Relative quantification of multi-components in Panax notoginseng (Sanqi) by high-performance liquid chromatography with mass spectrometry using mobile phase compensation

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Relative quantification of multi-components in complex mixture is significantly affected by the ionization variance caused by mobile phase composition in high-performance liquid chromatography with electrospray ionization mass spectrometry (HPLC–ESI-MS) analyses. The normalization methods for eliminating the variance are still less investigated. Herein, the mobile-phase compensation (MPC) method was applied to overcome the above problem. The developed method was firstly used for convenient evaluation of the coeluent interference and subsequently applied for relative quantification of the identified multi-components in Panax notoginseng (Sanqi) samples. The good linearity, precision and low limit of quantification of targeted analytes confirmed that the MPC–HPLC–ESI-MS method in gradient elution could achieve the isocratic test results compared with classical HPLC–ESI-MS. The established method was used for relative quantification of the minor Sanqi saponins by their detected peak areas divided by that of ginsenoside Rd. The results demonstrated the potential of the newly developed method for obtaining the normalized data shared in different laboratories.

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1. Introduction

Since the lack of the chemical standards, the relative quantification for the multi-components in complex mixtures is of great significance for pharmaceutical, nutritional and environmental analysis [1]. Recently, the liquid chromatography with electrospray ionization mass spectrometry (HPLC–ESI-MS) is a popular platform for multi-components quantification. In LC–MS analyses, numerous studies were conducted to decrease the signal suppression/enhancement effects [2–4], but the normalization methods for eliminating the variance caused by the mobile phase composition are still less investigated.

In general, the same instruments, chromatographic columns and separation conditions would produce the similar retention times and detection results [5]. However, the different brands of chromatographic columns can cause the inevitable retention time shifts [6] and the different elution solvent content will influence the accuracy of the observed intensities in LC–MS analysis [7]. As for traditional Chinese medicine, although many practical approaches have been established for correcting the retention time shifts [8], the mass spectrometric response fluctuation caused by organic solvent content has not been substantially resolved. Therefore, the obtained relative quantification data [1] could not be normalized and widely shared in inter-laboratory trials. The HPLC–MS with gradient elution system is a preferred tool for sensitive and selective characterization of multiple compounds in one-injection run [9]. However, to the best of our knowledge, the official quantification protocol always proposes the isocratic HPLC method for analytes without the specific standards [10]. For example, the active components in Coptidis Rhizoma ( Huanglian) are determined by quantitative analysis of multi-components by use of a single marker (QAMS) with isocratic separation. The QAMS is gradually adopted in the Chinese Pharmacopoeia and United States Pharmacopoeia to save costs in obtaining all chemical standards [5,11]. Therefore, development of a method simultaneously achieving gradient elution and isocratic detection could overcome the mass spectrometric response fluctuation and extend the QAMS applications with the consistently relative correction factors in LC–ESI-MS analyses.

Fortunately, a simple approach named mobile-phase compensation (MPC) could solve the above problems [7,12,13]. The MPC provides the potential to effectively eliminate the gradient-related effects [7] in LC–ESI-MS analyses by implementing an exactly
inverse mobile phase gradient to generate the same detection background in whole analysis process [14]. The ESI ionization efficiency of detected compounds can vary greatly among each other due to the different chemical properties and spray conditions. Although the structures of certain compounds are very similar, their relative signal strengths can be different [15]. Therefore, in whole or trace components quantitative analyses, the LC–ESI–MS coupled with MPC method (MPC–LC–ESI–MS) is an alternative to use less or no chemical standards (like QAMS analysis) for obtaining the normalized data [16]. Moreover, the MPC was a convenient method for evaluating the coelution interference in complex mixtures by using one single-run for all components, which was more effective than the classical methods [17].

In the present work, the MPC method was used to eliminate the solvent interference in LC–ESI–MS analysis and it was developed as a novel generic quantification approach for convenient evaluation of coelution interference and relative determination of the multicomponents in Panax notoginseng (Sanqi).

