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Liquid chromatography–hydride generation–atomic fluorescence spectrometry determination of arsenic species in dog plasma and its application to a pharmacokinetic study after oral administration of Realgar and *Niu Huang Jie Du Pian*

Yunjing Zhang, Shuping Qiang, Jing Sun, Min Song*, Taijun Hang*

Department of Pharmaceutical Analysis, China Pharmaceutical University, Nanjing 210009, PR China

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ABSTRACT

A high performance liquid chromatography–hydride generation–atomic fluorescence spectrometry (HPLC–HG–AFS) method was developed for the simultaneous determination of four arsenic species (As(III), dimethylarsinic acid (DMA), monomethylarsonic acid (MMA) and arsenate As(V)) in dog plasma. Good separation of the four arsenic species was achieved within 15 min on an anion-exchange column with isocratic elution using 15 mmol/L KH_2PO_4 (pH 5.9) as eluent at a flow rate of 1.0 mL/min. The assay was linear over the range of 1.25–200, 1.56–200, 1.34–172, and 2.50–200 ng/mL with the detection limits of 0.80, 1.00, 0.86 and 2.00 ng/mL for As(III), DMA, MMA and As(V), respectively. The method was validated for selectivity, precision, accuracy and recovery and then applied to a comparative pharmacokinetic study of the arsenic species in beagle dogs after a single oral administration of Realgar (24.32 mg/kg, equivalent to 11.31 mg As/kg) alone or *Niu Huang Jie Du Pian* (a patent traditional Chinese medicine (TCM), 380 mg/kg, equivalent to 28.45 mg As/kg), respectively. DMA was found to be the predominant species in the dog plasma after dosing, with As(V) appeared as the quickly eliminating one. No traces of MMA and As(III) were detected at any sampling time points. The main pharmacokinetic parameters found for DMA *p.o.* administration of Realgar and *Niu Huang Jie Du Pian* were as follows: C_{max} (14.7 ± 4.2) and (57.0 ± 32.0) ng/mL, T_{max} (2.4 ± 0.5) and (2.5 ± 0.5) h, AUC_{0-36} (151.1 ± 12.9) and (635.9 ± 418.2) ng h/mL, $AUC_{0-\infty}$ (206.0 ± 44.5) and (687.2 ± 425.1) ng h/mL, $t_{1/2}$ (16.2 ± 7.9) and (9.4 ± 2.2) h, respectively. The influence of compounding in *Niu Huang Jie Du Pian* on the pharmacokinetics of arsenics was shown with increased transformation of DMA and its faster elimination rate.

1. Introduction

Mineral arsenicals, such as Realgar, has long been used in traditional Chinese medicines (TCMs) and proved to have therapeutic effects. Realgar, containing 90% arsenic disulfide (As_2S_2) or tetra-arsenic tetra-sulfide (As_4S_4), has been applied for the treatment of carbuncles, boils, insect- and snake-bites, intestinal parasitosis, convulsive epilepsy, and psoriasis [1]. Recently it was also demonstrated to be clinically effective for acute promyelocytic leukemia and other hematopoietic malignancies [2].

Rather than being used alone, Realgar is commonly prescribed in compound Chinese medicines, such as *Niu Huang Jie Du Pian* (NHJDP), a traditional Chinese medicine with a long history of therapeutic use for fever relieving, detoxicating and anti-inflammation [3]. NHJDP officially listed in the *Chinese Pharmacopeia* consists of 6.4% Realgar along with seven other components: *Niuhuang* (*Calculus Bovis*), *Huangqin* (*Scutellariae Radix*), *Dahuang* (*Rhei Radix et Rhizoma*), *Shigao* (*Gypsum Fibrosum*), *Jiegeng* (*Platycodonis Radix*), *Bingpian* (*Borneolum Syntheticum*), and *Gancao* (*Glycyrrhizae Radix et Rhizoma*) [4].

However, many arsenic species are also potent poisons and even environmental carcinogens. Although Realgar is poorly soluble in water and thus considered to be less poisonous [5], upon ingestion of NHJDP, the potential of arsenic poisoning cannot be ignored. The recently reported adverse effects of NHJDP in humans include hyperpigmentation, hyperkeratosis, and hepatic lesion [6,7], especially after overdose or long-term uses, or in combination usages with other drugs. Thus the safety and rational uses of NHJDP, an arsenic-containing medicine, are of great concern.

