Simultaneous Determination of Eight Potential Q-Markers in Zishen Tongguan Capsules Based on UHPLC-MS / MS

Running title: UHPLC-MS / MS Analysis of Q-Markers in Zishen Tongguan Capsules

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Abstract

Background: Zishen Tongguan (ZSTG) Capsule was a hospital preparation of Affiliated Hospital of Nanjing University of TCM ，and has been clinically proven to be effective for treating pyelonephritis and benign prostatic hyperplasia. However, its quality standards are not perfect; a comprehensive study of the “quality marker” (Q-marker) of this medicine has not been carried out.

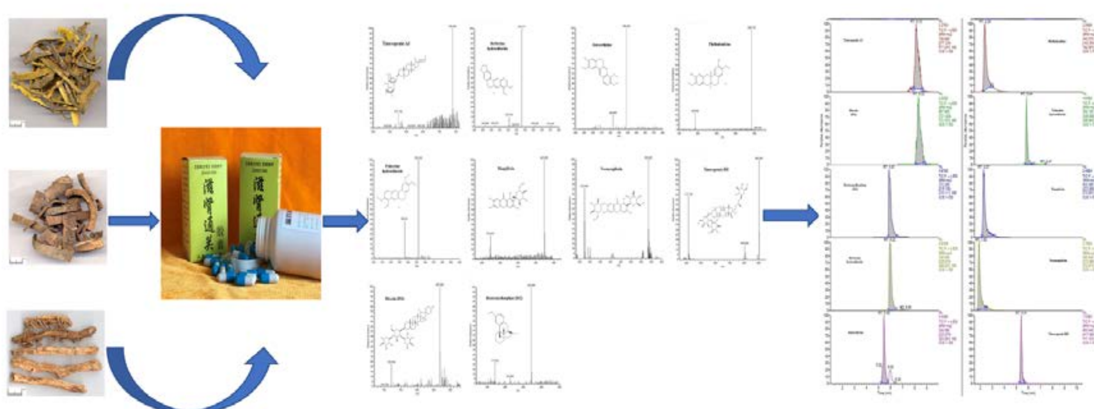
Experimental Methods: In this paper, a sensitive and specific ultra-high performance liquid chromatographic-tandem mass spectrometric (UHPLC–MS/MS) method was developed for the simultaneous determination of eight potential Q-Markers of ZSTG, including Timosaponin A3, Berberine hydrochloride, Jatrorrhizine, Phellodendrine, Palmatine hydrochloride, Mangiferin, Neomangiferin, and Timosaponin BII. Kromasil 100-3.5 C18 column, mobile phase is a mixture of 0.2% formic acid with acetonitrile by gradient elution at a flow rate of 0.2 mL/min achieved in 13 minutes was used for separation. Detection was performed on a positive/negative mode with multiple-reaction-monitoring (MRM).

Results: The analytical condition was validated in terms of the linearity, accuracy and precision, repeatability, and stability. The method established in this assay was successfully applied to the study of the potential Q-Markers in 8 batches of commercial samples, which supported the potential of this method for improving the quality control of ZSTG.

Conclusion: It is the first time to report the rapid and simultaneous analysis of eight Q-Markers in ZSTG by UHPLC-MS/MS and apply to determine 8 batches of ZSTG .

This method could be considered as good quality criteria to control the quality of ZSTG.

Graphical Abstract



In this paper, we have developed ultra-high performance liquid chromatographic-tandem mass spectrometric method for simultaneous determination of 8 Q-markers of Zishen Tongguan (ZSTG) Capsule.

Keywords: Zishen Tongguan Capsule; Q-Marker; Benign Prostatic Hyperplasia; Pyelonephritis; UHPLC–MS/MS; Quality Control

1. Introduction

Zishen Tongguan Capsule (ZSTG) is a hospital preparation of Affiliated Hospital of Nanjing University of Traditional Chinese Medicine，which is improved by the Tongguan Pills from Li Dongyuan's "Lanshi Micang ". ZSTG consists of three herbs: *Anemarrhenae Rhizoma*, *Phellodendri Chinensis Cortex* and *Cinnamomi Cortex*, with the effect of "Enriching Kidney Yin and Clearing Heat and Qi". It can be clinically used to remove "dampness-heat in lower energizer" and helped "promoting bladder activity", and has proven efficacy for the clinical treatment of acute and chronic pyelonephritis, benign prostatic hyperplasia [1, 2]. According to TCM Theory, *Anemarrhenae* and *Phellodendri* used as "Mutual promotion" to supplement deficiency of kidney-yin, and clear kidney-yang; with a small amount of *cinnamomi* to warm blood and supplement the vital fire, so as to help promoting bladder activity, enhance diuretic effect. Three herbs are used together to increase the efficacy for achieving "efficacy synergy".

