Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 502–507

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Rapid and sensitive quantitation of major constituents in Danggui Buxue Tang by ultra-fast HPLC–TOF/MS

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ARTICLE INFO

Article history:
Received 5 September 2008
Received in revised form 19 October 2008
Accepted 21 October 2008
Available online 28 October 2008

Keywords:
Rapid and sensitive quantitation
Ultra-fast HPLC
Time-of-flight mass spectrometry
Danggui Buxue Tang

ABSTRACT

A quantitative method by ultra-fast HPLC coupled with time-of-flight mass spectrometry (TOF/MS) has been developed for analysis of 15 major constituents in Danggui Buxue Tang (DBT) preparations. Its performance was compared with a conventional HPLC method with diode array detection/evaporative light scattering detection (DAD/ESLD). Accurate mass measurements within 3 ppm error were obtained for all the compounds. The analytical time by an ultra-fast system is 4 times faster than conventional HPLC, the limits of detection by TOF/MS are low to 0.004–0.08 ng compared with 1–200 ng for HPLC–DAD–ELSD, and acceptable linearity of response was demonstrated over two orders of magnitude ($r^2 > 0.99$) for all analytes. Intra-day reproducibility was below 3% and inter-day values were below 5% R.S.D. Robustness and applicability of the method was validated for the analysis of market DBT real samples, proposing a rapid, sensitive and validated method for routine analysis and quality control of DBT.

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1. Introduction

Danggui Buxue Tang (DBT), consisting of Radix Astragali and Radix Angelica Sinensis, has been used as a classical prescription since 1247 AD for nourishing the body blood and raising the vital energy. Nowadays DBT is used not only as an efficacious medicine but also as a common food supplement [1–3]. Chemical investigations and pharmacological studies on DBT have revealed that saponins (*e.g.*, astragaloside IV) and isoflavonoids (*e.g.*, calycoside), ferulic acid and ligustilide are the major bioactive compounds of DBT [4–6]. Developing a validated method that can assay qualitative and quantitative determination of these components in DBT is necessary for its quality control.

To date, various analytical methods have been reported for analysis of active ingredients in DBT, including HPLC with diode-array detection (DAD) [7], evaporative light scattering detection (ELSD) [8], etc. Time-consuming procedure, no structure information and insensitivity are the major obstacles for these methods. Because of the high speed of analysis, sensitivity and confirmation of structural information, ultra-fast HPLC system coupled with mass spectrometry (MS) has become the preferred analytical technique for herbal medicines [9–12]. Using time-of-flight (TOF) MS instruments for quantitation has recently gained in popularity [13,14]. TOF/MS shows its unique advantages in providing high selectivity with narrow mass windows (<0.01 Da mass range) over nominal mass chromatograms (1 Da), accurate mass measurements for elemental composition and structural information, resolutions of 9500 ± 500, and full-scan conditions without optimization of precursorto-product ion transitions which is not possible for nominal MS [15].

Previously, we have developed an ultra-fast HPLC–DAD–TOF/MS method for characterization of constituents in DBT [16], but quantitation information is absent. As a consecutive work, the aims of this paper are to develop a rapid and sensitive analytical method to determine major constituents in real DBT samples and to compare its performance with a conventional HPLC–DAD/ELSD technique.

2. Experimental

2.1. Samples, chemicals and reagents

DBT samples 1–3 (No. 20050709, 20060313 and 20060424) were produced by Xiehe Pharmaceutical Plant (Zhengzhou, Henan Province, China); sample 4 (batch No. 050102) was produced by Wuhan No. 4 Pharmaceutical Plant (Wuhan, Hubei Province, China); sample 5 (batch No. 060701) was produced by Liyuan Pharmaceutical Corporation (Changchun, Jilin Province, China); sample 6 was prepared with the extraction procedure by our laboratories as described by Yi et al. [8].

Isoflavonoid and saponin reference compounds were isolated previously from the dried roots of *Astragalus. membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao in our laboratories. Ligustilide

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doi:10.1016/j.jpba.2008.10.026

was isolated from *Angelica sinensis* (Oliv.) Diels. Ferulic acid and internal standards (not included in DBT samples) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The structures of these compounds are shown in Fig. 1.

The solvents acetonitrile (ACN) and methanol are of HPLC grade from Merck (Darmstadt, Germany), and formic acid with a purity of 96% is of HPLC grade (Tedia, USA). Deionized water (18 MΩ) was prepared using Milli-Q system (Millipore, Milford, MA, USA).

