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Qualitative and quantitative determination of complicated herbal components by liquid chromatography hybrid ion trap time-of-flight mass spectrometry and a relative exposure approach to herbal pharmacokinetics independent of standards

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ABSTRACT

To date, the pharmacokinetic research of herbal medicines (HMs) is still in its infancy and is facing critical technical challenges on the qualitative and quantitative analysis of complicated components from biological matrices. Additionally, the lack of authentic standards constitutes another bottleneck on assessing herbal pharmacokinetics. This present work contributes to the development of a powerful technical platform for both qualitative and quantitative pharmacokinetic analysis of herbal components, and a strategy of relative exposure that provides a practicable pharmacokinetic assessment independent of authentic standards, based on the use of liquid chromatography hybrid ion trap time-of-flight mass spectrometry (LC–IT-TOF/MS). Taking *schisandra* lignans extract (SLE) as an example, the LC–IT-TOF/MS assay was initially applied to the global qualitative analysis of components contained in SLE *per se* and in the rat plasma post SLE dosing. Afterwards, this study focused on validating the quantitative performance of LC–IT-TOF/MS assay by comparison with a well-established LC–Q/MS assay. For the absolute quantification of five lignans components with authentic standards, both assays showed very similar analytical figures of merit such as linearity, precision, accuracy, and pharmacokinetic parameters. Compared with LC–Q/MS, the prominent advantage of LC–IT-TOF/MS assay is its much higher sensitivity. Moreover, a 'relative exposure approach' (REA) that entails the use of sequentially diluted original herbal preparations to prepare the 'mixed calibration curves' was developed to assessing herbal pharmacokinetics independent of specific authentic compounds for each component. Such an approach was found capable of providing virtually identical pharmacokinetic parameters as that from the typical pharmacokinetic assay calibrated by authentic standards, except for the absolute plasma concentrations. The presently developed methodology and approach will find its wide use in, but not limited to, the qualitative and quantitative pharmacokinetic analysis of herbal medicines.

1. Introduction

Herbal medicine, the major carrier of traditional medicine, has been playing an important role on the human health and welfare for a long history. Despite the skepticism raised against their poor scientific evidences, the use of herbal medicines has still been expanding globally and gains more and more popularity across the world [1–3]. Herbal medicines are today still the primary form of health care for the poor in the developing countries, and also widely used as the supplement or substitute to conventional drugs in the developed countries. More importantly, herbal medicines are receiving renewed interest accompanied with the gradual recognition that the current 'one gene, one drug, one target' drug discovery paradigm is very questionable because of the significantly declined productivity of new drug discovery under such a paradigm in the last decade [4–6]. Therefore, it is reasonable to expect that the herbal medicine based and inspired holistic paradigm will be of great attraction in the future drug discovery.

It has been well acknowledged that pharmacokinetics (PK) plays an important role, and represents an integral part throughout the whole pipeline of new drug discovery. Similarly, the extensive PK study of herbal medicines is of certain importance for explaining and predicting their efficacy and toxicity, and the widely reported herb–drug interactions [7–10]. Unfortunately, the current research on herbal PK is still in its infancy and largely limited to the isolated components research by following the methodology for pharma-

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cokinetic study of single drug. As pointed out by WHO [11], there is very limited knowledge about PK of HMs, because of the following challenges: (1) HM is a complex system, in most cases, medicinal plants comprise hundreds of different constituents that belong to different compound classes with diverse chemical and physical properties; (2) most components are unknown (nontarget); (3) the lack of powerful techniques on the global qualitative and quantitative analysis of components constrained by the lack of standards, low plasma concentration, large range of concentration scales, etc.

For the pharmacokinetic studies of herbal medicines, the first step would be the global identification of components contained in the herbal preparations, which provides prerequisite information for the following choice of target components for detailed pharmacokinetic studies. More recently, we and other groups have developed some powerful qualitative tools and strategies for the global identification of components from various herbal preparations [12–15]. However, the case would be much more challenging to identify herbal resourced components from biological matrices. Furthermore, compared with qualitative analysis, the quantitative analysis of complicated herbal components in biological matrices would be much more difficult based on the current analytical platforms. To date, most quantitative analysis in the field of pharmacokinetics were carried out on a LC coupled to single or triple quadrupole (Q) mass spectrometers, which always provided satisfactory performances on quantifying single or a few number of drugs [16,17]. However, both LC–Q/MS and LC–Q-q-Q/MS may not meet the challenges of the quantitative analysis of complicated herbal components, because it is well acknowledged that their detecting sensitivity decreases dramatically with the increase of ions selected [18,19]. Such a sensitivity compromise will lead to a critical problem for the global quantifications of herbal components in plasma, because for most herbal components the plasma concentration is extremely low.

The hybrid ion trap and time-of-flight mass spectrometry (LC–IT-TOF/MS), that integrates the advantageous merits of ion trap in producing multistage tandem (MS^{1-10}) fragmentations and of TOF in high resolution and accurate mass measurement, has been recently confirmed by us and other groups to be a very powerful tool on the global identifications of both target and nontarget components [12–14,20]. However, its performance on quantitative analysis is largely unknown. In view of the procedures for pharmacokinetic analysis of herbal medicines, it will be very ideal and desirable that a single instrument could meet the requirements of both qualitative and quantitative analysis of complicated components. Driven by this idea, the present study was designed as an extension of our previous works to test the applicability of LC–IT-TOF/MS on both qualitative and quantitative pharmacokinetic analysis of complicated herbal components.

Schisandra chinensis extract (SLE), a well-known herbal medicine possessing a wide range of therapeutic effects including hepatoprotective effect, potent antioxidative property, detoxificant effect, anticarcinogenic activity was taken as a model herbal medicine in this study [21–23]. Global qualitative analysis of components contained in SLE *per se* and in the rat plasma post SLE dosing were carried out based on our previous strategies with slight modifications. More importantly, the potential quantitative capacity of LC–IT-TOF/MS was fully validated and compared with a well-established typical LC–Q/MS assay.

