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Original article

Novel assays for quality evaluation of XueBiJing: Quality variability of a Chinese herbal injection for sepsis management

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ABSTRACT

XueBiJing is an intravenous five-herb injection used to treat sepsis in China. The study aimed to develop a liquid chromatography-tandem mass spectrometry (LC-MS/MS)- or liquid chromatography-ultraviolet (LC-UV)-based assay for quality evaluation of XueBiJing. Assay development involved identifying marker constituents to make the assay therapeutically relevant and building a reliable one-point calibrator for monitoring the various analytes in parallel. Nine marker constituents from the five herbs were selected based on XueBiJing's chemical composition, pharmacokinetics, and pharmacodynamics. A selectivity test (for "similarity of response") was developed to identify and minimize interference by non-target constituents. Then, an intercept test was developed to fulfill "linearity through zero" for each analyte (absolute ratio of intercept to C response, <2%). Using the newly developed assays, we analyzed samples from 33 batches of XueBiJing, manufactured over three years, and found small batch-to-batch variability in contents of the marker constituents (4.1%–14.8%), except for senkyunolide I (26.5%).

1. Introduction

In China, herbal medicines are regulated as botanical drug products [1]. Herbal medicines, based on the longstanding use in traditional Chinese medicine or well-established medicinal use, are often formulated as tablets, capsules, and droplet pills for oral administration and as injectable formulations for parenteral administration and are required to be manufactured in compliance with good manufacturing practice by licensed pharmaceutical companies [2]. Most Chinese herbal medicines are prepared from multi-herb formulas and have complex chemical compositions with multiple bioactive constituents acting in concert. Although the quality of Chinese herbal medicines is pivotal for their clinical use and research, current quality evaluation of such medicines is based mostly on the quantification of one or more constituents as analytical markers (serving solely for analytical purposes) [3]. For most medicines, not all component herbs are monitored by measuring their constituents. Despite being regulated as drug products in China, the quality of herbal medicines is still a matter of concern, and data of associated quality characterization, such as batch-to-batch variability over multiple years, are limitedly available. Recently, the concept of quality marker (Q-marker) has been proposed for quality evaluation of Chinese herbal medicines by identifying and monitoring, on a case-by-case basis, the constituents that are key to the quality of the medicines [4–10]. For Q-marker-based assay, all the marker constituents should be measurable in the herbal medicine, should be relevant to the medicine's therapeutic action and safety, and should reflect the entirety of the medicine's formula (i.e., the presence, in the formula, of each component herb that is believed to contribute to the therapeutic action).

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Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection [11]. XueBiJing, an intravenous herbal medicine, was licensed in 2004 for the treatment of sepsis and multiple organ dysfunction syndrome (MODS) in China. XueBiJing is prepared from a combination of five herbs: *Carthamus tinctorius* flowers (Honghua in Chinese, acting as the "monarch" component herb), *Paeonia lactiflora* roots (Chishao, the "minister" component herb), *Ligusticum chuanxiong* rhizomes (Chuanxiong, another "minister" component herb), *Angelica sinensis* roots (Danggui, the "adjuvant" component herb), and *Salvia miltiorrhiza* roots (Danshen, another "adjuvant" component herb). Combining XueBiJing with conventional sepsis care was found to further reduce 28-day mortality of patients with sepsis and incidence of complications and improves prognosis, with low incidence of adverse effects [12–16]. Recently, XueBiJing has been officially recommended as an add-on therapy in management of coronavirus disease 2019 (COVID-19) when patients with severe diseases develop systemic inflammatory response syndrome (SIRS) and/or MODS [17]. Even though XueBiJing is extensively used in clinics, the current quality evaluation assays for XueBiJing that are stipulated by the Chinese National Medical Products Administration (NMPA) are based on liquid chromatography-visible (LC-Vis) and liquid chromatography-ultraviolet (LC-UV) quantification of three constituents for analytical purposes, i.e., one assay for measuring hydroxysafflor yellow A (originating from Honghua) at 402 nm and another one for measuring paeoniflorin and benzoic acid (both from Chishao) at 230 nm, and on LC-UV-based detection of six known and 11 unknown XueBiJing constituents at 280 nm with a chromatographic run time of 130 min [18]. However, the NMPA-stipulated assays do not consider the content levels of any marker constituents for the other component herbs Chuanxiong, Danggui, and Danshen, which are all believed to contribute to XueBiJing's therapeutic action as well.

Several assays have been developed for analyzing the constituents of XueBiJing [19–28]. However, these assays have several limitations as follows: 1) poorly defined associations between the analytes and therapeutic actions of XueBiJing, 2) no consideration of the potential interference of the analytes by co-eluting non-target constituents in LC-UV measurements, and 3) using multi-point calibration, which is relatively inefficient for routine analysis of XueBiJing samples. One-point calibration-based assay is widely used for quality evaluation of synthetic medicines. Chinese herbal medicines, unlike synthetic medicines, have complex chemical composition and are commonly prepared from multi-herb combinations. One-point calibration is also used for the analysis of many Chinese herbal medicines, but the vast majority of assays monitor only a single marker constituent per medicine [3]. The monitoring of only one compound hardly accounts for the therapeutic reality in the use of multi-herb formulations. For the few herbal medicines that are monitored for multiple constituents, multiple one-point-calibration-based assays are developed for each medicine, i.e., still one assay for one analyte. This study aimed to develop therapeutically relevant assays for quality evaluation of XueBiJing. With a one-point calibrator, liquid chromatography tandem-mass spectrometry (LC-MS/MS)- or LC-UV-based assay simultaneously measured nine relevant marker constituents (originating from across five component herbs). Using these newly developed assays, we evaluated the batch-to-batch quality variability among 33 batches of XueBiJing manufactured over three years.

2. Materials and methods

2.1. Study design

This study was designed to develop novel quality evaluation assays for XueBiJing, with the following attributes: therapeutically relevant, reflecting the entirety of the XueBiJing formula, and efficient and reliable for routine pharmaceutical analysis. Selecting the right marker constituents is crucial in developing a therapeutically relevant assay. Here, it was based on a comprehensive understanding of 1) the chemical composition of the injection, 2) the dose-dependent bioavailability of XueBiJing compounds and the pharmacokinetics and disposition of the major circulating compounds, and 3) the compounds' bioactivities related to the injection's antiseptic action. Such information was obtained from our prior and current investigations of XueBiJing and also by literature mining. Marker constituents were identified for each of the component herbs to reflect the entirety of the XueBiJing formula. LC-MS/MS- and LC-UV-based multi-analyte assays were developed using one-point calibration, according to the following conditions: similarity of response (i.e., similarity, for each marker constituent, of response corrected by concentration between the calibrator and XueBiJing samples) and linearity through zero (i.e., linear concentration-response function with near-zero intercept) [29,30]. Assay conditions were optimized by ensuring minimal compromise between assay selectivity and sample throughput. After being validated with respect to reliability and robustness, the newly developed assays were used to analyze XueBiJing samples from 2017 to 2019 and evaluate variation in batch-to-batch quality. Fig. 1 summarizes the study workflow.

2.2. Literature mining

Literature mining, comprising retrieval, extraction, and review of information, was performed to facilitate the selection of analytes from constituents of XueBiJing for developing therapeutically relevant assays for quality evaluation of the injection. Three types of information were obtained: 1) chemical composition of XueBiJing and assays for quality evaluation of the injection and for quality control in associated manufacturing; 2) pharmacokinetics and disposition of XueBiJing compounds, particularly the human data; and 3) antisepsis-related properties, adverse effects, and drug interaction potential of XueBiJing and its compounds. The information retrieval was performed to obtain research and review articles, limiting to those in English and Chinese, from the electronic databases Embase and China National Knowledge Infrastructure (CNKI) from January 2004 to January 2021. Search terms were "XueBiJing" or its component herbs combined with "constituent", "chemical composition", "chemical components", "chemical ingredient", "quality evaluation", "quality assessment", or "quality control" for retrieving type-(1) information; "XueBiJing" combined with "pharmacokinetic", "systemic exposure", "disposition", or "metabolism" for retrieving type-(2) information; and "XueBiJing" or names of XueBiJing compounds combined with "sepsis", "antiseptic", "inflammation", "anti-inflammatory", "anticoagulant", "immunoregulation", "immunomodulation", "endothelium protection", "organ protection", "anti-endotoxin", "toxicity", "safety", "drug-drug interaction", "herb-drug interaction", and "pharmacokinetic compatibility" for retrieving type-(3) information. The Embase was searched using the search terms in the title, abstract, and keywords, and the CNKI database was searched in the subject. Three of the authors independently screened the retrieved titles and abstracts (if applicable) for relevance, and the results of the screening were verified by another author. These authors independently extracted all of the relevant information from each article; two of the authors confirmed the extracted information. In addition, supplemental information on the constituents of XueBiJing component herbs was obtained from Traditional Chinese Medicine Integrated Database (http://47.100.169.139/tcmid/) and Traditional Chinese Medicine Database and Analysis Platform (https://tcmsp-e.com/) databases. Type-(1) information was used to update herb-

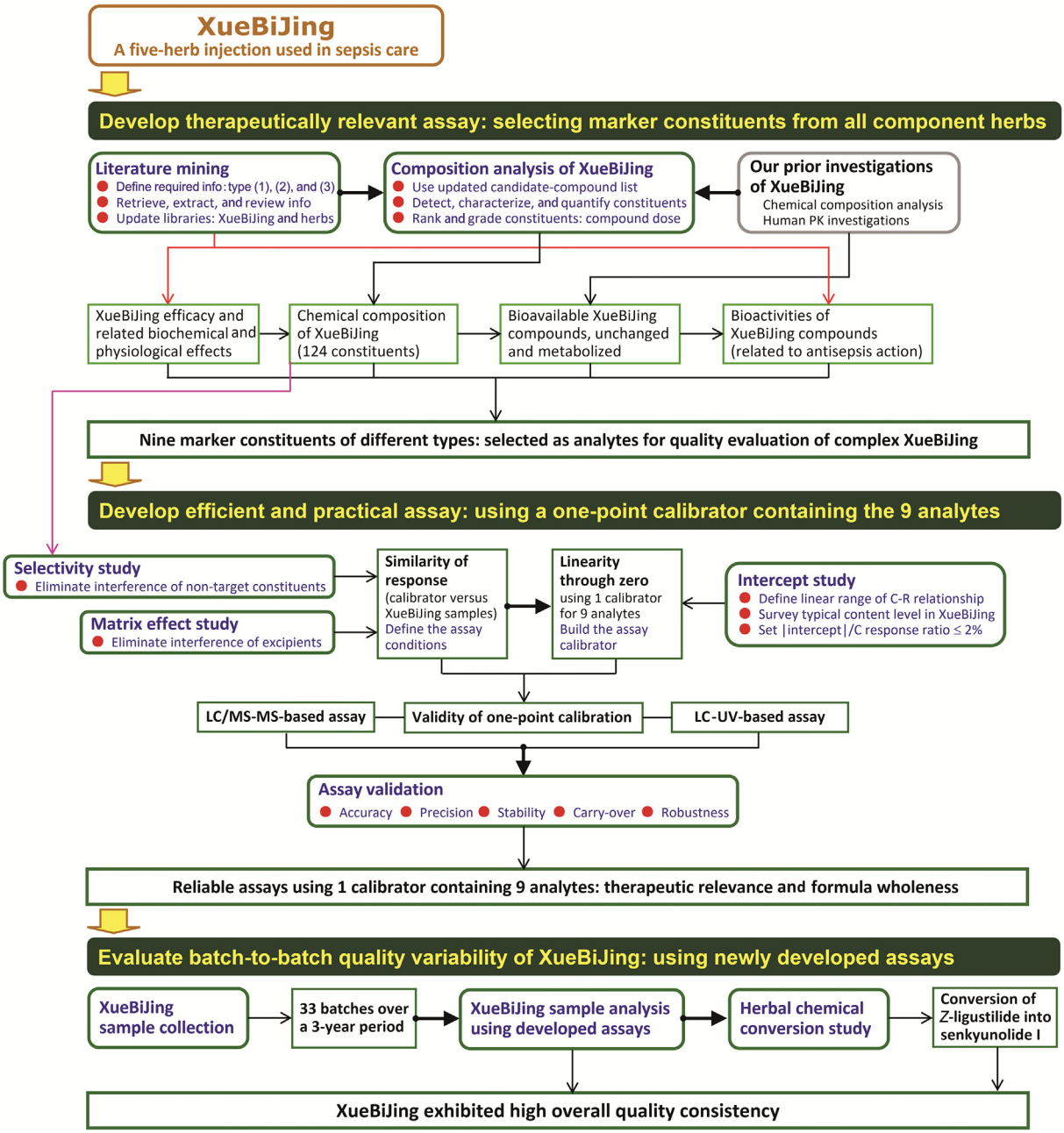

Fig. 1. An overview of the development of quality evaluation assays for XueBiJing. Info: information; PK: pharmacokinetic; C-R relationship: concentration-response relationship; C response: compound response obtained using XueBiJing sample diluted at the level that allows all the analytes measured to fall within their respective linear concentration ranges.

compound libraries of Honghua, Chishao, Chuanxiong, Danggui, and Danshen to guide composition analysis of XueBiJing in this investigation. Each of these herb-compound libraries included a compound list, containing the compound name, molecular formula (related to accurate molecular mass), and chemical structure, and was created using Waters UNIFI software (version 1.8; Milford, MA, USA).

