge ratio value as the fragment ion $[M+H-132]^+$ derived from P7, suggesting that P4 might be adenine (MW = 135), the aglycone of adenosine.

In addition to guanosine, the amino acids tyrosine and phenylalanine have been identified in banxia [26–28]. The peaks at *m/z* 165 and 188 detected in the APCI-MS–MS spectra of P2 and P8, respectively, potentially originated from the loss of NH_3 from the protonated molecules, while the peaks observed at *m/z* 136 for P2 and *m/z* 120 for P6 probably arise from the loss of HCOOH. The MWs of P2, P6, and P8 were the same as those of tyrosine (MW = 181), phenylalanine (MW = 165), and tryptophan (MW = 204), respectively.

To determine the elemental composition for the eight main constituents of banxia, accurate mass measurement of the protonated molecules was performed in high mass-resolution mode. The resulting mass spectra show $[M+H]^+$ at *m/z* 244.0912 for P1, 182.0808 for P2, 245.0761 for P3, 136.0627 for P4, 284.0997 for P5, 166.0855 for P6, 268.1021 for P7, and 205.0976 for P8. Since guanosine, tyrosine, and phenylalanine present in banxia have been reported, the limits set for calculating elemental composition were C[6–12] for P1, P3, P5, and P7, C[0–12] for P4, C[0–20] for P2, P6, and P8, H[0–30] for all, N[0–10] for all, O[4–10] for P1, P3, P5, and P7, O[0–10] for P4, O[2–10] for P2, P6, and P8. Also, proper constraints, such as the Nitrogen Rule, were used for formulae generation. By setting a tolerance of ±10 ppm on the accurate mass measurement, elemental formulae were determined for P1, P2, P3, P4, P5, P6, P7, and P8 as $C_9H_{13}O_5N_3$, $C_9H_{11}O_3N$, $C_9H_{12}O_6N_2$, $C_5H_5N_5$, $C_{10}H_{13}O_5N_5$, $C_9H_{11}O_2N$, $C_{10}H_{13}O_4N_5$, and $C_{11}H_{12}O_2N_2$, respectively.

^1H LC–NMR analysis of the banxia extract was performed to confirm the proposed structures. The same elution order of the analytes was maintained in LC–NMR experiments as that in the preceding LC–MS experiments. Because NMR is inherently less sensitive than MS or UV detection, the concentrated samples of banxia extract were used for the ^1H LC–NMR experiments. This resulted in slight overloading of the LC column. Prior to NMR analysis, chromatographic peaks of interest eluting from the column were trapped and stored in the 130-μL capillary loops under the control of Varian LC–NMR Analyte Collector. To prevent flat-top peak from triggering the loop change more than once, LC separation was monitored at 235 nm, and both the optimized threshold and the limited data collecting rate of the UV detector were optimized to collect each peak in only one loop.

Using this system, ^1H NMR spectra were acquired for P2, P3, P4, P5, and P6 after the individual peaks were transferred from the trapping loops to the LC–NMR flow probe (Fig. 3). To confirm the detection of all analyte signals, the ^1H LC–NMR analysis of the sample was also performed using another mobile phase system (MeOH/D_2O) with solvent suppression (data not shown). The use of LC–NMR technique in this study minimized our effort lost in the isolation of the compounds.

The loop-collection ^1H NMR analysis failed to give discriminable spectra for P1, P7, and P8 due to insufficient concentration of the eluent trapped in the loops. To obtain the ^1H NMR data of these compounds, we therefore used a traditional method involving HPLC isolation and purification to provide sufficient amounts of the pure analytes. These were dissolved individually in D_2O for standard NMR analysis. The experimental ^1H NMR values of the analytes (Table 1) were superimposable on the published ^1H NMR values (data not shown) from the integrated Spectral Database System for organic compounds of the National Institute of Advanced Industrial Science and Technology (Tsukuba, Ibaraki, Japan).