Abbreviations: As(III), arsenite; As(V), arsenate; DMA, dimethylarsinic acid; GLS, gas–liquid separator; HG–AFS, hydride generation–atomic fluorescence spectrometry; ICP–MS, inductively coupled plasma mass spectrometry; MMA, monomethylarsonic acid; NHJDP, *Niu Huang Jie Du Pian*; TCM, traditional Chinese medicine.

* Corresponding authors. Tel.: +86 25 8327 1090; fax: +86 25 8327 1090.
E-mail addresses: cqsongmin@sina.com (M. Song), hangtj@cpu.edu.cn (T. Hang).

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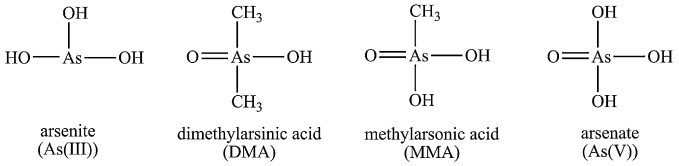

Fig. 1. Chemical structures of arsenite (As(III)), dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), arsenate (As(V)).

To promote the development of rational therapy schedules, thorough investigation of the pharmacokinetics of NHJDP is needed. Although pharmacokinetics of Realgar [8–10] and arsenic trioxide [11–13] have been reported, the pharmacokinetic data of arsenic in Chinese patent medicine are still very limited. One study of NHJDP in healthy volunteers showed that the cumulative urinary excretion rate of total arsenic during 7 days following ingestion of three tablets b.i.d. was less than 1% of the dose [14]. However, it has been widely recognized that the toxicity and biochemical functions of trace elements strongly depend on species [15]. In another study, arsenic speciation analysis in human urine was carried out, revealing that metabolism to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) took place after taking a single pill of NHJDP [16]. These studies were performed with single or short-term administration and focused only on urine excretion, though it was a generally accepted biomarker of arsenic exposure, arsenicals in blood could indicate the recent exposure of arsenic-containing drugs. In vitro studies showed that dissolution of arsenic from Realgar was significantly altered by the coexisting components in the TCM [17,18], suggesting that the possible influence of the drug–drug interactions and drug–excipient interactions on the pharmacokinetics of the arsenicals should also be investigated. However, it is a great challenge to determine trace levels of arsenicals from such complex matrix as plasma, especially for speciation studies. Therefore, more research is needed to elucidate the in vivo disposition of arsenic in NHJDP.

The common arsenic species in vivo include As(III), DMA, MMA and As(V) (Fig. 1). They can be neutral or negatively charged depending on the solution pH so that ion exchange chromatography can be a suitable method for their separation. The detection system commonly employed in speciation studies is inductively coupled plasma–mass (ICP–MS), which is highly sensitive and robust. AFS represents a suitable alternative to mass spectrometric techniques. The coupling of HPLC with hydride generation–atomic fluorescence spectrometry (HG–AFS), has been described to be similar to HPLC–ICP–MS regarding sensitivity and linear range, with further advantages such as simplicity, shorter warm up times and lower acquisition and running costs for arsenic speciation in routine analysis [19]. However, the hydride generation coupled systems are limited to the analysis of hydride forming elements. Some of the hydride inactive organic arsenicals, e.g., arsenobetaine and arsenocholine which do not form volatile hydrides have to be broken down with the help of online decomposition methods, which would jeopardize the sensitivity. In the present study, the four toxicologically important arsenic species are all hydride active. Thus, an HPLC–HG–AFS method was established for the quantification of the four arsenic species in dog plasma and was applied to a comparative pharmacokinetic study of the arsenic species in beagle dogs after oral administration of Realgar and NHJDP, respectively.

2. Materials and methods

2.1. Materials and reagents

NHJDP was manufactured by Huiren Pharmaceutical Co., Ltd. Realgar levigated with water was supplied by Melaluca wild mushroom Ganoderma lucidum Development Co., Ltd. (Jinhua, China). Contents of the total arsenic expressed as As in NHJDP and Realgar were quantified by validated atomic absorption spectrophotometric method and the arsenic species after HCl extraction was determined by HPLC–HG–AFS. Arsenic trioxide reference standard was provided by National Institutes for Food and Drug Control (Beijing, China). Monosodium methanearsonic acid was purchased from Laboratories of Dr. Ehrenstorfer GmbH (Augsbrug, Germany). Cacodylic acid sodium salt trihycrate was obtained from Huamaike Biotechnology Co., Ltd. (Beijing, China). Hydrochloric acid and nitric acid (Sinopharm Chemical Reagent Co., Ltd.) were of guaranteed grade and methanol (TEDIA Company, Inc.) was of HPLC grade. All other chemicals were of analytical-reagent grade. Water was freshly purified (18.3 MΩ cm, Millipore, USA) and filtered through a 0.22 μm membrane before use.