Besides various technical challenges, the lack of authentic standards for calibration constitutes another critical bottleneck of the pharmacokinetic analysis of herbal components. Although the relative quantification approach by directly measuring the peak area or perk area ratio to internal standards may be practicable to obtain some useful pharmacokinetic parameters independent of absolute plasma concentrations such as elimination half-life ($t_{1/2}$) and the time taken to achieve the peak concentration (T_{max}), the important pharmacokinetic parameters such as area under concentration–time curve (AUC) and maximum plasma concentration (C_{max}) obtained from this approach are not comparable among different components, and thus impracticable for the important quantitative structure pharmacokinetics relationship research of herbal components. To address such a critical problem, this study created a 'relative exposure approach' (REA) that was independent of specific authentic compounds of each component, whereas the sequentially diluted rude extraction of herbal medicines *per se* was used instead to prepare the 'mixed calibration curves' which was then used for calibrating all potential components in plasma. To validate such an approach, results obtained from REA were compared with those obtained from a conventional approach that was calibrated by the authentic compounds for five major components.

2. Experimental

2.1. Chemicals

Schizandrol A and B, schizandrin A and B, and schisantherin A, as well as internal standard, nimodipine (NMP, >99% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SLE was purchased from Nanjing Qingze Medical Technological Development Co. Ltd. (Nanjing, China). The content of major lignans components including schizandrol A and B, schizandrin A and B, and schisantherin A in SLE was 4.26%, 1.18%, 0.32%, 0.34% and 0.14%, respectively. HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water was collected from a Milli-Q Ultrapure water system with the water outlet operating at 18.2 MΩ (Millipore, Bedford, USA). Other chemicals and solvents were all of analytical grade.

2.2. Animals and treatment

Animal studies were conducted according to protocols approved by the Review Committee of Animal Care and Use of China Pharmaceutical University. Six female Sprague–Dawley rats weighing 200 ± 20 g were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, China). All the rats were acclimatized in an environmentally controlled breeding room for at least 3 days before experiments, fed with standard laboratory food and water and fasted overnight but with access to water before the test. For pharmacokinetic studies, rats were intragastrically administered SLE at a dose of 500 mg/kg. Heparinized blood samples of 200 μL were collected at 10, 20, 40 min, 1, 2, 3, 4, 5, 6, 8, 12, and 24 h from the ophthalmic veins by sterile capillary tube under anaesthesia. Blood samples were then shaken up and centrifuged at $2000 \times g$ for 10 min. The supernatants were decanted, and immediately frozen at −20 °C until analysis.

2.3. Sample preparation

The primary stock solutions of 5 lignans (schizandrol A and B, schizandrin A and B, and schisantherin A) were prepared in methanol at a concentration of 1 mg/ml and stored at 4 °C until analysis. Working solutions of the analytes were prepared by appropriate dilution of the primary stock solutions in methanol. Then, 10 μL of working solutions were spiked into 90 μL blank rat plasma in an Eppendorf tube to make calibration standard curves or quality control (QC) samples at appropriate concentrations for each compound.

The primary stock solution of SLE was prepared by ultrasonic extraction 100 mg SLE in 50 ml methanol for 1 h. Working solu-

tions were prepared by dilution of the primary stock solution in methanol to 1–2000 folds. Then, 10 μL of working solutions was spiked into 90 μL blank rat plasma to prepare the standard curves (0.1–200.0 μg/ml of SLE).

All the analytes were extracted by using a liquid–liquid extraction technique. To 100 μL of plasma, 10 μL of internal standard (NMP, 10.0 μg/ml) and 1.0 ml of ethyl acetate were added. The mixture was then vortexed for 2 min, and centrifuged for 10 min at 40,000 × *g*. The supernatant was evaporated to dryness in a rotary evaporator and the residue was dissolved in 200 μL of methanol.

2.4. LC–IT-TOF/MS and LC–Q/MS conditions

LC experiments were conducted on a Shimadzu (Kyoto, Japan) HPLC system consisting of an LC-10AD binary pump, DGU-14A degasser, SIL-20AC autosampler and a CTO-20AC column oven. Chromatographic separation was achieved on a SymmetryShield™ RP_8 column (3.5 μm, 50 mm × 2.1 mm I.D., Waters) at 40 °C. The mobile phase (delivered at 0.2 ml/min) consisted of solvent A, CH_3OH/H_2O (5:95, v/v, containing 1.0 μM CH_3COONa) and solvent B, CH_3OH/H_2O (95:5, v/v, containing 1.0 μM CH_3COONa). A binary gradient elution was performed: initial 55% B for 0.2 min, linear gradient 55–65% B from 0.2 to 25.0 min and 65 to 85% B from 25.0 to 40.0 min, then quickly returned to initial 55% B and maintained until 48 min for column balance.

The mass spectrometer of LC–IT-TOF/MS (Shimadzu, Japan) equipped with an ESI source in positive ion mode. The optimized analytical conditions were as follows: detector voltage, 1.60 kV; nebulizing gas (N_2) flow, 1.5 L/min; dry gas (N_2) flow, 50 kPa; pressure of TOF region, 1.5×10^{-4} Pa; ion trap pressure, 1.7×10^{-2} Pa; ion accumulated time, 30 ms; precursor ion selected width, 3.0 amu. Accurate mass determination was corrected by calibration using the sodium trifluoroacetate clusters as reference. For qualitative analysis: scan ranges were set at *m*/*z* 350–700 for MS^1, 100–600 for MS^2 and 50–500 for MS^3; ultra-high purity argon was used as the cooling gas and the collision gas for CID experiments, and the collision energy was set at 50% for MS^2 and 100% for MS^3, respectively. Accordingly, multiple stage fragmental energy was canceled when LC–IT-TOF/MS was used for quantitative analysis; in this case, mass spectrometry was conducted in the full scan mode at the range of *m*/*z* 350–700, and ultra-high purity argon was used only as the cooling gas.

The mass spectrometer of LC–Q/MS system consisted of a Shimadzu LCMS-2010A quadrupole mass spectrometer interfaced by an ESI probe in positive ionization mode. The $[M+Na]^+$ ions for the targeted analytes (*m*/*z* 455 for schizandrol A, *m*/*z* 439 for schizandrol B, *m*/*z* 559 for schisantherin A, *m*/*z* 439 for schizandrin A, *m*/*z* 423 for schizandrin B, and *m*/*z* 441 for NMP, respectively) were monitored simultaneously. The MS operating conditions were optimized as follows: drying gas 1.5 L/min, curved desolvation line (CDL) temperature 250 °C, heat block temperature 200 °C, and detector voltage 1.6 kV.

