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Rapid quantification of iridoid glycosides analogues in the formulated Chinese medicine Longdan Xiegan Decoction using high-performance liquid chromatography coupled with mass spectromentry

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ABSTRACT

Longdan Xiegan Decoction (LXD) is a formulated preparation composed of 10 ingredient herbs, with iridoids as the main bioactive components. In this study, a rapid, simple and reliable method of simultaneous determination of four iridoid glycosides in LXD using high-performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (MS) was first developed and validated. The four iridoid glycosides references were isolated from LXD extract and purified using a preparative HPLC chromatography. The sample preparation for quantification comprised of a simple ultrasonic extraction and the satisfactory chromatographic separation of the four structurally similar iridoid glycosides was effected in less than three minutes on a CAPCELL PAK C_{18} MGII column (3 μm, 100 mm × 2.0 mm), using an elution system of 10% methanol and their concentrations in different batches of LXD and ingredient herbs were simultaneously determined by HPLC–MS/MS using a multiple reaction monitoring (MRM) mode. The method was validated with respect to the overall intra- and inter-day variation (RSD less than 8%) and the limits of quantification for the four iridoid glycosides were 35, 20, 37 and 33 ng/mL, respectively.

1. Introduction

Traditional Chinese Medicines (TCMs) have been practiced in China and other eastern countries over long period of time. Usually, TCMs are administrated in the form of formulated preparations consisting of several even more than 10 ingredient herbs in a certain ratio, which may provide polyvalent biological actions and also reduce the side-effects caused by some toxic or high potent ingredients [1]. With the constant increase in the use of herbal medicines world-wide and the rapid expansion of its global market, the safety and efficacy of TCM herbs and their finished products have become a major concern for health authorities, pharmaceutical industries and the public due to the limited cognition on the bioactive components and the uncertainty of the quality of these herbal products. It is always a challenge to accurately identify and determine the target compounds from a complex matrix. High-performance liquid chromatography (HPLC) equipped with diode-array detector (DAD) has been more and more frequently applied for this purpose and become a routine method for quality criteria [2,3]. However, due to the extreme complexity and diversity of the co-existing components in herbal products, especially for formulated TCMs preparations, the establishment of a relatively ideal HPLC separation system may need a long running time and multiple sample preparation procedures, which is undoubtedly time-consuming, economy-costing and laboring task. In recent years, LC–MS, especially of LC–MS/MS, has become a powerful tool for the analysis of pharmaceuticals and herbal products as this hyphenated technique can achieve high sensitivity and selectivity without needing the baseline chromatographic separation of the target analytes from each other, which greatly facilitates the characterization of chemical markers in the complex matrixes especially in authenticating the structurally similar analogues in natural products mixtures. LC–MS provides a rapid, simple and feasible qualitative and quantitative analysis method, and applicable for the quality control of the herbal preparations. Nevertheless, till now, although LC–MS method has been largely utilized in qualitative identification of the multiple components in herbal products [4–6], only a few of reports dealt with the quantification of active compounds in TCMs [7].

Longdan Xiegan Decoction (LXD), a traditional Chinese formulation, has been widely practiced to treat jaundice, conjunctival congestion, earache, scrotum and extremitas inferior eczema, etc.,

