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Short communication

Simultaneous determination of senecionine, adonifoline and their metabolites in rat serum by UPLC–ESIMS and its application in pharmacokinetic studies

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ABSTRACT

A rapid, selective and sensitive ultra-performance liquid chromatography–electrospray ionization mass spectrometry (UPLC–ESIMS) method was firstly developed and validated for the simultaneous determination of two hepatotoxic pyrrolizidine alkaloids (HPAs), senecionine (SEN), adonifoline (ADO), and their *N*-oxides (SENNOX and ADONOX), the main metabolites in rat serum. The whole analysis was achieved within 4.5 min by gradient elution on an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm, i.d. 1.7 μm) following a solid phase extraction for serum samples. Good linearity was achieved using weighted ($1/x^2$) least squares linear regression over a 1600-fold dynamic range for SEN and ADO (LLOQ was about 0.006 μg/ml) and 800-fold dynamic range for SENNOX and ADONOX (LLOQ was about 0.012 μg/ml). The R.S.D. of intra- and inter-day precision was below 4.91% and 11.15% respectively, while the R.E. of accuracy was within 4.52%, 6.81%, 2.69%, and 7.12% for SEN, SENNOX, ADO, and ADONOX, respectively. The developed method was successfully applied to the *in vivo* pharmacokinetic study in rats after intravenous administration of SEN and ADO.

1. Introduction

Strict limits on consumption of hepatotoxic pyrrolizidine alkaloids (HPAs) and their *N*-oxides have been set in several countries and areas [1–3] since 1989, in which World Health Orgnization (WHO) issued the Health and Safety Guide No. 26 [3]. Adonifoline (ADO) was found to the main HPAs in *Senecio scandens* Buch.-Ham., a traditionally used Chinese material herb for the treatment of oral and pharyngeal infections. Senecionine (SEN), one of the most studied HPAs, was also reported to be present in *S. scandens*, LD_{50} of ADO and SEN was 153 and 57 mg/kg (mice, p.o., unreported data), respectively. Metabolism researches of HPAs revealed *N*-oxides to be the main metabolites and shown a hepatotoxicity similar to that of the parent alkaloids due to reduction in the alimentary tract prior to absorption [4–6].

Several analytical methods had been developed and published for the quantitation of PAs and their metabolites, including spectrophotometric methods, gas chromatography–mass spectrometry (GC–MS), liquid chromatography (LC). Recently, LC–MS/MS method [7] was used for its high sensitivity and selectivity. But the long analysis time (>20 min) may not meet the requirement for high throughput and speed in biological specimen analysis of pharmacokinetic (PK) study.

Ultra-performance liquid chromatography (UPLC) is famous for its high power in separation and analysis speed over traditional HPLC [8,9]. In present study, we have developed an improved, rapid, selective and sensitive UPLC–ESIMS analytical method for the simultaneous determination of SEN, ADO, and their *N*-oxides in rat serum, using monocrotaline (MONO) as an internal standard (IS) (see structures in Fig. 1). With a comparative sensitivity with those reported (LOQ on-column was 10 pg), the chromatographic run time per sample was 4.5 min, which was only fifth of that reported [7]. The method was fully validated and applied to the *in vivo* PK study in rats after intravenous administration of SEN (1.5 mg/kg) and ADO (4.0 mg/kg).

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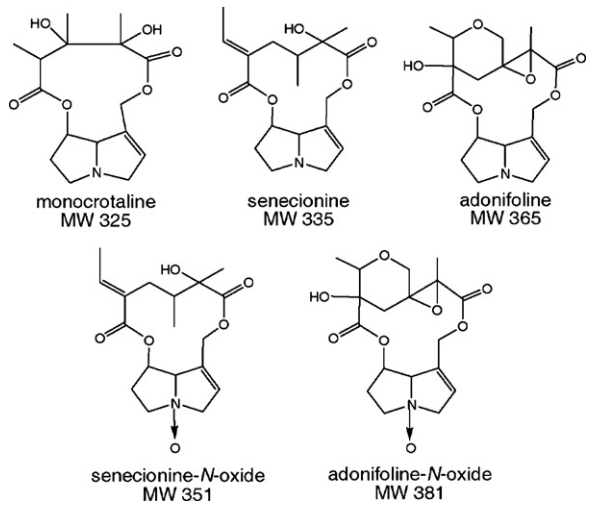

Fig. 1. Structures of MONO, SEN, ADO, SENNOX, and ADONOX.