2.5. Experimental design workflow

To show clearly the experimental design of this study, the experimental workflow is schematically depicted in Fig. 1. First, the lignan components contained in SLE were detected and identified based on our previous report with slight modifications [13]. Second, both lignan compounds and potential metabolites in the rat plasma at 3 time points (10 min, 1 and 6 h) after oral administration of SLE were profiled and characterized by comparing the retention time and accurate mass data of analytes in rat plasma with those of the lignans in SLE. Third, to determine the quantitative potential of the novel IT-TOF/MS approach, the quantitative behaviors of IT-TOF/MS for the simultaneous quantification of five lignan components in rat plasma were fully validated and compared with those of a typical LC–Q/MS method. Fourth, the quantitative performances of LC–IT-TOF/MS on the simultaneous determination of 31 lignans were further validated. Finally, to address the critical problem of lacking authentic compounds, a relative exposure approach (REA) independent of authentic compounds was developed based on preparing the calibration curves by sequential dilution of rude extracts (dosed pharmaceutics, SLE in this study). The linear regression equation for each component was expressed as: $y = bx + a$, where y represents the mass response ratio of targeted analytes to internal standard, and x represents the concentrations of rude extract. The plasma concentration of each component was then calculated from this calibration curve and the data expressed as the concentration of rude extract means that the actual plasma concentration of certain component is equivalent to its content contained in the rude extract at measured concentration. Pharmacokinetic parameters were then calculated from the relative concentrations versus time data.

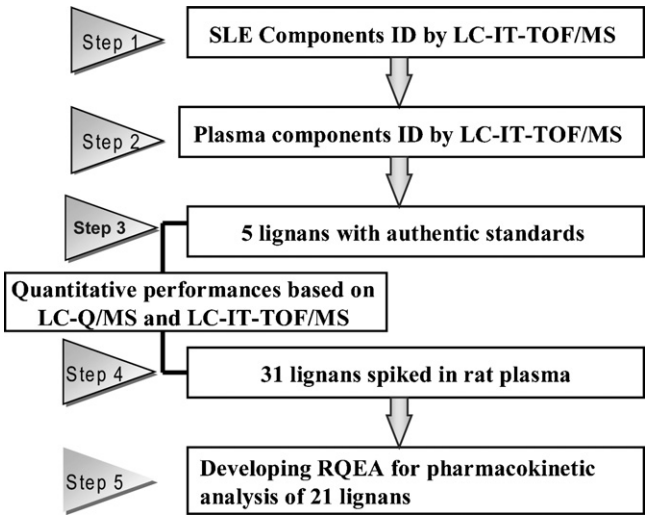

Fig. 1. Systematic workflow.

3. Results and discussion

3.1. Identification of lignans in SLE by LC–IT-TOF/MS

Lignans, composed of A, B and C rings, are the major and characteristic constitutes of *Schisandraceae* plants. Recently, we have developed a diagnostic fragment-ion-based extension strategy (DFIBES) for the rapid detection and structural characterization of 20 kinds of lignans components from '*Shengmai*' injection using LC–IT-TOF/MS analysis [13]. In this study, such a strategy was applied with slight modifications for characterizing the lignans components from SLE based on the newly developed and optimized LC–IT-TOF/MS assay conditions as described above. Herein, sodium acetate was added in the mobile phase to improve the detection sensitivity, and two different collision energies (50% and 100%) induced fragmentations were performed to obtain sufficient fragment ions and to select the most sensitive response for each product ion. Fig. S1 (supporting information) shows the TIC of SLE obtained from the positive ion scan mode. The detailed structural characterizations of 31 lignans are shown in Table S1 and classified into five subfamilies according to their skeleton structures. Except 2 pairs of isomers, most of the lignans components were successfully characterized in this study.

Table 1
Identification of parent lignans and metabolites in rat plasma after SLE dosage.

No.	Rt (min)	*m/z* [M+Na]+		Formula	Error (ppm)	Identification
		Measured	Exact			
M1	5.5	541.2055	541.205	C28H38O9	0.92	Hydrol Angeloylgomisin H
M2	6.2	457.1824	457.1838	C23H30O8	−3.06	Hydroxyl Gomisin-Tol
M3	7.6	555.2197	555.2154	C28H36O10	−3.90	Hydrogenation Gomisin D
M4	7.8	455.1666	455.1682	C23H28O8	−1.60	Hydroxyl Schisandrol B
M5	8.6	525.2089	525.2101	C27H34O9	−2.28	Demethyl & Hydroxyl Angeloylgomisin H
M6	10.4	525.2089	525.2101	C27H34O9	−2.28	Demethyl Tigloyl/Angeloyl gomisin Q
M7	11.9	471.199	471.1995	C24H32O8	−0.26	Hydroxyl Schisandrol A
M8	16.3	553.2053	553.205	C28H34O10	0.54	Hydroxyl Tigloyl/Angeloyl gomisin P
3	10.4	441.1876	441.1884	C23H30O7	−1.81	Gomisin T-ol
5	15.2	441.1875	441.1884	C23H30O7	−2.04	Gomisin
6	17	455.2035	455.204	C24H32O7	−1.10	Schisandrol A
7	19.8	553.2059	553.2044	C28H34O10	2.71	Gomisin D
9	22.2	523.2321	523.2302	C28H36O8	3.63	Tigloylgomisin H
10	23.5	439.1726	439.1727	C23H28O7	−0.23	Schisandrol B
11	24.6	537.2095	537.2095	C28H34O9	0.00	Schisantherin B
12	25	523.2303	523.2302	C28H36O8	0.19	Angeloylgomisin H
13	25.8	559.1935	559.1939	C30H32O9	−0.72	Gomisin G
14	25.8	553.2049	553.2044	C28H34O10	0.90	Angeloylgomisin Q
16	29.1	537.2097	537.2095	C28H34O9	0.37	Schisantherin C
18	32.8	537.211	537.2095	C28H34O9	2.79	Gomisin F
21	33.4	559.1932	559.1939	C30H32O9	−1.25	Schisantherin A
22	34.1	537.2098	537.2095	C28H34O9	0.56	Tigloylgomisin P
23	35.7	425.1932	425.1935	C23H30O6	−0.71	Gomisin K
24	36.9	409.1624	409.1622	C22H26O6	0.49	Gomisin M1/M2
25	37	409.1624	409.1622	C22H26O6	0.49	Gomisin M1/M2
26	37.2	537.2095	537.2095	C28H34O9	0.00	Angeloylgomisin P
29	39.2	439.2078	439.2091	C24H32O6	−2.96	Schizandrin A
30	42.3	423.1773	423.1778	C23H28O6	−1.18	Gomisin N
31	42.9	423.1773	423.1778	C23H28O6	−1.18	Schizandrin B

3.2. Identification of lignans and their metabolites in rat plasma

Plasma collected at 0, 20 min, 1 and 6 h after an intragastic administration of SLE (500 mg/kg) were subjected to the qualitative analysis of lignans components and their potential metabolites.

