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Identification and determination of major constituents in a traditional Chinese medicine compound recipe Xiongdankaiming tablet using HPLC-PDA/ESI-MS^n and HPLC-UV/ELSD*#

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Abstract: Xiongdankaiming tablet (XDKMT), a well-known compound in traditional Chinese medicine, is widely used for the treatment of acute iridocyclitis and primary open-angle glaucoma. In this paper, accurate and reliable methods were developed for the identification of 20 constituents using high-performance liquid chromatography with photo-diode array and electron spray ionization-mass spectrometry (HPLC-PDA/ESI-MS^n), and determination of nine of the constituents (chlorogenic acid, gentiopicroside, isochlorogenic acid B, diosmetin-7-*O*-β-D-glucopyranoside, apigenin, diosmetin, tauroursodeoxycholic acid, acacetin, and taurochenodeoxycholic acid) was developed using HPLC with ultraviolet absorption detector and evaporative light scattering detector (HPLC-UV/ELSD) for the first time. The best results were obtained on a Zorbax SB-C18 column with gradient elution using water (0.1% formic acid) (A) and methanol (0.1% formic acid) (B) at a flow rate of 0.7 ml/min. Tauroursodeoxycholic acid and taurochenodeoxycholic acid, owing to their low UV absorption, were detected by ELSD. The other seven compounds were analyzed by HPLC-UV with variable wavelengths. The calibration curves of all nine constituents showed good linear regression (R^2>0.9996) within the linearity ranges. The limits of detection and quantification were in the ranges of 0.0460–9.90 μg/ml and 0.115–24.8 μg/ml, respectively. The accuracy, in terms of recovery, varied from 95.3% to 104.9% with relative standard deviations (RSDs) less than 4.4%. Precision (with the intra- and inter-day variations less than 4.4%) was also suitable for its intended use. The developed method was successfully applied for the analysis of major components in XDKMT, which provides an appropriate method for the quality control of XDKMT.

Key words: Traditional Chinese medicine (TCM) compound recipe, Xiongdankaiming tablet, HPLC-UV/ELSD, HPLC-MS, Quality control

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1 Introduction

Traditional Chinese medicines (TCMs) and their preparations have been widely used whether in the field of treatment or health care in China for a long time. Such good effects may be attributed to obtaining synergistic effects or diminishing potential adverse reactions among dozens of chemical compounds in TCMs (Li *et al*., 2011). As for preparations, TCMs are usually composed of several crude drugs, which make these mixtures become more complex. It is challenging and valuable to cover most of the "markers" in quality control of TCMs (Li *et al*., 2011). More comprehensive strategies are urgently required for the determination of their chemical markers and quality control of a complex TCM system.

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Xiongdankaiming tablet (XDKMT), a well-known compound recipe of TCM, is usually used in clinical treatment for acute iridocyclitis and primary open-angle glaucoma. Other clinical uses of XDKMT, including an analgesic (Zhao *et al*., 1999), prevention of the postoperative complication of phacoemulsification and intraocular lens implantation (Liu *et al*., 2005), lowering intraocular pressure (Zhao and Qi, 2007), and relieving symptoms of visual fatigue caused by hepatobiliary swelter among young people (Hong *et al*., 2008), have also been reported. XDKMT is composed of seven medicinal materials or extracts, including bear bile powder, *Haliotidis concha*, *Chrysanthemi flos*, *Gentianae radix et rhizoma*, *Lycii fructus*, *Alismatis rhizoma*, and *Leonuri fructus*. The seven medicinal materials or extracts are used according to the theory of TCM of providing better efficacy through synergistic interaction and causing less severe side effects. However, it is uncertain which components are actually responsible for the therapeutic effects. Therefore, we identified the chemical constituents of XDKMT, which is the basis of investigating the mechanism of XDKMT and monitoring its quality. The pharmacologically active ingredient of XDKMT remains to be further studied.

The chemical constituents for the seven single medicinal materials or extracts have been reported. The main effective components include bile acids (e.g., tauroursodesoxycholic acid and taurochenodesoxycholic acid) (Xie *et al*., 1981; Cui *et al*., 2006; Gong *et al*., 2007), calcium carbonate, flavonoids (diosmetin-7-*O*-β-D-glucopyranoside, apigenin, diosmetin, acacetin, etc.), organic acids (e.g., chlorogenic acid) (Miyazawa and Hisama, 2003; Kim *et al*., 2003; Cheng *et al*., 2005), iridoids (e.g., gentiopicroside) (Kondo *et al*., 1994; Kumarasamy *et al*., 2003), terpenoids (e.g., alisol A, alisol B 23-acetate, and alisol C), and amino acid derivatives (e.g., betaine and stachydrine) (Yin *et al*., 2010). However, to our knowledge no investigation on the chemical constituents of XDKMT has been reported. Whether the constituents from the single medical materials are in fact the ones which make up XDKMT is still unknown.

There have been some reports on the determination of several effective components in the medicinal materials by high-performance liquid chromatography with evaporative light scattering detector (HPLC-ELSD), HPLC with ultraviolet absorption detector (HPLC-UV), or capillary electrophoresis (CE) methods (Zhao *et al*., 2006; Gao *et al*., 2007; Zhang *et al*., 2008; Wang, 2010; Cao and Wang, 2010; Qin and Wen, 2011). However, the combination of medicinal materials increased the complexity of constituents, so the reported methods could not be used for the analysis of XDKMT.