Table 1
^{1}H LC–NMR spectral data of the constituents of aqueous extract from banxia

Proton	Chemical shift[a] (coupling constant)
P1[b]	3.82 dd (4.28/12.8), 3.95 dd (2.56/12.8), 4.15 m, 4.22 dd (4.57/5.91), 4.32 dd (3.78/4.57), 5.91 d (3.78), 6.02 d (7.57), 7.84 d (7.57)
P2	3.00 dd (8.05), 3.15 dd (5.13), 3.87 dd (5.13/8.05), 6.84 d (8.06), 7.14 d (8.06)
P3	3.74 dd (4.52/12.63), 3.85 dd (2.96/12.63), 4.07 m, 4.17 dd (5.12/4.53), 4.29 dd (4.64/5.12), 5.84 d (8.06), 5.85 d (4.64), 7.82 d (8.06)
P4	8.12 s, 8.18 s
P5	3.68–3.84 (not resolved), 4.12–4.24 (not resolved), 4.34 dd (5.08/4.41), 5.84 d (5.08), 7.94 s
P6	3.08 dd (8.06/14.5), 3.24 dd (5.13/14.5), 3.94 dd (5.13/8.06), 7.39 dd (7.33), 7.41 dd (7.08/7.33), 7.33 d (7.08)
P7[b]	3.81 dd (3.54/12.9), 3.88 dd (2.69/12.9), 4.27 m, 4.40 dd (5.36/3.34), 4.77 dd (6.23/5.36), 6.03 d (6.23), 8.21 s, 8.29 s
P8[b]	3.28 dd (8.18/15.3), 3.46 dd (4.40/15.3), 4.03 dd (4.40/8.18), 7.17 dd (7.08/8.06), 7.18 dd (8.06/7.08), 7.27 s, 7.51 d (8.06), 7.75 d (8.06)

[a] Chemical shifts are referenced to TMS at 0 ppm for ^{1}H.
[b] The ^{1}H NMR date for P1, P7, and P8 were obtained by ^{1}H NMR analysis of the pure compounds isolated from banxia extract, respectively.

As the result of the study, P1, P2, P3, P4, P5, P6, P7, and P8 present in the banxia extract were identified as cytidine, tyrosine, uridine, adenine, guanosine, phenylalanine, adenosine, and tryptophan, respectively (Fig. 4). This is the first report of the presence of cytidine, tryptophan, uridine, adenine, and adenosine in banxia, while tyrosine, guanosine, and phenylalanine were previously known constituents of the herb [26–28].