2.2. Apparatus

The HPLC–HG–AFS system consists of an LC-2010C HT system (Shimadzu Corporation, Japan) and an HG–AFS detection system (AF-610D, Rayleigh, Beijing). All the glassware were soaked in 6 mol/L HNO_3 solution for at least 24 h and then cleaned and rinsed thoroughly with purified water.

2.3. Chromatographic and AFS conditions

The separation of the arsenicals was performed on a Hamilton PRP-X 100 anion-exchange column (250 mm × 4.1 mm I.D. 10 μm) at 30 °C with an isocratic elution by 15 mmol/L monopotassium phosphate solution (adjusted to pH 5.9 with 1 mol/L KOH solution) under a flow rate of 1.0 mL/min. The column effluent was first mixed at a T-joint with 1.2 mol/L HCl solution and then met with freshly prepared 0.37 mol/L KBH_4 stabilized in 36 mmol/L KOH solution as the reducing reagent in another T-joint, delivered by two peristaltic pumps at flow rates of 3.2 and 4.8 mL/min, respectively, where hydride generation took place as shown in Fig. 2. The mixture was separated by the gas–liquid separator (GLS). Arsenic hydrides, along with hydrogen generated were carried by a continuous flow of argon carrier gas at a flow rate of 200 mL/min. After being separated by the second and third GLS, the analytes in the gas phase were carried into the atomizer by the argon auxiliary gas flow of 900 mL/min and detected by the atomic fluorescence detector with the primary current, assistant current and PMT voltage set as 50 mA, 30 mA and 320 V, respectively.

2.4. Preparation of calibration standards and quality control samples

As(III) stock solution about 1.0 mg/mL as As was prepared by dissolving an accurately weighed amount of As_2O_3 about 0.13 g in 1 mL of 2.5 mol/L NaOH solution and then diluted with 0.3 mol/L HCl to 100 mL. As(V) was prepared by heating a known amount of As_2O_3 with excessive amount of nitric acid on a hot plate (maximum temperature of 110 °C) for 1 h and diluted with water to give a concentration of 0.8 mg/mL as As. Stock solutions of MMA and DMA were prepared by dissolving accurately weighted amount of sodium methylarsonate and cacodylic acid sodium reference standards in water to yield a concentration of 0.08 and 1.0 mg/mL as As, respectively. Series of working standard solutions were prepared by appropriate dilution of the above stock solutions with purified water to give the concentration ranges of 6.25–1000 ng/mL for As(III), 7.81–1000 ng/mL for DMA, 6.70–860 ng/mL for MMA, and 12.5–1000 ng/mL for As(V). All the stock solutions were stored in refrigeration at about 4 °C and the working standard solutions were freshly prepared before use.

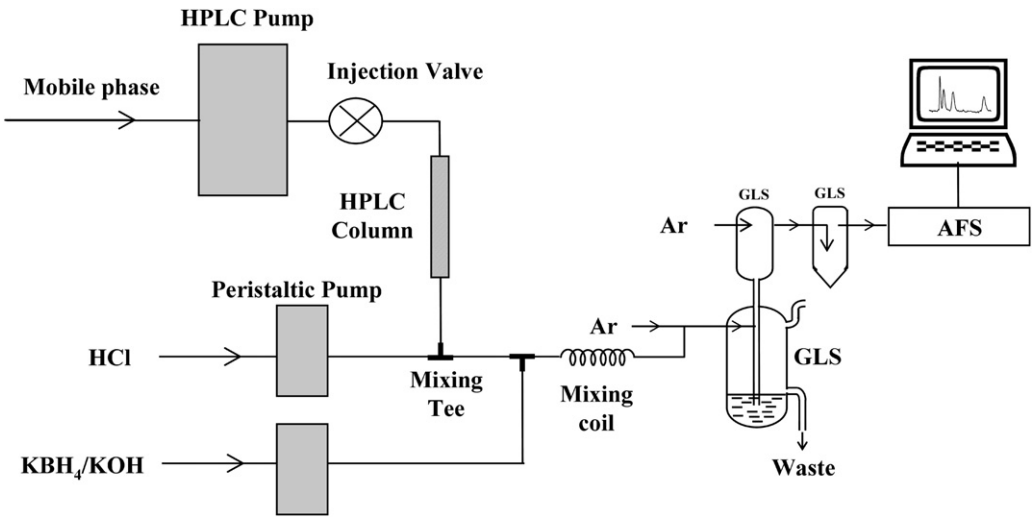

Fig. 2. Schematic diagram of the HPLC–HG–AFS system used for the determination of the arsenic species.