Until now, no method has been developed for the analysis of XDKMT. In this article, an HPLC with photo-diode array and electron spray ionization-mass spectrometry (HPLC-PDA/ESI-MS^n) method for the identification of 20 constituents in XDKMT was developed. These compounds have covered almost all the principal types of ingredients in the seven medicinal materials or extracts of XDKMT except calcium carbonate for *Haliotidis concha*. The content of calcium carbonate in *Haliotidis concha* should not be less than 93.0% according to the *Chinese Pharmacopoeia* (2010 Edition). Calcium carbonate is usually analyzed by titrimetry, and was not analyzed in this study. Moreover, an HPLC-UV/ELSD method was developed for quantitative determination of nine of the ingredients, including chlorogenic acid, gentiopicroside, isochlorogenic acid B, diosmetin-7-*O*-β-D-glucopyranoside, apigenin, diosmetin, tauroursodesoxycholic acid, acacetin, and taurochenodeoxycholic acid. This study represents a detailed investigation of the components in XDKMT for the first time and provides an applicable method for its quality control.

2 Materials and methods

2.1 Apparatus and chromatographic conditions

2.1.1 HPLC-UV/ELSD analysis

The analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies, USA), equipped with a quaternary pump, an on-line degasser, an auto-sampler, a column oven, and an ultraviolet detector coupled with a ChemStation Software Version B.04.01 (Agilent Technologies, USA). An Alltech ELSD-2000ES instrument as well as an Allchrom Plus Client/Server data operator (Alltech, USA) was connected to the liquid chromatography for detection of non-chromophoric substances.

The separation was carried out on a Zorbax

SB-C18 column (4.6 mm×250 mm, 5 μm; Agilent Technologies, USA) and temperature was set at 39 °C. The mobile phase consisted of water (0.1% formic acid) (A) and methanol (0.1% formic acid) (B) at a flow rate of 0.7 ml/min, using the following gradient elution: 0–50 min (3%–58% B), 50–80 min (58%–91% B), 80–90 min (91%–100% B). There was a 5-min wash with 100% B after each run and equilibrium time was 15 min. The injection volume was 20 μl. Based on variable wavelength detector (VWD), different detection wavelengths were set for different periods of time: 330 nm for 0–28 min, 280 nm for 28–33 min, or 340 nm for 33–95 min.

The ELSD was set up to a drift tube temperature of 100 °C at gain 1 and the N_2 flow rate was 2 L/min.

2.1.2 HPLC-PDA/ESI-MS^n analysis

The analysis was performed on a Finnigan LCQ Deca XP^{plus} ion trap mass spectrometer (Thermo Finnigan, USA) connected to the Agilent 1100 HPLC instrument via an electron spray ionization (ESI) interface. The chromatographic conditions were the same as described in Section 2.1.1. The MS spectra were acquired in both negative and positive modes and full scan mass spectra were acquired from the mass-to-charge ratio (*m*/*z*) of 100–1000. Parameters for MS were as follows: collision gas, ultrahigh-purity helium (He); nebulizing gas, high-purity nitrogen (N_2); sheath gas (N_2) and auxiliary gas (N_2) at a flow rate of 60 and 20 arbitrary units (AU), respectively; ion spray voltage −4.5 kV; capillary temperature 350 °C; capillary voltages in positive and negative modes 19 and −15 V, respectively; and, tube lens offset voltages 25 and −30 V, respectively.

2.2 Reagents, chemicals, and materials

Deionized water was purified by the Milli-Q System (Millipore, USA) for HPLC assay. Methanol (Merck, Germany) and formic acid (ROE SCIENTIFIC Inc., USA) were both of HPLC grade.

The reference standards are: stachydrine hydrochloride (**2**; batch No. 32650), chlorogenic acid (**3**; batch No. 39350), gentiopicroside (**4**; batch No. 101226), luteolin-7-*O*-β-D-glucoside (**5**; batch No. 110426), isochlorogenic acid B (**6**; batch No. 110317), cosmosiin (**7**; batch No. 101220), diosmetin-7-*O*-β-D-glucoside (**8**; batch No. 101207), luteolin (**9**; batch No. 110403), trifloroside (**10**), apigenin (**11**; batch No. 101207), diosmetin (**12**; batch No. 110405), (1*S*, 5*R*, 9*R*)-scabraside (**13**), (1*S*, 5*R*, 9*R*)-deglucosyltrifloroside (**14**), sodium tauroursodeoxycholate (**15**; batch No. 110816-200507), acacetin (**16**; batch No. 110330), alisol C (**17**), taurochenodeoxycholic acid (**18**; batch No. 110846-200506), alisol A (**19**), and alisol B-23-acetate (**20**; batch No. 110713). Compounds **2** and **3** were purchased from Aladdin reagent database Inc. (Shanghai, China). Compounds **15** and **18** were from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Compounds **4**, **5**, **6**, **7**, **8**, **9**, **11**, **12**, **16**, and **20** were provided by Shanghai Winherb Medical S&T Development Co. Ltd. (China). Compounds **10**, **13**, **14**, **17**, **19** were respectively isolated from *Gentianae radix et rhizoma* and *Alismatis rhizoma* in our laboratory with the purity higher than 98%. All these five compounds were identified by ESI-MS, proton nuclear magnetic resonance (^{1}H-NMR), and ^{13}C-NMR. The absolute configurations of compounds **13** and **14** were reported in Wang *et al*. (2013). The chemical structures of the 20 analytes are shown in Fig. 1.

XDKMTs (Xiuzheng Pharmaceutical Group, batch Nos. 110103, 110601, and 120102) were provided by the manufacturer.

