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Pharmacokinetic Studies of Chinese Medicinal Herbs Using an Automated Blood Sampling System and Liquid Chromatography-mass Spectrometry

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Abstract

The safety of herbal products is one of the major concerns for the modernization of traditional Chinese medicine, and pharmacokinetic data of medicinal herbs guide us to design the rational use of the herbal formula. This article reviews the advantages of the automated blood sampling (ABS) systems for pharmacokinetic studies. In addition, three commonly used sample preparative methods, protein precipitation, liquid-liquid extraction and solid-phase extraction, are introduced. Furthermore, the definition, causes and evaluation of matrix effects in liquid chromatography-mass spectrometry (LC/MS) analysis are demonstrated. Finally, we present our previous works as practical examples of the application of ABS systems and LC/MS for the pharmacokinetic studies of Chinese medicinal herbs.

Key words: Chinese medicinal herbs, Pharmacokinetics, Automated blood sampling systems, Liquid chromatography-mass spectrometry

Introduction

Chinese medicinal herbs have been used for treating ailments in China and Asia for several centuries, and are becoming more and more popular worldwide. The global market for phytopharmaceutical usage has been estimated at 60 billion US dollars in 2003, with an annual growth rate of at least 5% (Timmermans, 2003). People generally believe that medicinal herbs and botanical products have fewer adverse effects and are much safer than synthetic drugs. However, evidence-based verification of the safety and efficacy of herbal medicinal products is still not comprehensive enough (Bhattaram et al., 2002). The safety issue of herbal medicinal products is becoming more widely recognized due to the increasing cases of adverse drug reactions and herb-drug pharmacokinetic interactions (Zeng and Jiang, 2010). In addition, a better understanding of the bioavailability and pharmacokinetics of herbal medicinal products will be helpful in determining reasonable dosage regimens. For these reasons, more effort has been put into the bioavailability and pharmacokinetics investigations of medicinal herbs.

To examine the absorption, distribution, metabolism, and excretion of medicinal herbs and herbal products, we have to perform a serial blood sampling in rodents or other experimental animals to obtain the time-concentration profile of the test compound, and the selection of the suitable bleeding method for pharmacokinetic evaluations relies on the study aim and physicochemical properties of compounds. Though

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the stress caused by anesthetics and restraint on the physiological status of experimental animals and drug metabolism have been proved (Mahl et al., 2000; Tabata et al., 1998; Vachon and Moreau, 2001), this issue is usually ignored when conducting pharmacokinetic evaluations. Another concern is the analytical method sensitivity, because the concentrations of the major herbal compounds in biological samples (e.g., plasma, bile, urine and tissue) usually fall within or lower than the microgram per liter range. Therefore, the analytical method requires sufficient sensitivity to acquire accurate pharmacokinetic results. High-performance liquid chromatography coupled to mass spectrometry (LC/MS) offering high selectivity and sensitivity has proven it the most powerful analytical tool for pharmacokinetics and drug discovery (Korfmacher, 2005; Papac and Shahrokh, 2001; Tolonen et al., 2009).

In this article, we discussed two important technologies, the automated blood sampling (ABS) system and LC/MS, for the pharmacokinetic applications of Chinese medicinal herbs. A previous report had compared the impacts of tail-bleeding, cannula and retro-orbital bleeding on the pharmacokinetics of six marketed drugs, and the authors suggested that cannulation with the ABS system could become the most efficient way for pharmaceutical industry to perform rat bioavailability, pharmacokinetic and toxicokinetic studies (Hui et al., 2007). In addition, we introduced the frequently used methods of sample preparation. Furthermore, we reviewed the issue of matrix effects of LC/MS analysis of biological samples. Finally, pharmacokinetics of polyphenolic compounds from three Chinese medicinal herbs, including green tea (綠茶 lǜ chá), turmeric (薑黃 jiāng huáng) and Cistanches Herba (肉蓯蓉 ròu cōng róng), were summarized and discussed.