The calibration plasma standards were prepared by spiking dog blank plasma with the working standard solutions covering the ranges from 1.2 to 200 ng/mL for As(III), from 1.6 to 200 for DMA, from 1.3 to 172 for MMA, and from 2.5 to 200 ng/mL for As(V).

QC samples were prepared separately by spiking pooled blank dog plasma with four arsenic standards to yield final concentrations of 2.5, 25 and 100 ng/mL for As(III), 3.1, 25, and 100 ng/mL for DMA, 2.7, 22, and 86 ng/mL for MMA, 5.0, 50 and 100 ng/mL for As(V).

All the concentrations in the QCs and unknown plasma samples were quantified by external standard calibration method. The concentrations of the arsenicals were expressed as the concentrations of arsenic element (As) unless otherwise indicated.

2.5. Method validation

The method validation was carried out following the European Medicines Agency (EMA) guideline (Guideline on Bioanalytical Method Validation, EMEA/CHMP/EWP/192217/2009 of 21 July 2011) [20].

2.5.1. Specificity

The specificity was verified by comparing the chromatograms of plasma blanks from 6 dogs with those of the spiked by arsenic standards.

2.5.2. Linearity and sensitivity

The calibration curves of the HPLC–HG–AFS method were evaluated by analyzing a series of standard plasma samples at concentrations from 1.25 to 200 ng/mL for As(III), from 1.56 to 200 for DMA, from 1.34 to 172 for MMA, and from 2.50 to 200 ng/mL for As(V) using weighted least squares linear regression of the peak areas of each arsenic species obtained against the corresponding concentrations (C). The limits of detection (LOD) were determined as the plasma concentration giving a signal-to-noise ratio about 3. The lower limits of quantification (LLOQ) were defined as the plasma concentration giving a signal to noise ratio (S/N) >5.

2.5.3. Accuracy, precision and recovery

The intra-batch accuracy and precision were evaluated by analyzing five replicates at the QC concentration levels of the same batch. The inter-batch precision was determined by analyzing the respective QC samples in three different batches.

Recoveries of the four arsenicals from dog plasma were determined by comparing the arsenicals found in plasma carried through the complete preparation procedure with those spiked into the prepared blank plasma at the same concentration levels as those of QCs, respectively.

2.5.4. Stability test

The stability of stock solutions was evaluated at 4 °C for 90 days. The stabilities of the four arsenic species during the evaporation were evaluated by evaporation recovery which compares the responses of four species found in plasma carried through the complete preparation procedure with those freshly prepared in mobile phase at the same concentration levels as those of QCs, respectively. The plasma stability was assessed by analyzing triplicates of dog plasma samples containing the arsenicals respectively at low and high QC levels. Long-term stability was evaluated on samples stored at −80 °C for 30 and 60 days, respectively. Freeze–thaw stability was assessed by conducting three freeze–thaw cycles. Each cycle consisted of removing the samples from the freezer at −80 °C, thawing them unassisted to room temperature and refreezing them at −80 °C for 24 h. The bench-top stability was accessed on plasma samples kept at room temperature (25 ± 2 °C) for 6 h. Stabilities of the post-preparative samples kept at room temperature (25 ± 2 °C) and in the autosampler at 4 °C were evaluated for 6 h and 12 h, respectively. The time period is considered long enough to disclose any possible problems of sample stability during the sample storing and processing procedures.

2.6. Pharmacokinetic study application

2.6.1. Study design

Six beagle dogs, 3 males and 3 females, aged 8–9 months, weighing 11 ± 1.0 kg were supplied by Xingang Laboratory Animal Center (Shanghai, China) and housed in an air-conditioned room (temperature, 22–25 °C; relative humidity, 55 ± 5%). The dogs were previously determined to be clinically healthy on physical examination. Animal studies were approved by the Committee of Ethics for Animal Experimentation of China Pharmaceutical University.