2. Experimental

2.1. Chemicals and reagents

MONO was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). SEN and ADO were isolated from *S. vulgaris* L. (Jilin, P.R. China) and *S. scandens* (Guizhou, P.R. China), respectively. Senecionine-*N*-oxide (SENNOX) and adonifoline-*N*-oxide (ADONOX) were obtained by oxidation of SEN and ADO, respectively, following the reported method [10]. Their structures were elucidated by comparing their spectral data (UV, IR, MS, ^{1}H NMR and ^{13}C NMR) with references. And purities of them were determined to be more than 98% by normalization of the peak areas detected by HPLC–DAD as well as HPLC–MS. Acetonitrile and methanol of HPLC grade were purchased from Fisher Scientific Co. (Santa Clara, USA). 96% Formic acid of HPLC grade was purchased from Tedia Co. (Fairfield, USA). Potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH), perchloric acid ($HClO_4$), and 25% ammonia solution were of analytical grade and purchased from Shanghai Reagent Co. (Shanghai, P.R. China). Double-distilled water was used in all experiments.

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

The separation was performed on a Waters-ACQUITY^{TM} UPLC system (Waters Corp., Milford, MA, USA) using an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm, i.d. 1.7 μm) maintained at 45 °C. The mobile phase consisted of A (acetonitrile) and B (aqueous 0.1% formic acid) at a flow rate of 0.3 ml/min, using a gradient elution: 0–1 (5% A), 1–2.5 (5–17% A), 2.5–3 (17–20% A), 3–4 (20% A), and 4–5.5 min (80% A). The injection volume was 2 μl with partial loop mode.

2.2.2. Mass spectrometric conditions

A quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) interface was used for analytic detection. The mass spectrometer was operated in positive ionization mode using selective ion monitoring (SIM) of *m*/*z* 326.3 (MONO), *m*/*z* 366.3 (ADO), *m*/*z* 382.3 (ADONOX), *m*/*z* 336.3 (SEN), and *m*/*z* 352.3 (SENNOX). The tuning parameters were optimized and set as follows: capillary voltage: 4.2 kV; cone voltage: 45 V; source temperature: 120 °C; desolvation temperature: 300 °C; desolvation gas (nitrogen): 600 L/h; cone gas (nitrogen): 60 L/h. All data were acquired in centroid mode and processed using MassLynx ^{TM} 4.1 software with QuanLynx ^{TM} program (Waters Corp., Milford, MA, USA).

2.3. Stock solutions, standards and quality control samples

Stock solutions of SEN, SENNOX, ADO, and ADONOX with a concentration of 139, 146, 146, and 155 μg/ml were prepared by dissolving proper amount of each standard substance in 10 ml of methanol, respectively. A mixture solution contained these four standards was obtained and serially diluted with the initial mobile phase to provide working solutions of desired concentrations for standard curve and quality control (QC). An IS working solution (0.259 μg/ml) was also prepared by dissolving proper amount of MONO in methanol. Samples for standard curve and QC were prepared with working solutions following a solid phase extraction (SPE) procedure before processing of each analytical batch, and processed together with the biological specimen.

2.4. Sample preparation

IS (100 μl) was added to serum (100 μl) and mixed well before deproteinized with 100 μl of 6% $HClO_4$ (v/v). Then 300 μl of KH_2PO_4–KOH (1 M; pH 8.1) was added. The mixture was vortex-mixed and centrifuged at 10,000 × *g* for 10 min. The supernatant (500 μl) was applied to a C_{18} solid phase extraction (SPE) cartridge (Supelclean LC-18, 1 ml; Supelco Co. Lot) which had been conditioned with 2 ml of methanol followed by 2 ml of water. Each cartridge was then washed with 0.5 ml of water and 1.5 ml of 1% ammoniated methanol (v/v). The later was evaporated to dryness under a flow of nitrogen in a heating block at 40 °C. The residue was dissolved by 200 μl of initial mobile phase and centrifuged at 20,000 × *g* for 15 min. The supernatants were applied to the UPLC–ESIMS analysis.

2.5. Method validation

2.5.1. Selectivity

The selectivity was evaluated by comparing the SIM chromatograms of blank serum with IS-spiked serum samples after intravenous administration of SEN and ADO, respectively.