To facilitate the identification of parent lignans and metabolites from plasma, a simple four-step approach was developed. First, the endogenous interferences were rapidly excluded by comparing the mass profiles of the real plasma samples (SLE dosage) with that of the blank rat plasma using Met ID solution software (Shimadzu, Japan). Second, the parent lignans components in plasma were readily characterized by comparing the mass profiles of real plasma samples after SLE dosage with that of SLE *per se*. Third, remaining peaks in the TIC after the first and second step of exclusions were treated as potential metabolites and were subjected to structural characterizations. In this case, 21 parent lignans and 8 metabolites were found from the rat plasma after SLE dosage (Table 1). Finally, all metabolites were structurally characterized by comparing sequentially their accurate mass spectra data with those for the parent lignans components based on our previously reported strategy. Here we took the M1 identification as an example to describe the strategy in detail. M1, eluted at 5.5 min, showed the predominant quasi-molecular ion $[M+Na]^+$ at *m/z* 541.2055 ($C_{28}H_{38}O_9$) that is a H_2O more than tigloylgomisin H or angeloylgomisin H. The major fragment ion at *m/z* 495.1995 ($C_{26}H_{32}O_8$) was a C_2H_6O loss from the quasi-molecular ion and the same fragmentation mode also occurred on angeloylgomisin H to produce a major fragment ion at *m/z* 477.1894 through a C_2H_6O loss. In addition, the fragment ions found at *m/z* 441.1890 ($C_{23}H_{30}O_7$), 423.1413 ($C_{22}H_{28}O_6$), 325.0599 ($C_{16}H_{14}O_6$) were also same to angeloylgomisin H. All the fragment mode comparisons support that M1 was a hydration product of angeloylgomisin H. Furthermore, the fragment ion at *m/z* 425.1513 was the same to the loss of 2-methyl-3-butenoic acid on angeloylgomisin H, suggesting that the hydration position was on the 2-methyl-3-butenoic acid group. The possible structure and proposed fragment pathways of M1 are shown in Fig. S2.

3.3. Quantitative analysis of lignans in rat plasma by LC–IT-TOF/MS and LC–Q/MS

Current pharmacokinetic analyses are often carried out by single or triple quadrupole mass spectrometer platform in SIM or SRM mode. However, the sensitivity of the quadrupole mass not only depends on the compound structure, mobile-phase solvents and additives, but also depends largely on the number of ions detected. It has been well characterized that the signal level/noise level (S/N) decrease accompanied with the increasing of numbers of ions detected in the single and/or triple quadrupole mass spectrometer platform [18,19]. Therefore, quadrupole mass spectrometers may not be suitable for the pharmacokinetic study of complex HMs for which dozens of components are always necessary to be quantified simultaneously. Herein, we sought to investigate whether the LC–IT-TOF/MS technique would be a good choice for the pharmacokinetic analysis of multiple components in herbal medicines. For this purpose, the quantitative behaviors of LC–IT-TOF/MS on the simultaneous determination of five lignans (a mixture of five authentic compounds) in rat plasma were fully validated and compared with those of a well-established LC–Q/MS assay. The applicability and suitability of both LC–IT-TOF/MS and LC–Q-MS assay into the pharmacokinetic study of such five lignans in SLE were evaluated. Then, the quantitative performances of both assays were compared for the simultaneous determination of 31 lignans spiked in rat plasma.

3.3.1. Comparison of analytical performance of 5 lignans on LC–IT-TOF/MS and LC–Q/MS

For this comparison, all experimental parameters were standardized and the sample preparation method and chromatographic conditions were the same for both assays. Five lignans authentic

Table 2
Assay calibration data, precision and accuracy for schizandrol A and B, schizandrin A and B, and schisantherin A in rat plasma measured by LC–IT-TOF/MS and LC–Q/MS.

Schisandra lignans	$y = bx + a$ r^2 (dynamic range, ng/ml) LOQ		C (ng/ml)	Accuracy		Intra-day precision		Inter-day precision	
	I	II		I	II	I	II	I	II
Schizandrol A	$y = 0.0311x + 0.0089$	$y = 0.0109x + 0.0003$	2.0	6.5	9.0	1.39	10.05	2.44	4.76
	0.9989 (0.2–200)	0.9998 (0.5–500)	20.0	2.5	−8.2	1.12	4.63	3.75	3.87
	LOQ: 0.1 ng/ml	LOQ: 0.5 ng/ml	200.0	0.5	−3.5	1.05	0.59	1.66	6.61
Schizandrol B	$y = 0.0181x + 0.0014$	$y = 0.0143x + 0.0019$	2.0	2.0	2.5	3.75	19.01	2.83	3.62
	0.9979 (0.2–200)	0.9988 (1.0–500)	20.0	7.2	−6.5	0.31	11.81	3.23	1.96
	LOQ: 0.2 ng/ml	LOQ: 1.0 ng/ml	200.0	−8.4	9.5	0.59	3.12	2.26	5.43
Schisantherin A	$y = 0.0089x + 0.0032$	$y = 0.0094x + 0.0012$	2.0	1.0	6.0	3.92	6.22	5.40	15.44
	0.9993 (0.2–200)	0.9996 (1.0–500)	20.0	0.7	1.6	3.46	6.50	9.64	5.81
	LOQ: 0.1 ng/ml	LOQ: 1.0 ng/ml	200.0	−1.4	1.1	2.54	9.77	5.76	7.45
Schizandrin A	$y = 0.0103x + 0.0088$	$y = 0.0111x + 0.0008$	2.0	1.0	2.5	3.48	12.05	5.12	17.76
	0.9989 (0.2–200)	0.9987 (1.0–500)	20.0	7.2	−6.5	3.22	7.22	8.94	8.07
	LOQ: 0.2 ng/ml	LOQ: 1.0 ng/ml	200.0	−1.0	4.0	2.64	7.64	7.64	11.11
Schizandrin B	$y = 0.0073x + 0.0042$	$y = 0.0040x + 0.0003$	2.0	1.5	4.0	4.18	14.79	8.94	12.37
	0.9999 (0.5–200)	0.9987 (2.0–500)	20.0	3.0	5.4	0.50	8.10	2.97	11.43
	LOQ: 0.5 ng/ml	LOQ: 2.0 ng/ml	200.0	−0.5	−1.4	1.60	7.78	4.57	10.13

I: LC–IT-TOF/MS; II: LC–Q/MS.

compounds including schizandrol A and B, schizandrin A and B, and schisantherin A were spiked into rat plasma and were determined separately by LC–IT-TOF/MS and LC–Q-MS to validate their performances on the sensitivity, linearity, precision, accuracy, specificity, and matrix effect.