2.3. XueBiJing samples

XueBiJing, a sterile and nonpyrogenic injection for intravenous administration, is manufactured by Tianjin Chasesun Pharmaceutical Co., Ltd. (Tianjin, China; NMPA drug ratification number: GuoYaoZhunZi-Z20040033). The injection is prepared from a combination of Honghua (*Carthamus tinctorius* flowers), Chishao (*Paeonia lactiflora* roots), Chuanxiong (*Ligusticum chuanxiong* rhizomes), Danggui (*Angelicae sinensis* roots), and Danshen (*Salvia miltiorrhiza* roots), yielding a herb-to-injection ratio of 1:2 (*V/V*). XueBiJing is standardized to contain 200–500 μg/mL hydroxysafflor yellow A, 1000–1700 μg/mL paeoniflorin, and 10–200 μg/mL benzoic acid [18]. The following samples from 33 batches of XueBiJing were obtained: 1704181, 1710072, 1710311, 1712011, 1712041, 1712071, 1712081, 1712111, 1712141, 1712211, 1712261, and 1712281 (manufactured in 2017); 1805171, 1806241, 1806251, 1806261, 1807062, 1807101, 1807102, 1808292, 1812081, 1812091, and 1812101 (manufactured in 2018); and 1904011, 1904041, 1904171, 1904221, 1905071, 1905161, 1905251, 1905301, 1906021, and 1906281 (manufactured in 2019). All XueBiJing

samples were analyzed within their validity period (expected shelf life: 18 months).

2.4. Reference compounds and reagents

Hydroxysafflor yellow A, paeoniflorin, albiflorin, oxypaeoniflorin, senkyunolide I, senkyunolide G, ferulic acid, tanshinol, and protocatechuic aldehyde, with purity ≥98%, were obtained from Shanghai Standard Technology Co., Ltd. (Shanghai, China). All these compounds were initially dissolved in dimethyl sulfoxide (DMSO; except for tanshinol which was dissolved in 50% DMSO) at a concentration of 80 mg/mL to prepare their individual stock solutions (100 μL), which were combined and diluted with 100 μL of an excipient solution to yield a combined stock solution (8 mg/mL for each compound). The excipient solution (containing no herbal compound or extract) was prepared by dissolving Tween-80 and glucose in water at the same respective concentrations as for XueBiJing. *Z*-ligustilide was obtained from Shanghai Standard Technology Co., Ltd. as well.

A one-point calibrator (containing the nine selected marker constituents of XueBiJing, i.e., hydroxysafflor yellow A, paeoniflorin, oxypaeoniflorin, albiflorin, senkyunolide I, senkyunolide G, ferulic acid, tanshinol, and protocatechuic aldehyde) was used for the LC-MS/MS- and LC-UV-based assays. This calibrator was prepared by combining the individual stock solutions of the nine compounds (80 mg/mL for each) at a ratio of 80:300:8:4:12:3:6:6:3 and diluting with the excipient solution to yield final concentrations of 400, 1500, 40, 20, 60, 15, 30, 30, and 15 μg/mL, respectively. For assay validation purposes, a quality control (QC) sample was prepared independently to contain the nine compounds at the same concentrations as the corresponding ones in the calibrator.

High performance liquid chromatography (HPLC)-grade methanol, formic acid, and lithium acetate were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HPLC-grade water was prepared using a Millipore Milli-Q Integral 3 cabinet water purifying system (Bedford, MA, USA).

2.5. LC-MS/MS quantification of nine marker constituents in XueBiJing

An AB SCIEX Triple Quad 5500 mass spectrometer (Singapore), interfaced via a Turbo V ion source with an Agilent 1290 Infinity II UHPLC separation module (Waldbronn, Germany), was used to measure the nine marker constituents in the XueBiJing samples. Chromatographic separation was achieved on a YMC-Triart C_{18} column (50 mm × 2.1 mm i.d., 1.9 μm; Kyoto, Japan) with a mobile phase delivered at 0.3 mL/min, consisting of (A) water/methanol (99:1, *V/V*; containing 8 mM formic acid and 25 μM lithium acetate) and (B) water/methanol (1:99, *V/V*; containing 8 mM formic acid and 25 μM lithium acetate). An 8 min gradient program was used: 0–3 min, from 5% B to 40% B; 3–5.5 min, from 40% B to 80% B; 5.5–6.5 min, 80% B; 6.5–6.6 min, from 80% B to 5% B; and 6.6–8 min, at 5% B. The mass spectrometer generated deprotonated molecules $[M-H]^-$ or formate adducts $[M+HCOO]^-$ in the negative ion mode and lithiated molecules $[M+Li]^+$ in the positive ion mode, and the analytes were measured in a positive/negative polarity switching mode. The optimal parameters of the ion source included source voltage, −4.5 or +5.5 kV; curtain N_2 gas, 20 psi; GS1, 40 psi; GS2, 40 psi; GS1 and GS2 temperature, 500 °C; and entrance potential, 10 V. In the negative ion mode, the optimal precursor-to-product ion pairs for multiple reaction monitoring of hydroxysafflor yellow A, paeoniflorin, oxypaeoniflorin, albiflorin, ferulic acid, tanshinol, and protocatechuic aldehyde were *m/z* 611 → 491 (collision energy: 40 eV), 525 → 449 (20 eV), 541 → 495 (16 eV), 525 → 121 (28 eV), 193 → 134 (22 eV), 197 → 135 (24 eV), and 137 → 108 (30 eV), respectively. In the positive ion mode, the ion pairs for senkyunolides I and G were *m/z* 231 → 202 (29 eV) and 215 → 191 (30 eV), respectively. The LC-MS/MS-based assay used a one-point calibration without an internal standard. The XueBiJing samples and the calibrator were analyzed in triplicate. Before LC-MS/MS analysis, the XueBiJing samples (278 μL) and the one-point calibrator (278 μL) were diluted to an end volume of 500 mL with 50% methanol (i.e., 1800-fold dilution) and then 5 μL of the diluted solutions were injected from the autosampler (maintained at 10 °C) of the LC-MS/MS instrument.

2.6. LC-UV quantification of nine marker constituents in XueBiJing

A Waters Acquity UPLC separation module, coupled with a Waters tunable UV detector (Milford, MA, USA), was used to determine the content levels of hydroxysafflor yellow A, paeoniflorin, oxypaeoniflorin, albiflorin, senkyunolides I, senkyunolide G, ferulic acid, tanshinol, and protocatechuic aldehyde in the XueBiJing samples. Chromatographic separation was achieved on a Waters Acquity UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm; maintained at 40 °C; Milford, MA, USA) with a mobile phase delivered at 0.3 mL/min, consisting of (A) water/methanol (99:1, *V/V*; containing 50 mM formic acid) and (B) water/methanol (1:99, *V/V*; containing 50 mM formic acid). A 60 min gradient program was used: 0–2 min, at 2% B (gradient curve: 6); 2–50 min, from 2% B to 50% B (6); 50–55 min, at 98% B (1); and 55–60 min, at 2% B (1). The UV detector was set at 230 and 280 nm to measure the analytes of high signal intensities and selectivity. The LC-UV-based assay used a one-point calibration without an internal standard. The XueBiJing samples (5 μL) and the one-point calibrator (5 μL) were directly injected from the autosampler (maintained at 10 °C) onto the column and analyzed in triplicate.

2.7. Tests supporting assay development and data interpretation

2.7.1. Composition analysis of XueBiJing

In this investigation, composition analysis was a crucial step in developing the quality evaluation assay, because it facilitated the selection of appropriate marker constituents of XueBiJing and allowed for subsequent selectivity tests. Composition analysis, comprising detection, characterization and grading of the constituents, was performed to determine the chemical composition of XueBiJing. To guide the composition analysis, our candidate-compound lists for XueBiJing component herbs were updated using the literature-mined information (Section 2.2). The experimental configuration for the composition analysis was based on a previous study [31]. Briefly, we used a 42 min LC-MS/MS assay for accurate mass measurement on a Waters Synapt G2 high-definition time-to-flight (TOF) mass spectrometer (Manchester, UK) interfaced via a LockSpray source with the Waters Acquity UPLC separation module. All the MS data were processed using the UNIFI software.

2.7.2. Selectivity tests

To ensure the validity of the one-point calibration with respect to “similarity of response”, a selectivity test was performed by determining potential interference by non-target XueBiJing constituents fully or partially co-eluting with the nine marker constituents; the similarity of response (corrected by concentration) between the calibrator and the XueBiJing samples should be achieved for each marker constituent. The results of the selectivity test served to define assay conditions, particularly those for chromatography. The co-eluting non-target constituents were identified by using the results of composition analysis of XueBiJing. For LC-MS/MS measurement (initially using a 5 min LC gradient program), the selectivity test focused on isobaric non-target XueBiJing

constituents co-eluting with the analytes; interference was eliminated by achieving chromatographic separation between the co-eluted compounds. For LC-UV measurement, all non-target constituents co-eluted with the analytes were identified using the data in Table S1 (using a 42 min LC gradient program [25,31–34]). A typical sample of XueBiJing (Batch No.: 1805171) was analyzed in parallel by LC-UV and LC-MS/MS, both using a 90 min LC gradient program, to separate the analytes from their respective non-target co-eluting constituents; the interference was initially observed using the 42 min LC gradient program and the UV-interfering constituents exhibited significant UV absorption at 230 and 280 nm. The 90 min LC gradient program was modified for LC-UV measurement to shorten the chromatographic run-time without sacrificing resolution ($R_S \geq 1.0$) between analytes and their respective UV-interfering constituents.

2.7.3. Matrix effect tests

To fulfill the "similarity of response" condition, a post-column infusion study, based on a method by Bonfiglio et al. [35], was performed to assess interference by excipients in the LC-MS/MS measurement of the nine marker constituents of XueBiJing. In brief, the LC mobile phase was delivered at 0.3 mL/min onto the YMC-Triart C_{18} column under the 8 min gradient program. Meanwhile, a solution containing the marker constituents hydroxysafflor yellow A (600 ng/mL), paeoniflorin (60 ng/mL), oxypaeoniflorin (600 ng/mL), albiflorin (60 ng/mL), senkyunolide I (180 ng/mL), senkyunolide G (180 ng/mL), ferulic acid (60 ng/mL), tanshinol (120 ng/mL), and protocatechuic aldehyde (60 ng/mL) was infused at 5 μL/min using a syringe pump and combined, through an Upchurch tee, with the post-column LC eluent (0.3 mL/min), and the mixture was introduced into the Turbo V ion source of the 5500 mass spectrometer. Matrix effects of XueBiJing excipients were assessed by comparing the chromatograms of the nine test compounds after injecting, onto the LC column, 5 μL of the excipient solution (containing Tween-80 and glucose), diluted excipient solution (at 20-, 200-, or 2000-fold dilution with 50% methanol), Tween-80 solution (at the same concentration as in the excipient solution), or glucose solution (at the same concentration as in the excipient solution) with the respective compound chromatograms after injection of 5 μL of the negative control (water). Tween-80 was detected at *m/z* 744, 763, 766, 898, 940, 1008, and 1160 in the positive ion mode, while glucose was at *m/z* 179 in the negative ion mode [36,37].