2.5.2. Linearity and LLOQ

Ten calibration standards were prepared ranged from 0.005 to 10 μg/ml in triplicate on each validation run and the calibration curve plotted the peak area ratio (analyte/IS, *y*) versus the analyte concentration (*x*), using a least squares linear regression model with weighted ($1/x^2$). LLOQ is defined as the lowest concentration on the calibration curve with an acceptable accuracy (R.E., within ±20%) and precision (R.S.D., below 20%).

2.5.3. Precision and accuracy

Precision and accuracy were assessed by performing replicate analysis of QC samples ($n = 5$) at three levels (cal. 0.010, 0.100, and 1.00 μg/ml). The procedure was repeated on 5 different days for the serum matrix ($n = 5$) to determine inter-day precision values. The accuracy of the assay was expressed by recovery of QC samples at three levels. Amounts of SEN, SENNOX, ADO, and ADONOX were added into blank serum and extracted with the SPE procedure. Apparent concentrations of them were calculated by calibration curves. And recovery was determined as the ratio of the concentration measured versus the concentration added into the blank serum.

2.5.4. Matrix effect and stability

The matrix effect was examined by comparing the SIM peak response of each analyte (at three concentration levels of QC sam-

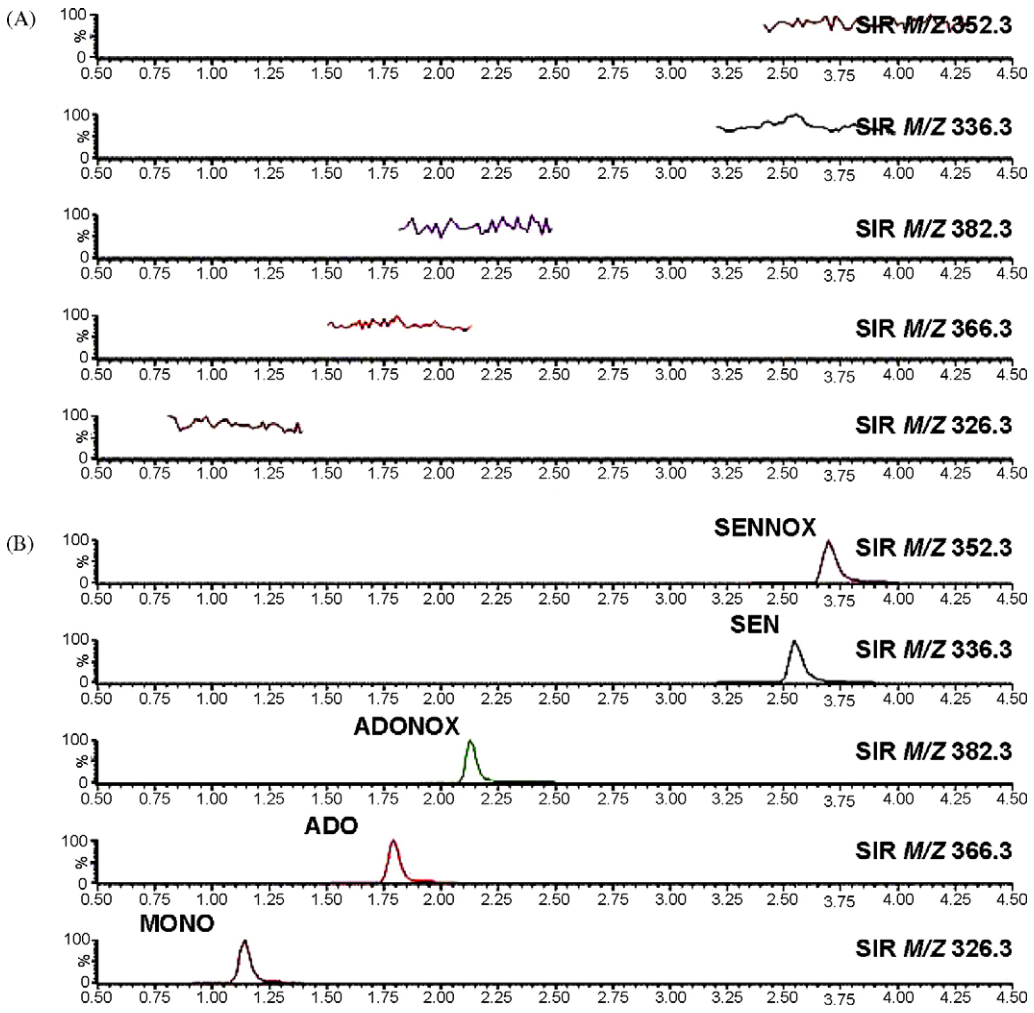

Fig. 2. Representative SIM chromatograms of (A) blank serum sample and (B) IS-spiked serum samples after intravenous administration of SEN (1.5 mg/kg) and ADO (4.0 mg/kg), respectively.

ples) or IS spiked into extracts originating from serum (A) to those of the same analyte presented in the neat mobile phase (B). The value A/B × 100% was considered as the matrix effect.