Blood sampling for pharmacokinetic studies

Blood can be obtained from animals using different bleeding techniques, such as the retro-orbital plexus sampling, tail tip amputation and jugular vein cannulation, which lead to various impacts on the animal's physiological conditions because of the use of anesthetics and restraining of the animal. Isoflurane and pentobarbital have been reported to increase the stress of animals as indicated by elevated plasma glucose and corticosterone concentrations (Mahl et al., 2000; Tabata et al., 1998; Vachon and Moreau, 2001). A suitable restrainer (e.g. a plastic tube) is used to immobilize conscious rodents for blood sampling to eliminate the influence of anesthetics, but this way is also very stressful to these animals, affecting animals' physiology, biochemistry, metabolism and protein expression (Balcombe et al., 2004; Menguy, 1960; Xie et al., 2003). To lower the stress and effects of stress-related hormones, it is needed to improve experimental procedures to lessen the stress caused by handling, restraint and sampling (Royo et al., 2004).

ABS systems have been proposed for pharmacokinetic studies of *trans*-resveratrol (Zhu et al., 2000), epigallocatechin gallate (Long et al., 2001), daphnoretin (Lin et al., 2005) and plumbagin (Hsieh et al., 2006) in rodents to minimize the stress due to the restrain and anesthesia. Generally, the ABS system consists of a robotic sampling module and an animal cage. The module equips with a syringe pump, a special 5-way joint, 4 pinch valves, and optical sensors and is controlled by a computer to withdraw sample from the jugular vein catheter of the animal. During the sampling process, blood is sandwiched by air gaps in order to decrease diffusion, clean the inner surface of the tubing and eliminate any cross contamination. Collected samples are placed in standard 1.5 mL centrifuge tubes, covered by airtight silicon cap and stored at a refrigerated fraction collector. A cross-over study had been designed to compare the effects of manual and ABS methods on the pharmacokinetic parameters of carbamazepine (Zhu et al., 2005). They found a significant difference of the concentration ratio of carbamazepine and its metabolite from the two sampling techniques. The authors concluded that the physical stress influenced blood flow and distribution impacting drug absorption, distribution and metabolism, resulting in the differences of pharmacokinetic parameters obtained by the two techniques. One drawback of ABS is that the catheter implantation of the rats clearly causes an initial stress response, but the animals usually recover quickly and gain a cyclical diurnal rhythmicity 18 h after surgery (Royo et al., 2004). Therefore, the ABS still provides a major advantage by allowing free movement, which can minimize the stress caused by the restraint or anesthesia.

Sample preparation

Sample preparation must be done before LC/MS analysis to improve the performance of assay. The goal of sample preparation is to selectively separate the analyte from the matrix, which diminishes interference

and thus increases sensitivity. Specific considerations include the removal of protein or non-protein material that may deteriorate column, the elimination of nonvolatile salts that suppress ionization, and the concentration of trace amounts of analyte (Chien et al., 2011; Dams et al., 2003). The common processes of sample preparation discussed in this section include protein precipitation (PPT), liquid-liquid extraction (LLE) and solid-phase extraction (SPE).

Protein precipitation

PPT of plasma sample by acetonitrile or methanol is a simple and quick method for biological sample preparation, because this method requires low cost and minimal method development procedures. Recently, the integration of robotic liquid handling module with peripheral devices realized automated PPT steps, such as sequential completion of the centrifugation, plate sealing, piercing and supernatant transferring, and reduced manual labor and reduced possibility of systematic error due to the manual volumetric transfers during sample preparation (Ma et al., 2008). The major drawback of PPT is the more severe matrix effect (signal suppression) when using LC- MS/MS, because PPT is a non-selective sample pretreatment and the cleanup of the bio-fluid is limited (Bonfiglio et al., 1999; Dams et al., 2003).

Liquid-liquid extraction

LLE utilizes the different solubilities of a compound in two different immiscible solvents to separate the compound from biological matrix. The manipulations of pH value of plasma and different extraction solvents can greatly enhance extraction recovery and increase the selectivity of method. Solvent-induced phase transition (SIPTE) is a combinational method using PPT and LLE for sample preparation, and has been proposed for the preparation of andrographolide, a hydrophobic major constituent from Andrographis Herba (穿心蓮 chuān xīn lián), in human plasma before introducing them to the LC/MS system (Liu et al., 2010). Briefly, a hydrophobic solvent like chloroform is added into the mixture of acetonitrile and plasma, and the upper chloroform layer is collected for analysis. The samples processed by SIPTE can be directly injected into the system because nonvolatile materials have been excluded by the chloroform, providing an alternative method for biological sample pretreatment.