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in Chinese medicine for thousands of years [1]. LXD are prepared from 10 ingredient herbal materials including Gentianae Radix, Gardeniae Fructus, Scutellarae Radix, Rehmanniae Radix, Alismatisi Rhizoma, Plantaginis Semen, Angelicae Sinensis Radix, Clematidis Armandii Caulis, Glycyrrizae Radix et Rhizoma and Bupleuri Radix, which are formulated in specified ratio. With respect to the biological active components, iridoid glycoside, as well as flavonoids, triterpenoids, and essential oils, etc., were reported from the ingredient herbs in LXD [8–12]. For the quality control purpose of such a mysterious TCM mixture, it is almost impossible to display the whole image and count every peaks contributed by each of the ingredient herbs, as those compounds are in diverse physical and chemical properties and various concentrations. Moreover, we still know little about the bioactive components in some of the individual herbs, such as Rehmanniae Radix and Alismatisi Rhizoma. Based on TCM theory or hypothesis, those 10 herbs are not in equivalent potency in LXD. Among them, Gentianae Radix and Gardeniae Fructus have been considered as the "King" and "Minister" agents, respectively, and the others play subsidiary and assistant roles. Both Gentianae Radix (King) and Gardeniae Fructus (Master) contain iridoid glycosides as the principal components contributing to their activities for treatment of jaundice and cystitis [8–10]. Consequently, iridoid glycosides in these two herbs have been chosen as the markers compounds for quality assessment of LXD. For instance, determination by HPLC-UV of an iridoid glycoside, gentiopicroside, has been specified for LXD in Chinese Pharmacopoeia (2005 edition). Besides, there are a few reports dealing with the simultaneous analysis of iridoids in a single herb using HPLC-UV with a time-consuming sample preparation procedure [13]. The iridoid glycosides existing in Gentianae Radix and Gardeniae Fructus are very similar in structure but disparate in concentration, which led to poor resolution on a conventional ODS column in a short analytical time. So, a rapid, simple and reliable analytical method for the simultaneous determination of different iridoid glycosides in the complicated LXD extract is highly demanded. In the present study, first, an effective preparative HPLC method was established for the isolation and purification of four marker iridoid glycosides. Then, a high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (LC–MS/MS) was developed and validated for simultaneous determination of the target iridoid glycosides in LXD and the ingredient herbal materials.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade of acetonitrile and methanol were provided by Yuwang Chemical Co., Ltd. (Shandong, China). Water was purified with a Milli-Q system (Millipore, Bedford, USA). The other solvents, purchased from Shuanhuan Chemical Co., Ltd. (Beijing, China) were of analytical grade.

2.2. Isolation of iridoid glycosides standards

The LXD extract for isolation was prepared in the same manner as described in our previous paper [14], but using 100-fold of quantity. Two hundred grams of the extract was subjected to column chromatography with macro-porous resin (HPD600), stepwise eluted with water–ethanol (80:20, 50:50 and 0:100) to get three fractions (Fr.1–3). Fraction 1 was evaporated in vacuum and 2 g was then applied to a preparative HPLC to yield four iridoid glycosides: genipin-1-*O*-gentiobioside (**1**, 20 mg), swertiamarin (**2**, 15 mg), gentiopicroside (**3**, 260 mg) and geniposide (**4**, 150 mg), respectively. The HPLC system used for the separation was a Waters Delta Prep 4000 Technologies Series (Waters, Milford, MA, USA) coupled with a waters 996 PDA detector set to 240 nm and a Waters Prep Nova-Pak C_{18} (200 mm × 20 mm, 6 μm) column was used for preparation. Chromatographic separation of genipin-1-*O*-gentiobioside (**1**) was carried out using 10% acetonitrile at a flow rate of 14 mL/min. Thirty-two percent MeOH at a flow rate of 14 mL/min was used in the prep-HPLC separation for swertiamarin (**2**) and gentiopicroside (**3**), 35% MeOH at a flow rate of 12 mL/min used for preparation of geniposide (**4**).

The structures of those compounds were elucidated by comparing their spectral data (UV, IR, MS, ^{1}H NMR and ^{13}C NMR) with Refs. [15–18] (Fig. 1). The purities of these glycosides were determined to be more than 98% by normalization of the peak areas detected by HPLC-DAD and further confirmed by quantitative NMR analysis [19].

2.3. Samples preparation

Four commercial batches of LXD pills were purchased from Hexi Medicine Corporation, Ltd., Gansu (HX, Batch No. 030109), Yaodu Medicine Corporation, Ltd., Hebei (YD, Batch No. 030901), Guoren Medicine Corporation, Ltd., Henan (GR, Batch No. 041201) and Zhongyi Medicine Corporation, Ltd., Guangzhou (ZY, Batch No. G00001), respectively. The 10 ingredient herbal materials were purchased from Huayu Crude Drug Corporation, Ltd., (Shanghai, China), authenticated by Dr. Lihong Wu, Shanghai R&D Centre for Sdandardization of Chinese Medicines. The voucher specimens were deposited in Institute of Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China. All the samples were dried at 60 °C for 12 h before use (Table 1).

Approximately 0.1 g of the pulverized samples, accurately weighed, were placed in a stoppered conical flask, added with 20.0 mL of MeOH:water (1:1, v/v), weighed, and macerated at 50 °C for 60 min, then extracted in a ultra-sonic bath for 45 min, weighed again and compensated the loss of weight with MeOH:water (1:1, v/v). Allowing to cool, 5.0 mL of the supernatant was transferred to a 10-mL volumetric flask, made to volume with the same solvent and mixed well.