The stability of analytes in serum was assessed by analyzing replicate samples ($n = 3$) which were spiked with standards at low and high QC concentrations and stored for 2 h at ambient temperature, three freezing (−20 °C) and thawing cycles, and 20 days at −20 °C. Post-preparation stability was estimated by analysis QC samples stored at 4 °C in 3 days. Chemical stability of standards was also examined by re-injecting the standards mixture dissolved in the pure mobile phase and stored at 4 °C in 5 days at high and low concentrations.

2.6. Application to pharmacokinetic study

The PK study was approved by the local Ethics Committee of Shanghai University of Traditional Chinese Medicine. Male Sprague–Dawley rats ($n = 16$, 240–260 g) were randomly classified into two groups and fasted for 12 h with free access to water prior to be intravenous administrated of SEN (1.5 mg/kg) and ADO (4.0 mg/kg). Blood samples (each cal. 0.3 ml) were collected via a jugular vein catheter using a disposable syringe before and at 2, 5, 10, 20, 30, 45, 60, 90, 120, 240, 480, and 720 min after administration. Serum samples were obtained by centrifugation of blood at 3000 × *g* for 10 min and stored at −20 °C until analysis.

The PK parameters such as apparent volume of distribution (V_d), clearance rate (CL), and mean residence time (MRT) were analyzed by using the non-compartmental pharmacokinetics data analysis software of PK solutions 2^{TM} (Summit Research Services, USA). The maximum peak concentration (C_{max}) and time of maximum serum concentration (T_{max}) values were obtained directly from the observed concentration versus time data. The area under the serum concentration–time curve from zero to infinity ($AUC_{0-\infty}$) was calculated by means of the trapezoidal rule with extrapolation to infinity with terminal elimination rate constant (K_e).

3. Results and discussion

3.1. Sample preparation

The protein precipitation of serum was carried out by adding aqueous $HClO_4$. An aliquot of KH_2PO_4–KOH was added to precipitate the ClO_4^- thus to protect the MS detector and a C_{18} SPE process was employed. Ammoniated methanol, which was optimized for the concentration (0.1, 1%) and volume (l, 1.5 ml), was used as the elute solvent. Multiple samples ($n = 5$) containing certain quantity of IS (0.259 μg/ml), SEN (0.111 μg/ml), SENNOX (0.117 μg/ml), ADO (0.117 μg/ml) and ADONOX (0.124 μg/ml) were prepared following the method similar to that described in Section 2.4 with ammoniated methanol of different concentration and volume and

Table 1
LLOQ and representative calibration curves of standards in UPLC–ESIMS method.

Analyte	LLOQ (μg/ml)	Linear range (μg/ml)	Slope	Intercept	r^2
SEN	0.006	0.006–8.896	0.8804	1.6468	0.9912
SENNOX	0.012	0.012–9.344	0.0564	0.1269	0.9977
ADO	0.006	0.006–9.328	0.3575	0.7105	0.9923
ADONOX	0.012	0.012–9.920	0.0098	0.0703	0.9941

compared with those in neat mobile phase. And the ratio was defined as the accuracy while the relative variation of multiple samples was defined as the precision. It was found that washing with 1.5 ml of 1% ammoniated methanol was sufficient to achieve an acceptable accuracy (between 93.38% and 111.23%) and precision (not more than 7.59%) for all the analytes.

3.2. Method validation

3.2.1. Selectivity

The representative SIM chromatograms of blank serum and IS-spiked serum after intravenous administration of SEN and ADO, respectively, were shown in Fig. 2. No interference from endogenous substance was observed at the elution times for each analyte SIM channel.

3.2.2. Linearity and LLOQ

The slopes, intercepts, and correlation coefficients obtained for typical calibration curves of all analytes were shown Table 1. The LLOQ was 0.006, 0.012, 0.006, and 0.012 μg/ml for SEN, SENNOX, ADO, and ADONOX, respectively, with acceptable limits of accuracy and precision.