According to the theory of Academician Changxiao Liu, Q-marker is defined as a chemical substance formed in the process of preparation or processing of TCM and TCM products (such as Decoction Pieces, Chinese herbs decoction, Chinese herbs extracts, and Chinese patent medicine preparations), which is closely related to the functional properties of TCM. It is therefore highly relevant for the quality control of TCM to ensure the safety and efficacy. The basic criteria for a Q-marker include: (1) They are chemical substances that are inherent in the processing or processing of TCM and TCM products; (2) They are closely related to the functional properties of TCM, and have clear chemical structure; (3) They can be qualitatively identified and quantitatively determined ;(4) They could reflect the theory of TCM combination, spanning components in the "Monarch" "Minister" and "Assistant and Guide" [3-7].

Therefore, Q-markers are inherent chemicals in pharmaceutical preparations and helpful to the practice of TCM quality control.

To treat pyelonephritis and benign prostatic hyperplasia, TCM has been used in patients for many years [8, 9]. Modern pharmacological studies have shown that *Anemarrhenae* mainly contains saponins, mangiferin, and neomangiferin, it has been used in the treatment of fever, ulcers, constipation, cough asthma and as an anti-inflammatory agent [10-14]. *Phellodendri* has the highest levels of alkaloids, which were its main pharmacological active ingredients, about anti-inflammatory, anti-nephritis, anti-ulcer, anti-oxidation, anti-gout, lowering blood pressure, lowering blood sugar, anti-tumor, and anti-heart failure, et al [15, 16]. This is also consistent with the Q-Marker's "property-effect-component" ternary theory.

ZSTG, as the clinical commonly used prescriptions under the guidance of TCM's "corrigent" theory, used TLC and ethanol extracts as its quality control standards; in Chinese Pharmacopoeia quality evaluation of the two medicinal materials are only TLC and content determination (mangiferin and timosaponin BII in *Anemarrhenae*; berberine and phellodendrine in *Phellodendri*) [17] .So it is important to determine the Q-marker in the preparation and to establish its assay method if it is to be evaluated from a safe, effective and quality controllable point of view. In this paper, the "Monarch" medicines of *Anemarrhenae* and *Phellodendri* typical compositions (Timosaponin A3, Berberine hydrochloride, Jatrorrhizine, Phellodendrine, Palmatine hydrochloride, Mangiferin, Neomangiferin, Timosaponin BII) were selected by literature review, source analysis of chemical composition and compositional specificity, these 8 components as ZSTG's potential quality markers (Q-markers), and to establish methods of multi-Q-markers of quantitative determination.

At present, determination of saponins from *Anemarrhenae* is mainly based on the

HPLC-ELSD method [18, 19]; Determination of alkaloids from *Phellodendri* is mainly based on HPLC-UV method [20]. The sensitivity of these two methods is relatively low, and the chromatographic separation difficulty, long analysis time [21]. The ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), which was established in this experiment, has the characteristics of simple, high efficiency, good specificity and high sensitivity, which can eliminate the interference of similar chemical components in a complex matrix. Therefore, in this study, UHPLC-MS/MS was employed to enable a high-throughput and senstivie analysis of the potential Q-markers of ZSTG Capsule. The results showed that the presently developed method are validated in sensitivity, precision, accuracy and can be used for the quality control of ZSTG Capsules. This technique is expected to provide technical support for multi-components' rapid analysis of Chinese herbs medicine and its compound preparation.

2. Material and methods

2.1 Reagents and materials

Timosaponin A3, Berberine hydrochloride, Jatrorrhizine, Phellodendrine, Palmatine hydrochloride, Mangiferin, Neomangiferin,Timosaponin BII, (purity>98%), Dioscin (internal standard (IS1) for Timosaponin A3, purity>98%), and Dextromethorphan (IS2 for other 7 markers, purity>98%),were purchased from Shanghai YuanYe Biological Technology Co., Ltd.

ZSTG (0.5g/tablet, batch No. 1708005, 1710007, 1712009, 1712010, 1808002, 1809004, 1809005, 1809006) was manufactured by Affiliated Hospital of Nanjing University of Traditional Chinese Medicine.

Methanol and acetonitrile were HPLC grade (Merck, USA). The additional reagents

were AR grade. The ultrapure water used for UHPLC-MS/MS was from a Milli-Q water purification system (Millipore, USA).

2.2 Standard solution and sample preparation

The standard stock solutions were prepared by dissolving Timosaponin A3 (999μg/ml), Berberine hydrochloride (4004μg/ml), Jatrorrhizine (1251μg/ml), Phellodendrine (1288 μg/ml), Palmatine hydrochloride (1775μg/ml), Timosaponin BII (1006μg/ml) in 70% acetonitrile, and Mangiferin (1258μg/ml), Neomangiferin (1114μg/ml) in dilute ethanol. Working solutions were prepared from the stock solutions by diluting in the initial mobile phase until use. A set of standard solutions with six different concentration levels were prepared by further dilution of the stock solution with initial mobile phase for assessment of linearity. All working solutions were stored at 4 ℃.