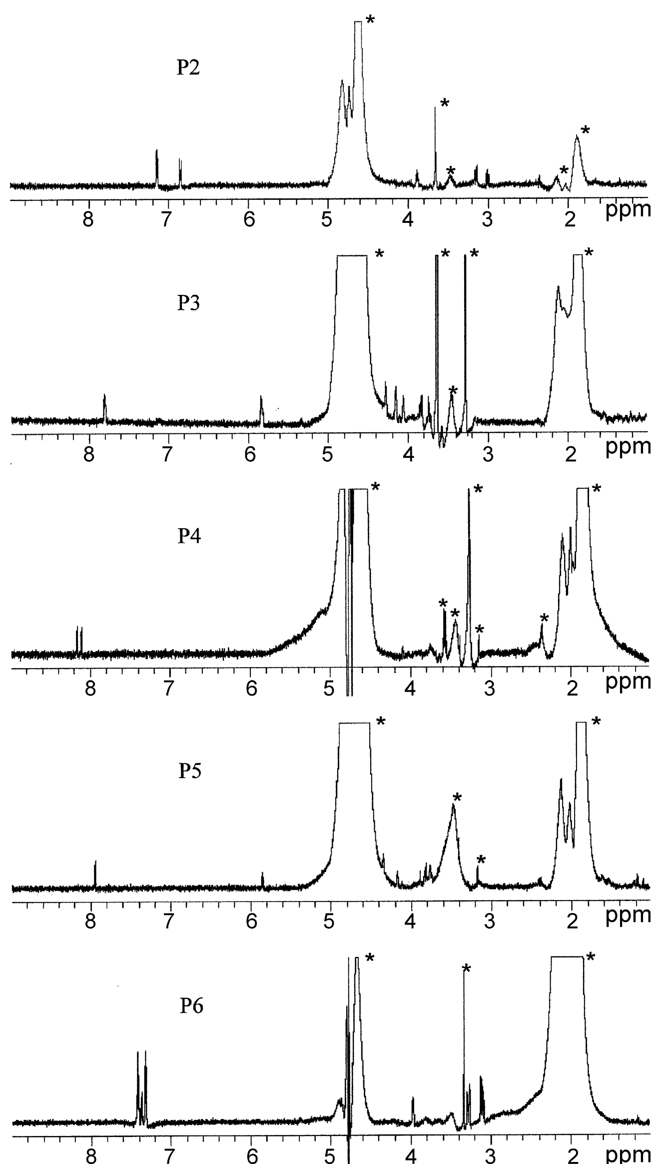

Fig. 3. ^{1}H LC–NMR (600 MHz) spectra of P2, P3, P4, P5, and P6 from aqueous banxia extract. Peak with asterisks are due to solvent or impurities therein.

On the basis of this analysis of banxia extract, chemical profiling was continued to quantify the identified water-soluble constituents in various banxia tubers and Xiaochaihu-tang products. As depicted in Fig. 5, the eight test compounds were analyzed in parallel within a 5-min chromatographic run. Specificity of the fast LC–MS–MS method for quantitative purposes was assessed by monitoring the ion transitions of the other seven analytes for appearance of the peak in an aqueous solution containing only one analyte. In addition, lack of cross-interference between the analytes was also established by measuring the peak area of each analyte signal in water spiked with all eight analytes and internal standard or with the individual analyte alone (data not shown). The results indicated that the method for measuring the test compounds in herb samples was specific and that the analytes did not interfere with each other. Calibration curve regression coefficients were greater than 0.99 over a wide concentration range (1.6–1000 ng/mL), indicating a good correlation between the analyte/internal standard peak area ratio and the concentration for all the eight analytes.

The accuracy and precision of the analytical method during a single analytical run ($n = 5$) and with time ($n = 3$) were assessed by replicating analysis of calibration standard samples containing known amounts of the eight analytes. As shown in Table 2, the accuracy ranged from 87 to 115% for the eight analytes. Meanwhile, the R.S.D. never exceeded 13.8% at the concentrations examined, indicating good assay precision. The method provided a lower limit of quantification of 16.0 pg on column for the eight analytes.

The quantified results for the three crude banxia tubers are present in Table 3. Each sample was analyzed in duplicate. The crude banxia samples contained a total of 216 ± 32 μg/g nucleosides as 113 ± 34 μg/g guanosine (P5), 58.3 ± 21.8 μg/g uridine (P3), 30.9 ± 9.3 μg/g cytidine (P1), and 13.4 ± 6.7 μg/g adenosine (P7). The low levels of adenosine in the samples were associated with the relatively high concentrations of its aglycone adenine (P4). Meanwhile, the levels of the individual amino acids tyrosine (P2), phenylalanine (P6), and tryptophan (P8) contained in the crude banxia samples were 294 ± 202, 252 ± 99, and 23.9 ± 14.0 μg/g, respectively. All the preceding compounds detected in the aqueous banxia extracts were also present in the Xiaochaihu-tang or Shosaiko-to granules

