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Short communication

# Determination of strychnine and brucine in rat plasma using liquid chromatography electrospray ionization mass spectrometry

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ABSTRACT

A simple, sensitive and selective liquid chromatography–electrospray mass spectrometric (LC–ESI-MS) method was developed and validated for simultaneous determination of strychnine and brucine in rat plasma, using tacrine as the internal standard (IS). Sample preparation involved a liquid–liquid extraction of the analytes with *n*-hexane, dichloromethane and isopropanol (65:30:5, v/v/v) from 0.1 mL of plasma. Chromatographic separation was carried out on a Waters $C_{18}$ column using a mobile phase of methanol–20 mM ammonium formate–formic acid (32:68:0.68, v/v/v). Positive selected ion monitoring mode was used for detection of strychnine, brucine and the IS at *m*/*z* 335.2, *m*/*z* 395.2 and *m*/*z* 199.2, respectively. Linearity was obtained over the concentration range of 0.5–500 ng/mL for strychnine and 0.1–100 ng/mL for brucine. The lower limit of quantification was 0.5 ng/mL and 0.1 ng/mL for strychnine and brucine, respectively. The intra- and inter-day precision for both strychnine and brucine was less than 7.74%, and accuracy ranged from −4.38% to 2.21% at all QC levels. The method has been successfully applied to a pharmacokinetic study of processed Semen Strychni after oral administration to rats.



## 1. Introduction

Semen Strychni, officially listed in Chinese Pharmacopoeia, is the dried mature seed of *Strychnos nux vomica* L., a tree native in India. In classic Chinese Materia Medicas, the herbal drug is listed as a toxic herb and its biological effects (pharmacological and toxic effects) are dose-dependent, needing processed to reduce the toxicity before clinical uses required by Chinese medicine prescriptions. Nowadays, processed Semen Strychni is clinically used as an important ingredient in various remedies of traditional herbal medicines to treat nervous diseases, vomiting, arthritic, traumatic pains [1] and to promote blood circulation and remove blood stasis [2]. The main bioactive components of Semen Strychni are alkaloids, and 16 of them have been isolated and identified by now [3]. The most abundant alkaloids existing in the processed Semen Strychni are strychnine and brucine (Fig. 1) [2], which have been reported to possess analgesic, anti-inflammatory and anti-tumor effects [4,5], despite their toxicity in nature [6,7].

Several analytical methods for quantitative determination of each or both of the two strychnos alkaloids have been described, including HPLC with UV detection [8,9], TLC [10], fluorescence spectrophotometric method [11], $^1$H NMR [12], GC–MS [13], LC–MS [14] and LC–MS/MS [15], etc. However, few reports are available regarding simultaneous determination of strychnine and brucine in biological matrix for pharmacokinetic studies of Semen Strychni. The published HPLC–UV method [8] was developed for simultaneous determination of four strychnos alkaloids including strychnine and brucine in rat plasma after i.v. administration, with a LLOQ of 250 ng/mL for both of strychnine and brucine. The method was not sensitive enough for pharmacokinetic studies of strychnine and brucine after orally administered low dosage of processed Semen Strychni, due to the low levels of both alkaloids in rat plasma. Besides, the method required time-consuming sample preparation procedure, using 1 mL of plasma sample. To investigate the ADME properties of processed Semen Strychni following oral administration, a more simple and sensitive bioanalytical method is required for simultaneous determination of strychnine and brucine.

In the present study a simple, sensitive and selective liquid chromatography–electrospray mass spectrometric (LC–ESI-MS) method for simultaneous determination of strychnine and brucine in rat plasma was established. The method has been successfully applied to a pharmacokinetic study after oral administration of processed Semen Strychni to Wistar rats.