2. Materials and methods

2.1. Chemicals and reagents

Protocatechuic aldehyde (PA), notoginsenoside R1 (NGR1) and ginsenosides Rg1 (GRg1), Rb1 (GRb1), Rd (GRd), F2 (GF2), and Re (GRe) were purchased from Chengdu Must Bio-technology Co., Ltd (Chengdu, China), and all their purities were above 98% determined by HPLC–UV. Acetonitrile and formic acid with HPLC grade were purchased from Merck (Darmstadt, Germany). Anhydrous ethanol was analytical grade and purchased from Nanjing Chemical reagent Co., Ltd (Nanjing, China). Milli-Q ultrapure deionized water was used for all experiments.

The raw materials of P. notoginseng (Sanqi) were collected from Yunnan Province of China in October, 2011, and authenticated by Professor Ping Li. The voucher samples were deposited in the Department of Pharmacognosy, China Pharmaceutical University.

2.2. Sample preparation

The air-dried roots of Sanqi were pulverized into powder and sieved through an 80 mesh (180 μm) screen. In order to determine the high-content components of Sanqi (NGR1, GRg1, GRb1 and GRd), 5.0 mg powder of each sample was individually added into 0.75 ml 75% (v/v) ethanol solution, and extracted at 100 Hz for 30 min. After being cooled at the room temperature, the samples were compensated with 75% (v/v) ethanol for the lost weight. The solutions were centrifuged at 13,000 rpm for 10 min before injecting 1 μl into LC–ESI–MS. On determining the minor Sanqi saponins, the extracted powder was 75.0 mg. The other operations were consistent with the above procedures.

2.3. HPLC–ESI–MS and HPLC–ESI–QTOF/MS analyses

The HPLC–ESI–MS was used as the control method to verify the accuracy of MPC–HPLC–ESI–MS. For the qualitative and quantitative analysis of Sanqi saponins, an Agilent 6120 single quadrupole mass spectrometer equipped with a Dual ESI source (Palo Alto, CA, USA) was coupled with an Agilent 1200 series HPLC, consisting of a binary pump, an online degasser, an autosampler and a thermostatically controlled column compartment. Samples were separated at 25 °C on an Agilent Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm) preceded by an Agilent guard column (12.5 mm × 4.6 mm, 5 μm) at a rate of 0.8 ml/min in split mode. The mobile phase was composed of 0.1% (v/v) formic acid water (A) and 0.1% (v/v) formic acid acetonitrile (B). The gradient elution program was 20% B at 0–9 min; 20–25% B at 9–19 min; 25–27% B at 19–29 min; 27–60% B at 29–49 min; 60–100% B at 49–52 min, and the composition of 100% B was maintained for 7 min to clean the column. The equilibration time was 8 min.

For MS detector, the optimized MS conditions were as follows: drying gas (N2) flow rate, 12.0 l/min; nebulizer pressure, 45 psig; drying gas temperature, 350 °C; capillary voltage, 3500 V; fragmentor voltage, 120 V. SIM was employed for quantification in negative mode. The detected mass for each analyte is as follows: m/z 137.1, PA (IS); m/z 1093.6, compound 1: m/z 845.5, GRg1; m/z 945.5, compound 2 (GRe), 10 and GRd: m/z 841.5, compound 3, 5, 6 and 9; m/z 901.5, compound 4 and 7; m/z 1107.6, GRb1; m/z 783.5, compound 8 and 11 (GF2); m/z 931.5, NGR1.

For qualitative analysis of Sanqi, 1 μl of high concentration of Sanqi extracted solution was injected into an Agilent 6530 QTOF/MS under autotarget and target mode. The collision energy was 30 V or 50 V, and the other main parameters were consistent with those of the HPLC–ESI–MS analysis.

2.4. MPC–HPLC–MS

Another Agilent 1260 pump at a flow rate of 0.8 ml/min was installed to avoid organic solvent composition fluctuations. Two PEEK tubings of 0.02 mm i.d. (T-1 and T-2, Shanghai Anpel Scientific Instrument Co., Ltd., Shanghai, China) was used for connecting with lines [16]. In order to obtain equal dead volumes for both flow paths before the T-1, the length of the connected lines were optimized [7]. The detailed schematic diagram could be seen in Fig. S1. All the connecting lines and the mobile phases were the same in this study, but the gradient elution program was exactly inverted [14]. The controlled system was finished in the same ChemStation software. It linked the postcolumn MPC (PMPC) pump with the analytical one for starting analysis at the same time [13], which ensured that this key operation process was precisely reproduced. To reduce the overall solvent volume, T-2 was used. Notably, in HPLC–ESI–MS analysis, only the analytical pump and its line system were used.