Solid-phase extraction

SPE is often recognized as the most effective method for sample preparation. A SPE cartridge can be regarded as a miniaturized chromatographic column, where analyte and matrix are retained on the surfaces of the solid packing material and are then eluted separately by manipulating different "mobile phases". The selection of SPE sorbents, such as normal phase, reversed phase, ion exchange type and mixed model, usually depends on the physicochemical properties of analytes (Hennion, 1999). The SPE method has been used to extract saponins of Ginseng Radix (人參 rén shēn), including ginsenoside Rg1, its secondary glycoside Rh1 and aglycone protopanaxatriol, in a pharmacokinetic study (Sun et al., 2005). The analytical sensitivity could be further improved by a 10-fold concentrating the eluate of SPE by sample evaporation and reconstitution.

The issue of matrix effects in LC/MS

Interference peaks in biological samples are easily observed when using HPLC coupled to ultraviolet, fluorescence and electrochemical detectors. These visible interference peaks can be removed by altering the chromatographic conditions, sample extraction method or detector. With the introduction of highly sensitive and selective LC-MS-based bioanalytical methods, sample preparation can usually be simplified to speed up the throughput of data. However, although those endogenous compounds in biological matrices are indiscernible in the chromatogram, they may induce matrix effects (ME). ME can be defined as a phenomenon observed when the signal of an analyte is either suppressed or enhanced because of the co-eluted components that come from the sample matrix and influence the ionization efficiency. The issue of ME had been noticed by the late 1990s (Matuszewski et al., 1998), and has now become an imperative item for analytical validation (Chambers et al., 2007; O'Halloran and Ilett, 2008; Van De Steene and Lambert, 2008). As described by guidelines of the U.S. Food and Drug Administration, ME must be investigated to ensure that accuracy, precision, selectivity, and robustness in the HPLC-MS-based procedures, so that sensitivity will not be compromised (FDA, 2001).

ME are recognized to be both analyte- and matrix-dependent, and exogenous compounds such as excipients, or the vehicle used for drug administration and mobile phase modifiers are all possible factors that can lead to ME (Schuhmacher et al., 2003). Though the detailed mechanism of ME is not fully understood, it is believed that ion suppression is a result of the

ionization competition between analytes and co-eluted components (Matuszewski et al., 1998, 2003; Taylor, 2005). One approach to the identification of ME is the method of post-column infusion (Bonfiglio et al., 1999). In brief, the analyte of interest is infused constantly into the ion source of a mass spectrometer to produce a steady signal. The blank matrix after sample preparation (e.g. PPT, LLE and SPE) is then injected into the HPLC/MS system, which helps to identify the region in the chromatogram suppressed or enhanced by the components of the matrix. However, this method cannot quantitatively determine the extent of ME. Another approach to quantitatively assess ME is the post-extraction fortification method (Matuszewski et al., 2003). In brief, two sets of the samples are prepared to evaluate the matrix effect and recovery in the quantitative bioanalytical method. Set 1 is a neat solution (analyte only) prepared in the mobile phase, and set 2 is the analyte fortified with plasma that has been extracted. The value of ME is expressed as the ratio of the response of set 2 to the response of set 1. A value of > 100 % indicates ionization enhancement, and a value of < 100 % indicates ionization suppression. The other recently proposed method for assessment of ME is to monitor phospholipids by a mass transition *m/z* 184 > 184 in the case of hydrocodone and pseudoephedrine determinations in human plasma (Ismaiel et al., 2007). Post-column infusion studies have indicated that the ion suppression for analytes correlates with the elution of phospholipids, and therefore chromatographic conditions were optimized to resolve the peaks of interest from the phospholipids. The authors concluded that this is useful in method development and in routine analysis to avoid the ME (Ismaiel et al., 2007). Generally, four aspects can be considered to reduce ME. The first way is to change the methods of sample preparation. PPT is likely to cause more ME than is produced by LLE or SPE, but the latter two methods require more time-consuming and tedious procedures (Dams et al., 2003). Secondly, ME can be avoided by adjusting chromatographic parameters. Many papers have pointed out that the appropriate chromatography is necessary to reduce ME (Chambers et al., 2007; Kloepfer et al., 2005; Niessen et al., 2006), and as mentioned above, using columns with different retention mechanisms (e.g. HILIC for polar compounds) may be of great help. Thirdly, trying different ionization interfaces such as atmospheric pressure chemical ionization and atmospheric pressure photoionization is also effective. Finally, the use of a stable-isotope-labeled compound as the internal standard can compensate for ME because the ion currents of the analyte and the internal standard are both affected by the matrix effect in a similar manner (Haubl et al., 2006; Schuhmacher et al., 2003).