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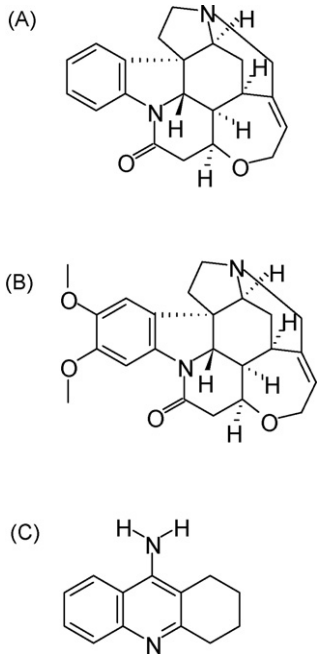


**Fig. 1.** Chemical structures of strychnine (A), brucine (B) and tacrine (C, internal standard).

## 2. Experimental

### 2.1. Chemicals and reagents

Reference substances of strychnine, brucine and tacrine (IS, Fig. 1) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Processed Semen Strychni, namely Semen Strychni pulveratum, was kindly provided by Tianjin Darentang Jingwanhong Pharmaceutical Co., Ltd. (Tianjin, China). HPLC grade of methanol, formic acid (88%) and isopropanol were from Tianjin Concord Tech Reagent Co., Ltd. (Tianjin, China). Ammonium formate and other reagents were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the experiment.

### 2.2. Instrumentation and LC–MS conditions

The LC–MS system consists of a Surveyor autosampler, a Surveyor LC pump, a TSQ Quantum Discovery Max$^{TM}$ triple–quadrupole mass spectrometer and Xcalibur 1.4 software (Thermo Finnigan, USA). A Waters Symmetry$^{TM}$ $C_{18}$ column (100 mm × 4.6 mm I.D., 5 μm, Waters, USA) kept at 30 °C was used for chromatographic separation. The mobile phase was composed of methanol, 20 mM ammonium formate and formic acid (32:68:0.68, v/v/v) at a flow rate of 0.4 mL/min.

Electrospray ionization (ESI) source in positive mode was used for mass spectrometric detection. Mass spectrometric conditions were optimized to achieve the maximum sensitivity. The ionspray voltage was set at 4000 V. The sheath and auxiliary gas was nitrogen, with the pressure and flow rate of 35 psi and 5 L/min, respectively. The heated capillary temperature was 300 °C. The extra energy of 10 V was added for source collision-induced dissociation (CID). Selected ion monitoring (SIM) mode was used for the quantification at $m/z$ 335.2 for strychnine, $m/z$ 395.2 for brucine and $m/z$ 199.2 for tacrine with a dwell time of 0.2 s.

### 2.3. Calibration standard and quality control preparation

Stock solutions of strychnine and brucine were prepared in methanol at concentrations of 1 mg/mL and 100 μg/mL. Both stock solutions were diluted with methanol to get a combined standard working solution of 500 ng/mL strychnine and 100 ng/mL brucine. Then the combined standard working solution was further diluted with methanol to provide a series of standard working solutions of desired concentrations. The internal standard was prepared in methanol at a concentration of 100 μg/mL and was further diluted to 50 ng/mL as a working solution. All solutions were stored at −20 °C.

The calibration standards were prepared by spiking blank plasma (0.1 mL) with appropriate amounts of working solutions to yield final concentrations of 0.5, 1, 5, 25, 50, 250 and 500 ng/mL for strychine, and 0.1, 0.2, 1, 5, 10, 50 and 100 ng/mL for brucine, respectively. Combined quality control (QC) samples were prepared at low, medium and high concentration levels of 1, 25 and 250 ng/mL for strychine, and 0.2, 5 and 50 ng/mL for brucine, respectively. The spiked samples were then treated following the sample preparation procedure indicated in Section 2.4.