2.3 Preparation of standard solutions

A mixed standard stock solution containing compounds **3**, **4**, **6**, **8**, **11**, **12**, **15**, **16**, and **18** were prepared in 50% methanol. The working standard solutions were obtained by diluting the mixed standard stock solution with 50% methanol to a series of appropriate concentrations for the calibration curves. Tauroursodeoxycholic acid and taurochenodeoxycholic acid were analyzed by an HPLC-ELSD method and the other seven analytes were analyzed by an HPLC-UV method with variable wavelengths. The standard stock and working solutions were all stored at 4 °C until use and centrifuged prior to injection.

2.4 Preparation of sample solutions

Twenty tablets were accurately weighed and finely powdered after removing the coating. The powder of XDKMT (0.5 g) was weighed accurately into a 25-ml volumetric flask, and 50% methanol was added to the volume mark. After accurately weighing, the mixed solution was ultrasonicated (40 kHz) at room temperature for 40 min. After that, the same

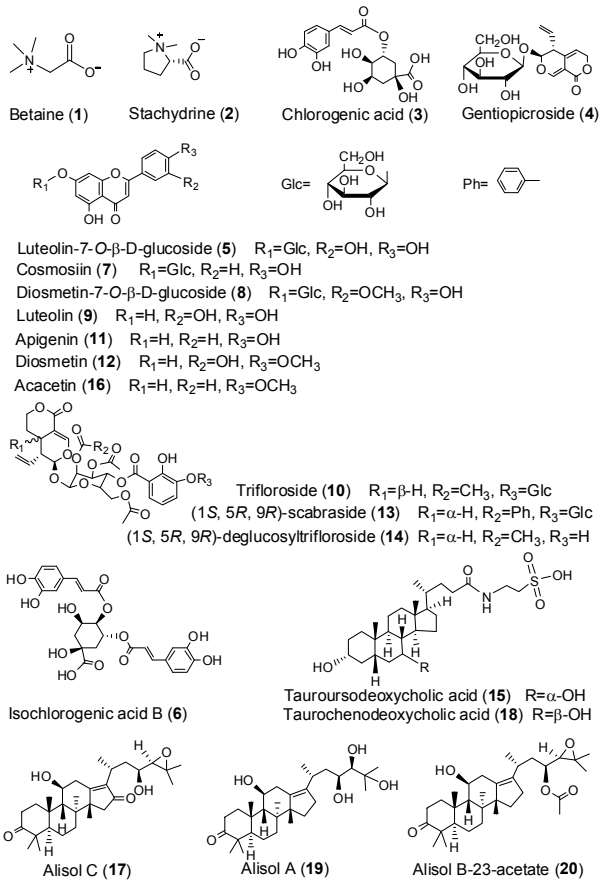

Fig. 1 Structures of the 20 compounds identified in Xiongdankaiming tablets

solvent was added to compensate for the lost weight during the extraction. The extraction was centrifuged at 12000 r/min for 10 min. The supernatant was prepared for injection.

For MS analysis, the pretreatment of sample was the same as described above. To get higher detection sensitivity, the supernatant was concentrated to 5 ml by vacuum evaporation process. A 1.0-g aliquot of powder of bear bile powder, *Chrysanthemi flos*, *Gentianae radix et rhizoma*, *Lycii fructus*, *Alismatis rhizoma*, and *Leonuri fructus* were separately sonicated in 10 ml of 80% methanol for 60 min followed by centrifugation at 12000 r/min for 10 min. The supernatant was prepared for analysis.

2.5 Validation of the HPLC method

2.5.1 Calibration curves and limits of quantification and detection

The working standard solutions were first restored to room temperature, and then eight concentrations were analyzed by plotting the peak area versus concentration for LC-UV. For ELSD, the response is usually a non-linear function, so it was calculated by the double logarithmic plots of the peak area versus the concentration of the reference solution injected. The limit of quantification (LOQ) and the limit of detection (LOD) were determined by diluting the standard solution progressively until the signal-to-noise ratios (S/N) of analytes were approximately 10 and 3, respectively.

2.5.2 Precision, stability, and accuracy

The precision of the developed method was determined by intra- and inter-day variability tests, which were investigated by determining the nine analytes for six parallel samples during one day and by repeating the experiments on six consecutive days, respectively. Variations of the concentrations of analytes were taken as the measure of precision and delivered as percentage relative standard deviations (RSDs). The sample solution was injected into the apparatus at 0, 4, 8, 12, 16, 20, and 24 h, respectively, to evaluate the stability of the solution. Recovery test was used to evaluate the accuracy of the method, which was performed by adding accurate amounts (high, medium, and low levels) of the nine standards into 0.25 g of XDKMT powder separately with three replicates at each level. The spiked samples were then extracted according to the method mentioned in Section 2.4, and quantified under the condition in Section 2.1.

3 Results and discussion

3.1 Optimization of sample preparation procedure

To achieve maximum extraction efficiency of the nine compounds, four important factors, namely, extraction solvents, the amount of extraction solvent, extraction time, and extraction number of times were investigated independently. XDKMT was extracted with different levels of each factor including extraction solvent, the amount of extraction solvent, extraction time, and extraction number of times, that were investigated individually by one-factor experimental design. The results revealed that for extraction solvents, methanol had significantly better extraction efficiency than ethanol and acetonitrile. Furthermore,

50% methanol reached the highest point of the extraction efficiency. Thus, 50% methanol was chosen as the suitable extraction solvent. Moreover, using 25 ml 50% methanol and 40 min extracting for one time was found to be adequate for the analysis. We decided that ultrasonic extraction for 40 min by 25 ml 50% methanol was appropriate for the analysis. Optimization process can be seen in the supplementary material.