Animals were assigned to two groups, using a single-dose, two-treatment, two-period cross-over design, with a washout period of 1-week. Both NHJDP and Realgar were ground as fine powders and suspended in 0.5% sodium carboxymethylcellulose solution and the suspensions were well-mixed before administration. After an overnight fast, animals received a single oral dose of 24.32 mg/kg of Realgar (equivalent to 11.31 mg As/kg) or 380 mg/kg NHJDP (equivalent to 28.45 mg As/kg). The contents of total arsenic and arsenic

Table 1
Contents of total arsenic and arsenic species in NHJDP and Realgar (mean ± SD, $n = 3$).

Sample	Total arsenic as As (g/kg)	As(III) (g/kg)	DMA (g/kg)	MMA (g/kg)	As(V) (g/kg)
NHJDP	74.9 ± 4.0	0.115 ± 0.001	ND	ND	0.070 ± 0.005
Realgar	465 ± 26	1.01 ± 0.41	ND	ND	0.337 ± 0.038

species in the extract of Realgar and NHJDP used in this study are shown in Table 1. Blood samples about 2 mL were withdrawn from the dog forelimb veins into heparinized polythene tubes prior to, 0.17, 0.33, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12, 24 and 36 h after the oral administration. Plasma was separated by centrifugation at 1000 × *g* for 5 min and stored at −80 °C until analyses.

2.6.2. Plasma sample pretreatment

An aliquot of 0.2 mL plasma was mixed with 0.6 mL methanol. After vortex-mixing for 1 min and centrifuging at 16,000 × *g* force for 10 min, the supernatant was separated and evaporated to dryness under reduced pressure (−0.09 MPa) at a temperature of 25 °C. The residue was reconstituted with 0.2 mL of mobile phase by vortex-mixing for 3 min and 100 μL of the supernatant obtained after centrifuged at 10,000 × *g* for 10 min was injected for the HPLC–HG–AFS analysis.

2.6.3. Pharmacokinetic analysis

The pharmacokinetic parameters of the arsenicals were calculated using the non-compartmental method with the aid of the DAS program (Drugs and Statistics version 2.0, Chinese Pharmacological Society).

3. Results and discussion

3.1. Sample preparation

One of the key steps for arsenic speciation is the sample pretreatment without any disturbance of the original species which is especially important for the biological samples due to the complexity of the matrix and the low level of the arsenicals. Several studies have been reported for the extraction of arsenic species from complex matrices such as seafood [21], urine [22] and environmental samples [23]. However, only a few reports dealt with the pretreatment of arsenics in plasma. Generally, sample extraction is carried out by protein precipitation with strong acids or organic solvent. In our preliminary study, $HClO_4$ solution was used as protein precipitant as reported [24]. But serious peak broadening was observed with inadequate resolution between the arsenic species, and the column had to be flushed and re-equilibrated with the mobile phase after each injection to remove interfering salts and organic species built up in the column to ensure repeatability of the analysis. Such a time consuming approach was not practical for high-throughput analysis required for a pharmacokinetic study. Mandal et al. [25] used trichloroacetic acid for the protein precipitation and HPLC–ICP–MS to determine the arsenic species in human plasma which was not suitable for HPLC–HG–AFS system, since massive amounts of contaminants and interferences were encountered. Protein precipitation with organic solvent such as acetonitrile and methanol was also investigated. Acetonitrile was excluded due to its interference with the analysis of As(III). Methanol in the ratio of triple the volume of plasma offered satisfactory recoveries for the arsenicals. However, direct injection of the supernatant after deproteinization with high content of organic solvent into the ion-exchange chromatographic system led to deformed peak shapes, fluctuating retention time and low sensitivity. Therefore, the supernatant obtained with protein precipitation by methanol was evaporated to dryness and the residue was reconstituted with the mobile phase for the sample analysis.