For Gentianae Radix herb, 5.0 mL of the supernatant was transferred to a 25-mL volumetric flask, made to volume and mixed well.

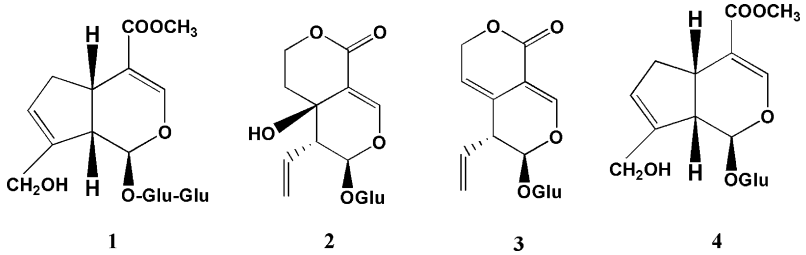

Fig. 1. Four iridoid glycosides genipin-1-*O*-gentiobioside (**1**), swertiamarin (**2**), gentiopicroside (**3**) and geniposide (**4**).

Table 1
Ten ingredients of LXD

No.	Name	Latin name	Voucher no.
1	Gentianae Radix	*Gentiana scabra* Bge.	05060001
2	Gardeniae Fructus	*Gardenia jasminoides* Ellis	05060002
3	Scutellarae Radix	*Scutellaria baicalensis* Georgi	05060003
4	Rehmanniae Radix	*Rehmannia glutinosa* Libosch	05080001
5	Alismatisi Rhizoma	*Alisma orientalis* (Sam.) Juzep	05080002
6	Plantaginis Semen	*Plantago asiatica* L.	05080003
7	Angelicae Sinensis Radix	*Angelica sinensis* (Oliv.) Diels	05080004
8	Clematidis Armandii Caulis	*Clematis armandii* Franch	05120001
9	Glycyrrizae Radix et Rhizoma	*Glycyrrhiza uralensis* Fisch	05120002
10	Bupleuri Radix	*Bupleurum chinense* D.C.	05120003

While for Gardeniae Fructus, 2.0 mL of the supernatant was transferred to a 100-mL volumetric flask, made to volume, and mixed well.

All solutions were filtered through a 0.45-μm membrane filter before LC–MS/MS analysis.

2.4. HPLC–MS/MS analysis

HPLC–MS/MS analysis was performed using a Waters 2690 system (Waters, Milford, MA, USA) coupled with a TSQ triple quadrupole (Finnigan MAT, San Jose, CA, USA) equipped with an ESI interface. A CAPCELL PAK C_{18} MG II column (100 mm × 2.0 mm, 3 μm, Shiseido, Japan) heated to 45 °C was used for analysis and the total separation was achieved within 3 min using a mixture of acetonitrile and water (10:90, v/v) as the mobile phase at a flow rate of 0.7 mL/min. A splitter was used to transfer only one-third of the flow into the mass spectrometer. The MS/MS was operated in positive ion mode, with multiple reaction monitoring (MRM). The mass spectrometric conditions were optimized by manual optimization using infusion with a syringe pump to select the most suitable ion transitions for the target analytes. And by automatically injecting standard solutions and comparing the ration of signal/noise, other operation parameters were also optimized as the heated capillary temperature was set to 320 °C, the electrospray voltage was 5.0 kV, sheath gas flow rate was 38 psig, and auxiliary gas flow was 20 arbitrary. The optimal collision energy was set at 40 V for **1** and **2**, while 30 V for **3** and **4**. Transitions of 551 → 209, 375 → 177, 357 → 177 and 389 → 227 were selected for MRM of **1**, **2**, **3** and **4**, respectively. HPLC–MS/MS data were acquired and processed using the Xcalibur 1.3 software.