3.2 Optimization of the HPLC chromatographic conditions

To optimize the condition of separation, different reverse-phase HPLC columns were compared to separate the target compounds, such as Zorbax SB-C18 (4.6 mm×250 mm, 5 μm) and Zorbax Eclipse Plus C18 (4.6 mm×100 mm, 1.8 μm). The latter had a shorter analysis time but a poor resolution. The better separation efficiency and peak shape were achieved by the Zorbax SB-C18. As for the mobile phase, we tested acetonitrile and methanol with different modifiers, such as formic acid and acetic acid. It was found that using methanol and water, both containing 0.1% formic acid achieved the optimal chromatographic conditions. Methanol can simultaneously separate more compounds in the samples than acetonitrile. And formic acid was used to improve the peaks shape and separation, which was also compatible to ELSD and MS detectors. Column temperature had a great effect on the resolution. Temperatures between 20 and 40 °C were tested, and 39 °C was deemed appropriate. Flow rate was also optimized. We found that under the flow rate of 0.7 ml/min, the nine marker constituents were successfully separated. To make each compound to achieve the maximum absorption wavelength, different detection wavelengths were set for different durations: 330 nm for 0–28 min (compound **3**); 280 nm for 28–33 min (compound **4**); 340 nm for 33–95 min (compounds **6**, **8**, **11**, **12**, and **16**).

Compounds **15** and **18** were detected by ELSD. As for ELSD, the drift tube temperature, flow rate of nebulizing gas, and detector gain are crucial to improve the S/N ratio. HPLC-ELSD analysis of the sample solution at different drift tube temperatures (110, 100, 90 °C), different gas flow rates (2.5, 2.0, 1.5 L/min), and different detector gains (3, 2, 1) were performed respectively, and optimal parameters were then selected for obtaining the optimal sensitivity. We found that when the drift tube temperature was set to 100 °C at gain 1 and the nitrogen flow rate was 2 L/min, S/N ratios achieved the maximum values.

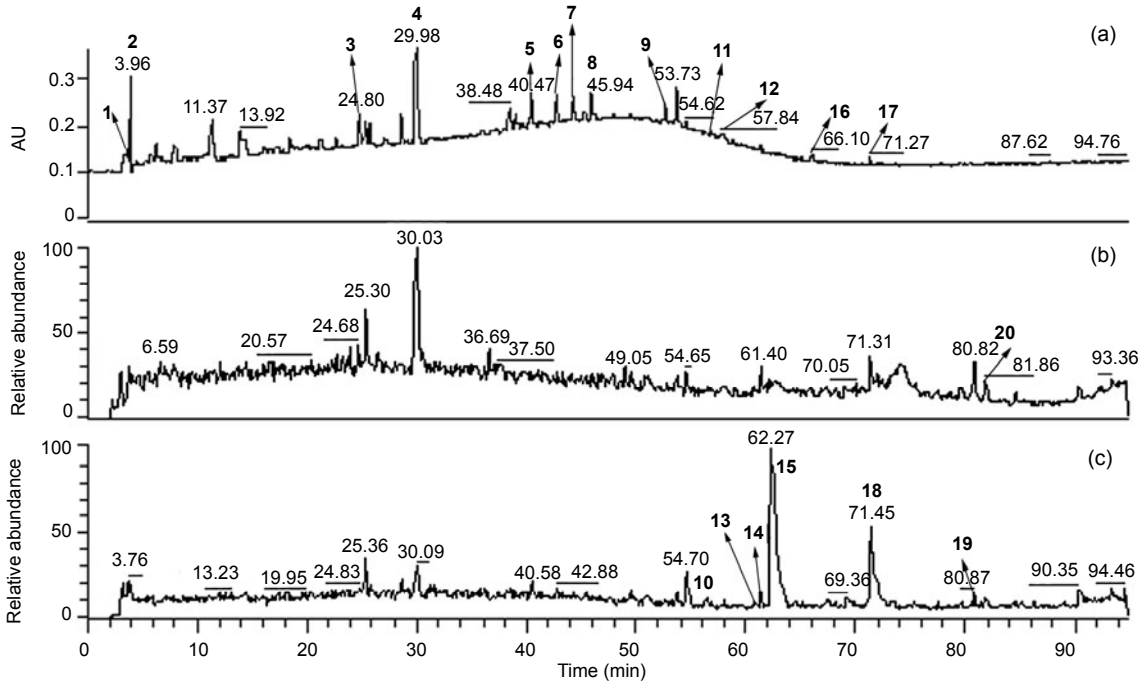

Fig. 2 Total scan PDA chromatogram (a), total ion chromatograms (TIC) in positive mode (b) and negative mode (c) of a Xiongdankaiming tablet sample (numbers of the compounds were the same as those in Fig. 1)