### 2.4. Sample preparation

To an aliquot (0.1 mL) of plasma sample, 100 μL of internal standard (50 ng/mL), 100 μL of methanol (or standard solutions for calibration curve or QC samples) and 500 μL of saturated $Na_2CO_3$ were added. The mixture was vortexed for 30 s and was extracted with 2 mL of *n*-hexane–dichloromethane–isopropanol (65:30:5, v/v/v) by thoroughly vortexing for 3 min. After centrifugation (3000 × *g*) for 5 min, the upper organic layer was taken and evaporated to dryness under a mild stream of $N_2$ at 45 °C. The residue was reconstituted in 100 μL of mobile phase. After centrifugation (12,000 × *g*) for 3 min, an aliquot of 10 μL was injected for LC–MS analysis.

### 2.5. Method validation

Selectivity was tested by comparison of blank plasma from six individual rats with corresponding spiked plasma samples. Calibration curves were obtained by plotting the peak–area ratio ($y$) of the analyte/internal standard against the spiked concentrations ($x$) of either strychnine or brucine, using a weighted (1/square of concentration) linear regression. Linearity of calibration was tested by analysis of three sets of calibration standards each day for three consecutive days. Deviations of the mean calculated concentrations were set at ±15% of nominal concentrations, except for the lower limit of quantification (LLOQ) where a deviation of ±20% was permitted.

Precision was expressed as the relative standard deviation (R.S.D.) and accuracy was calculated as the relative error (R.E.). QC samples at low, medium and high concentrations in six replicates were analyzed during the same day using the same calibration curve to determine the intra-day precision. Three batches of QC samples were analyzed on three consecutive days to evaluate the inter-day precision and accuracy.

Both of recovery and matrix effect were tested using triplicate of spiked samples at three QC levels. The absolute recoveries were determined by comparing the peak areas of extracted plasma samples with those of standard working solutions at equivalent concentration. The matrix effects were investigated by comparing the peak areas of post-extracted blank plasma spiked with working solutions of strychnine and brucine with those of corresponding standard solutions. The same procedure was performed for IS.