Pharmacokinetics of polyphenolic components from Chinese medicinal herbs

(-)-epigallocatechin-3-gallate from green tea

Green tea is a species of *Camellia sinensis*, an evergreen shrub belonging to Theaceae family, and the medicinal use of green tea can be dated back to thousands of years ago (Liu et al., 2008). Green tea has been demonstrated to have various biological activities, such as anti-oxidation (Serafini et al., 1996), anti-inflammation (Chan et al., 1998), cancer prevention (Nakachi et al., 2000) and increasing energy expenditure (Dulloo et al., 1999). We have developed a liquid chromatography with tandem mass spectrometry (LC-MS/MS) and applied ABS to investigate the pharmacokinetics of (-)-epigallocatechin-3-gallate (EGCG, Figure 1.), one of the major polyphenols contained in green tea, by a conscious and freely moving rat model (Lin et al., 2007). The precursor ion [M-H]- of EGCG (*m/z* = 457) was generated by a negative ionization mode, and the transition of the precursor ion to the product ion (*m/z* = 169) for EGCG was monitored by the multiple-reaction monitoring (MRM) used for quantification. The internal standard (theophylline) was monitored using the MRM mode at *m/z* transitions of 187 to 164. The limit of quantification (LOQ) of EGCG in rat plasma was 5 ng/mL, which was sensitive enough to determine EGCG after oral administration. We used a two-compartmental model to

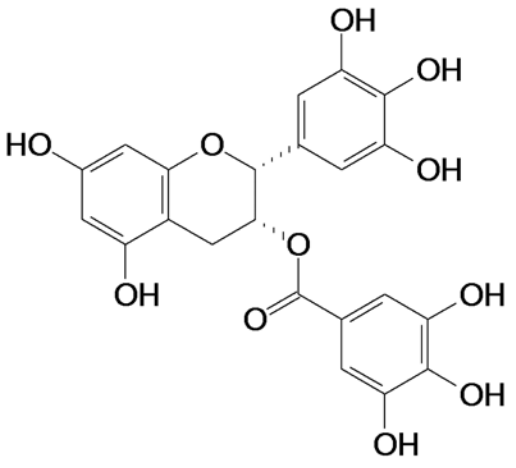

Figure 1. The chemical structure of (-)-epigallocatechin-3-gallate.

Table 1. Pharmacokinetics of (-)-epigallocatechin gallate (EGCG) obtained by different sampling methods in rodents

Animal/ blood sampling site	Dose (mg/kg)/ Administration route	Pharmacokinetic parameters	Analytical method	References
Sprague–Dawley rats/ from retro-orbital plexus	10 mg/kg, IV; 75 mg/kg, PO	PO group: C_{max} = 19.8 ± 3.5 ng/mL T_{max} = 85.5 ± 42.0 min $t_{1/2}$ = 16.8 ± 9.3 min AUC = 17.4 ± 7.0 μg min/mL BA = 1.6 ± 0.6 %	LC-EC, Applied potential: -10 mV LOQ: 6 ng/mL	(Chen et al., 1997)
Sprague–Dawley rats/ from jugular vein catheter by ABS	1 mg/kg, IV	$t_{1/2}$ = 1.55 ± 0.14 min AUC = 5147 ± 34 ng min/mL	LC-EC, Applied potential: +600 mV (vs. Ag/AgCl) LOQ: 5 ng/mL	(Long et al., 2001)
Sprague–Dawley rats/ from jugular vein catheter by ABS	10 mg/kg, IV; 100 mg/kg, PO	PO group: C_{max} = 1.52 ± 0.11 mg /mL T_{max} = 24 ± 7 min $t_{1/2}$ = 48 ± 13 min AUC = 80 ± 16 μg min/mL BA = 4.95 %	LC/MS, MRM: *m/z* 457→169 LOQ: 5 ng/mL	(Lin et al., 2007)
Sprague–Dawley rats/ from retro-orbital plexus	63.84 mg/kg, IV	$t_{1/2}$ = 68.6 min	LC-UV, Wavelength: 280 nm, LOD: 0.5 μg/mL	(Fu et al., 2008)
Wistar rats/ from retro-orbital plexus	101.76 mg/kg, PO	Cmax = 55.29 ± 1.70 ng/mL Tmax = 1 h $t_{1/2}$ = 2.04 ± 0.2 h AUC =510.16 ± 9.88 ng h/mL	TLC-UV, LOQ: not mentioned	(Kale et al., 2010)