3.3 Identification of the 20 constituents in XDKMT by HPLC-PDA/ESI-MS^n

The sample of XDKMT was analyzed by HPLC-PDA/ESI-MS^n in both negative and positive ionization modes. The total scan PDA chromatograms and total ion chromatograms (TIC) are shown in Fig. 2, and the mass spectra of the 20 compounds are in the supplementary material. By comparing the retention time, UV spectra and MS^n spectra of peaks in sample chromatogram with those of reference standards, 20 peaks were unambiguously identified as stachydrine (**2**), chlorogenic acid (**3**), gentiopicroside (**4**), luteolin-7-*O*-β-D-glucoside (**5**), isochlorogenic acid B (**6**), cosmosiin (**7**), diosmetin-7-*O*-β-D-glucoside (**8**), luteolin (**9**), trifloroside (**10**), apigenin (**11**), diosmetin (**12**), (1*S*, 5*R*, 9*R*)-scabraside (**13**), (1*S*, 5*R*, 9*R*)-deglucosyltrifloroside (**14**), tauroursodeoxycholic acid (**15**), acacetin (**16**), alisol C (**17**), taurochenodeoxycholic acid (**18**), alisol A (**19**), and alisol B-23-acetate (**20**). As for peak **1**, the quasi-molecular ions was 118 $[M+H]^+$ and dimer 235 $[2M+H]^+$. The quasi-molecular ion $[M+H]^+$ was odd number, which indicated that the chemical structure should contain one nitrogen atom. The fragment ions of 59 $[M+H-N(CH_3)_3]^+$ and 58 $[M+H-HCOOH]^+$ were observed in the MS^2 data. The MS data of peak **1** were consistent with those of Wang *et al*. (2011), so it was deduced to be betaine (compound **1**). The other peaks were not identified yet without accurate molecular weight data or reference compounds.

It is useful to observe the fragmentation patterns in the mass spectrum for the characterization of compounds. The quasi-molecular ions and characteristic fragment ions for the 20 compounds are all listed in Table 1.

For two amino acid derivatives, compounds **1** and **2**, the quasi-molecular ions were both $[M+H]^+$ and dimer $[2M+H]^+$. The $[M+H]^+$ ions of compounds **1** and **2** produced fragment ions of 58 and 84 after the loss of acetic acid, respectively. The fragment ion of $[M+H-HCOOH]^+$ was observed in the MS^2 data of compound **2** due to the side chain formyl group in the chemical structure.

The quasi-molecular ion 353 $[M-H]^-$ of peak **3** produced the fragment ions of 191 $[M-H-C_9H_6O_3]^-$, 179 $[M-H-C_7H_{10}O_5]^-$, 173 $[M-H-C_9H_6O_3-H_2O]^-$ after loss of caffeoyl group, quininyl group, or H_2O. The MS^2 data of peak **6**, 353 $[M-H-C_9H_6O_3]^-$ indicated that two caffeoyl groups exist in the chemical structure. The characteristic fragment ions of 191 $[M-H-2C_9H_6O_3]^-$, 179 $[M-H-C_9H_6O_3-C_7H_{10}O_5]^-$, and 173 $[M-H-2C_9H_6O_3-H_2O]^-$ were also observed.

Though compounds **4**, **10**, **13**, and **14** are all iridoid glycosides, the quasi-molecular ion of them were different. In the negative mode, the quasi-molecular ion of peak **4** was $[M+HCOO^-]^-$, but those of the other three peaks were all $[M-H]^-$. The fragment ion of $[M-H-C_2H_2O]^-$ was observed in the MS^2 data of peaks **10**, **13**, and **14** due to the existing of the acetyl group in the chemical structures. In addition, the fragment ion of $[M-H-Glc]^-$ was observed in MS^2 data of peaks **10** and **13**, while it was not found in those of peaks **4** and **14**. This indicated that the glucoside of R_3 was easy to lose.

For the flavonoid glycosides, compounds **5**, **7**, and **8**, $[M+H-Glc]^+$ or $[M-H-Glc]^-$ was observed in the MS^2 data of them. The fragment of $[M+H-CH_3]^+$ or $[M-H-CH_3]^-$ was observed for the flavonoid containing methoxy group, for example, peaks **12**, **16**, and **8**. The fragment ions of $[M-H-CO_2]^-$ in the MS^2 data of peaks **9** and **11** could be formed from the neutral loss of CO_2 on C-ring.

Compounds **15** and **18** are epimers. They yielded the same quasi-molecular ions and fragment ions but different retention time at 62.4 and 71.4 min, respectively, so it was easy to distinguish these two peaks.

Compounds **17**, **19**, and **20** are protostane type triterpenoids. The quasi-molecular ions and fragment ions formed from the loss of one or two molecules H_2O. Another significant feature was the fragment ions form the loss of C_4H_8O or C_3H_6O at the alkly chain.

Furthermore, six medicinal materials or extracts, including bear bile powder, *Chrysanthemi flos*, *Gentianae radix et rhizoma*, *Lycii fructus*, *Alismatis rhizoma*, and *Leonuri fructus* were also analyzed by HPLC-PDA/ESI-MS^n in both negative and positive ionization modes to confirm the attribution of the 20 compounds. As shown in Table 1, compound **1** was from *Lycii fructus*; compound **2** belonged to *Leonuri fructus*; compounds **3**, **5**, **6**, **7**, **8**, **9**, **11**, **12**, and **16** were derived from *Chrysanthemi flos*; compounds **4**, **10**, **13**, and **14** came from *Gentianae radix et rhizoma*; compounds **17**, **19**, and **20** were from *Alismatis rhizom*; and, compounds **15** and **18** were from bear bile powder.