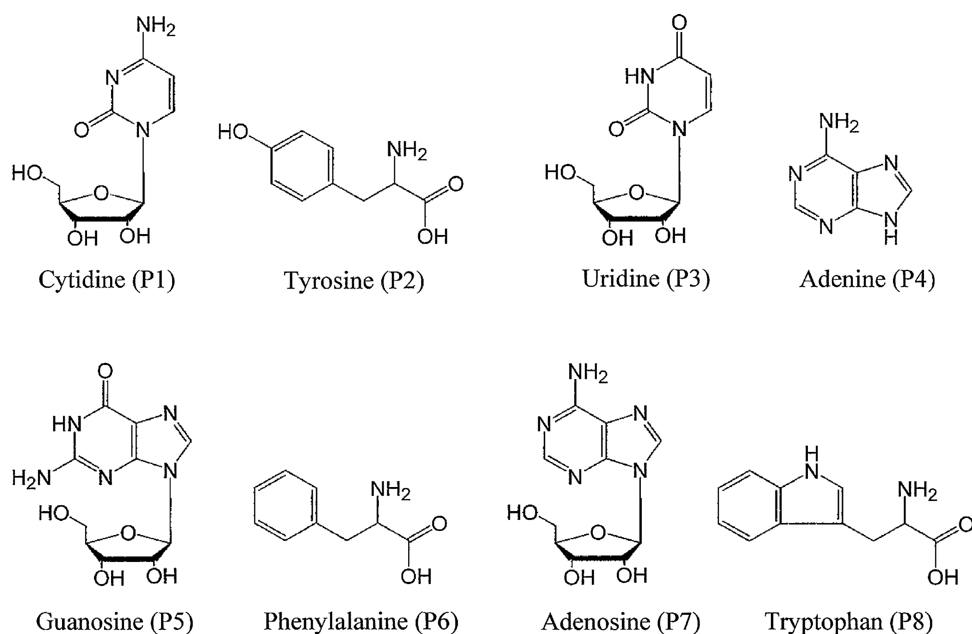

Fig. 4. Chemical structures of major water-soluble constituents from banxia.

from different sources (Table 4). The estimated daily intake from Xiaochaihu-tang was 0.630–1.80 mg for cytidine (P1), 0.140–1.59 mg for tyrosine (P2), 2.99–6.34 mg for uridine (P3), 0.146–0.701 mg for adenine (P4), 0.160–1.55 mg for guanosine (P5), 0.0198–0.876 mg for phenylalanine (P6), 0.488–1.75 mg for adenosine (P7), and 0.0078–0.0854 mg for tryptophan (P8). The amounts ingested per day from Shosaiko-to granules were 0.431–0.562 mg for cytidine (P1), 0.647–1.74 mg for tyrosine (P2), 0.855–1.06 mg for uridine (P3), 0.410–0.725 mg for adenine (P4), 1.01–1.21 mg for guanosine (P5), 0.358–1.01 mg for phenylalanine (P6), 0.673–1.81 mg for adenosine (P7), and 0.257–1.24 mg for tryptophan (P8). Malnutrition is frequent in patients with chronic liver damage, which may influence their clinical evolution and outcome. Nutritional therapy by providing amino acids, vitamins, minerals, and other nutrients may also play an important role in the management of these

Table 2
Within-run and between-run variations for calibrating the eight analytes derived from banxia

Compound[a]	Low concentration[b] (ng/mL)			Intermediate concentration (ng/mL)			High concentration (ng/mL)		
	FC[c]	R.S.D.[d]	*A*[e]	FC	R.S.D.	*A*	FC	R.S.D.	*A*
Within-run ($n = 5$)									
CYD (P1)	1.44 ± 0.05	3.8	90	40.3 ± 0.7	1.7	101	1007 ± 18	1.8	101
TYR (P2)	1.82 ± 0.25	13.7	114	41.0 ± 1.3	3.3	102	999 ± 20	2.0	100
URD (P3)	1.57 ± 0.22	13.8	98	41.1 ± 1.9	4.8	103	1002 ± 28	2.8	100
ADI (P4)	1.58 ± 0.07	4.6	99	45.9 ± 0.6	1.5	115	987 ± 25	2.5	99
GUO (P5)	1.38 ± 0.14	10.1	87	45.0 ± 1.0	2.6	112	990 ± 16	1.6	99
PHE (P6)	1.73 ± 0.14	8.0	108	41.3 ± 1.6	3.9	103	1002 ± 20	2.0	100
ADO (P7)	1.60 ± 0.05	3.1	100	44.1 ± 0.9	2.2	110	991 ± 12	2.3	99
TRP (P8)	1.85 ± 0.14	7.5	115	40.3 ± 0.9	2.3	101	1011 ± 33	3.3	101
Between-run ($n = 3$)									
CYD (P1)	1.48 ± 0.02	1.2	97	38.0 ± 1.5	3.9	95	974 ± 9	1.0	97
TYR (P2)	1.71 ± 0.04	2.2	107	39.2 ± 3.5	8.8	98	881 ± 92	10.4	88
URD (P3)	1.64 ± 0.06	3.9	102	39.1 ± 0.3	0.7	98	1000 ± 18	1.8	100
ADI (P4)	1.60 ± 0.01	0.5	100	43.3 ± 0.9	4.8	108	913 ± 1	0.1	91
GUO (P5)	1.52 ± 0.02	1.1	95	43.5 ± 1.0	2.1	109	990 ± 7	0.7	99
PHE (P6)	1.55 ± 0.07	4.8	97	37.3 ± 4.1	11.0	93	952 ± 44	4.6	95
ADO (P7)	1.69 ± 0.05	7.4	105	39.8 ± 4.3	0.8	99	865 ± 65	7.5	87
TRP (P8)	1.75 ± 0.14	2.2	109	34.4 ± 4.4	2.9	86	917 ± 37	4.1	92