2.2. Apparatus and chromatographic conditions

Chromatographic analysis was performed on an Agilent 1200 Series (Agilent, Germany) LC system. Chromatographic separation was performed at 25 °C on an Agilent ZorBax SB-C_{18} column

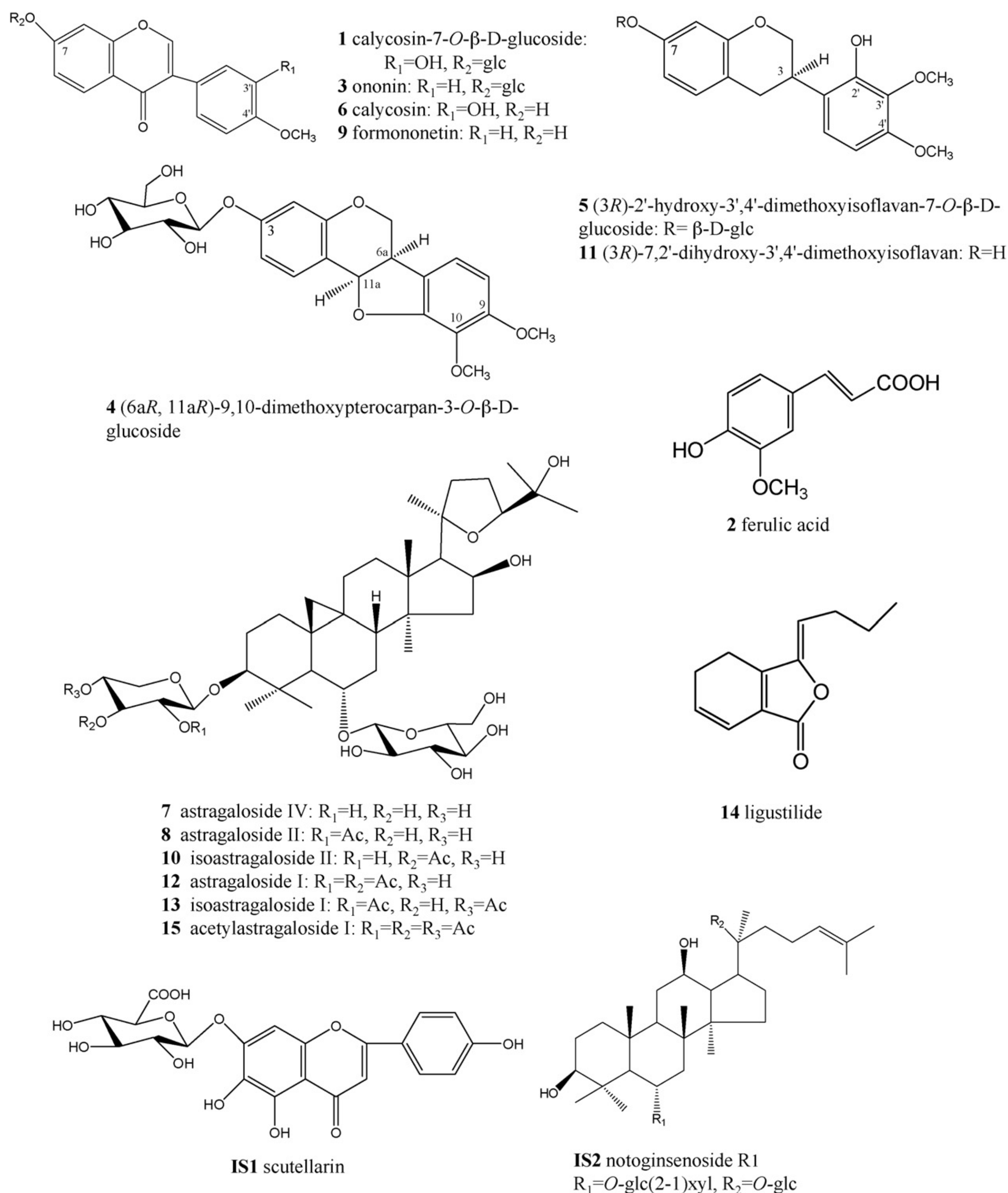

Fig. 1. Chemical structures of 15 marker constituents and two internal standards.