3.2.3. Precision and accuracy

The intra- and inter-day precision and accuracy of the method were summarized in Table 2. Overall, the intra-day precision (R.S.D.) were less than 4.91% and inter-day precision were less than 11.15% for each analyte at each QC level. The average recoveries at three concentration levels were 109.79 ± 4.52%, 93.56 ± 6.81%, 92.12 ± 2.69%, and 112.85 ± 7.12% for SEN, SENNOX, ADO, and ADONOX, respectively.

3.2.4. Matrix effect and stability

Matrix effect was conducted as described in Section 2.5.4. The value A/B × 100% of all analytes at three concentrations was within the acceptable limits (93.31–111.03%). Thus, ion suppression or enhancement from serum was negligible for this method.

In terms of stability, no significant difference of areas (R.S.D. < 8.20%) was detected for all standards in serum samples of both low and high QC levels during various storage and processing procedures. Good post-treatment stability as well as chemical stability was observed (R.S.D. < 18.38%, 9.00%, respectively).

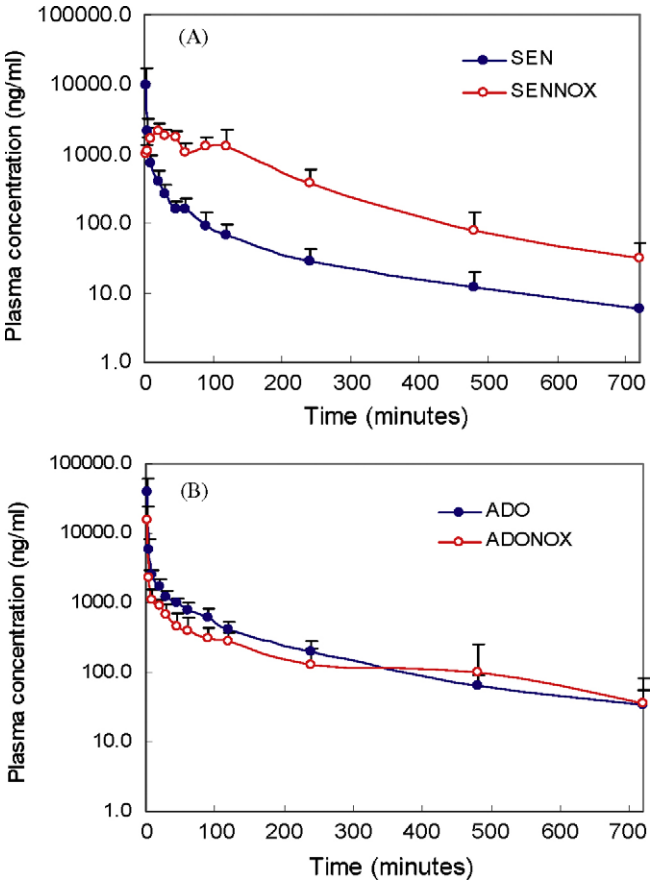

Fig. 3. Serum pharmacokinetic profiles of (A) SEN and SENNOX and (B) ADO and ADONOX in rats after intravenously administration of SEN (1.5 mg/kg) and ADO (4.0 mg/kg), respectively ($n = 8$).

3.3. Pharmacokinetics study

The validated UPLC–ESIMS method was successfully applied to 720-min period PK studies of ADO and SEN in rats. The concentration–time curves were shown in Fig. 3A and B. Based on these results the PK parameters of SEN, SENNOX, ADO, and ADONOX were calculated and summarized in Table 3.

Table 2
Summary of precision and accuracy for the UPLC–ESIMS method.