The stock solutions of IS1 and IS2 were mixed and diluted with ethanol to prepare an ISs working solution containing 7.01μg/ml IS1 and 11.11μg/ml IS2.

2.3 Sample preparation

An accurately weighed 0.2g sample of pulverized ZSTG was extracted in an ultrasonic bath (SK6200H, power: 500 W, frequencies: 40 kHz, Shanghai, China) with 10 ml methanol-water (50:50, v/v) for 30 min. And the extract was made up to its volume for the losing volume with the extraction solvent. Then the solution was filtered through a 0.22 μm membrane prior. Take 10 μl of the filtrate transferred into a 1.5 ml centrifuge tube, 10 μl of ISs working solution (7.01μg/ml IS1 and 11.11μg/ml IS2) and 80 μl initial mobile phases were added. The mixture was vortexed for 5 min and then centrifuged at 12000 rpm for 15 min. Subsequently, 2 μl supernatant was injected onto the UHPLC-MS/MS system for analysis.

2.4 UHPLC-MS/MS instrumentation and conditions

Analyses were performed with DIONEX Ultimate 3000 system (Thermo Scientific, MA, USA) consisting of an Ultimate 3000 pump, an Ultimate 3000 auto-sampler and a DIONEX Ultimate 3000 compartment. The chromatographic separation was performed on a Kromasil 100-3.5 C18, 3.5μm particle size, 100 Å, 150mm (length) ×2.1 mm (i.d.) reversed phase analytical column with a gradient elution of the mobile phase system consisting of an aqueous solution of 0.2% formic acid (A) and acetonitrile (B). The gradient elution program was programmed as follow: 0–1 min, 25% B; 1–4 min, 25-90% B; 4–9 min, 90% B; 9–10 min, 90–25% B; 10–13 min, 25% B. The flow rate was 0.2 ml/min, the auto-sampler temperature was maintained at 10 ℃ and the column temperature was kept at 30 ℃, respectively. The injection volume was 2 μl and the total LC run time was 13 min.

Detection of analytes and ISs were performed on TSQ Quantum™ Access MAX triple quadruple mass spectrometer (Thermo Fisher, MA, USA) outfitted with an electrospray ionization (ESI) source, interface in both positive and negative ion modes. The mass spectrometer parameters were: probe voltage, 4.0 kV (+), 3.0kV (-); source temperature, 200℃; capillary temperature, 270 ℃; Sheath gas, 30 psi; auxiliary gas, 5 psi. Quantification was performed using multiple reactions monitoring (MRM) method with the transitions of the parent ions to the product ions. Timosaponin A3 and Dioscin (IS1) were monitored in negative ionization mode by the transitions of m/z 739.99 → 577.24 and m/z 867.96 → 721.33, respectively. Analytes Berberine hydrochloride, Jatrorrhizine, Phellodendrine, Palmatine hydrochloride, Mangiferin, Neomangiferin, Timosaponin BII and Dextromethorphan (IS2) were detected in positive ionization mode by the transitions of m/z 336.08 → 320.02(Berberine hydrochloride), m/z 338.09 → 323.08(Jatrorrhizine), m/z 342.07 → 192.06(Phellodendrine) , m/z 352.11 → 336.09(Palmatine hydrochloride),m/z 423.39

→ 273.00(Mangiferin),m/z 585.64 → 272.97(Neomangiferin),m/z 903.94 → 417.10(Timosaponin BII),and m/z 272.2 → 171.08(IS2) respectively. The chemical structures and product ion MS spectra of eight potential Q-Markers and ISs are shown in Fig 1. The analytical data were processed using Xcalibur software (version 2.2, Thermo Fisher).

2.5 Method validation

The method was validated in terms of specificity, linearity, the lower limit of detection (LLOD), the lower limit of quantification (LLOQ), precision, repeatability, stability, and recovery according to US Food and Drug Administration for bioanalytical method validation (US Food and Drug Administration 2001)[22].

The specificity of the method was determined by the lack of *Anemarrhenae or Phellodendri* samples to avoid potential interfering peaks of endogenous substances within the range of the retention time of each analyte. The linearity of each standard curve was determined by plotting the peak area ratio(y) (analyte/IS) versus the nominal concentration (x) of the analytes with weighted ($1/x^2$) least square linear regression. The LLOD and LLOQ were defined as the lowest concentration of a signal-noise (S/N) ratio of 3:1 and 10:1, respectively.

The intra-day precisions were evaluated by determining the sample solution (batch No. 1712009) under the optimized condition six times in succession within a day. The relative standard deviation (RSD %) was taken as a measure of precision.