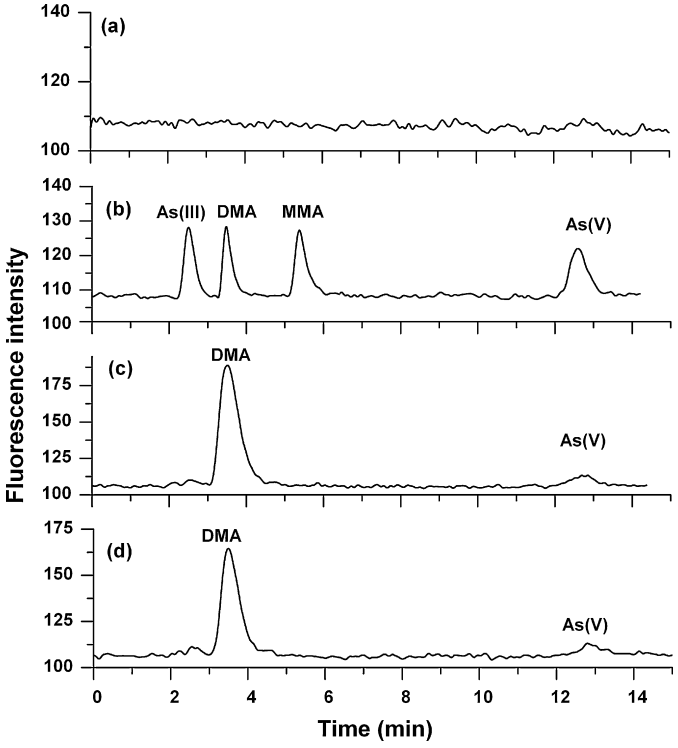

Fig. 3. HPLC–HG–AFS chromatograms of (a) blank plasma, (b) blank plasma spiked with the four arsenic species at LLOQ (As(III): 1.25 ng/mL, t_R = 2.6 min; DMA: 1.56 ng/mL, t_R = 3.5 min; MMA: 1.34 ng/mL, t_R = 5.7 min; As(V): 2.5 ng/mL, t_R = 12.8 min), (c) plasma sample collected 1 h after oral administration of 380 mg/kg of NHJDP (equivalent to 28.45 mg As/kg) and (d) 24.32 mg/kg of Realgar (equivalent to 11.31 mg As/kg).

3.2. Optimization of HPLC–HG–AFS system

As for HPLC–HG–AFS detection, the mobile phase of HPLC should not only be suitable for the separation but also compatible with the hydride-generation system. For arsenic speciation using ion-exchange chromatography and electro-chemical detection, acetonitrile or methanol is often added to the mobile phase as organic modifiers to improve the separation [26,27]. However, interferences have been demonstrated on As signals with HPLC–HG–AFS system upon the presence of organic solvents, such as methanol, even at low concentration, which can be attributed to the formation of a fine aerosol of organic solvent, which may be carried further toward the atomizer and then the arsine atomization [28]. Therefore, the use of organic solvents in the mobile phase for arsenic speciation by ion-exchange chromatography HG–AFS system should be avoided. In this study, a phosphate buffer was used for the ion-exchange separation of arsenic species which allowed the separation of four arsenic species and showed full compatibility with the HG–AFS system. To obtain the best separation and detection, the effects of KH_2PO_4 concentration in mobile phase were investigated and the pH of the mobile phase was tested over the range of 5.0–8.0. Baseline separations as shown in Fig. 3 were achieved for the arsenic species by using 15 mmol/L of KH_2PO_4 solution with pH at 5.9.

Table 2
Precision, accuracy and recoveries of As(III), DMA, MMA and As(V) in plasma by HPLC–HG–AFS method.

Arsenic species	Spiked (ng/mL)	Found (ng/mL) (mean ± SD, $n = 5$)			Overall mean (ng/mL)	Intra-batch RSD (%)	Inter-batch RSD (%)	RE (%)	Recovery (%) (mean ± SD, $n = 5$)
		Batch 1	Batch 2	Batch 3					
As(III)	2.5	2.46 ± 0.23	2.18 ± 0.04	2.31 ± 0.15	2.32	5.91	15.03	−7.78	84.0 ± 5.7
	25	25.50 ± 0.46	23.47 ± 2.81	26.63 ± 0.51	25.20	6.62	10.98	0.79	83.2 ± 6.4
	100	93.26 ± 2.46	94.51 ± 0.96	98.60 ± 1.41	95.45	1.81	5.07	−4.55	85.6 ± 4.4
DMA	3.1	3.45 ± 0.20	3.28 ± 0.31	3.12 ± 0.34	3.30	8.81	3.67	5.70	87.7 ± 3.6
	25	23.96 ± 1.14	24.23 ± 1.81	25.41 ± 1.20	24.28	5.85	5.83	−2.87	93.1 ± 6.6
	100	102.75 ± 1.77	99.96 ± 1.78	98.58 ± 3.19	100.48	2.00	2.08	0.48	89.8 ± 1.7
MMA	2.7	2.74 ± 0.09	2.67 ± 0.10	2.70 ± 0.18	2.71	3.27	6.10	0.68	82.0 ± 2.8
	22	19.65 ± 0.07	20.62 ± 0.12	20.45 ± 0.40	20.24	2.39	2.77	−5.88	82.4 ± 1.9
	86	79.63 ± 0.42	79.84 ± 0.52	84.23 ± 13.67	81.23	7.52	9.92	−5.57	87.0 ± 0.6
As(V)	5	4.83 ± 0.21	4.92 ± 0.01	5.33 ± 0.03	5.03	0.71	7.54	0.51	82.7 ± 3.4
	50	49.85 ± 1.00	48.45 ± 2.41	47.17 ± 1.11	48.98	3.38	3.91	−3.02	84.3 ± 6.2
	100	99.59 ± 1.20	100.31 ± 1.10	102.43 ± 1.21	100.78	1.15	2.07	0.78	83.1 ± 1.3