(4.6 mm × 50 mm, 1.8 μm). An in-line filter with 4.6 mm diameter frits and 0.2 μm pore size was used before the column. The mobile phase consisted of 0.2% formic acid water (A) and acetonitrile (B). The analysis was performed using a gradient elution of 23% B at 0–1.5 min, 23–30% B at 1.5–4.0 min, 30–35% B at 4.0–5.0 min, 35–40% B at 5.0–7.0 min, 40–43% B at 7.0–8.5 min, 43–51% B at 8.5–11.5 min, 51% B at 11.5–15.0 min, 51–56% B at 15.0–17.0 min, 56–100% B at 17.0–19.0 min. The flow rate was kept at 0.6 mL/min, and the sample volume injected was set at 2 μL. For comparison with conventional HPLC–DAD–ELSD, analysis was also carried out on an Agilent ZorBax Extend-C_{18} column (4.6 mm × 250 mm, 5 μm) (for experimental details see Yi et al. [7]).

Analytes were detected using an orthogonal TOF/MS (Agilent Corp., Santa Clara, CA, USA) equipped with ESI source. The mass range was set at *m*/*z* 100–1500. The conditions were as follow: drying gas (N_2) flow rate, 10.0 L/min; temperature, 330 °C; nebulizer, 35 psig; fragmentor, 120 V; capillary, 3000 V; skimmer, 60 V; OCT RF V, 250 V. All the acquisition and analysis of data were controlled by Agilent LC–MS/TOF Software Ver. A.01.00 (Agilent Technologies, USA) and Applied Biosystems/MDS–SCIEX Analyst QS Software (Frankfurt, Germany), respectively.

2.3. Sample preparation

Solid phase extraction (SPE) was selected as a convenient and timesaving pretreatment method to remove many highly poly-complex ingredients such as saccharides in DBT preparation [8]. Sample solution (0.1–0.5 mL) was loaded onto a SPE column (Supelclean C_{18}, 500 mg, 3 mL column volume), and then eluted with 100% methanol slowly. The eluant added with 50 μL of the internal standards solution was then transferred to a 5 mL volumetric flask with methanol. An aliquot of 2 μL was injected into LC system for analysis.

2.4. Calibration curves, limits of detection (LOD) and quantification (LOQ)

Methanol stock solution containing 15 accurately weighed reference compounds were prepared and diluted to appropriate concentration ranges for the construction of calibration curves. The concentration of the internal standards was 5 μg/mL for all analysis. The LOD and LOQ under the present chromatographic conditions were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively.

2.5. Precision and accuracy

Intra-day variations for 5 times within 1 day and inter-day variations for consecutive 3 days were chosen to determine the precision of the developed method. Recovery test was used to evaluate accuracy of this method. For the determination of the recoveries of analytes in pretreatment of DBT preparation, three different concentration levels of purified preparations were submitted to filtration and purification steps as described in Section 2.3. In addition, a purified DBT preparation was analyzed by ultra-fast HPLC–TOF/MS in comparison with HPLC–DAD–ELSD method for confirmation of the accuracy [7]. For determining matrix effects of MS detection, the DBT samples and samples added with different concentrations (low, middle and high) of standard solutions were analyzed with LC–TOF/MS, and the response was compared to standards in solvent (methanol without matrix). An exact determination of matrix effects was obtained by relative recoveries: Relative recoveries = (Sample contents after adding − Original contents)/Contents of standard solutions for adding.

3. Results and discussion

3.1. Optimum conditions for ultra-fast HPLC–TOF/MS analysis

The optimized conditions including gradient program, flow rate and column temperature adopted in this method produced short analytical time less than 18 min and moderate column pressure at about 120 Bar for DBT sample analysis (Fig. 2). Higher flow rate (2 mL/min) resulted in the backpressure increasing up to 300 Bar and the low resolution of adjacent peaks. The positive ion mode was compared with the negative mode for analysis of constituents in DBT. Negative mode was finally selected for sensitive detection of saponins (notoginsenoside R1 as the IS), while positive mode for isoflavonoids, ferulic acid and ligustilide (scutellarin as the IS). A low gentle fragmentor at 120 V was applied for quantitation of the major constituents in DBT, providing minimal fragmentation and maximum molecular ion intensity in most compounds. As shown in Fig. 3, by extracted ion chromatograms (XICs) mode with a narrow mass window (*e.g.*, ±0.01 Da), TOF/MS can alleviate the matrix interferences from background and co-eluting compounds, which are often encountered by conventional HPLC–DAD–ELSD and LC–MS methods.