2.7.4. Intercept test

To ensure the validity of the one-point calibration with respect to "linearity through zero," an intercept test was performed; this was a crucial step in building a calibrator containing multiple analytes at different concentrations. First, linear concentration ranges to MS/MS response (chromatographic peak area) and UV response were assessed for each marker constituent. To this end, a combined stock solution (containing the nine marker constituents at 8000 μg/mL each) was serially diluted with 50% methanol to yield a set of test solutions at 4000, 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.81, 3.91, 1.95, 0.977, 0.488, 0.244, 0.122, 0.0610, 0.0305, 0.0153, 0.00763, 0.00381, 0.00191, 0.000954, 0.000477, and 0.000239 μg/mL, for each compound. These test solutions were analyzed in triplicate by LC-MS/MS (using the 8 min gradient program) and in duplicate by LC-UV (using the 60 min gradient program) to assess linear range of concentration-response relationship and to estimate the associated intercept of the linear regression equation for each compound. Pilot regression equations were generated for the compounds using their corresponding concentration-response data that were visually in a linear dynamic range. Using these pilot equations, each measured concentration of compound in the test solution was examined to see whether it exhibited an acceptable accuracy of 85%–115%, which was calculated as the percent ratio of the measured concentration to the nominal concentration. Any concentration point exhibiting unacceptable accuracy, i.e., not within the range of 85%–115%, was removed and the remaining concentration points were used to generate a final regression equation.

Second, three samples of XueBiJing were analyzed to assess content levels of the marker constituents using their respective regression equations. Initial compound responses (C responses) were obtained using XueBiJing samples diluted at a level that allowed all nine analytes measured to fall within their respective linear concentration ranges, and an absolute ratio of intercept to initial C response was estimated for each analyte using the intercept in its final regression equation. When the absolute ratio of intercept to C response was ≤2% for an analyte, the intercept was considered to be sufficiently low for measurement of the analyte within its linear range (i.e., meeting the "linearity through zero" condition) and the associated concentration was used to set the concentration of the analyte in the calibrator. When the ratio was >2%, an enlarged response at a concentration (still within the linear concentration range) higher than that at the measured concentration should be used to achieve a ratio ≤2%; this response was used as a new C response for the analyte and the new C response-associated concentration was used to set the concentration of the analyte in the calibrator.

2.7.5. Z-ligustilide conversion study

To understand relatively large batch-to-batch variability of XueBiJing in content level of senkyunolide I, the chemical stability of *Z*-ligustilide (the major phthalide constituent present in the component herbs Chuanxiong and Danggui) was assessed in triplicate by incubating this oily compound (5 μL) in 1 mL of water at 80 °C for 120 min. The upper aqueous phase of the incubation (50 μL) was sampled at 0, 10, 30, 60, 90, and 120 min after starting the incubation and mixed with 50 μL of ice-cold methanol. After centrifugation, the supernatants were analyzed by LC-MS/MS for *Z*-ligustilide, senkyunolide I, senkyunolide G, and *Z*-6,7-epoxyligustilide.

2.8. Assay validation

To verify the suitability of the newly developed LC-MS/MS- and LC-UV-based assays for XueBiJing quality evaluation, their analytical procedures were validated in accordance with the Guidelines for Validation of Analytical Method Adopted in Pharmaceutical Quality Specification in the Pharmacopoeia of the People's Republic of China (2020 edition) [38] and the International Council for Harmonisation Guideline for Validation of Analytical Procedures (2005 edition) [39].

2.8.1. Accuracy assessment

Assay accuracy was assessed using a recovery test, which involved spiking a XueBiJing sample with 0-, 0.5-, 1-, or 1.5-vol of the QC, each in sextuplicate. The resulting mixture was analyzed using the LC-MS/MS- and LC-UV-based assays. The assay accuracy was calculated using the following equation:

$$\text{Accuracy}\ (\%) = [(A_{SS} - A_{CS})/A_{ns}] \times 100\% \quad (1)$$

Where A_{SS} is the observed amount of an analyte in the spiked sample, A_{CS} is the observed amount of an analyte in the control sample (without spiking), and A_{ns} is the nominal spiked amount of an analyte. The acceptance criterion for assay accuracy was the percent recovery 85%–115% for each analyte.

2.8.2. Precision assessment

Assay precision was assessed with regard to repeatability and intermediate precision. The repeatability was assessed by analysis of a XueBiJing sample, in sextuplicate, for the nine marker constituents. The intermediate precision was assessed by comparing triplicated analyses over three consecutive days. The acceptance criterion for assay precision was the relative standard deviation (RSD) of measurement response ≤15% for each analyte.

2.8.3. Analyte stability and carry-over assessment

Analyte stability was assessed, in sextuplicate, by maintaining at 10 °C for 24 h, a 50% methanol-diluted QC sample (mimicking XueBiJing samples prepared for LC-MS/MS analysis) and an undiluted QC sample (mimicking XueBiJing samples prepared for LC-UV analysis), and the sampling was performed at 0, 12, and 24 h. Carry-over was assessed for both LC-MS/MS- and LC-UV-based assays by analyzing a blank sample against the calibrator; analyte response from the blank sample was required to be ≤2% of that from the calibrator. Concentrations used for the carry-over assessment were 400, 1,500, 40, 20, 60, 15, 30, 30, and 15 μg/mL for the nine analytes, i.e., hydroxysafflor yellow A, paeoniflorin, oxypaeoniflorin, albiflorin, senkyunolide I, senkyunolide G, ferulic acid, tanshinol, and protocatechuic aldehyde, respectively.

2.8.4. Robustness assessment

Chromatographic separation was a determinant of high selectivity for both LC-MS/MS and LC-UV measurements. Assay robustness to small variations in chromatographic conditions was assessed using a typical XueBiJing sample (Batch No.: 1805171) and monitoring LC resolution (R_S). For the LC-MS/MS-based assay, R_S was related to the marker constituent senkyunolide I and non-target constituents 6,7-dihydroxyligustilide and senkyunolide H; for the LC-UV-based assay, R_S was related to the nine marker constituents and their respective potentially interfering, non-target constituents. The observed variations were the concentrations of formic acid (6, 8, and 10 mM) and lithium acetate (20, 25, and 30 μM) in the LC-MS/MS mobile phase, the concentrations (40, 50, and 60 mM) of formic acid in the LC-UV mobile phase, and the flow rates of the mobile phase (0.27, 0.30, and 0.33 mL/min) for both assays.

2.9. Data processing

In the composition analysis of XueBiJing, all the detected and characterized herbal constituents were ranked in a descending order of compound doses and graded, i.e., 10–100, 1–10, 0.1–1, and 0.01–0.1 μmol/person. The compound dose, calculated for each constituent of XueBiJing, was the product of the compound's content level and the injection's label dose (100 mL/person).

LC R_S of ≥1.5 indicates that two peaks achieve baseline separation. When two peaks exhibited a R_S of 1.0–1.5 (such as paeoniflorin with its interfering peak in the LC-UV chromatogram of XueBiJing samples), the peak area of the marker constituent (eluted before the non-target constituent) was calculated from the start point to the valley between the partially resolved peaks by the trapezium rule, with a baseline manually positioned between the start point of the first peak and the end point of the second peak. When the marker peak eluted after the non-target peak, the calculation considered specifically the valley to the end point. This method of calculating peak area is applicable when the size of the marker peak is comparable with or significantly greater than that of the non-target peak.

The Shapiro-Wilk test of normality was used to assess the distribution of content level for each marker constituent in the XueBiJing samples. Outliers and extremes were identified according to whether the values were 1.5–3 and >3 box lengths, respectively, from the upper or lower edge of the box of interquartile range (IQR). Normally distributed data use the mean and standard deviation (SD) as measures of central tendency and dispersion, respectively, while non-normally distributed data use the median and IQR as measures of central tendency and dispersion, respectively. The content levels of each marker constituent in the XueBiJing samples were compared between years. When data of both groups of XueBiJing samples were normally distributed, a Student's *t*-test was performed to compare data between the two groups; when data of one or two groups were non-normally distributed, the Mann-Whitney *U* test was performed. Pearson's correlation coefficient (r) was used to measure the relationship between content levels of XueBiJing marker constituents measured by LC-MS/MS and the respective levels measured by LC-UV. A $P<0.05$ was considered to indicate statistical significance. IBM SPSS Statistics software (version 19.0; IBM, Chicago, IL, USA) was used for statistical analysis.

3. Results

3.1. Selected marker constituents of XueBiJing

Information obtained through literature mining and experimental studies of XueBiJing was categorized into three types to facilitate the selection of marker constituents (analytes) to develop therapeutically relevant assays. Regarding type-(1) information (i.e., chemical composition of XueBiJing), two research groups, i.e., Zhang's group [25] and our group, analyzed the chemical composition of XueBiJing. Zhang's group detected and characterized 162 XueBiJing constituents, and quantified 38 of them (using commercially available reference compounds for calibration). We had previously detected, characterized, and quantified 104 XueBiJing constituents (the compound's dose ≥0.01 μmol/person; this level was higher than the assay's lower limits of detection) and defined their origins among the component herbs [31–34]. We used the compounds' doses from the injection, rather than the compounds' content levels in the injection, because such doses can be used as a measure to predict systemic exposure to the compounds after dosing a herbal medicine like XueBiJing. Based on our previous multi-compound pharmacokinetic investigations of Chinese herbal injections [31–34,40–45], a herbal compound with a dose of ≥1 μmol/person, from the dosed injection, often generates measurable systemic exposure in its unchanged and/or metabolized forms. When a herbal compound has a dose of 0.01–1 μmol/person, its circulating forms, unchanged and/or metabolized, are often detectable, but measurements are limited. Minor constituents with doses <0.01 μmol/person normally exhibit undetectable or negligible systemic exposure. In addition, using such compound doses allows for data comparison among different herbal medicines.

In this study, we performed a more comprehensive composition analysis of XueBiJing using an updated candidate-compound list. A total of 176 constituents were detected and characterized, with 124 constituents having a dose ≥0.01 μmol/person (Table S1 and Fig. S1). In addition to the 111 compounds previously analyzed by one or both research groups (61, by both research groups; 7, only by Zhang's group [25]; 43, only by our group), 13 novel compounds were detected in this investigation. The 20 newly detected constituents (7 + 13 compounds that had not been previously detected specifically by our research group) exhibited doses of <1 μmol/person, except for vanillic acid that exhibited a dose of 1–10 μmol/person. There were 94 other constituents (reported by Zhang's group) that were not included in the 124 constituents, because they were either detected at levels <0.01 μmol/person (9 compounds) or

not detected (85 compounds, occurring in the lower limits of detection of the Synapt G2 mass spectrometer). Several assays for analyzing constituents of XueBiJing have been developed in previous studies [19–28], as summarized in Table S2.

Type-(2) information (i.e., significantly bioavailable XueBiJing compounds and their pharmacokinetics and disposition) was obtained mainly from our previous pharmacokinetic investigations of XueBiJing [31–34]. Several other research groups have developed bioanalytical assays to measure unchanged herbal compounds in the systemic circulation in rats receiving XueBiJing [22,46–49]. Based on an understanding of chemical composition of XueBiJing, we performed systematic pharmacokinetic investigations of XueBiJing to identify major circulating herbal compounds, unchanged and metabolized, in humans receiving the injection and to characterize their pharmacokinetics and disposition. The pharmacokinetic data provide an important basis for the selection of marker constituents for XueBiJing. The major circulating compound is hydroxysafflor yellow A, which originates from the component herb Honghua. Other major circulating compounds from Chishao are paeoniflorin, oxypaeoniflorin, and albiflorin; from Chuanxiong and Danggui are senkyunolide I, senkyunolide G, and ferulic acid; and from Danshen is tanshinol. Unlike these unchanged XueBiJing compounds, protocatechuic acid is a circulating metabolite of the constituent protocatechuic aldehyde (originating from Danshen). In addition, the 20 XueBiJing constituents newly detected in this investigation were negligible or not detected in plasma samples (after dosing the injection; the samples obtained in our prior human pharmacokinetic study). Therefore, the XueBiJing constituents, i.e., hydroxysafflor yellow A, paeoniflorin, oxypaeoniflorin, albiflorin, senkyunolide I, senkyunolide G, ferulic acid, tanshinol, and protocatechuic aldehyde, were considered to have high levels of bioavailability, either in the unchanged or metabolized form (Table 1) [31–34,50–77].