Analytes	Concentration added (μg/ml)	Concentration measured (μg/ml)	Accuracy (%)	Mean accuracy (%)	Intra-day precision (R.S.D. %)	Inter-day precision (R.S.D. %)
SEN	0.01112	0.0127 ± 0.0012	113.99 ± 10.83	109.79 ± 4.52	1.92	2.99
	0.1112	0.1227 ± 0.0026	110.37 ± 2.32		2.10	2.82
	1.112	1.1680 ± 0.0060	105.01 ± 0.54		0.52	4.00
SENNOX	0.01168	0.0114 ± 0.0005	97.85 ± 4.66	93.56 ± 6.81	4.76	8.84
	0.1168	0.1001 ± 0.0049	85.70 ± 4.21		4.91	7.75
	1.168	1.1340 ± 0.0310	97.13 ± 2.69		2.77	9.53
ADO	0.01166	0.0111 ± 0.0001	95.07 ± 1.23	92.12 ± 2.69	1.30	6.68
	0.1166	0.1047 ± 0.0018	89.81 ± 1.54		1.72	8.83
	1.166	1.0660 ± 0.020	91.47 ± 1.73		1.89	7.02
ADONOX	0.01240	0.0143 ± 0.0004	115.08 ± 3.00	112.85 ± 7.12	2.61	8.02
	0.1240	0.1471 ± 0.0049	118.59 ± 3.93		3.32	11.15
	1.240	1.3010 ± 0.020	104.88 ± 1.63		1.55	7.55

Table 3
Pharmacokinetic parameters of SEN, SENNOX, ADO, and ADONOX in rat after intravenous administration of SEN and ADO at 1.5 mg/kg and 4.0 mg/kg, respectively ($n = 8$).

Pharmacokinetic parameters	SEN	SENNOX	ADO	ADONOX
C_{max} (μg/ml)	9.83 ± 6.85	2.34 ± 0.53	40.29 ± 18.74	15.73 ± 8.28
T_{max} (min)	2.00 ± 0.00	22.78 ± 10.93	2.00 ± 0.00	2.00 ± 0.00
$AUC_{(0-\infty)}$ (μg min/ml)	72.12 ± 24.37	310.10 ± 84.37	334.03 ± 85.36	176.61 ± 47.84
MRT (min)	128.33 ± 99.61	168.29 ± 41.24	128.69 ± 46.20	191.51 ± 79.82
V_d (ml/kg)	10,864.55 ± 8,736.35	1,250.52 ± 850.13	4,124.91 ± 1,270.22	9,175.61 ± 6,180.51
CL (ml/min/kg)	23.23 ± 8.39	5.30 ± 2.07	12.74 ± 3.42	24.36 ± 7.50

4. Conclusions

Combined with a C_{18} SPE process, an UPLC–ESIMS method for the simultaneous analysis of SEN, ADO and their main metabolites in rat serum was developed and validated. Compared with the analytical methods reported previously, the method proved to be time-saving (cal. 4.5 min vs 20 min) with a comparable on-column sensitivity, thus can provide reliable data for the *in vivo* pharmacokinetic studies of SEN, ADO and their main metabolites and help promoting the toxicokinetics research of HPAs. The method was successfully applied to a pharmacokinetic study of SEN, ADO and their main metabolites in rats.

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References

[1] J.A. Edgar, E. Roeder, R.J. Molyneux, J Agric. Food Chem. 50 (2002) 2719–2730.
[2] Pyrrolizidine alkaloids in food: a toxicological review and risk assessment, Technical Report Series No. 2, Australia New Zealand Food Authority, 2001.
[3] Pyrrolizidine Alkaloids, Health and Safety Guide No. 26, World Health Organization, Geneva, 1989.
[4] A.R. Mattocks, Chemistry and Toxicology of Pyrrolizidine Alkaloids, Academic Press, London, 1986.
[5] M.W. Chou, Y.P. Wang, J. Yan, Y.C. Yang, R.D. Beger, L.D. Williams, D.R. Doerge, P.P. Fu, Toxicol. Lett. 145 (2003) 239–247.
[6] Y.P. Wang, J. Yan, P.P. Fu, M.W. Chou, Toxicol. Lett. 155 (2005) 411–420.
[7] F. Zhang, C.H. Wang, W. Wang, L.X. Chen, H.Y. Ma, C.F. Zhang, M. Zhang, S.W.A. Blight, Z.T. Wang, Phytochem. Anal. 19 (2008) 25–31.
[8] M. Liu, Y.G. Li, G.X. Chou, X.M. Cheng, M. Zhang, Z.T. Wang, J. Chromatogr. A 1157 (2007) 51–55.
[9] L. Yang, A.Z. Xiong, Y.Q. He, Z.Y. Wang, C.H. Wang, Z.T. Wang, W. Li, L. Yang, Z.B. Hu, Chem. Res. Toxicol. 21 (2008) 2280–2288.
[10] J. Tang, M. Zhang, T. Akao, N. Nakamura, M. Hattori, Z.T. Wang, J. China Pharm. Univ. 34 (2003) 499–502.