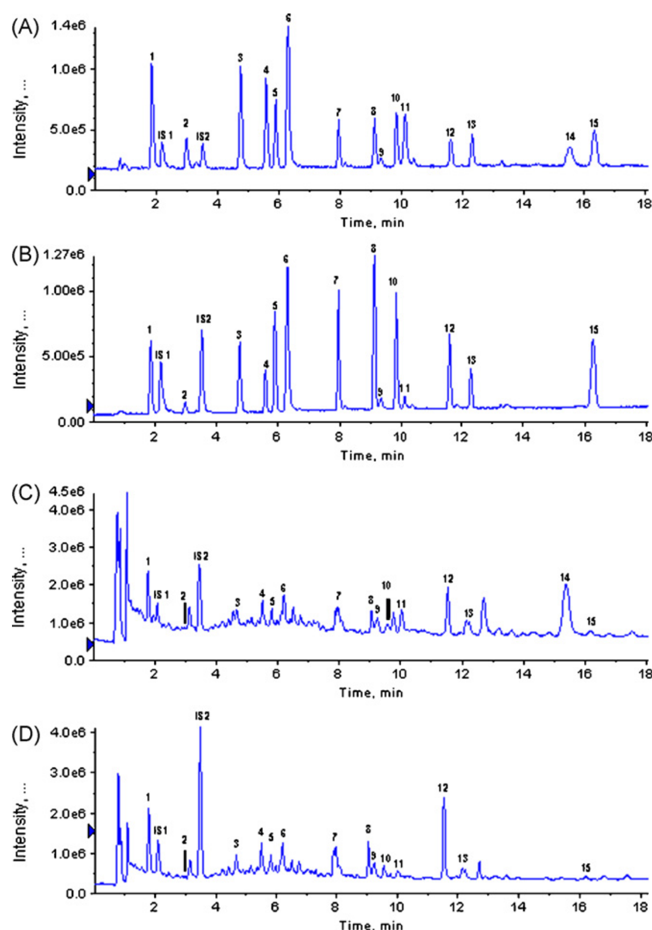

Fig. 2. Total ion chromatograms (TICs) of the reference stock solution and DBT samples. Chromatographic conditions are described in Section 2. The standard solutions of all analytes were stored in a refrigerator at 4 °C, except for ligustilide at −20 °C owing to its volatility. (A) TIC of reference stock solution in positive ion mode; (B) TIC of reference stock solution in negative ion mode; (C) positive TIC of DBT preparation from sample 6; (C) negative TIC of DBT preparation from sample 6.