Repeatability of the method was evaluated by analyzing six independently prepared samples of ZSTG (batch No. 1712009), and stability of sample solution was conducted by analyzing samples at the time interval of 0, 2, 4, 6, 8,10and 12h under room temperature.

Recovery of the analytical method was evaluated by adding the known quantities of

the 8 reference standards at three different concentration levels (approximately equivalent to 0.5, 1.0 and 1.5 times of the concentration of the matrix) to the sample of ZSTG (batch No. 1712009). The fortified samples were then extracted and analyzed by the proposed UHPLC-MS/MS method.

3. Results and Discussion

3.1 Extraction selection

For efficient extraction of all compounds from ZSTG powder, the extraction methods were used with ultrasonic extraction (UE), and continuous reflow extraction (CRE) at the same time. In comparison with CRE, UE was more convenient with a high extraction recovery, safety, easy to operate and the relative low-level matrix interference.

In addition, extraction solvents methanol-water and acetonitrile-water (v/v) at different ratios (30:70, 50:50, 70:30), the number of repetitions (1, 2, 3) and the extraction time (15, 30, 45min) were also compared and optimized. The results showed that the highest values of the peak areas for these potential Q-Markers were obtained by using methanol-water (50:50, v/v) as the extraction solvent. Therefore, UE method with methanol-water (50:50, v/v) for 30 min was employed.

3.2 UHPLC–MS/MS optimization

To achieve good resolution within a short analysis time, the mobile phase was optimized through comparisons of different solvents, solvent ratio, and gradient profile. It was found that comparing with other solvents, acetonitrile and acidic aqueous solution lead to a significant improvement in the resolution of the different constituents and minimize peak tailing. Thus, the optimal mobile phase, consisting of acetonitrile and water (0.2% formic acid), was finally employed with 0.2 ml/min in

the above gradient, which produced high resolution, high sensitivity, and symmetrical peak shape.

In this experiment, we investigated the response intensity of the analyte in positive and negative ion scanning mode and compared it. The three alkaloids in *Phellodendri* and the three saponins in *Anemarrhenae* have a higher response in the positive ion mode, and TimosaponinA3 in the negative ion mode has a higher response. Therefore, this paper established a method for simultaneous determination of eight potential Q-Markers in ZSTG by positive and negative two ion mode monitoring at the same time.

In the experiment, the detection was positive and negative ion modes, so dextromethorphan and dioscin were selected as internal standards in positive and negative ion mode, respectively. Dioscin was used in the negative ion detection mode of the internal standard for the determination of Timosaponin A3, dextromethorphan for positive ion detection mode, used to quantify the other seven potential Q-Markers. These two internal standards' retention time was appropriate, peak shape is good, and the experiment process is stable and reliable.

3.3 Method validation

The specificity of this method was determined by comparing the chromatograms of negative and ZSTG Capsule samples. As a result, no interfering peaks from endogenous substances were observed at the retention times of each analyte. It suggested that the UHPLC–MS/MS conditions in this study provided sufficient selectivity for the analytes. Retention times of the relative compounds were 8.14 (Timosaponin A3), 8.32(IS1), 5.86(IS2), 5.89(Berberine hydrochloride), 5.46(Jatrorrhizine), 2.45(Phellodendrine), 5.81(Palmatine hydrochloride), 2.33(Mangiferin), 1.92(Neomangiferin), and 5.89(Timosaponin BII)min.

Representative chromatograms of the eight potential Q-markers and ISs were shown in Fig 2.

The standard curve for each analyte was obtained by injecting 2μl of different combined standard solutions (with six different concentration levels) into the UHPLC–MS/MS. As shown in Table 1, all of the standard curves exhibited good linearity in the concentration ranges with correlation coefficients (r) ≥0.9990. The LLOQ and LLOD which were summarized in Table 1 are sufficient to support the pharmacokinetic study of the eight potential Q-Markers for the future.

The intra-day precisions, repeatability, and stability (expressed as the relative standard deviation; RSDs) for the investigated compounds were all less than 5%. Those were shown in Table 2.

As shown in Table 3, the recoveries of 8 constituents were in the range of 95.50–104.60% with their overall RSD less than 5.0%, indicating that the method was accurate and reliable.

All the data demonstrated that this method was sufficiently accurate, reproducible and satisfactory for the quantitative analysis of eight potential Q-Markers in ZSTG. Representative chromatograms of the eight potential Q-markers in ZSTG Capsule and ISs were shown in Fig 3.

3.4 Sample analysis

The proposed method was successfully applied to quantitatively analyze the Q-Markers in 8 batches samples. Each sample was analyzed three times to determine the mean content (μg/g) of these chemical compounds, and the results were shown in Table 4, and its mean&SD were shown in Fig 4. The results showed that the varying contents of the Q-markers are as follows: Timosaponin A3 1361.36-4777.04μg/g, Berberine hydrochloride 10176-26297.91μg/g, Jatrorrhizine 18.70-138.04μg/g,

Phellodendrine 1614.70-4584.94μg/g, Palmatine hydrochloride 123.39-181.30μg/g, Mangiferin 1425.22-2916.65μg/g, Neomangiferin 460.64-1761.26μg/g, and Timosaponin BII 795.23-3147.39μg/g.