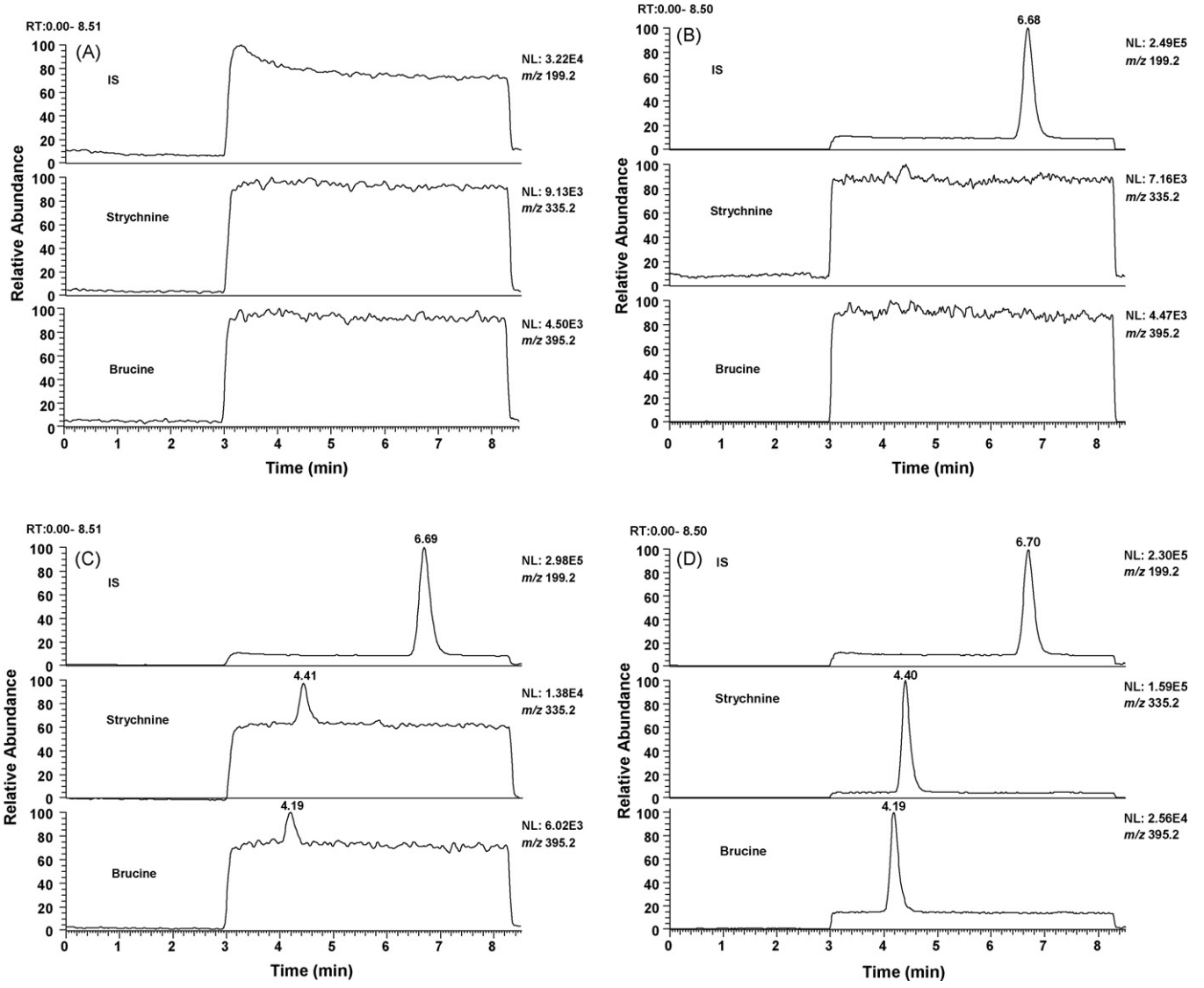


**Fig. 2.** Representative SIM chromatograms of (A) blank rat plasma; (B) blank rat plasma spiked with strychnine (LLOQ, 0.5 ng/mL), brucine (LLOQ, 0.1 ng/mL) and tacrine (IS, 50 ng/mL); (C) blank rat plasma spiked with 50 ng/mL of tacrine (IS); (D) a rat plasma sample at 2 h after oral administration of 150 mg/kg of processed Semen Strychni.

**Table 1**
Linearity for determination of strychnine and brucine in rat plasma ($n$ = 3 days, triplicates at each concentration per day).

| Nominal concentration (ng/mL) | Measured concentration (ng/mL, mean ± S.D.) | Intra-day R.S.D. (%) | Inter-day R.S.D. (%) | R.E. (%) |
|---|---|---|---|---|
| Strychnine | | | | |
| 0.5 | 0.49 ± 0.03 | 7.37 | 6.76 | −1.14 |
| 1 | 1.01 ± 0.05 | 4.80 | 4.64 | 0.55 |
| 5 | 5.26 ± 0.32 | 6.33 | 5.05 | 5.20 |
| 25 | 25.88 ± 0.81 | 3.27 | 2.60 | 3.52 |
| 50 | 50.08 ± 1.99 | 4.48 | 1.71 | 0.16 |
| 250 | 243.08 ± 8.66 | 2.75 | 5.30 | −2.77 |
| 500 | 470.34 ± 21.25 | 4.61 | 4.24 | −5.93 |
| Brucine | | | | |
| 0.1 | 0.10 ± 0.00 | 5.35 | 2.93 | −1.52 |
| 0.2 | 0.21 ± 0.01 | 6.92 | 5.74 | 2.72 |
| 1 | 1.01 ± 0.06 | 5.96 | 5.38 | 1.46 |
| 5 | 5.07 ± 0.15 | 2.13 | 4.68 | 1.35 |
| 10 | 9.93 ± 0.36 | 2.39 | 5.86 | −0.65 |
| 50 | 49.58 ± 2.42 | 3.73 | 7.31 | −0.84 |
| 100 | 97.49 ± 3.67 | 4.32 | 0.80 | −2.51 |