In this study, we found that the novel LC–IT-TOF/MS method gave a relatively wide and sufficient dynamic range (10^3) for all the five lignans components determined with the correlation coefficients (r^2) exceeding 0.996, and was comparable with that of LC–Q-MS assay (Table 2). Besides, the LLOQ, defined as the concentration producing a S/N ratio over 10 and a deviation less than 20%, for schizandrol A, schizandrol B, schisantherin A, schizandrin A, and schizandrin B in the LC–IT-TOF/MS analysis was 0.1, 0.2, 0.1, 0.2 and 0.5 ng/ml, respectively, and was 5, 5, 10, 5 and 4 times lower than that of the LC–Q/MS assay. This result suggests that the sensitivity of LC–IT-TOF/MS operated under scan mode is much higher than that provided by LC–Q/MS operated under SIM. For LC–IT-TOF/MS assay, the accuracy expressed as relative errors (% RE) was always lower than ±10% for all the five lignans, and was comparable with that for LC–Q/MS assay. The inter-batch and intra-batch precision values were below 5% RSD in the LC–IT-TOF/MS assay for all the five lignans and were found much better than that of LC–Q/MS for which approximately 10% RSD was observed. Matrix effect that determines the potential signal suppression or enhancement effect by biological matrix on the targeted analytes was also assessed and compared between both assays. The matrix effect, expressed as the percent difference of average extracted plasma peak area to average neat peak area, in the LC–Q/MS assay was within ±20% for the five lignans, whereas the LC–IT-TOF/MS assay provided much better result with the value within ±12%, indicating that the LC–IT-TOF/MS was much less susceptible to matrix ion suppression than the LC–Q/MS.

3.3.2. Relative quantitation assay of 31 lignans by LC–IT-TOF/MS

From the quantitative assay of five lignans with authentic standards, the LC–IT-TOF/MS provided at least comparable performances with LC–Q/MS, suggesting the LC–IT-TOF/MS was also suitable for the conventional quantitative analysis besides its well-proven powerfulness on qualitative assay. As have previously described, the sensitivity of LC–Q/MS and LC–Q-q-Q/MS were likely to decrease dramatically accompanied with the increasing numbers of ions detected. However, such a compromise of sensitivity would not occur in the LC–IT-TOF/MS assay, because it has been well proven that the TOF analyzer was merited with high sensitivity for full spectrum scan [18,24,25]. To confirm such a proposition, we conducted a relative quantitation assay on the simultaneous determinations of 31 lignans in rat plasma on both LC–Q/MS and LC–IT-TOF/MS. Rat plasma was spiked with the sequentially diluted SLE to prepare the calibration curve with a concentration range from 0.1 to 200.0 μg/ml. Calibration curves of SLE were analyzed for each lignan component by a linear regression analysis of the analyte to internal standard peak area ratio against SLE concentrations (C_{SLE}, μg/ml). The accuracy and precision were evaluated at 0.5, 10.0, and 50.0 μg/ml of SLE. The results are presented in Table 3. The LC–IT-TOF/MS assay provided a 200 to 1000 folds dynamic linear range with the correlation coefficients (r^2) exceeding 0.99 for all the 31 lignans components. The sensitivity of this assay was extremely high considering the low content of most lignans components in SLE. Taking schisantherin A as an example, its content in SLE was only 0.14%, which means that its LLOQ in this assay is about 0.14 ng/ml by calculating from the determined C_{SLE} at 0.1 μg/ml. The accuracy expressed as relative errors (%, RE) for all the 31 lignans were within ±15% and the intra-day and inter-day precision RSD were below 10%, supporting that the LC–IT-TOF/MS assay is a powerful quantitative approach for determining dozens of components. In contrast, extremely high noise was observed in LC–Q/MS when 31 lignans ions were simultaneously detected. Actually, the S/N ratio decreased dramatically accompanied with the increased numbers of ions detected in the LC–Q/MS assay. For example, the S/N of schizandrol A decreased 10 folds when 20 lignans were detected simultaneously as compared with that when schizandrol A alone was detected (shown in Fig. S3). These results indicate that quadrupole mass spectrometer may not be applicable for the pharmacokinetic study of complex HMs for which dozens of components are always necessary to be quantified at one analysis.

3.4. Application to rat PK studies

To determine the applicability of LC–IT-TOF/MS assay on the pharmacokinetic study of herbal medicines, rat plasma samples collected after a single intragastric dosage of SLE (500 mg/kg) were subject to both LC–IT-TOF/MS and LC–Q/MS assays. For LC–Q/MS assay, only the five lignans with authentic standards were determined, whereas the LC–IT-TOF/MS operated under full scan mode produced quantitative information for all the 31 lignans, from which the plasma concentrations were calculated by the calibration curves of either a mixture of five authentic compounds (absolute

Table 3
Test ranges, calibration curves, intra-day and inter-day variability for the 31 lignans determined by LC–IT-TOF/MS.