This technique doesn't require complete separation of the components in the assay and can be distinguished by the high selectivity of mass spectrometry. That provides a certain reference of quality control for Chinese herbal compound preparation.

In future studies, our team will continue the research on ZSTG Capsule's "property-effect-component", further quantitative analysis of eight potential Q-Markers, and explore its quality control methods through pharmacodynamics, pharmacokinetics and cell pharmacokinetics and other methods combined with metabolomics research. Therefore, the rapid determination of the complex system of TCM and the quantitative study of its potential Q-marker is the first stage of the Q-Marker study, which will directly affect the feasibility and reliability of the follow-up study. In this study, we will explore the rapid identification of the composition of the TCM complex system, and further lay the foundation for the study of ZSTG Capsule Q-Marker.

4. Conclusions

Chinese herbal compound formula is the main form of Chinese clinical medicine, and their efficacy was a combined effect produced by a variety of chemical compositions, so qualitative and quantitative analysis of a variety of chemical composition (Q-Marker) is the main method to control the compound and its preparation quality. The application of UHPLC-MS/MS technology provides a rapid, sensitive and convenient method for the determination of multi-components (Q-Marker) with certain pharmacological activity in the Chinese herbal compound preparation. In this

study, the method for the simultaneous determination of eight Q-Markers in ZSTG Capsule by UHPLC-MS/MS was established. The quantitative determination of various components can be accomplished accurately within 13min. The results showed that the components have a good linearity in the range of concentration respectively, the analysis time was shorter than that of HPLC-UV/HPLC-ELSD method. The accuracy, precision, recovery, stability all met the quantitative analysis requirements, and this method is expected to be widely used for the quality control of ZSTG Capsule samples.

List of Abbreviations

CRE, continuous reflow extraction

ESI, electrospray ionization

LLOD, lower limit of detection

LLOQ, lower limit of quantification

MRM, multiple reactions monitoring

Q-markers, quality markers

UE, ultrasonic extraction

UHPLC-MS/MS, ultra-high-performance liquid chromatography-tandem mass spectrometry

ZSTG, Zishen Tongguan Capsule

Ethics Approval and Consent to Participate

Not applicable.

Human and Animal Rights

Not applicable.

Conflict of Interest

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant number: 81603310).

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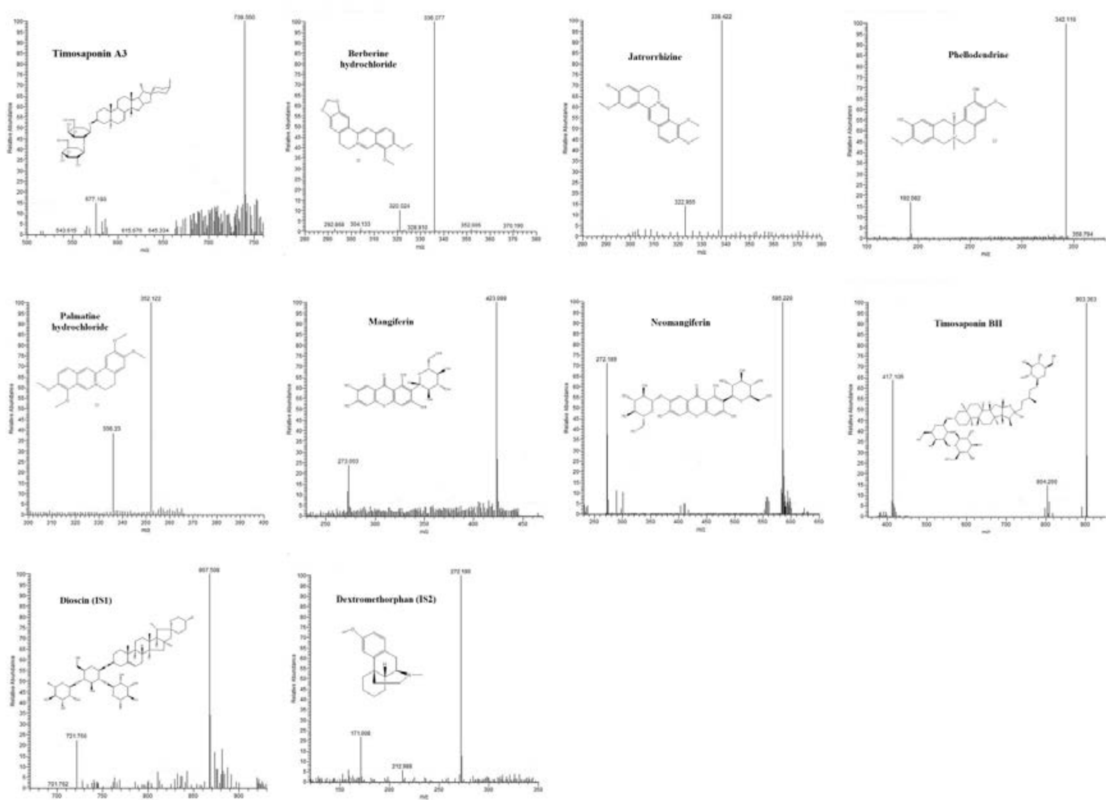