Literature mining for type-(3) information (i.e., antisepsis-related activities, adverse effects, and drug interaction potential) focused on the major circulating XueBiJing compounds, unchanged or metabolized. The antisepsis-related activities of these compounds include anti-inflammatory, anticoagulant, antithrombotic, endothelium protection, immunoregulatory, antioxidant, and organ protection activities (Table 1). Reports regarding the adverse effects of XueBiJing are scarce [78]. A recent investigation by our group suggested that these herbal compounds have low potential to take part, as perpetrator or victim, in therapeutic pharmacokinetic interactions with antibiotics when co-administered with XueBiJing in sepsis care [34].

Although benzoic acid (originating mainly from Chishao) was an analytical marker in the NMPA-stipulated XueBiJing assay, this compound is found in many foods and medicinal herbs. Benzoic acid could be detected in human plasma samples before dosing XueBiJing, at levels exhibiting large inter-individual variation. This compound has not been found to have any antisepsis-related activity and is generally considered safe in foods, with an acceptable daily intake of up to 5 mg/kg [79]. Therefore, benzoic acid was not selected as a marker constituent in developing assays for the quality evaluation of XueBiJing. A total of nine compounds (i.e., hydroxysafflor yellow A, paeoniflorin, oxypaeoniflorin, albiflorin, senkyunolide I, senkyunolide G, ferulic acid, tanshinol, and protocatechuic aldehyde) were selected as marker constituents for assay development, and their chemical structures are shown in Fig. 2 .

3.2. One-point calibration assay: similarity of response

Quantification of the nine selected marker constituents in XueBiJing (also containing 115 non-target herbal constituents, as well as the excipients Tween-80 and glucose) was based on LC-MS/MS and LC-UV assays. To use a one-point calibration, the assays to be developed should possess high selectivity for the analytes in complex injection samples. Given that the assay calibrator (only containing the analytes and the excipients, but no non-target constituent) differed from complex XueBiJing samples, high assay selectivity was necessary to meet “similarity of response”, a condition for using one-point calibration. Here, high assay selectivity was achieved by identifying and minimizing interference from the non-target constituents and excipients co-eluted with the marker constituents.

To develop the LC-MS/MS-based assay, several isobaric constituent pairs of XueBiJing were found, i.e., (1) hydroxysafflor yellow A (molecular mass, 612.1690 Da; ID in Table S1, *1*) and the non-target constituents saffloquinoside D (612.1690 Da; *4*)/saffloquinoside C (612.1690 Da; *5*), (2) the marker constituents paeoniflorin (480.1632 Da; *52*) and albiflorin (480.1632 Da; *54*), and (3) the marker constituent oxypaeoniflorin (496.1581 Da; *53*) and the non-target constituent oxypaeoniflorin isomer I (496.1581 Da; *61*). The compounds in these pairs were well separated under initial LC conditions with a chromatographic run time of 5 min. By adjusting the LC gradient program, baseline chromatographic separation was achieved for the marker constituent senkyunolide I (224.1049 Da; *83*) from the two untargeted constituents 6,7-dihydroxyligustilide (224.1049 Da; *90*) and senkyunolide H (224.1049 Da; *84*). As a result, under the final optimized LC conditions with a chromatographic run time of 8 min, the R_S values between 6,7-dihydroxyligustilide and senkyunolide I and between senkyunolides I and H, R_S were 2.2 and 1.9, respectively (Fig. 3A). The matrix effects of the excipients on electrospray ionization of the marker constituents were found to be negligible, except for the ionization of senkyunolide G (Fig. S2). The ion enhancement for senkyunolide G (mainly caused by the excipient Tween-80) was significantly minimized by diluting the sample with 50% methanol before injection onto the column. The positive/negative polarity switching function on the 5500 mass spectrometer allowed for simultaneous acquisition of precursor-to-product ion pair data in both the positive (lithiated senkyunolides I and G) and the negative ion modes (deprotonated hydroxysafflor yellow A, ferulic acid, tanshinol, and protocatechuic aldehyde and formate adducts of paeoniflorin, oxypaeoniflorin, and albiflorin). Efficient ionization of these analytes was achieved by optimizing electrolyte modifiers in the mobile phase; the optimized conditions are described in Subsection 2.5.

Unlike MS/MS detection, the non-specific nature of UV detection for analyzing complex XueBiJing samples needed high-resolution LC with a long chromatographic run time to give sufficient peak capacity. In the first step in developing the LC-UV-based assay, we identified non-target constituents co-eluted with each XueBiJing marker constituent; here, a typical sample of XueBiJing (Batch No.: 1805171) was analyzed by LC-MS/MS and LC-UV, in parallel, using the 42 min LC gradient program (Table S1). UV spectra were measured for the marker constituents, Tween-80 solution, glucose solution, and excipient solution (Fig. S3), and 230 and 280 nm were selected for the LC-UV-based assay. To minimize interference of the identified UV-interfering constituents on the target constituents, the XueBiJing sample (1805171) was analyzed again by LC-UV and LC-MS/MS, in parallel, using a modified LC gradient program with a chromatographic run time of 90 min. As a result, all the identified UV-interfering constituents were resolved from the marker constituents and no new UV-interfering constituent was found to co-elute with any marker constituent. The LC gradient was adjusted to shorten the chromatographic run time while maintaining the required separation between the marker constituents and UV-interfering constituents; as a result, a 60 min LC gradient was developed for the LC-UV-based assay (Fig. 3B). Under the optimized

Table 1
Human pharmacokinetics and antisepsis-related activities of nine marker constituents of XueBiJing.

Marker constituent (component herb of XueBiJing)	Systemic exposure parameter			Cell- or animal-based antisepsis-related activity			
	c_{max} (μM)	$AUC_{0-\infty}$ (μM·h)	$t_{1/2}$ (h)	Activity	Model	Effective dose	Refs.
Hydroxysafflor yellow A (Honghua)	3.8	15.8	4.0	Anti-inflammatory	LPS-induced RAW264.7 cells	25 μM	[34,50]
					LPS-induced acute lung injury mouse model	6 mg/kg; i.v.	[51]
				Anticoagulant, antithrombotic	ADP/PAF-induced platelet aggregation in rabbit blood	5 mg/kg; i.v.	[52]
					Shunt thrombosis in rat	20 mg/kg; i.v.	[52]
				Endothelium protection	Hypoxia-induced Eahy926 human endothelium cells	1 μM	[53]
				Organ protection	I/R-induced acute kidney injury rat model	92 mg/kg; i.p.	[54]
Paeoniflorin (Chishao)	18.3	36.4	1.1	Anti-inflammatory	LPS-induced THP-1 cells	1 μM	[32,55]
					LPS-induced RAW264.7 cells	0.01 μM	[56,57]
					LPS- or CLP-induced rat sepsis model	50 mg/kg; i.v.	[56]
					LPS-induced human Caco-2 cells	50 μM	[58]
					LPS-induced human mesangial cells	5 μM	[59]
				Endothelium protection	Ox-LDL-induced injury in HUVECs	208 μM	[60]
				Organ protection	H/R-injured rat intestinal epithelial cells	12.5 μM	[61]
				Anticoagulant, antithrombotic	Rat thrombosis model	10 mg/kg; i.v.	[62]
					ADP-induced platelet aggregation in rabbit blood	36 mM	[63]
				Immunoregulatory	LPS-induced peritoneal macrophages from MRL/lpr mouse	50 μM	[64]
Oxypaeoniflorin (Chishao)	0.6	1.1	0.7	Anti-inflammatory	LPS-induced THP-1 cells	10 μM	[32,55]
Albiflorin (Chishao)	0.8	0.6	0.7	Anti-inflammatory	LPS-induced RAW264.7 cells	0.01 μM	[32,57]
Senkyunolide I (Chuanxiong/Danggui)	0.4	0.6	0.8	Anti-inflammatory	LPS-induced THP-1 cells	10 μM	[31,55]
				Anticoagulant	ADP-induced platelet aggregation in rabbit blood	5 mM	[63]
				Organ protection/ antioxidant	I/R-induced focal cerebral injury rat model	36 mg/kg; i.v.	[65]
Senkyunolide G (Chuanxiong/Danggui)	0.5	1.7	2.3	Selectively and extensively bound to plasma albumin and likely to be a pharmacokinetic marker indicating decreased plasma albumin level in patients with sepsis			[31]
Ferulic acid (Chuanxiong/Danggui)	0.12	0.1	0.7	Anti-inflammatory	LPS-induced RAW 264.7 cells	50 μM	[66]
				Antioxidant	CLP-induced rat sepsis model	100 mg/kg; i.p.	[67]
				Endothelium protection	^{60}Co gamma radiation-injured HUVECs	125 nM	[68]
					Streptozotocin-induced vascular endothelial dysfunction rat model	50 mg/kg; i.p.	[69]
				Anticoagulant	Thrombin-induced acute thromboembolism mouse model	10 mg/kg; i.v.	[70]
				Antithrombotic	THR-induced acute thromboembolism mouse model	10 mg/kg; i.v.	[70]
Tanshinol (Danshen)	0.1	0.1	0.3	Anti-inflammatory	LPS-induced THP-1 cells	1 μM	[33,55]
					CLP-induced rat sepsis model	5 mg/kg; p.o.	[71]
				Antioxidant	H_2O_2-induced damage in HUVECs	25 μM	[72]
				Endothelium protective	Atherosclerotic $ApoE^{-/-}$ mouse model	15 mg/kg; p.o.	[73]
Protocatechuic aldehyde (Danshen)[a]	0.04	0.04	0.5	Anti-inflammatory	LPS-induced RAW 264.7 cells	1 μM	[33,74]
					LPS-induced acute lung injury mouse model	5 mg/kg; i.p.	[75]
					LPS/D-galactosamine (GalN)-induced mouse sepsis model	50 mg/kg; i.p.	[76]
				Antioxidant	H_2O_2-induced oxidative damage in BNLCL2 cells	130 μM	[77]
				Organ protection	LPS-induced acute lung injury mouse model	5 mg/kg; i.p.	[75]

[a] Protocatechuic acid is the oxidized metabolite of protocatechuic aldehyde; the pharmacokinetic and pharmacodynamic data in the columns to the right are those of protocatechuic acid. c_{max}: maximum plasma concentration; AUC: area under concentration-time curve; $t_{1/2}$: elimination half-life; LPS: lipopolysaccharide; ADP: adenosine diphosphate; PAF: paeoniflorin; I/R: ischemia/reperfusion; CLP: cecal ligation and puncture; LDL: low-density lipoprotein; HUVEC: human umbilical vein endothelial cell; H/R: hypoxia/reoxygenation; i.v.: intravenous administration; i.p.: intraperitoneal administration; p.o.: oral administration. The pharmacokinetic data of the nine marker constituents of XueBiJing are shown in previously published work [31–34] and our work intended for publication elsewhere.

LC conditions, all nine marker constituents were well-resolved. It should be noted that the marker constituents of XueBiJing did not necessarily exhibit peaks of high abundance in the LC-UV chromatograms (Fig. 3B). Although many non-target constituents exhibited peaks of high abundance, they were not deemed therapeutically relevant and could be minor constituents with high response-to-concentration ratios (Fig. S4 and Table S1). The excipients Tween-80 and glucose, at their respective concentrations in XueBiJing, did not interfere with UV measurement of the marker constituents (Fig. S5).