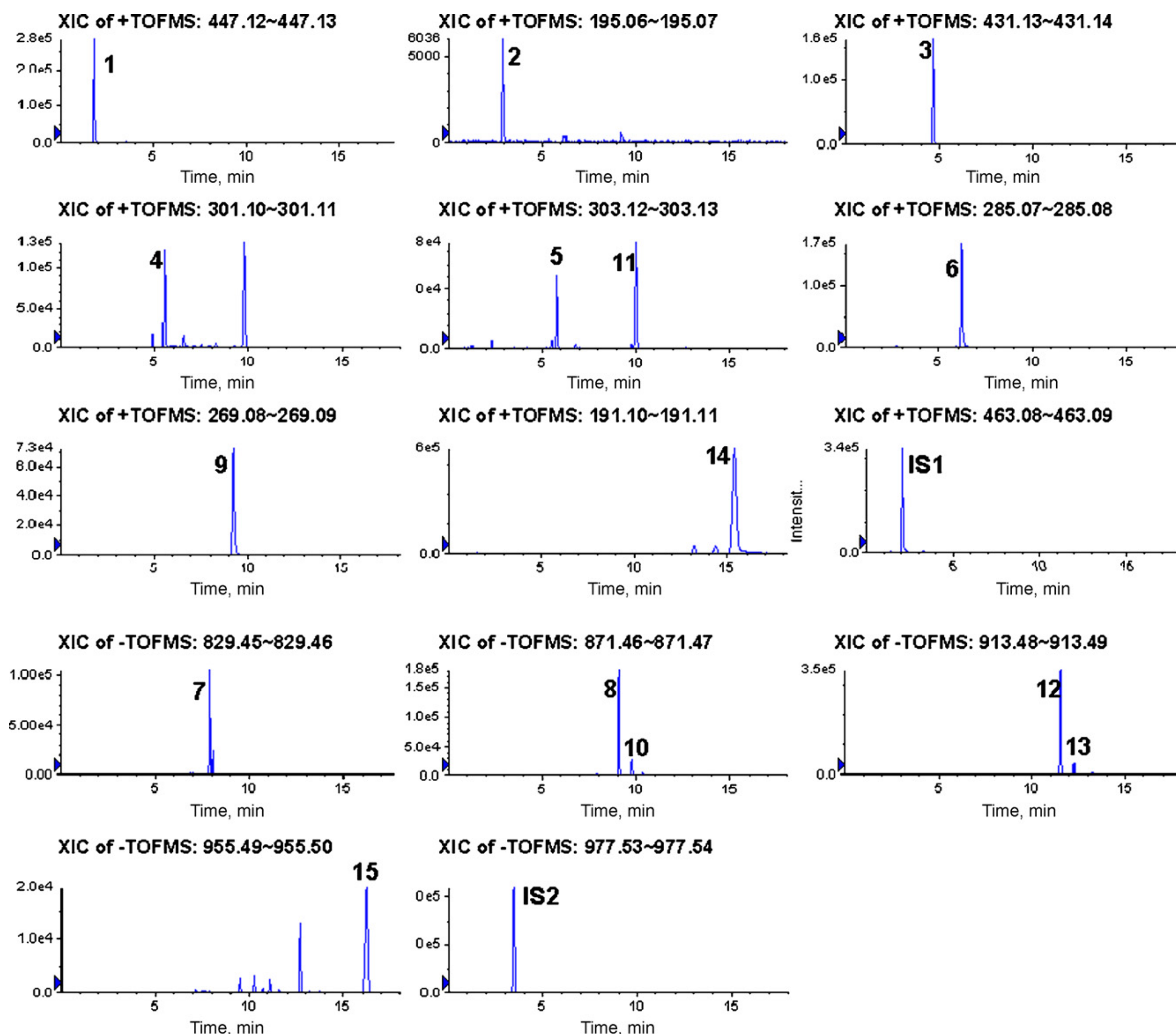

Fig. 3. Extracted ion chromatograms of 15 marker compounds and internal standards from the DBT matrix for quantitation with a 0.01 Da mass window in positive or negative modes.

3.2. Performance of ultra-fast HPLC compared with conventional HPLC

The major benefit from the use of the 1.8-μm porous particles packed into short columns is the increased column efficiency that results in narrow peaks and an improved separation. By using a 4.6 mm × 50 mm × 1.8 μm C_{18} column at a flow rate of 0.6 mL/min, the average peak width was 5–10 s at base, giving a peak capacity for 18 min separation of approximately 50–80. In comparison, with conventional HPLC, using a 4.6 mm × 50 mm × 5 μm C_{18} column, the average peak width was 30–60 s at the base giving a total peak capacity of approximately 65–75 min separation of the same sample. (For experimental details on HPLC see Yi et al. [7].)

3.3. Identification of the 15 target analytes in DBT samples by TOF/MS

Table 1 summarizes the accurate mass measurements for the selected ions of the 15 marker components in DBT. Retention times, formula, experimental and theoretical masses and ppm errors are included. The errors obtained were less than 3 ppm in most cases. Mass-measurement accuracy, along with characteristic retention time, provides highly reliable identification for the target markers. In addition, a high voltage (which provides an intense in-source collision-induced dissociation fragmentation) was applied to obtain product ion spectra that are matched with the ones for a standard solution, thus, providing two sets of information for unequivocal identification [15].

3.4. Quantitative validation

As seen in Table 2, linearity of analytical response was acceptable with correlation coefficients higher than 0.99, offering a dynamic range of about two orders of magnitude. The R.S.D. values of peak areas obtained from run-to-run experiments ranged from 0.7 to 2.7% and for day-to-day from 1.5 to 4.1%. The sensitivity is greatly improved using ultra-fast HPLC–TOF/MS instead of conventional HPLC–DAD–ELSD. The typical LODs of most compounds observed are low to 0.004–0.08 ng, compared with 1–200 ng by conventional HPLC–DAD–ELSD, and LOQs fall in the range of 0.015–0.450 ng compared with 5–300 ng for HPLC–DAD–ELSD.

Table 1
Accurate mass measurements for the marker constituents in DBT by ultra-fast HPLC-TOF/MS.