No.	Calibration curves			Intra-day variability (RSD % $n=6$)			Inter-day variability (RSD % $n=6$)			Accuracy RE (%)
	Linear range (μg/ml)	Regression equation	r^2	I	II	III	I	II	III	10 μg/ml of SLE
1	1.0–200.0	$y=0.0111x-0.0076$	0.9993	Nd	4.27	3.76	Nd	3.94	8.59	−0.53
2	0.5–200.0	$y=0.0150x+0.0036$	0.9992	5.78	3.10	1.46	Nd	3.63	3.07	−9.67
3	0.5–200.0	$y=0.0093x+0.0033$	0.9964	3.08	6.51	3.31	4.17	3.63	2.79	2.20
4	2.0–200.0	$y=0.0077x+0.0006$	0.9993	Nd	1.91	5.01	Nd	2.47	1.46	−6.31
5	1.0–200.0	$y=0.0045x+0.0014$	0.9993	Nd	3.54	3.58	Nd	3.6	3.58	9.31
6	0.1–50.0	$y=0.6417x-0.1581$	0.9998	2.34	1.95	4.5	3.03	1.71	4.89	7.43
7	0.5–200.0	$y=0.0477x-0.005$	0.9992	6.19	2.82	2.24	12.27	2.06	9.19	7.84
8	0.2–200.0	$y=0.0357x+0.0222$	0.9995	2.05	0.38	2.5	1.49	0.31	1.7	5.36
9	0.2–200.0	$y=0.0317x-0.0103$	0.9998	0.19	3.00	2.63	1.65	2.21	2.2	−6.98
10	0.2–200.0	$y=0.2884x-0.0087$	0.9981	2.66	1.54	0.89	11.65	2.06	0.38	3.82
11	1.0–200.0	$y=0.0107x+0.0003$	0.9997	Nd	4.87	2.44	Nd	1.77	1.21	−2.12
12	0.2–200.0	$y=0.1315x+0.0441$	0.9993	3.29	1.41	2.04	3.09	1.18	3.04	−0.32
13	0.2–200.0	$y=0.0297x+0.0176$	0.9993	1.7	3.27	2.88	8.61	1.42	17.27	11.22
14	0.5–100.0	$y=0.028x-0.0083$	0.9992	8.68	3.02	2.82	9.43	1.29	3.19	−11.93
15	0.2–200.0	$y=0.0099x-0.0047$	0.9993	2.79	1.92	2.43	4.27	1.11	1.65	−2.74
16	0.2–100.0	$y=0.0115x+0.0004$	0.9978	3.16	2.59	3.22	3.71	2.79	4.62	−6.81
17	0.5–200.0	$y=0.0087x-0.0079$	0.9997	1.1	1.44	8.65	13.3	1.16	6.78	9.22
18	0.2–200.0	$y=0.0151x-0.0035$	0.9991	3.32	0.97	7.76	4.35	7.59	9.21	6.76
19	0.5–200.0	$y=0.0261x+0.0039$	0.9999	3.57	1.65	9.38	2.07	1.28	2.45	−3.83
20	1.0–200.0	$y=0.0075x-0.0019$	0.9994	Nd	2.73	2.2	Nd	1.96	1.45	−14.09
21	0.2–200.0	$y=0.0147x-0.0034$	0.9997	4.24	1.64	1.51	6.18	1.62	1.15	−9.39
22	0.2–200.0	$y=0.0377x+0.0156$	0.9995	1.97	1.37	1.34	2.53	1.47	5.5	−13.82
23	0.2–200.0	$y=0.0075x-0.0025$	0.9996	9.94	2.69	2.21	3.83	1.93	1.57	−5.00
24	0.2–200.0	$y=0.0006x+0.0003$	0.9994	6.4	9.54	3.47	7.24	7.16	1.57	−9.56
25	1.0–200.0	$y=0.0006x-0.0001$	0.9966	Nd	10.43	4.31	Nd	8.27	4.11	−7.36
26	1.0–200.0	$y=0.0021x+0.0006$	0.9982	Nd	6.25	1.82	Nd	3.88	1.86	−8.89
27	1.0–200.0	$y=0.0011x-0.0003$	0.9994	Nd	3.15	4.4	Nd	15.37	4	6.31
28	1.0–200.0	$y=0.0015x+0.0004$	0.9995	Nd	6.11	7.46	Nd	6.59	4.49	−5.20
29	0.5–200.0	$y=0.0364x-0.0167$	0.9982	4.32	2.73	2.76	2.4	3.2	5.56	−6.11
30	0.2–200.0	$y=0.0118x-0.0017$	0.9993	4.36	2.42	0.73	3.27	2.42	0.73	−2.80
31	0.2–200.0	$y=0.0125x+0.0114$	0.9990	7.99	5.81	5.83	4.26	5.91	5.4	−2.39

I: 0.5 μg/ml of SLE; II: 10.0 μg/ml of SLE; III: 50.0 μg/ml of SLE. Nd: below the lowest limit of quantitation.

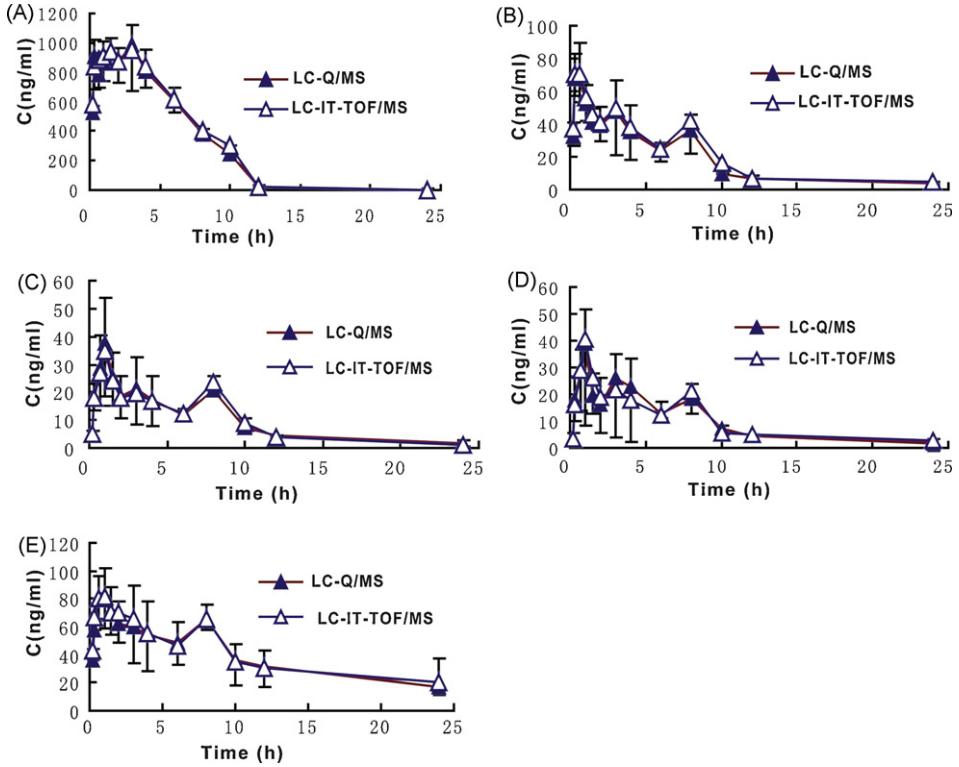

Fig. 2. The plasma concentration versus time profiles of all the five lignans measured by LC–IT-TOF/MS and LC–Q/MS. (A) Schizandrol A, (B) Schizandrol B, (C) Schizandrin A, (D) Schizandrin B, (E) Schisantherin A.