**Table 2**
Precision and accuracy for strychnine and brucine in rat plasma ($n$ = 3 days, six replicates at each concentration per day).

| Compound | Spiked concentration (ng/mL) | Measured concentration (ng/mL, mean ± S.D.) | Intra-day R.S.D. (%) | Inter-day R.S.D. (%) | R.E. (%) |
|---|---|---|---|---|---|
| Strychnine | 1 | 1.01 ± 0.07 | 7.74 | 2.39 | 0.74 |
| | 25 | 25.55 ± 1.47 | 5.82 | 5.31 | 2.21 |
| | 250 | 239.06 ± 12.70 | 5.54 | 3.15 | −4.38 |
| Brucine | 0.2 | 0.20 ± 0.02 | 7.55 | 7.50 | 0.12 |
| | 5 | 5.00 ± 0.28 | 5.30 | 7.70 | −0.06 |
| | 50 | 48.86 ± 2.46 | 4.88 | 6.13 | −2.28 |

Stability of analytes was assessed using triplicate of spiked samples at three QC levels, which were subjected to three freeze (−20 °C) to thaw (room temperature) cycles, stored at −20 °C for 1 month and at room temperature for 4 h. Stability in standard solutions (−20 °C) for 1 month and processed samples in the autosampler at 4 °C for 24 h were also evaluated.

### 2.6. Application to a pharmacokinetic study

Six male Wistar rats, weighting 190–220 g, were housed under controlled environmental conditions (temperature 20–22 °C; humidity 45–65%) with free access to food and drinking water. Animals were fasted for 12 h prior to administration. Dosing solutions were prepared by suspending accurately weighted amount of processed Semen Strychni in sodium carboxymethyl cellulose (1%, w/v, water) and each rat was given an oral gavage of 10 mL/kg (150 mg/kg). Blood samples were collected from ophthalmic venous plexus in heparinized tubes pre-dose and at 0.08, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8 and 12 h post-dose. Plasma samples were obtained by centrifugation at 12,000 × $g$ for 10 min. All plasma samples were stored at −20 °C and analyzed within 1 month.

## 3. Results and discussion

### 3.1. Method development

The LC–ESI-MS conditions were tested using reference standards. Due to the two nitrogen atoms in the chemical structures of strychnine, brucine and tacrine, the signal intensities obtained in positive mode were much stronger than those in negative mode. In full scan mass spectra, both alkaloids and the IS predominantly formed protonated quasi-molecular ions. The extra energy of 10 V was added for source CID potential to improve the signal sensitivity and stability of both alkaloids by reducing other adduct ions. Other parameters of the MS detector were optimized to get maximum response. Positive SIM mode was used for the quantification at $m/z$ 335.2, $m/z$ 395.2 and $m/z$ 199.2 for strychnine, brucine and tacrine, respectively.

The mobile phase was composed of methanol–20 mM ammonium formate (containing 1% formic acid). The addition of formic acid was found to enhance the sensitivity and to get better peak shape. A mixture of $n$-hexane, dichloromethane and isopropanol was used for the liquid–liquid extraction by virtue of its satisfactory recoveries. The addition of isopropanol was an important factor to achieve high recoveries because it increased the polarity of the extraction solvent and prevented the extraction solvent from emulsification when vortex-mixed. Due to the alkaline nitrogen atoms in the structures of strychnine and brucine, plasma alkalization with saturated $Na_2CO_3$ before extraction could help improve the extraction efficiency.