3.3. One-point calibration assay: linearity through zero

To meet "linearity through zero", another condition to validate the one-point calibration assay, the linear range and intercept of the concentration-response function were determined for the nine XueBiJing marker constituents. Both concentration-MS/MS response and concentration-UV response relationships exhibited linear concentration ranges, as well as the associated intercepts of the regression equation, and the linear ranges differed across analytes and across measurement techniques (Table 2). Here, the test XueBiJing samples were diluted 1800-fold with 50% methanol for LC-MS/MS measurement, while these samples were not diluted for LC-UV measurement; these treatments made measurements of all analytes fall within their respective linear concentration ranges. As shown in Table 2, using the initial C responses (i.e., responses of marker constituents in the 1800-fold diluted XueBiJing samples) and associated intercept of the LC-MS/MS measurement, absolute ratios of intercept to C response of hydroxysafflor yellow A, paeoniflorin, oxypaeoniflorin, albiflorin, ferulic acid, and protocatechuic aldehyde were <2%, while the ratios of senkyunolide I, senkyunolide G, and tanshinol were >2%. By enlarging the C response of senkyunolide I, senkyunolide G, and tanshinol, their absolute ratios of the intercept to C response were reduced to <2%. For LC-UV measurement, all compounds exhibited absolute ratios of the intercept to C response of <2%, using their initial C response (i.e., responses of marker constituents in XueBiJing sample without any dilution) and associated intercept. Based on the results of the intercept test (i.e., acceptable C response-associated concentrations), calibrator concentrations of XueBiJing marker constituents were established for both LC-MS/MS- and LC-UV-based assays using one-point calibration.

3.4. Reliability of the developed assays

As shown in Table 3, although no internal standard was used, both the LC-MS/MS- and LC-UV-based assays exhibited good accuracy for quantification of the nine XueBiJing marker constituents, as indicated by the percent recoveries 87.3%–112.7% and 89.1%–108.7%, respectively. Both assays also exhibited good repeatability (1.2%–6.5% and 0.5%–4.0%, respectively) and good intermediate precision (2.9%–8.4% and 1.1%–6.0%, respectively). Reproducibility, assessed by means of an inter-laboratory trial, should be considered in future research. Consistent with the assays' accuracy and precision, the marker constituents were stable at 10 °C for 24 h, and carry-over in the blank samples following the calibrators was negligible. Fluctuations in the electrolyte concentrations and flow rate of the mobile phases were found, for both assays, to have limited influences on peak resolutions among the marker constituents and their respective potentially interfering non-target constituents.

3.5. Batch-to-batch quality variability of XueBiJing

Table 4 and Fig. 4 summarize the content levels of the nine marker constituents measured using the LC-MS/MS-based assay across 33 batches of XueBiJing (manufactured from 2017 to 2019). The results obtained by LC-UV measurement are summarized in Table S3. All content levels of hydroxysafflor yellow A and paeoniflorin were in agreement with their respective officially stipulated levels for XueBiJing (i.e., 200–500 and 1000–1700 μg/mL, respectively). The content levels of the marker constituents in the XueBiJing samples were normally distributed within the years 2017, 2018, and 2019 and over the 3-year period of 2017–2019 (except for hydroxysafflor yellow A, senkyunolide I, senkyunolide G, ferulic acid, and protocatechuic aldehyde with P values of 0.0002–0.047). Given that the mean values of these five compounds in the three-year samples were close to the respective median values (92.0%–103.6% of the median), RSD was used to indicate their three-year batch-to-batch variability, similar to that of the other four compounds with normal distribution.

Based on the results of LC-MS/MS measurements of the nine marker constituents for 2017–2019, the nine marker constituents

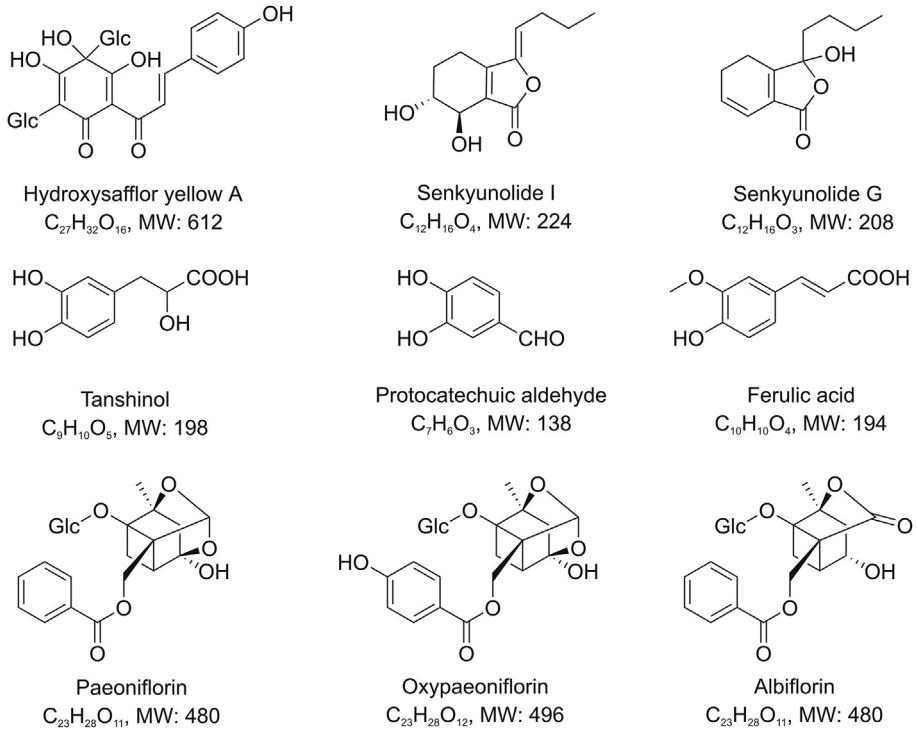

Fig. 2. Chemical structures of the nine marker constituents of XueBiJing.

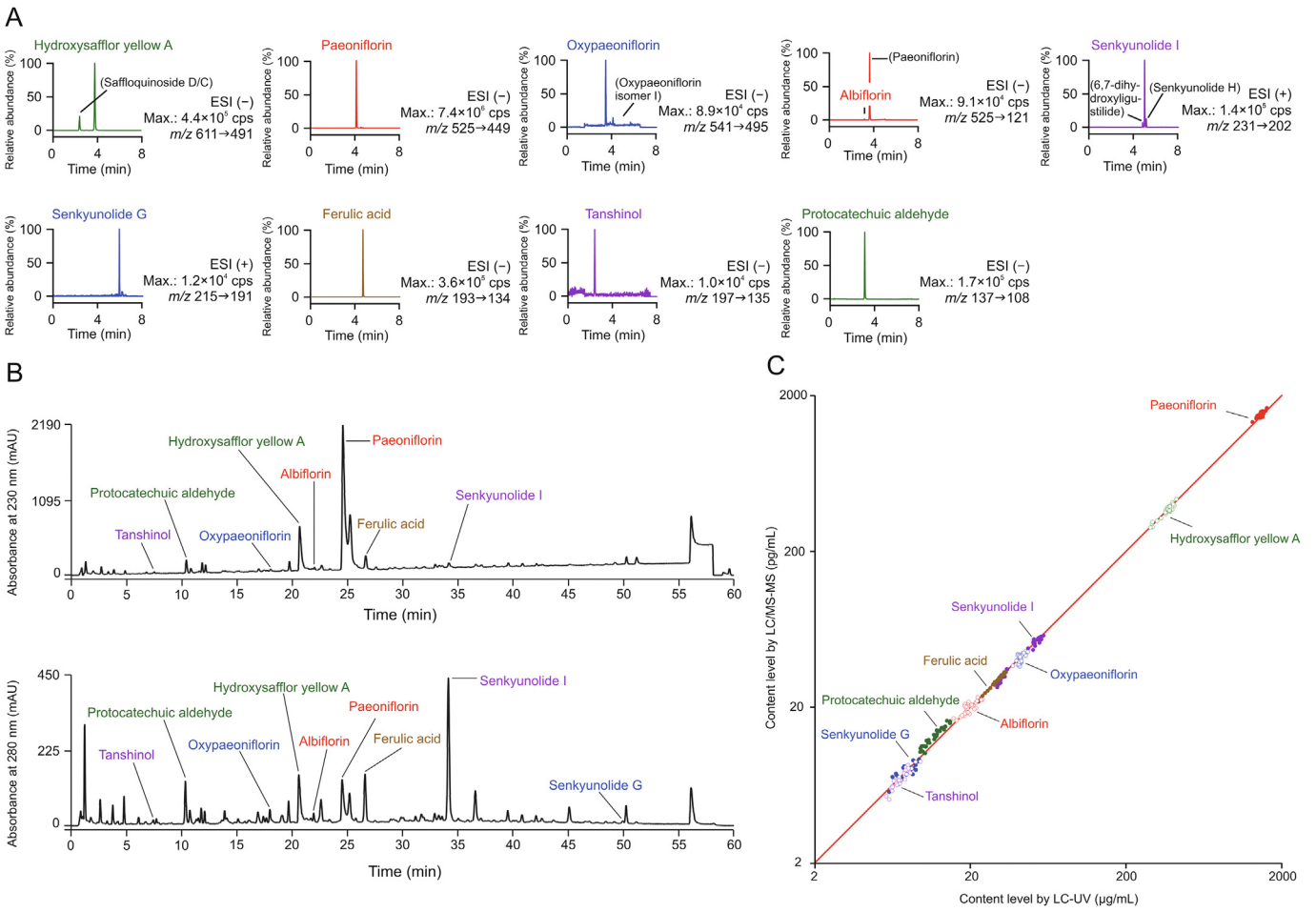

Fig. 3. (A) LC-MS/MS and (B) LC-UV chromatograms of the nine marker constituents in a typical XueBiJing sample. (C) Correlations in content levels of these constituents in samples from the 33 batches of XueBiJing measured using the two assays. The marker constituents were measured at 280 nm, except for albiflorin, which was measured at 230 nm for high selectivity.

were ranked as: paeoniflorin (mean content level, 1486 μg/mL) > hydroxysafflor yellow A (359 μg/mL) > senkyunolide I (42.4 μg/mL) ≈ oxypaeoniflorin (40.5 μg/mL) > ferulic acid (28.9 μg/mL) > albiflorin (19.3 μg/mL) > protocatechuic aldehyde (12.7 μg/mL) > senkyunolide G (7.7 μg/mL) ≈ tanshinol (7.0 μg/mL). The samples of XueBiJing exhibited three-year batch-to-batch variability of 4.1%–14.8% (RSD values), except for senkyunolide I (26.5%). Statistically significant differences were found in the content levels of some marker constituents: between 2017 and 2018 samples for hydroxysafflor yellow A, senkyunolide I, ferulic acid, tanshinol, and protocatechuic aldehyde (P=0.001–0.02); between 2018 and 2019 samples for oxypaeoniflorin, senkyunolide I, and protocatechuic aldehyde (P=0.0003–0.046); and between 2017 and 2019 samples for hydroxysafflor yellow A, oxypaeoniflorin, senkyunolide I, tanshinol, and protocatechuic aldehyde (P=0.000000000000002–0.03). As a percentage of that of the data of preceding year, mean content level of each marker constituent for each year ranged from 55% to 115%, with senkyunolide I exhibiting the greatest difference between 2017 and 2019 (55%).

Using the LC-UV-based assay, the three-year and the yearly mean content levels of the marker constituents were not statistically different from the respective levels measured by the LC-MS/MS-based assay. As shown in Fig. 3C, individual content levels measured by LC-UV positively correlated well with the respective levels measured by LC-MS/MS, as indicated by a correlation coefficient (r) of 0.9995 (n=297) with a slope of 1.009.