IV, intravenous; PO, per os; Cmax, maximum drug concentration; T_{max}, time to reach peak concentration; $t_{1/2}$, elimination half-life; AUC, area under the concentration versus time curve; BA, oral bioavailability; LC-EC, liquid chromatography with electrochemical detection; LOQ, limit of quantification; LC/MS, liquid chromatography with mass spectrometric detection; MRM, multiple-reaction monitoring; LC-UV, liquid chromatography with ultraviolet detection; LOD, limit of detection; TLC-UV, thin-layer chromatography with ultraviolet detection.

describe the pharmacokinetics of EGCG in the rat blood after intravenous administration (10 mg/kg, iv). The average oral bioavailability of EGCG obtained in the work was 4.95%, which was higher than another study obtained by blood sampling from retro-orbital plexus (Chen et al., 1997). The pharmacokinetics of EGCG obtained by different sampling methods in rodents was summarized in Table 1.

Curcumin from turmeric

Turmeric is a culinary spice derived from the root *Curcuma longa*, a member of the ginger family, Zingiberaceae. Turmeric is broadly used in south and southeast tropical Asia as a culinary spice and a remedy against skin diseases and colic inflammatory disorders in folk medicine (Villegas et al., 2008). Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione, Figure 2.] is a natural polyphenolic compound derived from the rhizomes of turmeric, and curcumin possesses antioxidant, anti-inflammatory and potential cancer chemopreventive activities (Ammon and Wahl, 1991; Conney et al., 1997; Sharma, 1976). Our previous study have presented a LC/MS method and an ABS system to investigate the pharmacokinetics of curcumin by a conscious and freely moving rat model (Yang et al., 2007). The precursor ion [M-H]- of curcumin (*m/z* = 367) was generated by a negative ionization mode, and the transition of the precursor ion to the product ion (*m/z* = 217) for curcumin was monitored by the MRM used for quantification. The internal standard (honokiol) was monitored using the MRM mode at *m/z* transitions of 265 to 224. The limit of detection and LOQ of curcumin in the rat plasma were 1 and 5 ng/ml, respectively. We used a non-compartmental model to describe the pharmacokinetics of curcumin in the rat blood after intravenous administration (10 mg/kg, iv). The average oral bioavailability of curcumin obtained in the work was 1 %. The pharmacokinetics of curcumin obtained by different sampling methods in rodents was summarized in Table 2.

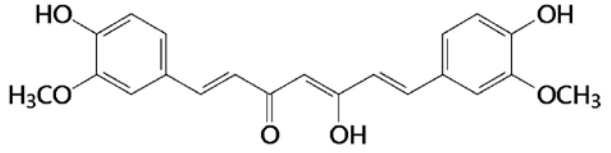

Figure 2. The chemical structure of curcumin.

Acteoside from Cistanches Herba

Cistanches Herba is the dried succulent stems of the Cistanche plants, and has been known as a superior tonic used for the treatment of impotence, female infertility, and senile constipation (Jiang and Tu, 2009). The

Table 2. Pharmacokinetics of curcumin obtained by different sampling methods in rodents

Animal/ blood sampling method	Dose (mg/kg)/ Administration route	Pharmacokinetic parameters	Analytical method	References
Sprague–Dawley rats/ not mentioned	100 mg/kg, PO	C_{max} = 266.7 ng/mL T_{max} = 1.62 h $t_{1/2}$ = 5.7 h AUC = 2609.04 ng/mL h	LC /MS, MRM: *m/z* 373.2→137.1 LOQ: 0.5 ng/mL	(Liu et al., 2006)
Sprague–Dawley rats/ from jugular vein catheter under halothane anesthesia	10 mg/kg, IV	$t_{1/2}$ = 0.57 ± 0.19 h AUC = 1.67 ± 0.52 mg h/mL	LC-UV, Wavelength: 428 nm LOQ: 20 ng/mL	(Ma et al., 2007)
Wistar rats/ from jugular vein under ether anesthesia	1 g/kg, PO	C_{max} = 0.50 μg/mL T_{max} = 0.75 h $t_{1/2}$ = 1.45 h	LC-UV, Wavelength: 425 nm, LOD: 25 ng/mL	(Maiti et al., 2007)
Sprague–Dawley rats/ from jugular vein catheter by ABS	10 mg/kg, IV 500 mg/kg, PO	PO group: C_{max} = 0.06 ± 0.01 μg/mL T_{max} = 41.7 ± 5.4 min $t_{1/2}$ = 44.5 ± 7.5 min BA = 1 %	LC /MS, MRM: *m/z* 367→217 LOQ: 2.5 ng/mL	(Yang et al., 2007)
Wistar rats/ from retro-orbital plexus	1 g/kg, PO	C_{max} = 258.64 ng/mL T_{max} = 1.72 h $t_{1/2}$ = 1.28 h AUC = 2483.32 ng/mL h	Not mentioned	(Gupta and Dixit, 2011)