Table 1 Identification of the twenty chemical constituents in XDKMT

No.	t_R (min)	MS (*m*/*z*)		MS^2 (*m*/*z*)	MS^3 (*m*/*z*)	Identification	Plant source
		ESI^+	ESI^-				
1	3.5	118 (59) $[M+H]^+$ 235 (100) $[2M+H]^+$	–	118@40: 59 (100) $[M+H-N(CH_3)_3]^+$ 58 (86) $[M+H-HOAc]^+$	–	Betaine	a
2	4.0	144 (100) $[M+H]^+$ 287 (87) $[2M+H]^+$	–	144@40: 98 (32) $[M+H-HCOOH]^+$ 84 (100) $[M+H-HOAc]^+$ 58 (53) $[M+H-C_4H_6O_2]^+$	–	Stachydrine	e
3	24.8	–	353 (74) $[M-H]^-$ 399 (53) $[M+HCOO^-]^-$ 707 (100) $[2M-H]^-$	353@25: 191 (100) $[M-H-C_9H_6O_3]^-$ 179 (6) $[M-H-C_7H_{10}O_5]^-$	353@25→191@35: 173 (34) $[M-H-C_9H_6O_3-H_2O]^-$ 127 (46) $[M-H-C_9H_6O_3-H_2O-HCOOH]^-$ 85 (44) $[M-H-C_9H_6O_3-H_2O-HCOOH-C_2H_2O]^-$	Chlorogenic acid	b
4	30.1	357 (5) $[M+H]^+$	401 (100) $[M+HCOO^-]^-$	401@30: 355 (61) $[M-H]^-$ 225 (28) $[M+HCOO^--C_{10}H_8O_3]^-$ 179 (100) $[M-H-C_{10}H_8O_3]^-$	401@30→179@20: 161 (84) $[M-H-C_{10}H_8O_3-H_2O]^-$	Gentiopicroside	c
5	40.6	449 (100) $[M+H]^+$	447 (85) $[M-H]^-$	449@35: 287 (100) $[M+H-Glc]^+$ 447@30: 285 (100) $[M-H-Glc]^-$	447@30→285@48: 241 (63) $[M-H-Glc-CO_2]^-$	Luteolin-7-*O*-β-D-glucoside	b
6	42.9	–	515 (100) $[M-H]^-$	515@30: 353 (100) $[M-H-C_9H_6O_3]^-$	515@30→353@35: 191 (32) $[M-H-2C_9H_6O_3]^-$ 179 (100) $[M-H-C_9H_6O_3-C_7H_{10}O_5]^-$ 173 (79) $[M-H-2C_9H_6O_3-H_2O]^-$	Isochlorogenic acid B	b
7	44.4	433 (100) $[M+H]^+$	431 (100) $[M-H]^-$ 863 (80) $[2M-H]^-$	433@25: 271 (100) $[M+H-Glc]^+$ 431@35: 269 (100) $[M-H-Glc]^-$	433@25→271@43: 225 (8) $[M+H-Glc-H_2O-CO]^+$ 153 (15) $[M+H-Glc-H_2O-2CO-CO_2]^+$	Cosmosiin	b
8	46.0	463 (100) $[M+H]^+$	–	463@25: 301 (100) $[M+H-Glc]^+$	463@25→301@38: 286 (71) $[M+H-Glc-CH_3]^+$	Diosmetin-7-*O*-β-D-glucoside	b
9	52.8	–	285 (100) $[M-H]^-$	285@50: 241 (50) $[M-H-CO_2]^-$ 175 (32) $[M-H-C_3O_2-C_2H_2O]^-$	285@50→241@55: 213 (77) $[M-H-CO_2-CO]^-$ 197 (88) $[M-H-2CO_2]^-$	Luteolin	b
10	54.7	–	781 (100) $[M-H]^-$	781@35: 739 (69) $[M-H-C_2H_2O]^-$ 619 (100) $[M-H-Glc]^-$ 577 (41) $[M-H-C_2H_2O-Glc]^-$	781@35→739@40: 697 (70) $[M-H-2C_2H_2O]^-$	Trifloroside	c
11	57.0	271 (100) $[M+H]^+$	269 (100) $[M-H]^-$ 315 (31) $[M+HCOO^-]^-$	269@50: 225 (100) $[M-H-CO_2]^-$	269@50→225@55: 197 (47) $[M-H-CO_2-CO]^-$ 181 (100) $[M-H-2CO_2]^-$	Apigenin	b
12	57.9	301 (100) $[M+H]^+$	–	301@40: 286 $[M+H-CH_3]^+$	301@40→286@45: 258 (100) $[M+H-CH_3-CO]^+$	Diosmetin	b
13	60.9	–	843 (100) $[M-H]^-$	843@35: 801 (56) $[M-H-C_2H_2O]^-$ 681 (100) $[M-H-Glc]^-$ 639 (27) $[M-H-C_2H_2O-Glc]^-$	843@35→681@40: 639 (100) $[M-H-Glc-C_2H_2O]^-$ 621 (16) $[M-H-Glc-C_2H_2O-H_2O]^-$	(1*S*, 5*R*, 9*R*)-scabraside	c
14	61.4	621 (74) $[M+H]^+$ 643 (100) $[M+Na]^+$	619 (100) $[M-H]^-$	619@35: 577 (100) $[M-H-C_2H_2O]^-$	619@35→577@40: 535 (100) $[M-H-2C_2H_2O]^-$	(1*S*, 5*R*, 9*R*)-deglucosyl-trifloroside	c