2.5. Method validation

The NGR1, GRg1, GRb1 and GRd were selected for testing the accuracy of MPC–HPLC–ESI–MS by comparing with the methodological parameters of the HPLC–ESI–MS, including precision, regression equation, limit of quantification (LOQ), limit of detection (LOD), repeatability, stability and recovery. The detection method and some other notes were stated in Table 1.

2.6. Studies on the effect of organic solvent ratio on mass spectrometric response

Two studies were performed to assess the effect of organic solvent ratio on the mass spectrometric response, as reflected by the relative response for the peak area of determined components to the PA (IS). The new gradient elution programs maintained 20% B at the beginning of 9 min. The organic solvent ratios were rapidly increased to 40% B, 60% B, 80% B and 100% B at 9.05 min, respectively, and the corresponding ratios were maintained until 19 min. 200 μl NGR1, GRg1, GRb1 and GRd solutions (0.75 mg/ml each) were individually mixed with 20 μl PA (1.0 mg/ml) and then injected into HPLC–MS and MPC–HPLC–ESI–MS. This analysis was similar to the experimental section for MPC–HPLC–MS except for the exactly inverted elution programs.
3. Results and discussion

3.1. Elimination of mass spectrometric response fluctuation by MPC

In LC–MS analysis, the internal standard is always required for accurate quantification, which is different from the LC–CAD [13] or LC–ELSD analysis [18]. According to the literatures, β-estradiol [4] and digoxin [3] were ever chosen as the IS for quantification of saponins in herbal medicines. However, in the present study, both β-estradiol and digoxin would be coeluted with the Sanqi saponins by the developed method. Therefore, the mass spectrometric response of IS could not be consistent, which indirectly influenced the quantification. In this study, PA was finally selected as the IS. It could also be rapidly eluted by the pulse isocratic organic solvent within the first 9 min, which could avoid the response interference between PA and the determined saponins. The relative response of the PA was stable in the MPC and common analyses with different solvent ratios. The RSD of intensities were below 3.9%. In our pre-experiments, the flow injection method was used, but the satisfactory peak shape could not be obtained in LC–ESI–MS analysis. Therefore, the pulse isocratic flows for IS and detected analytes were adopted for investigating the interference from the mobile phase composition. The developed method could avoid the delayed effect of elution solvents, which was different from that of the published instrumental setup [7].

In Fig. 1A, about two folds enrichment for the intensities of all determined Sanqi saponins was observed from 40% to 100% organic solvent, which is consistent with the previous studies [19], indicating that the significant chromatographic peak position drift in different columns [6] would cause inaccurate quantification of the relative abundance. The ESI signal was generally enhanced by organic solvents, especially for positive ions [2]. This phenomenon is closely related to the mechanism of ionization process in mass spectrometry with ESI source. The organic solvents are easily volatile and then the unpaired analyte ions can obtain more charge on droplets surface [20]. Therefore, the ESI signal intensities at high-content organic solvents are generally larger than those at high aqueous content.

Notably, the common HPLC is different from the micro- and nano-HPLC in electroospray process, which cannot provide a safe and constant source voltage to account for the variability caused by high aqueous content. In contrast, the micro- and nano-HPLC only provide minor solvent, so the analytes can usually obtain a large excess of charge and produce uniform response [16]. Since the ESI source mass spectrometry is more difficult to produce the similar response than other source mass spectrometry for structurally different compounds [12,15], the stable mass spectrometric response was important for determining multi-components of Chinese herbal medicine by using classical HPLC–ESI–MS.