No.	t_R (min)	Formula	Selected ion	Experimental (m/z)	Theoretical (m/z)	Error (ppm)
TOF/MS in positive mode						
1	1.79	$C_{22}H_{23}O_{10}$	$[M+H]^+$	447.1291	447.1285	1.18
IS1	2.17	$C_{21}H_{19}O_{12}$	$[M+H]^+$	463.0877	461.0871	1.29
2	2.97	$C_{10}H_{11}O_4$	$[M+H]^+$	195.0651	195.0651	−0.44
3	4.73	$C_{22}H_{23}O_9$	$[M+H]^+$	431.1348	431.1336	2.65
4	5.56	$C_{17}H_{17}O_5$	$[M+H\text{-glc}]^+$	301.1075	301.1070	1.50
5	5.88	$C_{17}H_{19}O_5$	$[M+H\text{-glc}]^+$	303.1231	303.1227	1.32
6	6.28	$C_{16}H_{13}O_5$	$[M+H]^+$	285.0762	285.0757	1.58
9	9.33	$C_{16}H_{13}O_4$	$[M+H]^+$	269.0812	269.0808	1.35
11	10.12	$C_{17}H_{19}O_5$	$[M+H]^+$	303.1234	303.1227	−1.80
14	15.48	$C_{12}H_{15}O_2$	$[M+H]^+$	191.1071	191.1066	2.32
TOF/MS in negative mode						
IS2	3.52	$C_{48}H_{81}O_{20}$	$[M+HCOOH\text{-}H]^-$	977.5333	977.5326	0.65
7	7.96	$C_{42}H_{69}O_{16}$	$[M+HCOOH\text{-}H]^-$	829.4594	829.4591	0.35
8	9.13	$C_{44}H_{71}O_{17}$	$[M+HCOOH\text{-}H]^-$	871.4698	871.4696	0.14
10	9.84	$C_{44}H_{71}O_{17}$	$[M+HCOOH\text{-}H]^-$	871.4705	871.4696	0.95
12	11.62	$C_{46}H_{73}O_{18}$	$[M+HCOOH\text{-}H]^-$	913.4809	913.4802	0.72
13	12.31	$C_{46}H_{73}O_{18}$	$[M+HCOOH\text{-}H]^-$	913.4807	913.4802	0.50
15	16.28	$C_{48}H_{75}O_{19}$	$[M+HCOOH\text{-}H]^-$	955.4906	955.4908	−0.20

Table 2
Calibration curves, LODs and LOQs for 15 marker compounds and instrument accuracy by spiking 15 stock solutions before analysis.

No.	Calibration curve	r^2	Test range (μg/mL)	LOD (ng)	LOQ (ng)	Precision	
						Intra-day R.S.D. (%) ($n=5$)	Inter-day R.S.D. (%) ($n=3$)
1	$y=0.1129x+0.0329$[a]	0.9976	0.15–57.20	0.005	0.020	0.8	1.5
2	$y=0.0655x+0.0016$	0.9987	0.14–54.00	0.004	0.015	2.5	4.1
3	$y=0.1593x+0.0262$	0.9993	0.13–50.80	0.005	0.025	0.9	2.5
4	$y=0.0969x-0.0163$	0.9915	0.14–53.20	0.010	0.050	0.4	1.5
5	$y=0.0690x-0.0093$	0.9978	0.13–50.80	0.005	0.040	0.5	2.0
6	$y=0.2482x+0.0904$	0.9985	0.10–37.20	0.006	0.035	1.7	3.5
7	$y=0.0612x+0.0040$	0.9988	0.11–18.10	0.009	0.055	0.5	2.7
8	$y=0.0685x+0.0019$	0.9991	0.11–21.60	0.007	0.050	1.5	3.1
9	$y=0.0600x+0.0024$	0.9994	0.11–43.60	0.009	0.040	1.0	2.6
10	$y=0.0518x+0.0008$	0.9990	0.12–23.20	0.005	0.035	2.0	3.5
11	$y=0.1368x-0.0673$	0.9981	0.13–50.40	0.010	0.05	1.2	2.8
12	$y=0.0838x+0.0055$	0.9992	0.09–17.10	0.009	0.045	1.2	2.9
13	$y=0.0734x+0.0033$	0.9932	0.06–11.50	0.009	0.038	1.5	2.3
14	$y=0.2341x+0.0363$	0.9969	0.18–57.20	0.080	0.450	2.7	4.5
15	$y=0.0955x-0.0051$	0.9958	0.02–10.50	0.010	0.060	1.8	2.5

[a] y: peak area ratio of the analyte/internal standard; x: concentration of analyte (μg/mL).