Fig 1. Chemical structures and product ion MS spectra of eight potential Q-Markers and ISs: Timosaponin A3, Berberine hydrochloride, Jatrorrhizine, Phellodendrine, Palmatine hydrochloride, Mangiferin, Neomangiferin ,Timosaponin BII , (purity>98%), Dioscin (IS1)，Dextromethorphan (IS2)

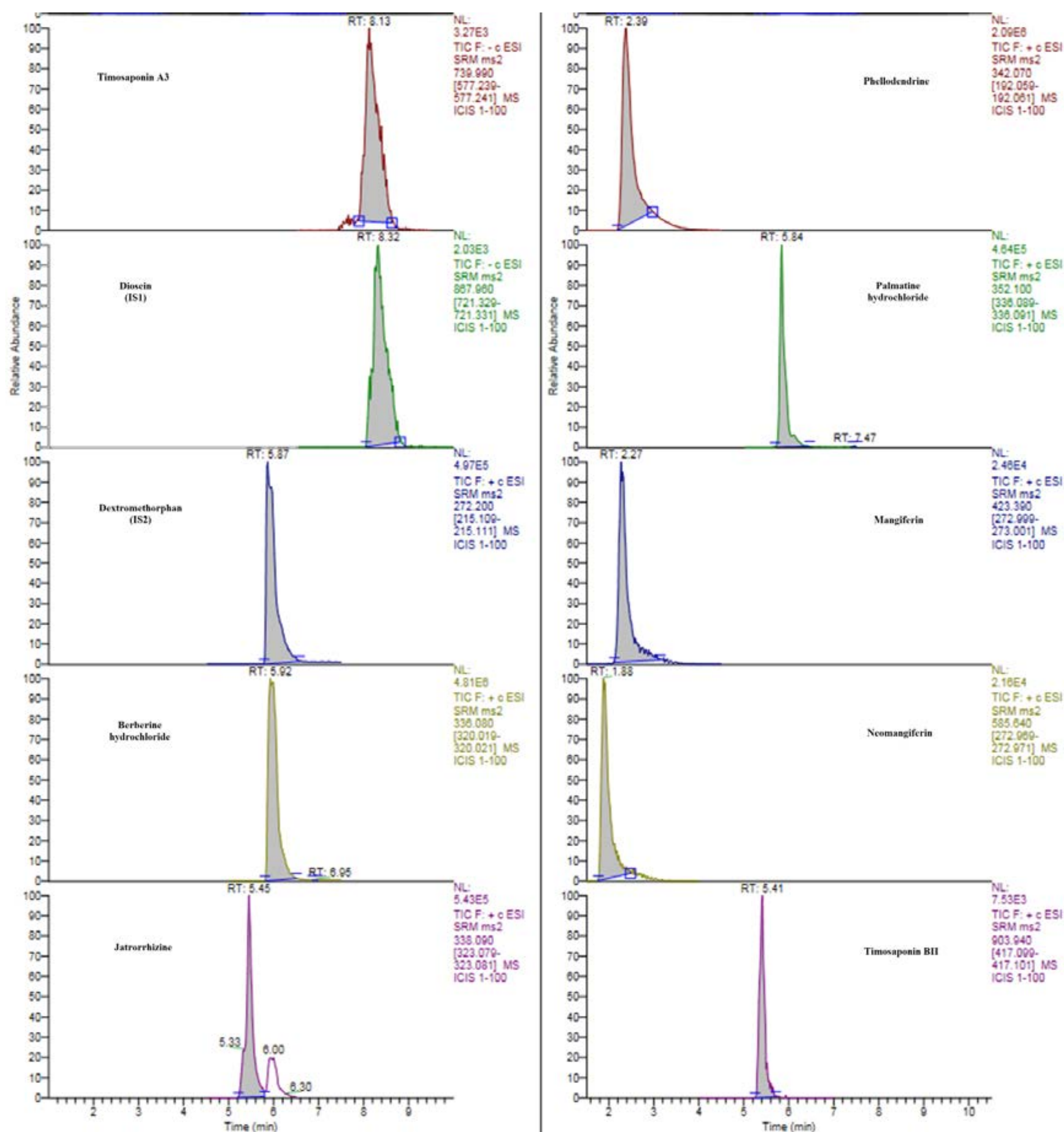

Fig 2. Representative chromatograms of the eight potential Q-markers and ISs: Timosaponin A3 , Berberine hydrochloride, Jatrorrhizine, Phellodendrine , Palmatine hydrochloride, Mangiferin, Neomangiferin,Timosaponin BII , (purity>98%), Dioscin (IS1), Dextromethorphan (IS2)