To be continued

Table 1

No.	t_R (min)	MS (*m*/*z*)		MS^2 (*m*/*z*)	MS^3 (*m*/*z*)	Identification	Plant source
		ESI^+	ESI^-				
15	62.4	–	498 (100) $[M-H]^-$ 997 (67) $[2M-H]^-$	498@55: 480 (52) $[M-H-H_2O]^-$ 432 (100) $[M-H-H_2SO_2]^-$ 414 (43) $[M-H-H_2SO_2-H_2O]^-$	–	Taurourso-deoxycholic acid	f
16	66.1	285 (100) $[M+H]^+$	283 (100) $[M-H]^-$ 329 (55) $[M+HCOO^-]^-$	285@45: 270 (100) $[M+H-CH_3]^+$ 283@45: 268 (100) $[M-H-CH_3]^-$	285@45→270@50: 242 (100) $[M+H-CH_3-CO]^+$	Acacetin	b
17	71.3	487 (38) $[M+H]^+$ 995 (100) $[2M+Na]^+$	–	487@25: 469 (99) $[M+H-H_2O]^+$ 451 (37) $[M+H-2H_2O]^+$ 397 (100) $[M+H-H_2O-C_4H_8O]^+$	487@25→469@30: 451 (98) $[M+H-2H_2O]^+$ 397 (100) $[M+H-H_2O-C_4H_8O]^+$	Alisol C	d
18	71.4	–	498 (100) $[M-H]^-$ 997 (48) $[2M-H]^-$	498@55: 480 (70) $[M-H-H_2O]^-$ 432 (69) $[M-H-H_2SO_2]^-$ 414 (100) $[M-H-H_2SO_2-H_2O]^-$	–	Taurocheno-deoxycholic acid	f
19	80.9	–	489 (6) $[M-H]^-$ 535 (100) $[M+HCOO^-]^-$	535@25: 471 (100) $[M-H-H_2O]^-$	535@25→471@30: 413 (32) $[M-H-H_2O-C_3H_6O]^-$ 395 (89) $[M-H-H_2O-C_3H_6O-H_2O]^-$ 339 (100) $[M-H-H_2O-C_3H_6O-H_2O-C_3H_4O]^-$	Alisol A	d
20	82.0	515 (100) $[M+H]^+$ 497 (18) $[M+H-H_2O]^+$	–	515@25: 497 (29) $[M+H-H_2O]^+$ 437 (75) $[M+H-H_2O-HOAc]^+$ 419 (29) $[M+H-2H_2O-HOAc]^+$ 383 (81) $[M+H-C_4H_8O-HOAc]^+$ 365 (100) $[M+H-H_2O-C_4H_8O-HOAc]^+$	515@25→383@30: 365 (100) $[M+H-H_2O-C_4H_8O-HOAc]^+$	Alisol B-23-acetate	d

Plant source: a, *Lycii fructus*; b, *Chrysanthemi flos*; c, *Gentianae radix et rhizoma*; d, *Alismatis rhizoma*; e, *Leonuri fructus*; f, bear bile powder; –. No MS response; t_R: retention time

3.4 Validation of the HPLC method

3.4.1 Linearity, LOD, and LOQ

As shown in Table 2, all calibration curves of the nine compounds showed good linear regression (R^2>0.9996) within the test ranges. The LODs and LOQs were in the range of 0.0460–9.90 and 0.115–24.8 μg/ml, respectively.

3.4.2 Accuracy, precision, and stability

The accuracy, in terms of recovery, was from 95.3% to 104.9% with RSD less than 4.4%. The intra- and inter-day variations that were to determine the precision of the method were less than 4.4%. For the stability test, the same sample solution was analyzed every 4 h in a day (n=7). The RSD values of the peak area and retention time were less than 3.8% and 0.1%, respectively. The solution was therefore proved to be stable within 24 h.

3.5 Quantitative determination of major constituents in XDKMT by HPLC-UV/ELSD

The proposed HPLC-UV/ELSD method was subsequently applied to simultaneous determination of nine components including chlorogenic acid, gentiopicroside, isochlorogenic acid B, diosmetin-7-*O*-β-D-glucopyranoside, apigenin, diosmetin, tauroursodeoxycholate acid, acacetin, and taurochenodeoxycholic acid in XDKMT of three batches. The HPLC-UV/ELSD chromatograms of the standard mixture and sample are presented in Fig. 3. In addition, the peak purity of nine compounds was proved using HPLC-PDA/ESI-MS^n. The contents of the nine components are listed in Table 3. It can be seen from the average contents that gentiopicroside (0.800–1.60 mg/tablet), tauroursodeoxycholic acid (1.61–1.76 mg/tablet), and taurochenodeoxycholic acid (0.864–0.992 mg/tablet) were the predominant

Table 2 Linearity, limits of detection (LOD), and limits of quantitation (LOQ) of nine compounds by HPLC-UV/ELSD method

Compound	Calibration curve[a]	R^2	Linear range (μg/ml)	LOQ[b] (μg/ml)	LOD[c] (μg/ml)
3. Chlorogenic acid	y=84.6944x−20.3658	0.9999	0.644–103	0.129	0.0516
4. Gentiopicroside	y=36.4927x+2.5304	0.9999	0.919–147	0.149	0.0596
6. Isochlorogenic acid B	y=89.5979x−47.1398	0.9999	0.625–100	0.186	0.0744
8. Diosmetin-7-*O*-β-D-glucopyranoside	y=57.9145x−2.3414	0.9998	0.323–51.6	0.190	0.0760
11. Apigenin	y=124.4984x−27.0302	0.9998	0.212–34.0	0.130	0.0520
12. Diosmetin	y=120.2592x−17.9481	0.9999	0.223–35.7	0.115	0.0460
15. Tauroursodeoxycholic acid	Y=1.5284X+3.0893	1.0000	39.5–158	20.0	7.98
16. Acacetin	y=138.2031x−25.7620	0.9999	0.238–38.0	0.180	0.0720
18. Taurochenodeoxycholic acid	Y=1.4636X+3.1675	0.9996	50.5–202	24.8	9.90