Table 4
PK results for LC–IT-TOF/MS and LC–Q/MS analyses of schizandrol A and B, schizandrin A and B, and schisantherin A in rats.

Schisandra lignans		C_{max} (ng/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC_{0-t} (ng h/ml)
Schizandrol A	I	1159.33 ± 61.34	2.67 ± 0.58	2.23 ± 0.71	7248.2 ± 1147.6
	II	1068.44 ± 44.54	2.67 ± 0.58	2.70 ± 1.29	6899.7 ± 851.2
Schizandrol B	I	78.46 ± 28.54	3.78 ± 3.89	7.30 ± 1.51	506.4 ± 114.8
	II	89.97 ± 16.45	3.78 ± 3.89	8.10 ± 1.17	432.4 ± 109.5
Schisantherin A	I	82.02 ± 20.08	1.72 ± 1.18	12.60 ± 4.39	977.2 ± 302.1
	II	84.22 ± 20.42	1.72 ± 1.18	12.82 ± 2.45	932.1 ± 345.6
Schizandrin A	I	43.37 ± 21.15	3.89 ± 3.75	5.77 ± 1.38	243.8 ± 66.1
	II	50.50 ± 18.16	3.89 ± 3.75	4.95 ± 2.92	232.7 ± 50.1
Schizandrin B	I	44.80 ± 33.32	3.89 ± 3.75	6.16 ± 3.65	237.1 ± 79.6
	II	46.43 ± 26.35	3.89 ± 3.75	5.05 ± 2.16	231.7 ± 110.8

I: LC–IT-TOF/MS; II: LC–Q/MS.

Table 5
Pharmacokinetic parameters of *Schisandra* lignans after oral dosing SLE at 500 mg/kg.

No.	C_{max} (μg/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC_{0-t} (μg h/ml)	REP_{AUC} (%)
3	340.14 ± 195.38	3.00 ± 1.00	2.75 ± 1.65	2226.8 ± 762.1	95.57
5	354.84 ± 341.96	3.00 ± 0.00	2.88 ± 1.13	2061.7 ± 961.1	88.49
6	22.56 ± 3.02	2.67 ± 0.58	2.23 ± 0.71	155.7 ± 22.6	6.68
7	167.77 ± 40.05	6.33 ± 2.89	8.25 ± 4.60	2329.9 ± 446.1	100.00
9	38.90 ± 40.05	4.00 ± 3.61	6.27 ± 2.47	249.1 ± 90.1	10.69
10	7.26 ± 0.82	3.78 ± 3.89	7.30 ± 1.51	43.7 ± 9.8	1.88
11	12.12 ± 1.78	3.89 ± 3.75	7.86 ± 1.61	91.0 ± 24.6	3.91
12	9.32 ± 2.24	3.78 ± 3.89	4.73 ± 2.61	44.9 ± 18.6	1.93
13	4.52 ± 1.07	3.89 ± 3.75	6.55 ± 0.24	35.1 ± 11.9	1.51
14	17.12 ± 3.94	6.33 ± 2.89	8.03 ± 1.93	172.6 ± 36.8	7.41
16	40.61 ± 14.36	3.78 ± 3.89	5.62 ± 3.57	353.2 ± 21.4	15.16
18	46.16 ± 9.47	1.72 ± 1.18	9.22 ± 4.07	501.3 ± 249.6	21.52
21	48.93 ± 11.81	1.72 ± 1.18	12.60 ± 4.39	534.3 ± 182.7	22.93
22	66.79 ± 8.22	6.33 ± 2.89	17.21 ± 2.22	943.9 ± 222.7	40.51
23	36.81 ± 28.90	6.67 ± 2.31	4.06 ± 1.18	277.6 ± 106.0	11.92
24	116.35 ± 35.88	4.00 ± 3.61	5.27 ± 3.46	1078.5 ± 186.2	46.29
25	130.17 ± 31.02	3.89 ± 3.75	5.62 ± 5.28	1540.8 ± 142.8	66.13
26	72.08 ± 35.50	6.33 ± 2.89	6.59 ± 3.63	599.3 ± 47.5	25.72
29	22.47 ± 7.98	3.89 ± 3.75	5.77 ± 1.38	113.7 ± 29.1	4.88
30	3.06 ± 0.63	3.89 ± 3.75	13.28 ± 0.43	17.4 ± 2.9	0.75
31	13.73 ± 9.80	3.89 ± 3.75	6.16 ± 3.65	64.9 ± 14.7	2.78

quantitation for the five lignans) or SLE (relative quantitation for all the 31 lignans).

3.4.1. LC–Q/MS and LC–IT-TOF/MS pharmacokinetic assays for five target lignans

To confirm that the new LC–IT-TOF/MS assay provided accurate measurement of the pharmacokinetics of components, the results obtained from the five target lignans with authentic standards were compared with those from the well-established LC–Q/MS. As clearly shown in Fig. 2, the plasma concentration versus time profiles of all the five lignans in both assays largely overlapped, suggesting identical pharmacokinetic profiles from both assays. A further correlation analysis of the plasma concentrations from all QC samples as well as all animal experiments resulted in a very good agreement between LC–Q/MS and LC–IT-TOF/MS assays (Fig. S4). All the important pharmacokinetic parameters including C_{max}, T_{max}, $t_{1/2}$ as well as the AUC values derived from the time profiles were also in very good agreement for all the compounds between two assays (Table 4).

3.4.2. Relative exposure approach (REA) to pharmacokinetic assays of lignans in rats

Compared with the typical LC–Q/MS assay, the prominent advantage of LC–IT-TOF/MS quantitation lies in the simultaneous determination of dozens of components without compromising sensitivity. Therefore, we sought to develop a LC–IT-TOF/MS assay based methodology for following the pharmacokinetics of all potential plasma components after herbal ingestions. One of the major bottlenecks for the pharmacokinetic studies of herbal medicines is that the authentic standards of many herbal components prove very difficult to obtain. Because of such a bottleneck, the previous studies of herbal pharmacokinetics were largely limited to very few components for which the authentic standards are readily accessible, and thus prevented deep insights into herbal pharmacokinetics. Therefore, it is very important to develop a generally applicable approach for assessing the herbal pharmacokinetics independent of authentic standards. In the present study, a REA to assessing the pharmacokinetics of all potential plasma components was carried out by a method of calculating the relative plasma concentrations of certain components from the corresponding calibration curves of dosed pharmaceutical preparations (SLE in this case) and expressed as "of C_{SLE}". For example, the determined C_{SLE} at 1.0 μg/ml for the plasma concentration of one component means that the actual plasma concentration of this component is equivalent to its content in 1.0 μg/ml SLE calibration samples. Based on the powerful LC–IT-TOF/MS assay and this approach, the plasma pharmacokinetic profiles of 21 out of a total of 31 lignans contained in SLE were successfully characterized (Fig. 3). Ten lignan components found in SLE were not detected in plasma after an intragastric administration of SLE, possibly because of their low contents in SLE and/or poor oral absorptions. From these plasma profiles, the relative pharmacokinetic parameters including AUC, C_{max}, T_{max}, and $t_{1/2}$ were calculated (Table 5). It is important to note that all these parameters are comparable among different compo-