The recovery experiment results showed acceptable losses in pretreatment procedure of DBT preparations in three different levels with recoveries no less than 85%. A good correlation (slope = 1.0647, $r^2=0.9968$) was observed for all analytes quantified between ultra-fast HPLC–TOF/MS and HPLC–DAD–ELSD method, indicating an adequate accuracy for both analytical methods. The relative recoveries for all 15 compounds ranged between 90 and 110%, thus show-

Table 3
Mean contents of 15 bioactive components in DBT products.

No.	Mean concentration ± S.D. of commercial products (μg/mL) ($n=3$)					
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
1	86.0 ± 1.9	168.4 ± 3.8	101.6 ± 2.1	12.5 ± 0.4	53.8 ± 1.2	127.4 ± 2.5
2	7.7 ± 0.2	12.7 ± 0.4	10.6 ± 0.2	0.8 ± 0.1	5.9 ± 0.1	5.9 ± 0.1
3	29.9 ± 0.8	39.9 ± 0.9	21.8 ± 0.6	3.4 ± 0.1	8.2 ± 0.2	35.0 ± 0.9
4	37.2 ± 0.5	56.3 ± 1.0	41.5 ± 1.2	6.3 ± 0.2	17.1 ± 0.3	43.7 ± 0.9
5	18.3 ± 1.5	22.3 ± 0.6	27.8 ± 0.4	6.9 ± 0.3	9.0 ± 0.5	28.3 ± 1.2
6	45.3 ± 1.6	23.3 ± 0.6	21.2 ± 0.5	22.1 ± 0.4	20.5 ± 0.4	48.4 ± 1.2
7	80.3 ± 2.1	34.8 ± 0.8	31.7 ± 1.0	45.5 ± 0.8	19.8 ± 0.5	19.2 ± 0.4
8	72.7 ± 1.2	52.7 ± 1.5	29.6 ± 1.0	39.5 ± 0.5	31.1 ± 0.9	33.5 ± 0.7
9	42.8 ± 1.5	13.7 ± 0.3	13.9 ± 0.4	37.7 ± 0.9	27.4 ± 0.5	123.7 ± 2.8
10	40.5 ± 1.2	25.5 ± 0.5	14.6 ± 0.4	19.2 ± 0.5	12.8 ± 0.3	5.3 ± 0.1
11	8.5 ± 0.2	6.0 ± 0.1	6.2 ± 0.2	3.8 ± 0.1	2.6 ± 0.1	34.0 ± 0.8
12	13.3 ± 0.3	17.6 ± 0.4	7.6 ± 0.2	5.1 ± 0.1	9.9 ± 0.2	112.1 ± 2.5
13	22.9 ± 0.8	27.8 ± 0.6	13.5 ± 0.8	8.2 ± 0.4	14.1 ± 0.2	11.7 ± 0.4
14	nd	nd	nd	nd	nd	166.7 ± 4.5
15	1.0 ± 0.1	1.0 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	7.1 ± 0.2

nd = not detected.

ing minimal matrix suppression or enhancement of this method.

3.5. Application to real samples

To demonstrate the applicability of the proposed method, 6 DBT samples from different manufactures or batches were analyzed. Each sample was analyzed three times and the mean contents determined for compounds 1–15 are shown in Table 3. In our previous analytical experiment for DBT samples by conventional HPLC–DAD–ELSD, several marker compounds fell in "trace" or "not detected" owing to the low sensitivity of the instrument [8]. In comparison, using an ultra-fast HPLC–TOF/MS, all constituents were readily determined with the greatly increasing sensitivity.

4. Conclusion

Compared with conventional HPLC–DAD–ELSD, significant advantages of the use of ultra-fast HPLC system with TOF/MS include the high speed of chromatographic separation, high sensitivity and selection, and structural information. Ultra-fast HPLC–TOF/MS use is likely to increase exponentially in the near future, proposing a rapid, sensitive and validated method for routine analysis and quality control of complex herbal medicines and preparations.

Acknowledgements

The authors greatly appreciate the financial support from the great research project (No. 90709020) of the National Science Foundation of China, and the Innovative Project from Jiangsu Province awarded to Dr. Lian-Wen Qi.

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