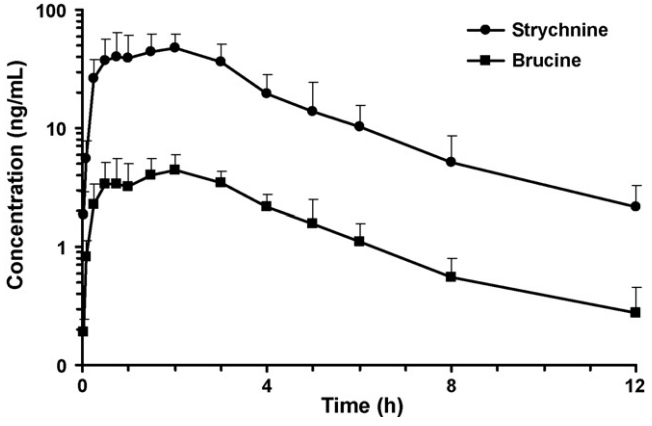


**Fig. 3.** Mean plasma concentration–time profiles of strychnine and brucine after a single oral dose of 150 mg/kg of processed Semen Strychni to rats ($n$ = 6, mean ± S.D.).

### 3.2. Method validation

The detection of strychnine, brucine and tacrine by SIM was highly selective with no interference. Typical SIM chromatograms are given in Fig. 2. Observed retention times for strychnine, brucine and tacrine were 4.2, 4.4 and 6.7 min, respectively. No ion suppression or ion cross-over between the two analytes was caused by their similar retention times.

Linearity for determining strychnine and brucine in rat plasma is described in Table 1. Calibration curves were linear over the concentration range of 0.5–500 ng/mL for strychnine and 0.1–100 ng/mL for brucine. Typical regression equations of the calibration curves were $y = 0.0141x + 0.0033$ ($r = 0.9984$) for strychnine and $y = 0.0204x + 0.0011$ ($r = 0.9985$) for brucine. LLOQ was measured to be 0.5 ng/mL for strychnine and 0.1 ng/mL for brucine with R.S.D. less than 12.34% for both analytes, which was within acceptable limits. Precision and accuracy were satisfactory at three levels of QC samples (Table 2).

The extraction solvent used in the experiment showed good extraction efficiency. Mean absolute recoveries of strychnine and brucine from rat plasma were 89–94% and 85–99% at three QC levels. The mean absolute recovery of the IS was 88%. For strychnine and brucine at three QC levels in rat plasma, the degree of ion suppression ranged from −1.8% to 2.1%, indicating a neglectable matrix effect on the ionization of the analytes.

Results of the stability tests for strychnine and brucine showed that both analytes were stable in plasma samples after three freeze–thaw cycles, and after being stored at room temperature for 4 h or being stored at −20 °C for 1 month. Both analytes were also stable in standard solutions at −20 °C for 1 month or when processed samples were kept in the autosampler at 4 °C for 24 h. The accuracy (R.E.) ranged from −0.44% to 5.18%, −8.35% to 12.55%, −10.07% to −2.28%, −7.14% to 3.90% and −6.61% to

0.84%, respectively after three freeze–thaw cycles, stored at room temperature for 4 h and at 4 °C in the autosampler for 24 h, as well as plasma samples and standard solution at −20 °C for 1 month.

### 3.3. Application to a pharmacokinetic study

Mean ($n$ = 6) plasma concentration–time profiles after oral administration of processed Semen Strychni are shown in Fig. 3. The mean maximum plasma concentrations ($C_{max}$) of strychine and brucine detected in the rats were 54.93 and 4.95 ng/mL, respectively. Obviously the previously reported method based on HPLC–UV could not satisfy the requirements of pharmacokinetic studies of both alkaloids following oral administration, whereas the present method based on LC–ESI-MS was sensitive enough for the oral pharmacokinetic research of strychnine and brucine.

## 4. Conclusion

A simple, sensitive and selective LC–ESI-MS method for simultaneous determination of strychnine and brucine in rat plasma was established. Compared with the previously reported methods, the present method employed a simple and rapid extraction procedure for sample preparation, and offered higher sensitivity, with the LLOQ of 0.5 and 0.1 ng/mL for strychnine and brucine, requiring only 0.1 mL of plasma. The validated method has been successfully applied to an oral pharmacokinetic study of processed Semen Strychni in Wistar rats.

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