2.5. Method validation

The method for the quantitative analysis was validated to determine the linearity, sensitivity, precision and accuracy for each analyte. Calibration curves were constructed using a range of concentrations of working standard as shown in Table 3. Each line is based on six concentrations of standard. The limits of detection (LOD) and quantification (LOQ) were estimated by baseline noise method with a signal 3 and 10 times higher than that of the baseline noise, respectively. Both precision and accuracy were determined with control samples prepared in triplicates at three different concentration levels, by spiking different amounts of reference standards to the LXD powder with known concentration of the target analytes. Then the control samples were treated according to the same procedure. The precision of the assay was determined by intra-day and inter-day variations and reported as RSD%. The accuracy was evaluated as the percentage recovery of analytes referred to the spiked samples.

3. Results and discussion

3.1. Isolation of iridoid glycosides standards by pre-HPLC

As no preparative HPLC method has been reported for the isolation of iridoids before the present study, an analytical grade column was used firstly to optimize the chromatographic conditions for the separation of the four target iridoid glycosides using a simple isocratic elution system consisting of a mixture of acetonitrile–water or MeOH–water. As the results, chromatographic separation of genipin-1-*O*-gentiobioside (**1**) was carried out using 10% acetonitrile at a flow rate of 0.8 mL/min. Thirty-two percent MeOH at a flow rate of 0.8 mL/min was used for swertiamarin (**2**) and gentiopicroside (**3**), and 35% MeOH at a flow rate of 0.65 mL/min applied for separation of geniposide (**4**).

For the preparative purpose, a scale up procedure using a HPLC column packed with the same stationary phase was performed. About 16 times of the injection volume were loaded and 18 times of the flow rate were used to get the same separation performance referring to the established analytical HPLC separation condition. The preparative isolation and purification of the four target iridoid glycosides were achieved for the first time using a preparative HPLC method. This optimized method can provide high speed and applicable technique for the batch size preparation of iridoid glycosides with high purity.

3.2. Optimization of sample preparation procedure

Because of the high sensitivity of mass spectrometry detector, only small amount (0.1 g) was needed for each extraction of the uniformly pulverized sample of LXD and herbal materials. Based on the polarity of target analytes, MeOH–H_2O (1:1, v/v) were used as the extracting solvent. Accordingly, as the iridoid glycosides are unstable, a relative soft extraction method, ultrasonication, was conducted instead of refluxing or Soxhlet's extracting on boiling water bath. Furthermore, the extraction efficiency for the four analytes was estimated by repeating extracting samples, three times by ultrasonication, and all more than 97% (Table 2), which confirm the completeness of the extraction for iridoids under the extraction conditions.

3.3. Optimization of the HPLC–MS/MS analysis conditions

The mass spectrometric conditions were optimized by automatically injecting the standard solutions and comparing the ratio of signal/noise. The MS/MS analysis was operated in positive ion mode. The total separation was finished within only three minutes and good integrated peaks were achieved relied on the high selectivity of mass spectrometry. By manual optimization using infusion with a syringe pump, the most suitable ion transitions and collision energy with MRM for the target analytes was selected. Transitions of 551 → 209, 375 → 177, 357 → 177 and 389 → 227 were selected for MRM of **1**, **2**, **3** and **4**, respectively, and the optimal collision energy was set at 40 V for **1** and **2**, while 30 V for **3** and **4** (Figs. 2 and 3).

Table 2
Concentration (mg/g) of iridoids in LXD products from three extracts

Extracts	Genipin-1-*O*-gentiobioside	Swertiamarin	Gentiopicroside	Geniposide
1st	0.0885	0.0162	2.27	1.63
2nd	–	–	0.082	0.054
3rd	–	–	0.016	0.014

–: not detectable.