To understand the relatively large batch-to-batch variation (26.5%) in the content level of senkyunolide I in XueBiJing samples, *Z*-ligustilide, the major constituent of Chuanxiong and Danggui, was assessed under conditions mimicking extraction of the two component herbs in the manufacturing process of XueBiJing. *Z*-ligustilide did not occur in XueBiJing due to the compound's hydrophobicity. As shown in Fig. 5, *Z*-ligustilide could be converted, at 80 °C in a time-dependent manner, into senkyunolide I (detected in the water phase), while senkyunolide G was negligibly detected; these findings were consistent with those by Kaouadji et al. [80]. In addition, the content levels of *Z*-ligustilide were reported to vary in raw materials of Chuanxiong and Danggui [81]. Therefore, the relatively large batch-to-batch variation of senkyunolide I level in XueBiJing samples was attributed, at least in part, to the *Z*-ligustilide conversion during the manufacturing process of XueBiJing.

4. Discussion

Regulation and use of complex Chinese herbal medicines as drug treatments necessitate the development of therapeutically relevant quality evaluation assays. For the assays to be therapeutically relevant, the medicine's efficacy, as well as the adverse effects and/or drug interaction potential (if any), should be appropriately defined. As shown in Table 5, we proposed criteria for defining the efficacy of a Chinese herbal medicine and summarized

Table 2
Linear concentration ranges of LC-MS/MS- and LC-UV-measured responses and associated intercepts for nine XueBiJing marker constituents.

Marker constituent	Linear concentration range (μg/mL)	Regression equation of response to analyte (Y; peak area) against concentration of analyte (X) [a]	Typical content level of marker constituent in XueBijing [b] (μg/mL)	Dilution of XueBiJing sample for measurement	Initial C response (μg/mL) [c]	Enlarged C response (μg/mL) [d]	Concentration in calibrator (μg/mL) [e]
LC-MS/MS measurement [f]							
Hydroxysafflor yellow A	0.00190–0.488	$Y = 8397386X + 1668$ [0.99999]	395	1800-fold dilution	1842213 [0.09%; 0.22]	–	400 [0.22]
Paeoniflorin	0.000477–0.975	$Y = 25481564X + 969$ [0.99998]	1495		21160473 [0.005%; 0.83]	–	1500 [0.83]
Oxypaeoniflorin	0.000954–0.0305	$Y = 12358726X + 795$ [0.99997]	40.4		278116 [0.29%; 0.02]	–	40 [0.02]
Albiflorin	0.000477–0.488	$Y = 17533452X - 1187$ [0.99997]	19.8		230651 [0.51%; 0.01]	–	20 [0.01]
Senkyunolide I	0.00190–0.0609	$Y = 18955057X + 11776$ [0.99990]	45.7		493048 [2.39%; 0.025]	643611 [1.83%; 0.03]	60 [0.03]
Senkyunolide G	0.00190–0.244	$Y = 5390860X + 646$ [0.99990]	6.91		21328 [3.03%; 0.004]	45570 [1.42%; 0.008]	15 [0.008]
Ferulic acid	0.000954–0.122	$Y = 48251860X + 9215$ [0.999995]	33.9		917660 [1.00%; 0.019]	–	30 [0.017]
Tanshinol	0.0038–0.244	$Y = 7070658X + 1913$ [0.99994]	9.11		37713 [5.07%; 0.005]	119757 [1.60%; 0.017]	30 [0.017]
Protocatechuic aldehyde	0.000477–0.0152	$Y = 71234182X + 9147$ [0.9998]	15.0		601661 [1.52%; 0.008]	–	15 [0.008]
LC-UV measurement [g]							
Hydroxysafflor yellow A	31.3–2000	$Y = 83X + 302$ [0.99996]	385	No dilution	32262 [0.94%; 385]	–	400
Paeoniflorin	62.5–2000	$Y = 21X - 244$ [0.99994]	1466		30537 [0.80%; 1466]	–	1500
Oxypaeoniflorin	0.488–125	$Y = 129X + 14$ [0.99992]	49.9		6451 [0.22%; 49.9]	–	40
Albiflorin	7.81–500	$Y = 88X - 25$ [0.99998]	16.8		1451 [1.72%; 16.8]	–	20
Senkyunolide I	0.488–125	$Y = 1381X + 130$ [0.999990]	46.4		64257 [0.20%; 46.4]	–	60
Senkyunolide G	0.122–31.3	$Y = 190X + 2$ [0.99998]	7.89		1500 [0.13%; 7.89]	–	15
Ferulic acid	0.488–125	$Y = 815X - 20$ [0.999996]	26.9		21942 [0.10%; 26.9]	–	30
Tanshinol	0.488–62.5	$Y = 208X - 12$ [0.999990]	8.04		1660 [0.73%; 8.04]	–	30
Protocatechuic aldehyde	0.244–250	$Y = 1198X + 67$ [0.999997]	13.0		15604 [0.43%; 13.0]	–	15

[a] Value in square brackets indicates correlation coefficient.
[b] The batch No. of the typical XueBiJing sample was 1805171.
[c] Values in square brackets represent [absolute ratio of intercept to initial C response; C response-associated concentration].
[d] Values in square brackets represent [absolute ratio of intercept to enlarged C response; enlarged C response-associated concentration].
[e] Value in square brackets indicates concentration in 1800-fold diluted calibrator for LC-MS/MS measurement.
[f] For hydroxysafflor yellow A, paeoniflorin, oxypaeoniflorin, albiflorin, ferulic acid, tanshinol, and protocatechuic aldehyde at m/z 611 → 491, 525 → 449, 541 → 495, 525 → 121, 193 → 134, 197 → 135, and 137 → 108, respectively, in the negative ion mode; for senkyunolides I and G at m/z 231 → 202 and 215 → 191, respectively, in the positive ion mode.
[g] At 280 nm, except for albiflorin at 230 nm.

antisepsis efficacy of XueBiJing based on previous studies [12–17,31–34,50–77,82–93]. XueBiJing is a herbal medicine with well-defined efficacy of antisepsis, particularly its efficacy demonstrated by a well-designed and effectively executed multicenter, randomized, double-blind, placebo-controlled clinical investigation, which found that combining XueBiJing with the conventional treatment reduced 28-day mortality in septic patients with severe community-acquired pneumonia [12]. Understanding of the

Table 3
Accuracy and precision of LC-MS/MS-based and LC-UV-based assays for measuring nine marker constituents of XueBiJing.

Marker constituent	Accuracy (%, n=6) [a]		Repeatability (RSD%, n=6) [b]		Intermediate precision (RSD%, n=3) [c]	
	LC-MS/MS-based assay	LC-UV-based assay	LC-MS/MS-based assay	LC-UV-based assay	LC-MS/MS-based assay	LC-UV-based assay
Hydroxysafflor yellow A	94.3	100.6	1.3	0.5	3.0	1.7
	101.6	101.7				
	94.2	96.6				
Paeoniflorin	96.8	102.2	1.5	2.7	3.0	2.4
	98.9	106.4				
	95.3	108.7				
Oxypaeoniflorin	105.7	108.7	3.9	1.6	7.3	1.4
	101.0	103.5				
	96.8	96.3				
Albiflorin	100.2	89.1	3.4	3.8	5.0	6.0
	110.3	94.7				
	100.0	99.0				
Senkyunolide I	95.5	99.4	4.9	0.8	8.4	1.1
	97.7	97.8				
	94.0	100.4				
Senkyunolide G	99.8	100.5	2.5	4.0	6.1	3.9
	103.3	90.3				
	99.2	92.7				
Ferulic acid	103.4	101.4	1.2	1.5	2.9	1.5
	112.7	100.0				
	105.5	100.7				
Tanshinol	90.4	95.3	6.5	1.7	5.1	3.1
	92.8	91.7				
	99.4	91.8				
Protocatechuic aldehyde	87.3	99.6	2.5	1.8	5.9	4.1
	95.1	98.2				
	89.5	96.6				

[a] Test samples: XueBiJing + 0.5 volume of QC, XueBiJing + 1.0 volume of QC, and XueBiJing + 1.5 volume of QC.
[b] RSD% of measurement responses to marker constituents by repeatedly analyzing a XueBiJing sample in a day; test sample: XueBiJing.
[c] RSD% of measurement responses to marker constituents by analyzing a XueBiJing sample over consecutive days.
RSD: relative standard deviation.

efficacy-associated physiological and biochemical effects of XueBiJing enables us to decide which bioactivities of the injection's compounds are related to the overall therapeutic action. Selecting appropriate marker constituents is a crucial step in developing a therapeutically relevant assay. To this end, a comprehensive understanding of the medicine's chemical composition should be combined with the available information on pharmacokinetics and pharmacodynamics. For XueBiJing, the understanding of the chemical composition was achieved to provide basis for our multi-compound pharmacokinetic investigations of the injection [31–34]; this was to avoid missing any XueBiJing compound with significant systemic exposure in the pharmacokinetic investigation. Given that the antisepsis efficacy of XueBiJing is most likely governed by its compounds with favorable pharmacokinetic and pharmacodynamic properties, we selected as markers those constituents (originating from each component herb of the injection) with high systemic exposure and antisepsis-related bioactivities. Although protocatechuic acid is a bioavailable and bioactive XueBiJing compound, it is a metabolite of protocatechuic aldehyde; thus, the parent compound (rather than the metabolite) was selected for analysis as a marker constituent. Here, we also proposed criteria for selecting marker constituents in assay development for a Chinese herbal medicine (Table 5). The non-target constituents were not deemed to be therapeutically important, because they were negligible or not detected in plasma samples of humans intravenously receiving XueBiJing, according to our pharmacokinetic investigations.

For efficiency and practicability of assay for routine quality evaluation of XueBiJing, one-point calibration was used for assay development. Different from conventional one-point-calibration-based assays for synthetic medicines, assays for complex Chinese herbal medicines (particularly those formulated from a multi-herb formula) are used to analyze samples that contain various non-target constituents co-eluted with the marker constituents in LC. Marker constituents, originating from different component herbs, normally have varying structural classes and content levels. Despite the chemical complexity, the validity of herbal medicine assays using one-point calibration relies on all the marker constituents measured under the conditions of “similarity of response” and “linearity through zero”. To achieve “similarity of response”, we developed a selectivity test by eliminating the interference of co-eluted non-target constituents on the measurement of marker constituents; information on the co-eluted constituents was obtained from composition analysis of XueBiJing. For LC-MS/MS measurement, interfering co-eluted non-target constituents were those isobaric to marker constituents; for LC-UV measurement, interfering co-eluted non-target constituents exhibited a UV absorbance at 230 or 280 nm. Eliminating the interference caused by co-eluted non-target constituents was achieved by adjusting the LC gradient program, and the sample throughput was determined by the results of the LC optimization.

To ensure “linearity through zero” for each analyte, we developed an intercept test, which was also a crucial step in generating an optimal calibrator with the appropriate concentrations of analytes. In the intercept test, we determined the linear concentration ranges of the analytes, and the associated intercepts of the concentration-response function, while the analyte content levels in XueBiJing were assessed. Here, we proposed an absolute ratio of intercept to C response of $\leq$2% to determine whether the intercept was sufficiently “near-zero” for each analyte. This was a reasonable criterion as demonstrated by the accuracy results of assay validation. When the absolute ratio of intercept to C response >2%, there are two methods for reducing the ratio: reducing the linear concentration range to

Table 4
LC-MS/MS-measured content levels of nine marker constituents in samples of 33 batches of XueBiJing manufactured from 2017 to 2019.