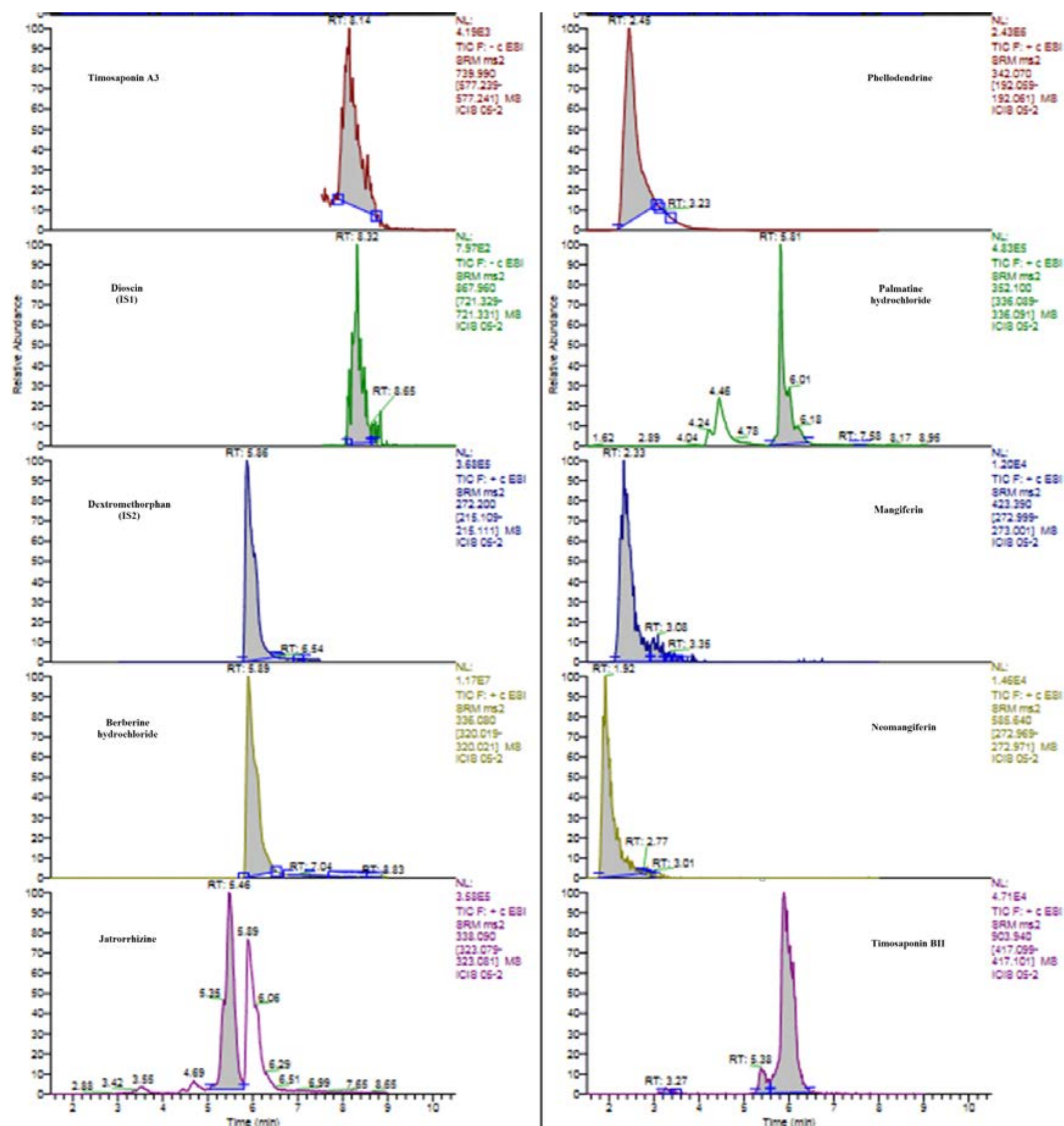

Fig 3. Representative chromatograms of the eight potential Q-markers in ZSTG Capsule and ISs: Timosaponin A3 , Berberine hydrochloride , Jatrorrhizine, Phellodendrine , Palmatine hydrochloride, Mangiferin, Neomangiferin ,Timosaponin BII, Dioscin (IS1), Dextromethorphan (IS2)