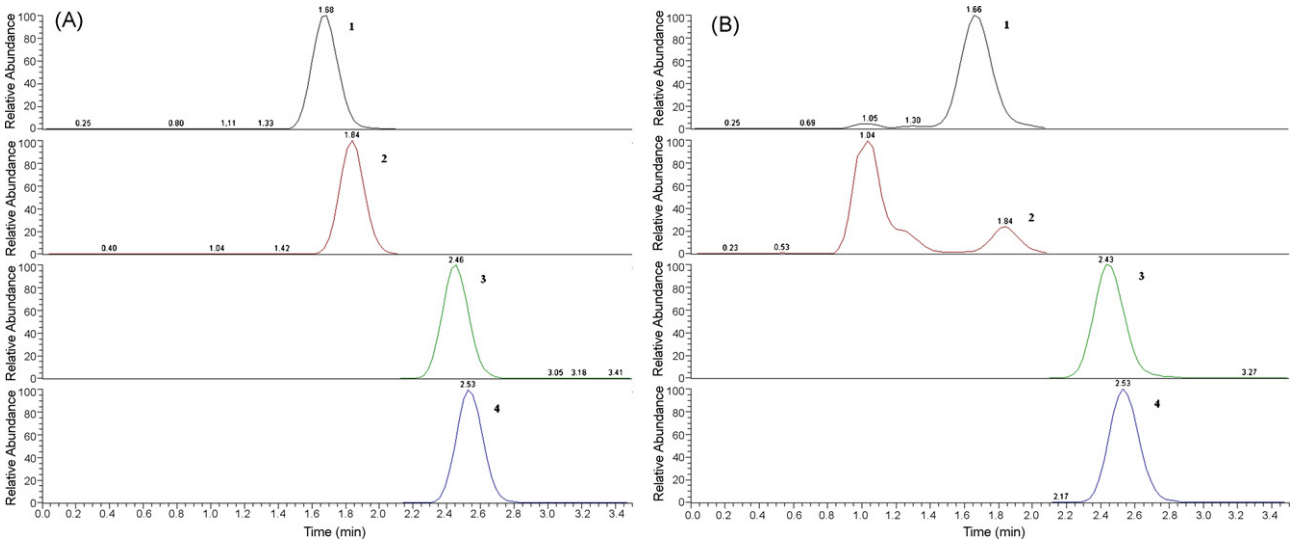

Fig. 2. MRM chromatograms of (A) reference standards and (B) LXD samples: (**1**) genipin-1-*O*-gentiobioside, (**2**) swertiamarin, (**3**) gentiopicroside and (**4**) geniposide.

3.4. Calibration and method validation

Calibration curves were constructed for genipin-1-*O*-gentiobioside, swertiamarin, gentiopicroside and geniposide and the least-squares linear calibration data were summarized in Table 1. Moreover, the LOD and LOQ for each analyte were calculated as 3 and 10 times of the signal-to-noise ratios (Table 3). Compound **1**, **3** and **4** had the similar LOD (11–13 ng/mL) and LOQ (33–37 ng/mL) values, whereas, the high sensitivity for **2** was obtained with LOD and LOQ values of 7 and 20 ng/mL, respectively.

The precision and accuracy of the method were assessed at three concentrations of spiked analytes in triplicate. The sample solutions were spiked with known amounts of the mixed standards of genipin-1-*O*-gentiobioside, swertiamarin, gentiopicroside and geniposide at three different concentration levels. The precision of the method was assessed by calculating the intra-day and inter-day variations of three replicates, verified by determining the samples with relative standard deviations (RSD) as listed in Table 3. The overall intra- and inter-day variations are less than 4.8% (ranging 0.58–4.76%), indicating satisfactory precision of the instrumentation and the stability of the samples. Furthermore, the analytical method developed in this study has good accuracy with the overall recovery ranging from 92.3 to 110.0% as the analytes concerned (Table 4).

There were several reports dealt with the analysis of iridoids in single plant materials such as gentiopicroside in Gentianae Radix or geniposide in Gardeniae Fructus using the routine HPLC-UV methods, which tolerated longer separation times for one run (16 min) with the LOD values ranged from 0.25 to 30 μg/mL [20–22]. Our developed HPLC–MS/MS method demonstrated excellent resolution capacity in a shortest time (3 min) and higher sensitivity with a lower LOD value (13 ng/mL), as shown in Table 3.

3.5. Analysis of iridoid glycosides in LXD products and ingredient herbs

The established quantitative method was applied to determine the concentrations of genipin-1-*O*-gentiobioside, swertiamarin,

Table 3
Linearity data and quantitation ranges obtained for **1**, **2**, **3** and **4** ($n = 5$)

Compounds	Concentration range (μg/mL)	Slope(±SD)	Intercept(±SD)	R^2	LOD (ng/mL)	LOQ (ng/mL)
1	0.035–21	$(5.0829 \pm 0.0832) \times 10^5$	−0.4126 ± 0.0076	0.9925	12	35
2	0.020–12.1	$(6.2241 \pm 0.1208) \times 10^6$	−0.1085 ± 0.0024	0.9945	7	20
3	0.037–22.2	$(0.9846 \pm 0.0306) \times 10^5$	0.2822 ± 0.0035	0.9905	13	37
4	0.033–18.2	$(5.1162 \pm 0.1318) \times 10^6$	0.0547 ± 0.0011	0.9999	11	33