Batch No.	Content level (μg/mL) [a]								
	Honghua	Chishao			Chuanxiong/Danggui			Danshen	
	Hydroxysafflor yellow A	Paeoniflorin	Oxypaeoniflorin	Albiflorin	Senkyunolide I	Senkyunolide G	Ferulic acid	Tanshinol	Protocatechuic aldehyde
Samples in 2017									
1704181	322	1439	40.7	16.8	51.2	8.21	31.1	7.64	12.3
1710072	388	1548	43.8	21.2	48.8	8.06	29.4	7.83	14.8
1710311	365	1427	39.1	20.6	46.1	7.48	26.2	7.76	14.5
1712011	360	1555	43.5	21.4	56.9	7.96	31.2	7.45	15.6
1712041	296	1455	43.4	20.2	51.4	7.81	24.4	8.84	16.2
1712071	304	1498	42.7	19.6	54.4	7.33	25.3	8.17	14.1
1712081	353	1534	40.9	20.7	56.0	8.16	33.2	6.64	15.6
1712111	283	1418	40.4	19.2	53.5	7.26	23.4	8.43	13.4
1712141	343	1531	40.3	17.9	53.9	8.48	27.9	7.85	16.1
1712211	280	1327	36.5	16.3	53.1	6.78	22.2	7.37	12.1
1712261	310	1396	39.5	17.2	54.0	7.70	21.5	8.88	14.0
1712281	362	1508	38.9	17.3	53.2	7.42	29.7	6.61	13.2
Mean ± SD	331 ± 36	1470 ± 71	40.8 ± 2.2	19.0 ± 1.8	52.7 ± 3.0	7.72 ± 0.49	27.1 ± 3.8	7.79 ± 0.73	14.3 ± 1.4
RSD (%)	10.9	4.8	5.4	9.7	5.7	6.3	14.2	9.4	9.8
Samples in 2018									
1805171	343	1460	40.5	21.2	53.5	6.69	29.7	6.41	12.9
1806241	363	1508	43.7	20.1	51.0	7.76	28.8	8.02	14.9
1806251	349	1547	42.4	19.9	55.4	7.79	28.7	7.50	14.2
1806261	378	1504	45.2	20.4	47.2	7.89	32.0	6.81	13.9
1807062	377	1555	44.0	20.0	50.7	8.29	33.2	6.76	14.6
1807101	365	1414	38.2	17.6	42.4	5.68	28.4	5.08	10.3
1807102	376	1456	40.5	19.3	44.1	6.32	27.7	6.19	10.0
1808292	377	1635	44.9	22.9	30.7	7.69	30.8	8.04	12.7
1812081	426	1491	39.2	17.4	34.7	8.06	30.9	6.75	11.1
1812091	406	1563	42.4	19.1	32.9	8.09	30.7	5.98	11.0
1812101	406	1508	39.4	18.0	34.7	7.44	31.0	6.02	11.1
Mean ± SD	379 ± 25	1513 ± 61	41.9 ± 2.4	19.6 ± 1.6	43.4 ± 8.9	7.43 ± 0.83	30.2 ± 1.7	6.69 ± 0.90	12.4 ± 1.8
RSD (%)	6.7	4.0	5.8	8.3	20.6	11.2	5.5	13.5	14.5
Samples in 2019									
1904011	366	1451	38.0	20.0	27.7	8.00	28.3	6.95	11.7
1904041	355	1430	36.7	18.2	28.2	8.04	27.5	5.33	10.2
1904171	383	1479	42.4	19.9	27.4	7.86	27.8	6.67	11.2
1904221	395	1541	38.7	21.7	28.3	7.85	28.6	7.05	12.2
1905071	367	1464	38.1	18.0	27.8	7.36	30.4	5.77	11.1
1905161	385	1534	41.6	20.5	31.1	9.05	30.8	6.67	10.4
1905251	356	1420	36.2	18.4	30.8	8.44	31.2	6.11	10.8
1905301	367	1451	37.3	19.4	29.5	8.18	28.4	6.74	11.1
1906021	383	1522	38.6	19.9	31.7	7.88	32.1	6.45	11.1
1906281	360	1459	39.1	17.5	26.6	7.96	31.8	6.29	11.7
Mean ± SD	372 ± 14	1475 ± 43	38.7 ± 2.0	19.4 ± 1.3	28.9 ± 1.8	8.06 ± 0.44	29.7 ± 1.7	6.40 ± 0.54	11.2 ± 0.6
RSD (%)	3.7	2.9	5.1	6.7	6.1	5.5	5.9	8.4	5.4
All samples of 33 batches in 2017–2019 [b]									
Mean ± SD	359 ± 34	1486 ± 61	40.5 ± 2.5	19.3 ± 1.6	42.4 ± 11.2	7.73 ± 0.65	28.9 ± 3.0	7.00 ± 0.95	12.7 ± 1.9
RSD (%)	9.6	4.1	6.2	8.2	26.5	8.4	10.2	13.5	14.8
Median (IQR)	365 (35)	1491 (89)	40.4 (3.9)	19.6 (2.5)	46.1 (22.6)	7.86 (0.66)	29.4 (3.3)	6.76 (1.45)	12.3 (3.3)
Mean/median (%)	98.3	99.7	100.2	98.5	92.0	98.3	98.3	103.6	103.3

[a] The sample of each batch of XueBiJing was measured in triplicate. The relative standard deviations (RSDs) of measured content levels for the injection samples ranged from 1.1% to 11.1%.
[b] Only paeoniflorin, oxypaeoniflorin, albiflorin, and tanshinol exhibited normally distributed three-year content levels.
IQR: inter-quartile range; SD: standard deviation.

minimize the intercept and using an adjusted C response at a concentration higher than the associated content level in the medicine (but still within the linear concentration range). In developing a multi-analyte assay, reducing the linear concentration range is normally unfavorable, as a wide range is required for a multi-analyte calibrator. In this investigation, responses at higher concentrations were used as the C responses for senkyunolide I, senkyunolide G, and tanshinol in developing the LC-MS/MS-based assay to make their ratios ≤2%. Both XueBiJing samples and calibrator were diluted 1800-fold with 50% methanol to allow LC-MS/MS measurement of the analytes within their respective linear concentration ranges; this dilution also minimized the effect of Tween-80 on electrospray ionization of senkyunolide G (a matrix effect enhancing compound ionization).

A comprehensive understanding of the chemical composition of XueBiJing was fundamental in developing a multi-analyte assay for quality evaluation of this complex herbal injection. It allowed for the selection of marker constituents and facilitated the selectivity test by identifying non-target constituents co-eluted with the selected analytes. In this investigation, composition analysis of XueBiJing was performed via LC-MS/MS, and a total of 124 constituents (compound dose, ≥0.01 μmol/person) were detected and characterized. Assays for composition analysis need to have high analyte capacity. The 42 min LC-MS/MS-based assay used in this investigation showed high analyte capacity in our previous composition analyses of many other herbal medicines [41–45,94–96]. The comprehensiveness of our composition analysis was verified by assessing whether all discernible peaks in the 60 min

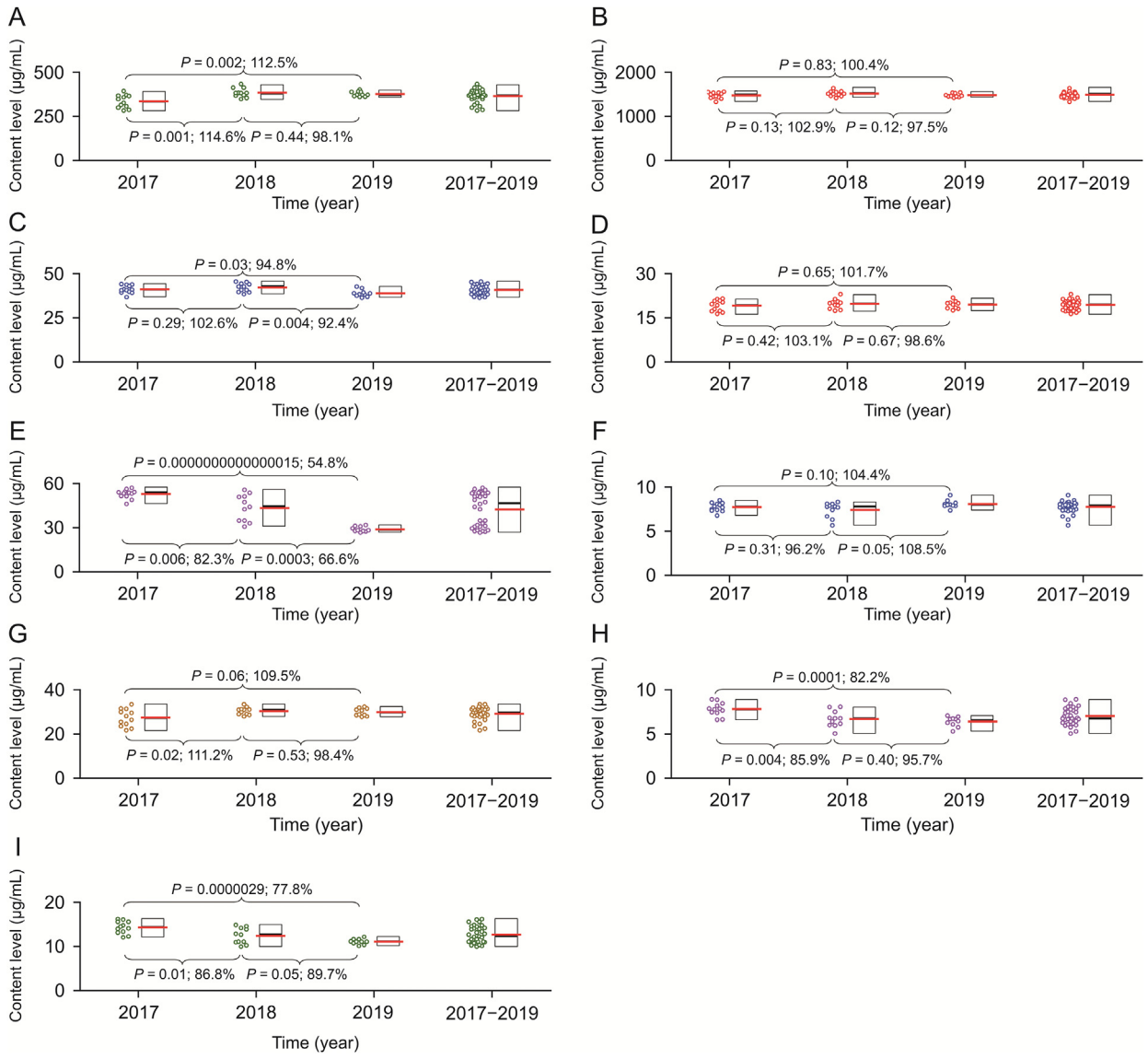

Fig. 4. LC-MS/MS-measured content levels of the nine marker constituents (A) hydroxysafflor yellow A, (B) paeoniflorin , (C) oxypaeoniflorin , (D) albiflorin, (E) senkyunolide I, (F) senkyunolide G, (G) ferulic acid, (H) tanshinol, and (I) protocatechuic aldehyde from samples of 33 batches of XueBiJing manufactured from 2017 to 2019. On the right side of the vertical scatter plot, the box plot shows the median as the black horizontal line inside the box and theinter-quartile range (IQR) as the length of the box. The associated red horizontal line (longer than the median line) represents the mean value.

LC-UV chromatogram of the injection (Fig. 3B) were also detected and characterized by our 42 min LC-MS/MS composition analysis (Table S1). Our results suggested that the LC-MS/MS analysis reliably determined the chemical composition of XueBiJing (Fig. S4).

As shown in the latest edition of the Pharmacopoeia of the People's Republic of China [3], LC-UV is still the most commonly used technique in the pharmaceutical analysis of herbal medicines, accounting for 81% of assays. Although these assays use one-point calibration, most of them monitor only a single marker constituent per medicine. However, when the LC-UV-based assay uses one-point calibration to simultaneously monitor multiple marker constituents of different structural classes at different content levels for a multi-herb medicine, high sample throughput of assay is sacrificed for the level of selectivity required to meet "similarity of response". In the LC-UV measurement of this investigation, the compromise was minimized by using composition information of XueBiJing and the complementary LC-MS/MS technique for identifying and eliminating interference of co-eluted non-target constituents that possessed UV absorbance at 230 or 280 nm. Despite these efforts, sample throughput of the LC-UV-based assay (using a 60 min gradient program) was significantly lower than that of the LC-MS/MS-based assay (using an 8 min gradient program), due to structurally specific data obtainable without a high LC resolution. High sensitivity for compound measurement by LC-MS/MS normally requires substantial dilution of both the medicine samples and calibrator; the dilution may cause not all marker constituents to meet "linearity through zero". To address this issue, we developed the intercept test. Although developing a therapeutically relevant LC-MS/MS- or LC-UV-based multi-analyte assay using a one-point calibrator requires a rigorous experimental setup and a wealth of supportive information, the validated assay used in this study is easy to apply in routine pharmaceutical analyses.