The concentrations and flow rates of HCl and KBH_4 solutions were optimized since they affected the efficiency of the hydride generation. The HCl solution was tested in the range from 0.6 to 1.8 mol/L at a flow rate of 3.2 mL/min, and the KBH_4 solution was investigated from 0.18 to 0.56 mol/L at a flow rate of 4.8 mL/min. Results showed that the largest responses were obtained when the concentrations of HCl and KBH_4 solutions were of 1.2 and 0.37 mol/L, respectively. The flow rate of argon that used as carrier and auxiliary gas was demonstrated to influence the peak responses. Results showed that the flow rates, 200 mL/min for carrier gas and 900 mL/min for auxiliary gas, produced the maximum peak responses, thus chosen for subsequent experiments.

3.3. Method validation results

Under the proposed HPLC–HG–AFS conditions, good separations were achieved between the arsenicals with no obvious interferences from endogenous substances. Typical chromatograms are shown in Fig. 3 for blank plasma, blank plasma spiked with As(III), DMA, MMA and As(V) at LLOQs, and plasma samples collected 1 h after oral administration of NHJDP and Realgar.

Calibration curves were obtained with linear regression over the ranges of 1.25–200, 1.56–200, 1.34–172, and 2.50–200 ng/mL for As(III), DMA, MMA and As(V), respectively. And the corresponding limits of detection were 0.80, 1.00, 0.86 and 2.00 ng/mL. Suitable weighting factors were selected for linear regression because the homoscedasticity tests by plotting residuals vs. concentration and *F*-tests demonstrated the heteroscedasticity. Empirical weights of $1/Y$, $1/C$, $1/Y^2$ and $1/C^2$ were evaluated. The best weighting factor was chosen according to the percentage relative error (%RE), which compares the regressed concentration computed from the regression equation obtained for each weighting factor, with the nominal standard concentration [29]. Results showed that the weighting factor of $1/C^2$ gave the least sum of absolute %RE across the whole concentration range. Thus $1/C^2$ was selected as the weighting factor. Typical equations were: $Y = 144.3C + 94.32$ ($r = 0.9989$), $Y = 111.7C + 9.220$ ($r = 0.9993$), $Y = 204.9C + 155.4$ ($r = 0.9964$), $Y = 84.27C + 31.67$ ($r = 0.9987$) for As(III), DMA, MMA and As(V), respectively, where C is the concentration of the arsenicals in the plasma (ng/mL) and Y is the corresponding peak area. For all the calibration curves obtained during validation, the bias of each back-calculated value at the LLOQ was all within ±20%, and the biases were all within ±15% for other levels above the LLOQ with the correlation coefficient (r) >0.990. Therefore, the calibration curves would be acceptable even the intercepts were non-zero. The proposed method is sensitive and suitable for the characterization of the pharmacokinetic profiles of the arsenic species in the beagle dogs.

The precision and accuracy data are shown in Table 2. Good repeatability and accuracy were obtained with the proposed method for all the analytes at each QC levels with the extraction recovery over 80%. The intra- and inter-batch precisions were ranged from 0.71% to 8.81% and 2.07% to 15.03%, respectively.

The stability test results are displayed in Table 3. The arsenic species in stock solutions were stable under the specified conditions for at least 90 days. The four species of As is stable in the solvent evaporation process for the evaporation recovery of the four arsenic species at each QC levels were over 80% and no evidence of species inter-conversion was observed. The arsenic species spiked in blank dog plasma were found to be stable at −80 °C for 60 days, after three freeze–thaw cycles, and on the bench top at room temperature for at least 6 h. The post-preparative samples were stable

Table 3
Stability of As(III), DMA, MMA and As(V) in dog plasma (mean ± SD, $n = 3$).