Table 4
Accuracy and precision for quantitation of **1**, **2**, **3** and **4**

Compounds	Conc. found (μg/mL)	Conc. added (μg/mL)	Conc. detected (μg/mL)	Recovery (%)	Intra-day ($n = 6$) (RSD (%))	Inter-day ($n = 18$) (RSD (%))
1	0.22	0.1	0.325	105.0	1.20	2.56
		0.2	0.416	98.0	0.84	1.35
		0.3	0.531	103.7	2.96	1.46
2	0.04	0.02	0.062	110.0	2.87	3.46
		0.04	0.079	97.5	1.82	3.25
		0.06	0.105	108.3	2.67	4.76
3	5.65	2.8	8.47	100.7	1.79	2.86
		5.6	10.82	92.3	2.68	4.79
		8.4	13.74	96.3	2.95	4.12
4	4.11	2.0	6.13	101.0	1.36	2.57
		4.0	8.12	100.2	0.58	2.44
		6.0	10.18	101.2	1.84	3.19

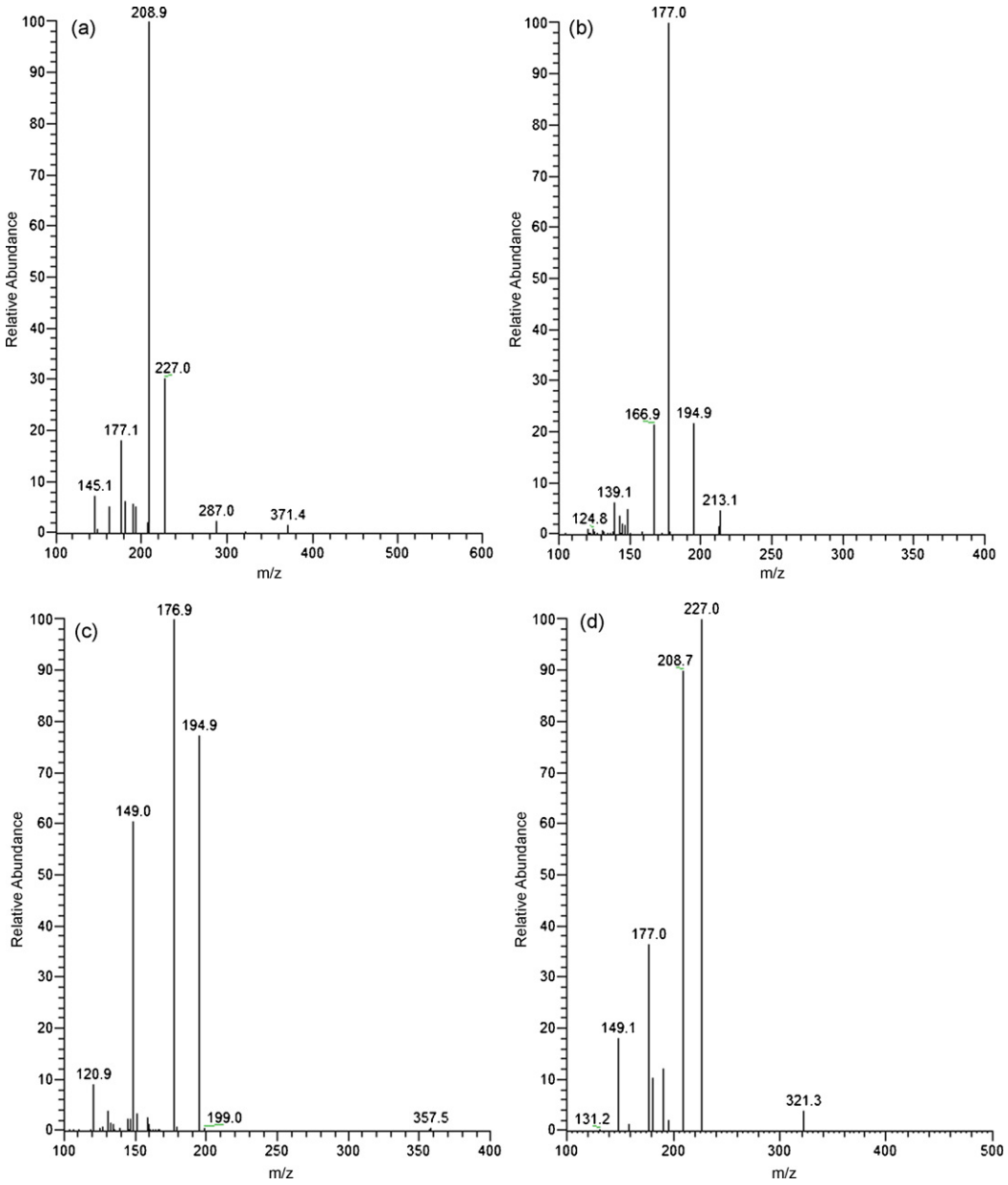

Fig. 3. Product ion mass (MS–MS) spectra of (a) *m/z* 551, genipin-1-*O*-gentiobioside, (b) *m/z* 375, swertiamarin, (c) *m/z* 357, gentiopicroside and (d) *m/z* 389, geniposide.

gentiopicroside and geniposide in four commercial LXD products and two ingredient herb materials, Gentianae Radix and Gardeniae Fructus (Table 5). Among the four iridoid glycosides, gentiopicroside and geniposide from different commercial LXD products were detected in a much higher level, ranged from 0.820 to 2.26 mg/g and from 1.64 to 3.96 mg/g, respectively. Comparatively, much smaller amounts of genipin-1-*O*-gentiobioside and swertiamarin were observed in the four analyzed LXD samples, the former ranged from 0.0375 to 0.0883 mg/g, and the latter, swertiamarin, only detectable in one batch of LXD sample with a very low concentration of 0.0166 mg/g. Gentiopicroside is specific for Gentianae Radix crude herb with a concentration of 9.42 mg/g, while geniposide is characteristic for Gardeniae Fructus crude drug, with a concentration of 20.0 mg/g.

Table 5
Concentration (mg/g) of iridoids in LXD products, Gentianae Radix and Gardeniae Fructus

Samples	Genipin-1-*O*-gentiobioside	Swertiamarin	Gentiopicroside	Geniposide
1	0.0883	0.0166	2.26	1.64
2	0.0401	–	0.820	3.89
3	0.0375	–	1.16	2.65
4	0.0525	–	1.71	3.96
5	–	0.127	9.42	0.0421
6	0.359	–	–	20.0

(1) LXD-1 (HX); (2) LXD-2 (YD); (3) LXD-3 (GR); (4) LXD-4 (ZY); (5) Gentianae Radix; (6) Gardeniae Fructus. –: not detectable.