[a] CYD: cytidine; TYR: tyrosine; URD: uridine; ADI: adenine; GUO: guanosine; PHE: phenylalanine; ADO: adenosine; TRP: tryptophan.
[b] Nominal low, intermediate, and high concentrations were 1.6, 40, and 1000 ng/mL, respectively.
[c] FC: found concentration in ng/mL.
[d] R.S.D.: relative standard deviation in %.
[e] *A*: accuracy in %.

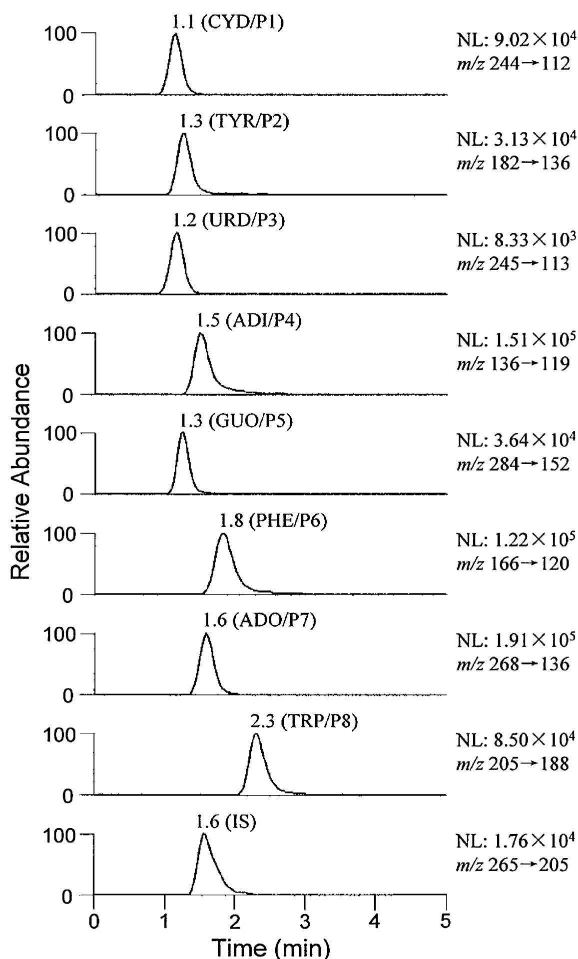

Fig. 5. High-throughput quantitative analysis of the eight water-soluble constituents present in a Xiaochaihu-tang extract using LC–ESI-MS–MS in selected reaction monitoring mode.

patients and may improve their prognosis [29–31]. In addition, mixed nucleoside tablets [WS-10001-(HD1197)-2002], containing adenosine, guanosine, uridine, and cytidine with an overall daily dose of 40–60 mg/day, has been officially listed in the Chinese National Formulary as adjuvant to aid the treatment of various liver dysfunctions [32]. Although our study presented in this report demonstrated the presence of nucleosides