Using the newly developed assays to analyze samples from the 33 batches of XueBiJing manufactured over three years, the herbal injection exhibited high overall quality consistency, which has contributed to its effective, safe, and growing medicinal use in the treatment of sepsis over the past 16 years, as well as its officially recommended use in the management of COVID-19 in China. This

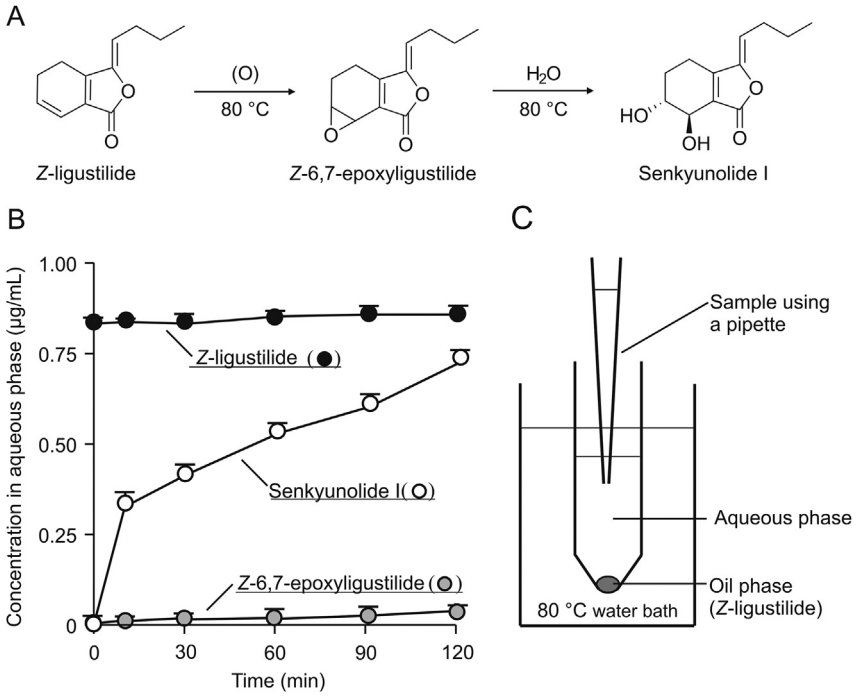

Fig. 5. (A and B) Chemical conversion of *Z*-ligustilide into senkyunolide I. (C) *Z*-ligustilide was poorly dissolved in water and had a greater density than water.

Table 5
Preconditions and criteria for developing a therapeutically relevant assay for quality evaluation of a Chinese herbal medicine: XueBiJing.

Feature	Criteria	XueBiJing	Refs.
Precondition (1) [a]			
Therapeutic action of the medicine	Intended for use in treatment, prevention, mitigation, cure, or diagnosis of a specific disease in humans	Licensed in China as a treatment for sepsis	N/A
Clinical efficacy of the therapeutic action	Well-designed and effectively executed clinical trials (multicenter, randomized, double-blind, placebo-control) Meta-analysis of several similarly designed trials Recommendation by authoritative treatment guidelines and/or expert consensuses	A prospective, randomized controlled trial in 710 patients with severe community-acquired pneumonia (sepsis) showed that combining XueBiJing with conventional sepsis care reduced 28-day mortality from 24.6% (conventional treatment only) to 15.9% (XueBiJing + conventional treatment), increased the percentage of patients with improved pneumonia severity index from 46.3% to 60.8%, improved SOFA scores from 4.44 to 3.65, and improved APACHE II scores from 11.12 to 9.19 (P<0.01 for all). Meta-analyses: combining XueBiJing with conventional sepsis care further reduced 28-day mortality and incidence of complications and improved prognosis. "Chinese guidelines for emergency management of sepsis and septic shock 2018": recommends combining XueBiJing with conventional sepsis care. "Clinical practice guideline on traditional Chinese medicine therapy alone or combined with antibiotics for sepsis": recommends combining XueBiJing with conventional sepsis care. "Chinese emergency medicine expert consensus on diagnosis and treatment of sepsis complicated with disseminated intravascular coagulation": recommends combining XueBiJing with conventional sepsis care. "Diagnosis and Treatment of Adults with Coronavirus Disease 2019 (COVID-19)": recommends XueBiJing as an add-on therapy in management of COVID-19 when patients with severe disease develop SIRS and/or MODS.	[12–17, 82–84]
Physiological and biochemical effects relevant to the therapeutic action	Pathophysiology of the disease, providing a scientific basis for the use of the medicine Physiological and biochemical effects observed in clinical and/or animal studies, on which treatment efficacy is based	Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. The pathophysiology of the septic response is characterized by overabundant innate immune response, uncontrolled release of inflammatory mediators,	[13,55,85–93]

(*continued on next page*)

Table 5 (*continued*)

Feature	Criteria	XueBiJing	Refs.
		inefficient use of the complement system, coagulation abnormalities, endothelial capillary leakage syndrome, immunosuppression, and organ dysfunction. XueBiJing inhibits the uncontrolled release of inflammatory mediators, relieves an early overabundant innate immune response and potentially cumulative immunosuppression, attenuates the crosstalk between inflammation and coagulation, protects endothelial cells, and maintains physiological functions of vital organs.	
Precondition (2) [b]			
Chemical composition	Based on comprehensive composition analysis, rather than detection or measurement of multiple constituents or fingerprint analysis Composition analysis: high analyte-measurement capacity and involving detection, characterization, and quantification of constituents Multi-herb medicine: assay reflecting the entirety of the medicine's formula, because all the component herbs are believed to contribute to the therapeutic action Exclusion of minor constituents: compounds with a dose <0.01 μmol/person, normally exhibiting undetectable or negligible systemic exposure	Composition analysis of XueBiJing: a total of 124 constituents (compound dose ≥0.01 μmol/person) were characterized and quantified; 51 constituents originate from the component herb Honghua, 31 from Chishao, 18 from Chuanxiong and Danggui, 20 from Danshen, and 4 from more than one component herb.	
Precondition (3) [c]			
Significantly bioavailable herbal compounds, originating from each component herb, after dosing the medicine	Chemical composition-based pharmacokinetic research on the medicine: human pharmacokinetic data substantiated by several types of supportive studies, determination of circulating compounds (unchanged and metabolized), and reflecting the entirety of the medicine's formula	As the first step in our prior pharmacokinetic research, chemical composition of XueBiJing was analyzed and a total of 104 herbal constituents (compound dose ≥0.01 μmol/person) were detected, characterized, and quantified. The human pharmacokinetic investigations (by dosing at the XueBiJing label dose) identified 11 major circulating herbal compounds, i.e., hydroxysafflor yellow A originating from Honghua, paeoniflorin, oxypaeoniflorin, and albiflorin from Chishao, senkyunolide I, senkyunolide I-7-*O*-β-glucuronide, senkyunolide G, and ferulic acid from Chuanxiong and Danggui, tanshinol, 3-*O*-methyltanshinol, and protocatechuic acid from Danshen, with their disposition characterized also by rat studies and *in vitro* metabolism and transport studies. Among these compounds, senkyunolide I-7-*O*-β-glucuronide, 3-*O*-methyltanshinol, and protocatechuic acid were metabolites of senkyunolide I, tanshinol, and protocatechuic aldehyde, respectively. In addition, there were 42 minor XueBiJing compounds detected in human plasma. The other XueBiJing constituents were not detected, in unchanged or metabolized forms. Systemic exposure to the newly characterized XueBiJing constituents (i.e., 20 = 124−104) was analyzed in human plasma samples (obtained from the previous human pharmacokinetic study). Their systemic exposure was limited, either in the unchanged or metabolized form.	[31–34]
Related bioactivities of the compounds	Focuses on the bioavailable constituents, with significant exposure as unchanged and/or metabolized forms Relates to the medicine's therapeutic action Bioavailable compounds should possess a maximum concentration (c_{max}; measured in a related pharmacokinetic investigation by dosing the medicine) higher than the minimum concentration necessary to elicit bioactivity (c_{EA}). Given that multiple compounds of a herbal medicine likely act on different targets to gain a multiple mode of perturbation of the pathogenic cascade (rather than to fully antagonize a single target), lower concentrations can be used.	Antisepsis-related activities of the major circulating XueBiJing compounds, unchanged and metabolized, included anti-inflammatory, anticoagulant, endothelium protective, immunoregulatory, antioxidant, and organ protection activities (see Table 1 for more details). Taken together, hydroxysafflor yellow A, paeoniflorin, oxypaeoniflorin, albiflorin, senkyunolide I, senkyunolide G, ferulic acid, tanshinol, and protocatechuic aldehyde were selected as marker constituents to develop therapeutically relevant assays for quality evaluation of XueBiJing.	[50–77]

[a] An effective herbal medicine, for which a therapeutically relevant assay to evaluate variability in quality will be developed.
[b] Well defined chemical composition of a medicine, for which a quality evaluation assay will be developed.
[c] Bioavailable compounds with bioactivities related to therapeutic effects.
SOFA: sequential organ failure assessment; APACHE II: acute physiology and chronic health evaluation II; SIRS: systemic inflammatory response syndrome; MODS: multiple organ dysfunction syndrome.

batch conformance can mainly be attributed to regulation of XueBiJing as a drug product in China. In addition to the strict regulation of Chinese herbal medicines, rapid expansion of the industry and a marked increase in scientific research are also necessary to ensure quality and safety of the medicines [97]. Over the years, an extensive amount of published data (relating to clinical efficacy and safety [12–16], pharmacodynamics [55,87–93], analytical chemistry [19–28], pharmacokinetics [31–33], drug interaction potential [34], and real-world adverse reactions [78]) has helped to define the therapeutic benefits and conditions for the safe use of XueBiJing. Similarly, developing therapeutically relevant assays, as in the current investigation, significantly contributes to ensuring the consistent quality of XueBiJing.

5. Conclusion

Chinese herbal medicines have been used for millennia for their health and therapeutic benefits and are still used as a treatment for many multifactorial diseases, including COVID-19, in China. Regulation as medicinal products requires manufacturing herbal medicines to be in line with contemporary pharmaceutical standards. Reproducible quality of complex Chinese herbal medicines requires the development of therapeutically relevant assays with efficiency and practicality. In this investigation, therapeutically relevant assays were developed for XueBiJing quality evaluation, based on LC-MS/MS and LC-UV measurement of nine marker constituents that originate from the five component herbs and that show high systemic exposure and bioactivity for the injection's antisepsis action. To make a one-point-calibration applicable for simultaneously monitoring the nine analytes of different structural classes at different content levels, “similarity of response” and “linearity through zero” were achieved for each analyte; this enabled the assays to be reliable and efficient for routine quality evaluation of XueBiJing. Developing therapeutically relevant assays for herbal medicines not only involves analytical chemistry, but also requires a multidisciplinary effort. Based on the newly developed assays, XueBiJing exhibited high overall quality consistency, which has probably contributed to its efficacy, safety, and popularity in medicinal use.

CRediT author statement

Xuan Yu: Investigation, Validation, Formal analysis, Writing - Original draft preparation, Visualization; **Wei Niu**: Investigation, Validation, Visualization; **Ya-Ya Wang**: Investigation, Validation, Visualization; **Olajide E. Olaleye**: Writing - Reviewing and Editing; **Jia-Nan Wang**: Investigation; **Meng-Yuan Duan**: Investigation; **Jun-Ling Yang**: Investigation; **Rong-Rong He**: Investigation; **Zi-Xuan Chu**: Investigation; **Kai Dong**: Resources; **Gui-Ping Zhang**: Resources; **Chang-Xiao Liu**: Writing - Reviewing and Editing; **Chen Cheng**: Conceptualization, Methodology, Formal analysis, Writing - Original draft preparation, Reviewing and Editing, Project administration, Funding acquisition; **Chuan Li**: Conceptualization, Methodology, Formal analysis, Writing - Original draft preparation, Reviewing and Editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2022.01.001.

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