[a] y and x stand for peak area and concentration (μg/ml), respectively; Y=lg$y$, X=lg$x$. [b] S/N=3. [c] S/N=10

Table 3 Contents of nine components in three batches of XDKMT

Compound	Batch No. 110103 (n=3)		Batch No. 110601 (n=3)		Batch No. 120102 (n=3)	
	Content (mg/tablet)	RSD (%)	Content (mg/tablet)	RSD (%)	Content (mg/tablet)	RSD (%)
3. Chlorogenic acid	0.1520	0.6	0.2270	0.3	0.2820	0.9
4. Gentiopicroside	1.6000	0.1	1.1400	0.3	0.8000	0.5
6. Isochlorogenic acid B	0.1090	1.6	0.1160	2.1	0.1110	1.7
8. Diosmetin-7-*O*-β-D-glucopyranoside	0.1310	1.0	0.1040	1.6	0.0599	2.3
11. Apigenin	0.0148	2.5	0.0328	1.2	0.0341	2.5
12. Diosmetin	0.0123	2.2	0.0175	3.5	0.0106	2.9
15. Tauroursodeoxycholic acid	1.7600	0.5	1.6200	1.0	1.6100	2.7
16. Acacetin	0.0175	0.6	0.0335	1.6	0.0216	2.1
18. Taurochenodeoxycholic acid	0.9920	0.8	0.8640	2.3	0.8880	2.7

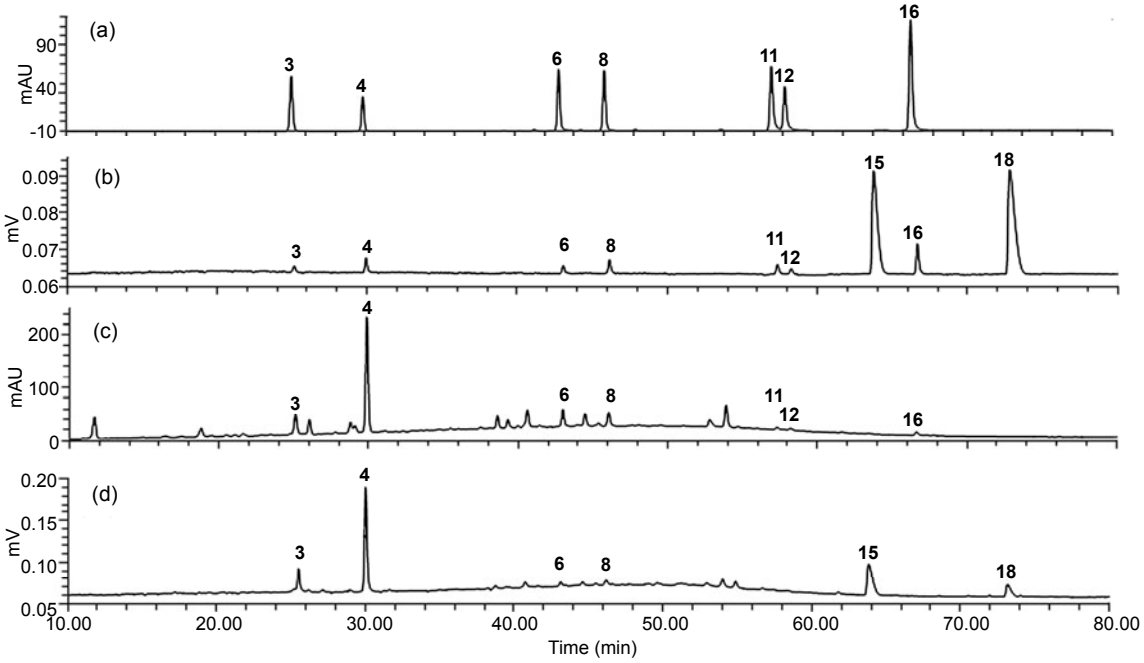

Fig. 3 HPLC chromatograms of standard mixture (a, b) and XDKMT (c, d) by LC-UV/ELSD method
(a, c) refer to UV detection; (b, d) refer to ELSD. The concentrations (μg/ml) of the standards: **3**: 9.42; **4**: 11.9; **6**: 9.42; **8**: 13.3; **11**: 8.51; **12**: 6.19; **15**: 63.3; **16**: 12.7; **18**: 80.7. Numbers of the compounds were the same as those in Fig. 1

components in XDKMT; and, there was much fluctuation in the component contents between different batches. Therefore, it is necessary to monitor the inter-batch variations of components.

4 Conclusions

This is the first report for the identification of 20 constituents by HPLC-PDA/ESI-MS^n and the simultaneous determination of the nine major components in XDKMT using HPLC-UV/ELSD. These compounds have covered almost all the principal types of active ingredients in XDKMT, such as bile acids, calcium carbonate, flavonoids, organic acids, iridoids, terpenoids, and amino acids. Therefore, selecting these active constituents as "markers", we will obtain more comprehensive information for the quality control of XDKMT. The quantitative method has the excellent linearity, precision, stability, and accuracy, and it was proven to be a simple and powerful tool for the quality control of XDKMT.

Compliance with ethics guidelines

Shu-fang WANG, Jing LENG, Yi-min XU, and Mei-ling FENG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

The electronic supplementary materials mainly cover: (1) identification of 20 constituents in XDKMT by HPLC-PDA/ESI-MS^n; (2) optimization of sample preparation procedure; and, (3) optimization of the HPLC chromatographic conditions.

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