Table 3
Quantification[a] of the eight major water-soluble constituents present in a variety of crude banxia tubers

Compound[b]	Banxia-1	Banxia-2	Banxia-3
CYD (P1)	31.3 ± 1.1	21.5 ± 1.4	40.0 ± 3.5
TYR (P2)	521 ± 8	132 ± 5	229 ± 4
URD (P3)	50.7 ± 0.7	41.4 ± 2.7	82.9 ± 4.9
ADI (P4)	93.1 ± 3.2	129 ± 6	106 ± 6
GUO (P5)	83.5 ± 7.8	151 ± 4	106 ± 6
PHE (P6)	364 ± 12	174 ± 9	218 ± 13
ADO (P7)	17.5 ± 1.4	5.70 ± 0.38	17.1 ± 1.3
TRP (P8)	9.98 ± 0.40	23.7 ± 0.7	38.0 ± 1.8

[a] Measured levels are expressed in μg/g.
[b] CYD: cytidine; TYR: tyrosine; URD: uridine; ADI: adenine; GUO: guanosine; PHE: phenylalanine; ADO: adenosine; TRP: tryptophan.

Table 4
Quantification[a] of the eight water-soluble constituents in a variety of tea granules from Xiaochaihu-tang or Shosaiko-to

Compound[b]	X1	X2	X3	X4	S1	S2	S3
CYD (P1)	10.5 ± 0.1 [0.630][c]	26.1 ± 0.6 [1.56]	74.9 ± 6.1 [1.80]	19.7 ± 1.3 [1.18]	57.9 ± 1.2 [0.434]	71.9 ± 3.1 [0.431]	75.0 ± 5.2 [0.562]
TYR (P2)	2.34 ± 0.08 [0.140]	7.12 ± 0.15 [0.427]	66.4 ± 4.1 [1.59]	5.02 ± 0.28 [0.301]	232 ± 5 [1.74]	178 ± 1 [1.07]	86.3 ± 0.9 [0.647]
URD (P3)	52.7 ± 2.16 [3.16]	57.2 ± 2.9 [3.43]	264 ± 24 [6.34]	49.8 ± 3.3 [2.99]	141 ± 3 [1.06]	170 ± 12 [1.02]	114 ± 0 [0.855]
ADI (P4)	2.44 ± 0.07 [0.146]	7.85 ± 0.31 [0.471]	29.2 ± 1.8 [0.701]	9.40 ± 0.14 [0.564]	96.7 ± 5.4 [0.725]	68.4 ± 1.1 [0.410]	70.5 ± 5.0 [0.529]
GUO (P5)	2.67 ± 0.05 [0.160]	19.7 ± 1.2 [1.18]	61.6 ± 3.2 [1.48]	25.9 ± 1.3 [1.55]	161 ± 2 [1.21]	168 ± 1 [1.01]	150 ± 0 [1.13]
PHE (P6)	0.33 ± 0.01 [0.0198]	3.97 ± 0.37 [0.238]	36.5 ± 2.3 [0.876]	3.37 ± 0.22 [0.202]	134 ± 2 [1.01]	135 ± 6 [0.810]	47.8 ± 4.5 [0.358]
ADO (P7)	8.13 ± 0.02 [0.488]	29.1 ± 2.1 [1.75]	39.5 ± 3.2 [0.948]	12.6 ± 0.0 [0.756]	241 ± 12 [1.81]	226 ± 8 [1.36]	89.7 ± 1.1 [0.673]
TRP (P8)	0.13 ± 0.01 [0.0078]	0.81 ± 0.05 [0.0486]	3.56 ± 0.20 [0.0854]	0.67 ± 0.02 [0.0402]	145 ± 10 [1.09]	207 ± 11 [1.24]	34.3 ± 2.1 [0.257]