4. Conclusions

As a continuation of our systematic study on interpretation of the biological active components and the quality assessment of a Chinese formulated preparation (LXD) [14], in the present study, a high-performance liquid chromatography coupled with mass spectrometry was successfully established for the quantitation of bioactive iridoidal compounds in LXD.

Firstly a simple and reliable preparative HPLC method was established for the scale up isolation and purification of genipin-1-*O*-gentiobioside, swertiamarin, gentiopicroside and geniposide. Then a HPLC–MS/MS method in a MRM mode was developed and successfully applied to the simultaneous determination of those targets in commercial LXD products and the two key ingredient herb materials. The developed method proved rapid, simple, sensitive and reliable for the analysis of the structurally similar iridoid glycosides in a very complicated matrixes on comparison with the routine HPLC-DAD method, which normally needs time-consuming sample pretreatment procedure prior to loading to the column and longer running time to get well enough separation of the iridoid analogues.

Based on our present analysis result, four iridoid glycosides were chosen as the markers for the quality assessment of LXD. At least the two main iridoids, gentiopicroside for Gentianae Radix and geniposide for Gardeniae Fructus, should be considered as the marker compounds for the quality criteria for LXD in the next revision of Chinese Pharmacopoeia, instead of only gentiopicroside being specified. The other two trace amount compounds, genipin-1-*O*-gentiobioside and swertiamarin, are not practicable to serve as the chemical marker for quantitation. Conclusively, LC–MS or LC–MS/MS has become an advanced quantitative tool for quality assessments of TCMs as well as other herbal products, teas, and food supplements due to its high capacity, high sensitivity, high selectivity and shorter analysis time.

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