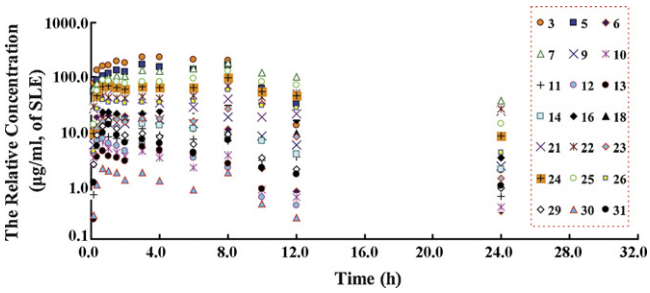

Fig. 3. The plasma pharmacokinetic profiles of 21 lignans determined by LC–IT-TOF/MS.

nents, and thus the presently developed REA provides as a very useful tool for evaluating the quantitative structure pharmacokinetics/metabolism relationships. As observed from this study, the relative oral exposures (AUC) of the 21 lignans varied up 0.75–100% folds, suggesting the structurally related different pharmacokinetic properties of lignans components. Although the relative pharmacokinetic profile of certain compound can be also created directly from the peak area ratios, it should be noted that such results obtained were incomparable among different components, because the mass responses for different components are completely different. Calibrated by the corresponding components contained in dosed preparation, the presently developed approach provides a comparable result among different components, which is similar to that calibrated by authentic standards.

To confirm the reliability of this approach, the obtained relative pharmacokinetic parameters including AUC and C_{max}, and the absolute parameters T_{max} and $t_{1/2}$ for the five lignans were compared with those obtained from the LC–Q/MS assay which was calibrated by the authentic standards. For comparison of the relative pharmacokinetic parameters, the component with the largest value in each of parameters was taken as the reference and assigned the relative value as 1, then; the relative values of other components were calculated and expressed as the percent of the reference. As clearly shown in Fig. 4, the REA based on LC–IT-TOF/MS assay provides almost identical results of all the pharmacokinetic parameters with the absolute exposure approach based on LC–Q/MS assay. These results indicated that, except for the absolute plasma concentrations of specific components, the presently developed REA provided necessary, sufficient, and reliable pharmacokinetic characteristics of all the followed components, and was especially useful for the rapid comparative pharmacokinetic study of dozens of components.

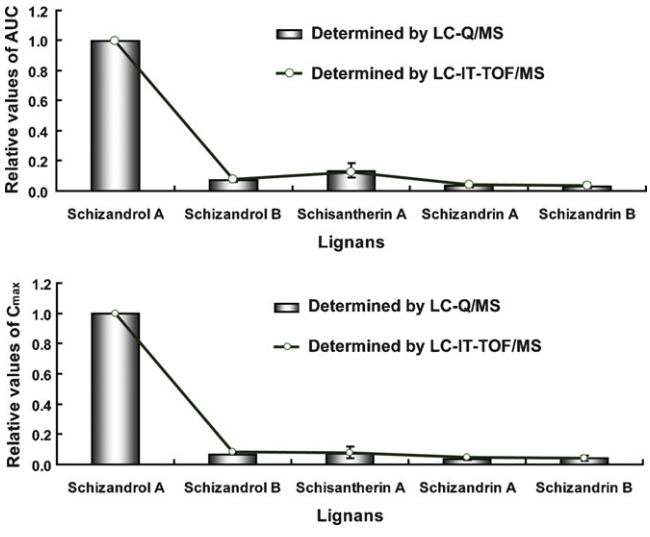

Fig. 4. The AUC and C_{max} values calculated by the REA based on LC–IT-TOF/MS assay and the absolute exposure approach based on LC–Q/MS assay.

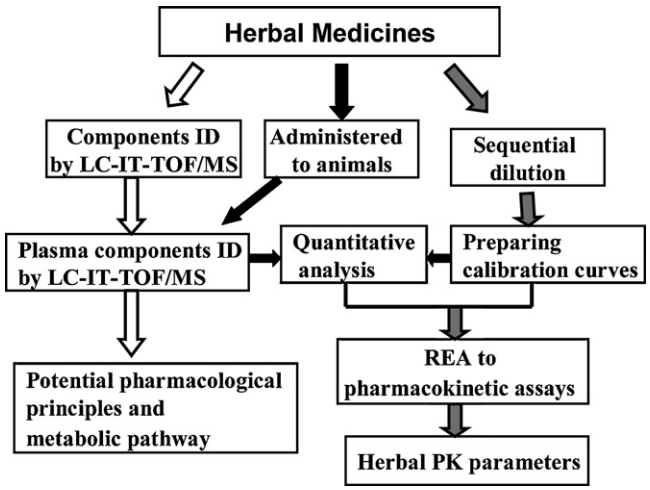

Fig. 5. Suggested herbal PK analysis platform and workflow based on LC–IT-TOF/MS.

4. Conclusion

The lacking of powerful analytical platform is still the major stumbling block for assessing the pharmacokinetics and dispositions of complicated herbal medicines. Besides the technical challenges, the lacking of authentic standards constitutes another great and longstanding obstacle on charactering herbal pharmacokinetics. To address this problem, we developed in this study a relative exposure approach based on calculating the relative plasma concentrations of each component from the corresponding calibration equation created from the calibration samples spiked with the dosed herbal preparations. Except for the absolute plasma concentrations, this approach was successful to obtain all the important pharmacokinetic parameters; and the results obtained were almost identical with those from a typical assay calibrated with authentic standards. Thus, the present study contributes to develop a systematic methodology on addressing the critical problems underlined in the field of herbal pharmacokinetics, including the qualitative and quantitative determinations of dozens of target and nontarget components, and the lacking of authentic compounds for verification and calibration, based on a single LC–IT-TOF/MS platform (Fig. 5).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.05.056.

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