In MPC experiments, the solvents were mixed at T-1 to stably produce 50% acetonitrile containing 0.1% (v/v) formic acid to the ESI mass spectrometry detector with split mode. Therefore, their

![Fig. 1](image-url) The mass spectrometric response was significantly affected by organic solvents ratio (A), and this effect was eliminated by the MPC method (B).
responses were stable over the wide range of polarities (Fig. 1B), which indicated that the MPC could effectively eliminate the mass spectrometric response fluctuation caused by the gradient related effects of LC–ESI-MS. The response reproducibility of MPC was estimated by running each analysis in triplicate. According to the above results, the MPC–LC–ESI-MS could be applied to analysis of the complex samples. Notably, the proposed MPC method would cause the dilution effect due to additional flow adopted and larger volume of solvents delivered to the ion spray source, which would decrease the sensitivities of certain trace compounds. Moreover, the precipitation for hydrophobic compounds should be stressed in practice.

3.2. Evaluation of coeluent interference by MPC–HPLC–MS

In most crude medicine analysis, the coeluent interference was ignored [21]. In contrast, the in vivo analysis was more reasonable since the interference caused by the matrix effect or other factors was evaluated [22]. When some analytes were coeluted by gradient elution, the classical method confirms the mass spectrometric response interference by individual injection of single compound and mixtures, which might be relatively inefficient when covering numerous compounds [3]. In this study, the MPC–HPLC–MS was found to be a convenient method for comprehensively evaluating the coeluent interference for all components under single run (Fig. 2A). Since the detected background in whole process of analyses for the coeluted and complete determinations was stable, it could overcome the shortcomings of the traditional methods with multi-injection of single compound. To evaluate the coeluent interference, all selected saponins were coeluted by 60% B. Compared with the coeluted method, the complete separation could significantly increase the mass response of all components (Fig. 2B).

Depending on the MPC method, we could more efficiently evaluate the coeluent interference and developed the satisfactory separation method to eliminate above interference. Moreover, in LC–MS analyses, the MPC could find out the false positive results, like the molecules with high molecular weight produced some fragment ions equaled to the parent ions of the determined small ones.

3.3. Validation of MPC–HPLC–ESI-MS for quantification

To evaluate the analytical capability and ensure the accuracy of the MPC–HPLC–ESI-MS, the developed method was compared with

![Chemical structures of minor saponins identified by HPLC-ESI-QTOF/MS.](Image)

Fig. 4. Chemical structures of minor saponins identified by HPLC-ESI-QTOF/MS.
the classical LC–MS method. In our study, the four saponins were completely separated for mixed standards solution (Fig. 3A), but the minor saponin GRE was still coeluted with GRg1 in Sanqi samples (Fig. 3B) due to the capacity limitation of the commercial column [24]. Since the content of GRE was about one-tenth the level of GRg1 in Sanqi samples and the determined concentration was smaller than the set concentration in Section 2.6, it could not significantly influence the absolute quantification of GRg1. Moreover, there were no matrix effects for the PA (Fig. S2).

In Table 1, the different slope of each calibration curve indicated that the development of the normalized method was important for the complex mixtures analysis. The order of the slope was GRg1 > NCGR1 > GRd > GRb1 in the concentration range of 7.5–75.0 μg/ml, which indicated that the protopanaxatriol (PPT) type ginsenosides had better response than the protopanaxadiol (PPD) type ginsenosides and the large molecules might show worse response [22]. This difference was believed to be closely related to the ionization efficiency for the hydroxyl group-containing compounds. The PPT ginsenosides always formed the [M+HCOO]⁻ to increase the response under the LC-electrolyte modifier [25].

The MPC obtained much better MS response (Fig. S3) and achieved the identical capability of classical LC–ESI-MS analysis in coefficient values ($R^2$). LOD, LOQ, precision, stability, accuracy, and recovery. The determined $R^2$ in the above method were above 0.9974, and their LOD and LOQ were smaller than those of LC–UV method [26]. The intra-day precision and inter-day precision were less than 6.0% at 750.0, 75.0 and 7.5 μg/ml, which indicated that the PMPC pump did not induce the solvent fluctuations. Their repeatability also validated these results. The extracted Sanqi samples were stable within 24h, and the concentration of analytes was agreed with that of the published results [26] and the data determined by the conventional HPLC–ESI-MS. These results verified the accuracy of the MPC–HPLC–ESI-MS and it could be widely applied to the relative quantification of minor components with stable mass response in different gradient elution systems.