PO, per os; Cmax, maximum drug concentration; T_{max}, time to reach peak concentration; $t_{1/2}$, elimination half-life; AUC, area under the concentration versus time curve; LC/MS, liquid chromatography with mass spectrometric detection; MRM, multiple-reaction monitoring; LOQ, limit of quantification; IV, intravenous; LC-UV, liquid chromatography with ultraviolet detection; LOD, limit of detection; BA, oral bioavailability.

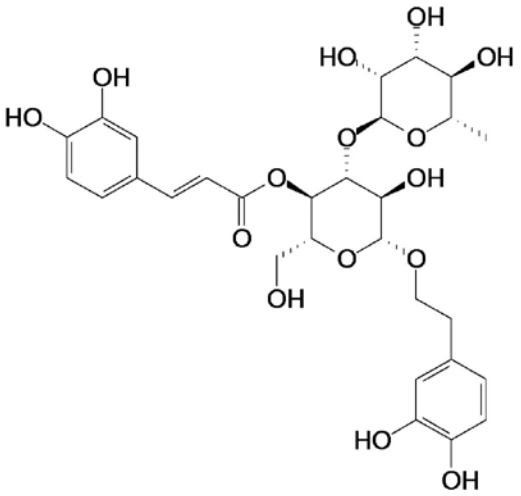

Figure 3. The chemical structure of acteoside.

major components of cistanche include phenylethanoid glycosides (PhGs), iridoids, lignans, alditols, oligosaccharides and polysaccharides. Acteoside (Figure 3) is one of the PhGs found in cistanche (Jiang and Tu, 2009). In order to know the pharmacokinetics of acteoside after intravenous administration and the bioavailability after oral administration, we applied an ABS system for blood sampling from freely moving rats to avoid anesthesia or restraining stress (Wu et al., 2006). A catheter placed in the jugular vein was exteriorized, fixed in the dorsal neck region and connected to the ABS. A 150-μL blood was collected after acteoside administration, and the resulting plasma was prepared by a solid-phase extraction process. Acteoside concentrations were determined by a LC/MS based on the MRM (*m/z* 623 to 161). In this study, the ABS system eliminated the extensive animal handling and restraint associated with manual blood sampling. Actually, a rat in this system could wander in a cage and access food and water freely. When blood sampling was conducted, no interference affects their normal activities. This sensitive LC/MS method can achieve very low quantification limit (5 ng/mL) of acteoside in plasma, and accurate and detailed pharmacokinetic profile of acteoside can be acquired. The oral bioavailability (BA) of acteoside was 0.12 %., and the low BA can be assorted to its phenolic structure, which is susceptible to first-pass effects in the intestinal tract (Donovan et al., 2001; Scalbert and Williamson, 2000).

Conclusions

This article reviews the development of blood sampling methods for preclinical pharmacokinetic investigations and critical issues for bioanalysis using LC/MS. ABS systems have several advantages when compared with the conventional manual blood sampling technique, such as reduced animal use and reduced animal stress during blood collection. LC/MS is a sensitive analytical tool to determine trace amount of analytes in plasma, which is beneficial for researchers to observe the detailed pharmacokinetic profiles of drugs. The safety of herbal products is one of the important issues for the modernization of traditional Chinese

medicine, because people have been aware of potential adverse drug reactions due to inappropriate use of herbs or herb-drug interactions. Pharmacokinetic studies help us to understand the absorption, distribution, metabolism and elimination of medicinal herbs, which is very useful to design the rational and optimal dose regimen of the herbal formula.

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