[a] The values are the measured levels in μg/g granules.
[b] CYD: cytidine; TYR: tyrosine; URD: uridine; ADI: adenine; GUO: guanosine; PHE: phenylalanine; ADO: adenosine; TRP: tryptophan.
[c] The amounts of the granules for ingestion per day were 60 g/day for X1, X2, and X4, 24 g/day for X3, 7.5 g/day for S1 and S3, and 6 g/day for S2. The amounts (mg) of the test compounds for administration per day were calculated by multiplying the measured level by the amount of the granules for daily ingestion and are shown in square brackets.

in various Xiaochaihu-tang products with overall nucleoside daily doses of 3.2–10.6 mg/day, whether this herbal remedy exerts some nutrition and/or adjuvant actions in addition to its direct treatment of chronic liver diseases remains to be further studied.

Although increasing numbers of people are using herbal remedies, quality control and the knowledge of how they work remain important issues that hinder their wider clinical use. Most of the recent phytochemical investigations have been performed for individual herbs rather than multiherb remedies. Efficiently run investigations of traditional remedies require the ability to rapidly analyze chemicals present in monoherb as well as in more complex multiherb extracts, and LC-hyphenated techniques used in a complementary manner allow for an efficient analysis for such purposes. It should be noted that successful chemical profiling of herbal extracts also lies on an informative database search.

In summary, a combination of LC–UV–MS, LC–MS–MS, and LC–NMR was applied to rapid chemical profiling of herbal extracts, which provided information about the nature and content of eight main water-soluble constituents in crude extracts of banxia and their presence in various Xiaochaihu-tang products, including both the known banxia compounds tyrosine (P2), guanosine (P5), and phenylalanine (P6) and the newly identified banxia compounds cytidine (P1), uridine (P3), adenine (P4), adenosine (P7), and tryptophan (P8). All these compounds were identified for Xiaochaihu-tang for the first time. The chemical profiling was initiated by the LC–UV-based detection of main constituents present in the aqueous banxia extracts. LC combined with mass spectrometry played an important role in the identification and quantification of the targeted banxia constituents specified by the initial detection, but LC–MS and LC–MS–MS alone does not allow unequivocal structure confirmation. Thus, we turned to NMR spectroscopy to provide conclusive structural information. The use of LC–NMR technique minimized our effort lost in the isolation of the compounds P2, P3, P4, P5, and P6, which is what one needs when analyzing multiple chemicals from complex herb extracts. However, the usefulness of LC–NMR was limited by the inherent insensitivity of NMR spectroscopy, the dilution of the isolated analyte in the post-column plumbing, and the low availability of the isolated analyte in the NMR detection cell. Due to this reason and the relative low concentrations present in the banxia extract, we failed to obtain discriminable ^{1}H LC–NMR spectra for P1, P7, and P8. Instead, we used a traditional method involving HPLC isolation and purification to obtain the ^{1}H NMR data of these compounds. Recently, Sharman and Jones gave a detailed discussion on the factors principally responsible for the difficulties in LC–NMR spectroscopy in pharmaceutical impurity identification [33]. One of the most significant advances in NMR spectroscopy is cryogenic cooling of the NMR radio frequency coils and electronics to give increased sensitivity [34]. On-line sample concentration is another alternative for resolving the sensitivity problem of LC–NMR. Exarchou et al. demonstrated the usefulness of on-line solid-phase extraction in LC–NMR for peak storage after the LC separation prior to NMR analysis [35]. Constituents present in an herb remedy often include completely unknown, partially unknown, and known chemicals. To avoid paying the same amounts of effort for the identification of partially unknown or known molecules as that for unknown compounds, the use of LC-hyphenated techniques to profile the chemicals present in herbal extracts aims to perform a overall more efficient and intelligent study by minimizing the effort lost in compound isolation and purification.

Acknowledgements

This study was supported by the Grant 30271596 of the National Natural Science Foundation of China, Grants 01DJ19008 and 04DZ19215 of the Science & Technology Commission of Shanghai Municipality, and Grants 2004CB720305 and 2005CB523403 of the Chinese Ministry of Science and Technology. The authors thank Y.-L. He, J.-P. Li, W. Cheng, and L. Maljer for their assistant.

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