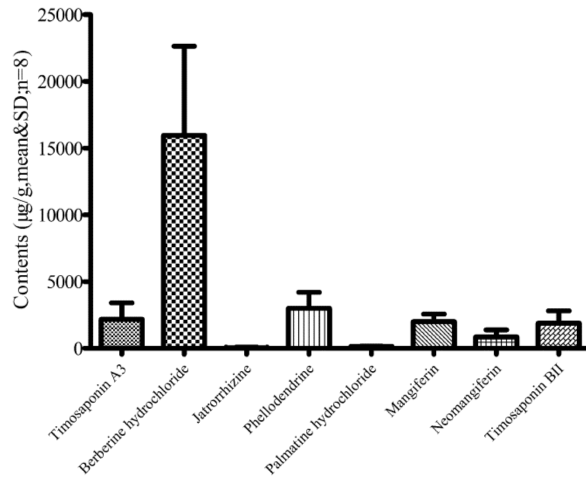

Fig 4. Contents of the Q-markers in 8 batches of ZSTG Capsules.

Table 1 Standard curves, correlation coefficient (r) and linear range of the 8 analytes in ZSTG

Analytes	Standard curves	R^2	Linear range (μg/mL)	LLOQ (ng/mL)	LLOD (ng/mL)
Timosaponin A3	y=1.5253x+0.1445	0.9999	1.259-125.874	19.980	1.999
Berberine hydrochloride	y=0.7611x+5.6103	1	4.505-450.450	1.360	0.272
Jatrorrhizine	y=1.4668x+0.6975	0.9991	0.252-25.220	0.161	0.053
Phellodendrine	y=1.5614x+3.3781	1	1.623-162.288	1.030	0.344
Palmatine hydrochloride	y=1.2825x+0.4522	0.9994	0.179-17.892	0.114	0.057
Mangiferin	y=0.0266x-0.0316	0.9999	2.807-280.728	2.568	1.027
Neomangiferin	y=0.0153x-0.0007	0.9998	1.132-113.220	4.400	1.760
Timosaponin BII	y=0.0057x-0.0027	0.9999	1.268-126.756	20.120	8.048

Table 2 Precisions, repeatability, stability of the 8 analytes in ZSTG

Analytes	Intra-day precision (n=6) RSD (%)	Repeatability(n=6) RSD (%)	Stability(n=6) RSD (%)
Timosaponin A3	4.4	4.2	3.8
Berberine hydrochloride	3.7	3.9	3.9
Jatrorrhizine	3.9	3.0	3.3
Phellodendrine	4.5	1.6	3.1
Palmatine hydrochloride	2.3	4.0	3.6
Mangiferin	3.5	3.6	3.6
Neomangiferin	4.1	2.9	3.7
Timosaponin BII	4.2	3.6	3.9

Table 3 Recoveries of the 8 analytes in ZSTG (n=6)

Analytes	Spiked amount(μg)	Mean recovery (%)	RSD (%)
Timosaponin A3	299.70	97.83	3.2
	599.40	101.35	2.4
	899.10	102.25	1.5
Berberine hydrochloride	5005.00	96.54	2.3
	10010.00	100.24	1.8
	15015.00	103.31	2.5
Jatrorrhizine	12.51	103.43	4.2
	25.02	99.32	3.4
	37.53	102.57	2.1
Phellodendrine	927.36	97.58	1.2
	1854.72	97.67	1.9
	2782.08	102.35	0.8
Palmatine hydrochloride	22.19	98.59	2.6
	44.38	96.22	2.2
	66.56	101.63	3.1
Mangiferin	578.68	98.57	1.4
	1157.36	100.86	0.9
	1736.04	103.42	2.6
Neomangiferin	356.48	98.64	3.2
	712.96	98.91	2.6
	1069.44	100.47	1.7
Timosaponin BII	528.75	100.72	2.8

	1257.50	101.06	1.3
	1786.25	99.47	3.2

Table 4 Results of 8 batches samples determination

Batch NO / Analytes	1708005	1710007	1712009	1712010	1808002	1809004	1809005	1809006
Timosaponin A3	2368.96	3178.65	1523.25	1396.29	4777.04	1361.36	1415.17	1386.09
Berberine hydrochloride	21559.35	23707.36	26297.91	12394.25	11556.59	10176.56	11027.19	10919.62
Jatrorrhizine	31.29	40.56	138.04	103.40	18.70	39.60	49.64	52.83
Phellodendrine	3555.59	4293.44	4584.94	3866.04	1614.70	1798.52	2102.28	2206.13
Palmatine hydrochloride	126.43	148.41	152.13	123.39	181.30	139.43	155.08	167.07
Mangiferin	1885.96	2050.35	2916.65	2760.02	1970.88	1425.22	1559.53	1519.77
Neomangiferin	715.91	852.41	1761.26	1571.76	573.89	460.64	495.47	461.13
Timosaponin BII	2139.14	2329.33	3147.39	2871.00	2018.00	876.55	850.89	795.23