3.4. HPLC–ESI-QTOF/MS identification

In HPLC–ESI-QTOF/MS analysis, the Sanqi saponins always lose sugar moieties to produce desugaring ion signals and sapogenin signals. In this case, 11 interested minor saponins were found in Sanqi extracted samples by using accurate mass, MS/MS ions (Fig. S4A and Table S1), retention time and chemical standards (GRE and GF2) [9,23]. The compound 11 was selected as an example for elucidation of the characterization of Sanqi saponins. In Fig. S4B, the abundant ion at $m/z$ 783 ($t_R = 48.22$ min) generated diagnostic ion at $m/z$ 459 by successive loss of two glucose mass units (−162 Da) in the negative mode, which indicated that the compound 11 contained PPD sapogenin and two glucose groups. This compound was preliminarily identified as GF2 or its isomers. Since their polarities and MS/MS spectra were similar, the compound 11 was finally verified as GF2 by using chemical standard. The characterizations of other Sanqi saponins were similar to those of GF2. Their structures were shown in Fig. 4, including PPT, PPD and ocottillol (OT) type ginsenosides. Some isomers were observed in this study, such as GRE (2) and GRd isomer (10), 6'-O-AcO-ginsenoside Rg1 (3, 5), its isomer (6) and 24(R)-vinaginsenoside R1 (9), PPT-20-glcp-xyl-6-xyl (4) and PPT-6-glcp-xyl-xyl (7), as well as PPD-20-glcp(2→1)glcp (8) and GF2 (11). The identification was consistent with that of the
3.5. MPC–HPLC–ESI-MS applied to relative quantification of minor components

The MPC–HPLC–ESI-MS was applied for relative quantification of 11 identified minor saponins in 100 mg/ml Sanqi samples. As the content of GRd was the lowest in the four absolutely determined saponins, it was selected as the control compound, which was expressed as 100%. In LC–ESI-MS analysis, it has been verified that the sensitivity would rapidly decrease when the number of the detected ions increased above 12 [27], which was mainly caused by the extremely high noise. In Fig. 5, a total of nine ions were detected by MPC–HPLC–ESI-MS. Most of their contents were less than 10% and their RSD were below 6.5%, which indicated that this method met the quantification requirements. Since the absolute intensity variations in the different instruments or operations, the reported relative form was a better way to decrease the errors in the ionization efficiency. In this study, all saponins were determined at the same solvent environment, and the relative content of all saponins was reported for the first time except GRe and GF2. The peak area ratio of GRε to GRd was 84.6%, which was in the range interval of the reported content ratios [26]. Therefore, the newly developed method demonstrated acceptable performance for relative quantification of other Panax species or formula. Moreover, the MPC–LC–MS method could overcome the data variations caused by the above conditions and increase the repeatability and MS signals’ normalization in targeted metabolomics with label-free method [28].

In the targeted metabolomics or complex samples analysis, the developed method could not directly quantify numerous ions in a single run. However, using LC–MS based on two ion channels [29] could significantly improve the detection efficiency and sensitivity. The multiple injections analysis was also an alternative method for quantification when it exceeded to the detection capability of LC–MS. The LC hybrid ion trap TOF/MS operated under full scan mode is verified to be a powerful approach for overcoming the above shortcomings for determination of multi-components [27]. Therefore, the MPC method coupled with those statistical LC–MS could be widely applied to relative quantification analysis with universal and stable results under different chromatographic conditions, and might provide some contributions to normalization of LC–MS detected data in different laboratories.

4. Conclusion

In the present study, we proposed MPC–HPLC–ESI-MS to eliminate the gradient related effects and to obtain the universal response for relative quantification, which was complementary to methodology studies for relative quantification of Sanqi saponins. The newly developed method equipped with a compensating pump could significantly decrease the mass spectrometric response fluctuation induced by the different mobile phase composition. It was convenient to evaluate the coelution interference with smaller operation errors and less time consumption. The accuracy and precision of the MPC–HPLC–ESI-MS method indicated it was suitable for relative quantification of various types of Sanqi saponins under the same detection background. These above results confirmed that the quantitative assay was robust and reliable potential to allow the different laboratories to share the normalized data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2014.09.004.

References