Analyte	Added (ng/mL)	Found (ng/mL)				
		At −80 °C for 60 days	After three freeze–thaw cycles	On the bench top at room temperature for 6 h	Post-preparative at room temperature for 6 h	Post-preparative in the autosampler at 4 °C for 12 h
As(III)	2.5	2.23 ± 0.08	2.47 ± 0.11	2.45 ± 0.04	2.36 ± 0.09	2.34 ± 0.07
	100	90.9 ± 6.2	95.6 ± 2.7	95.2 ± 0.5	93.7 ± 2.6	94.1 ± 1.0
DMA	3.1	2.79 ± 0.08	3.01 ± 0.05	2.89 ± 0.14	2.89 ± 0.09	2.86 ± 0.10
	100	93.0 ± 9.4	100.1 ± 0.5	91.1 ± 3.6	94.8 ± 3.5	95.0 ± 0.6
MMA	2.7	2.17 ± 0.25	2.25 ± 0.18	2.59 ± 0.21	2.38 ± 0.18	2.50 ± 0.09
	86	73.76 ± 4.06	92.5 ± 2.4	76.47 ± 1.40	80.4 ± 4.8	78.75 ± 11.55
As(V)	5	4.84 ± 0.16	5.03 ± 0.31	4.96 ± 0.05	4.93 ± 3.7	4.90 ± 0.14
	100	100.5 ± 5.8	99.6 ± 3.5	99.0 ± 2.8	98.1 ± 3.7	97.5 ± 2.5

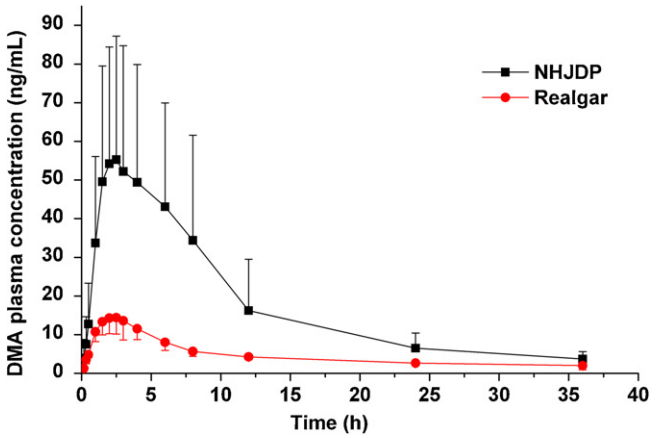

Fig. 4. Plasma concentration–time profiles of DMA in beagle dogs after single oral dose of 24.32 mg/kg Realgar (equivalent to 11.31 mg As/kg) (●) and 380 mg/kg NHJDP (equivalent to 28.45 mg As/kg) (■).

Table 4
Pharmacokinetic parameters of DMA in beagle dogs after single oral administration of 24.32 mg/kg Realgar (equivalent to 11.31 mg As/kg) and 380 mg/kg NHJDP (equivalent to 28.45 mg As/kg) (mean ± SD, $n = 6$).

Parameters	Realgar	NHJDP
C_{max} (ng/mL)	14.7 ± 4.2	57.0 ± 32.0
T_{max} (h)	2.4 ± 0.5	2.5 ± 0.5
$t_{1/2}$ (h)	16.2 ± 7.9	9.4 ± 2.2
$MRT_{0-\infty}$ (h)	22.4 ± 11.4	13.6 ± 3.0
AUC_{0-36} (ng h/mL)	151.1 ± 12.9	635.9 ± 418.2
$AUC_{0-\infty}$ (ng h/mL)	206.0 ± 44.5	687.2 ± 425.1
C_{max}/dose (ng/mL)/(mg/kg)	1.4 ± 0.4	2.1 ± 1.1
AUC_{0-36}/dose (ng h/mL)/(mg/kg)	13.2 ± 3.0	23.9 ± 14.7
$AUC_{0-\infty}$/dose (ng h/mL)/(mg/kg)	17.2 ± 3.9	25.4 ± 14.9

at room temperature for at least 6 h and at 4 °C in the autosampler for at least 12 h.

The reported toxic blood-plasma/serum concentration range of total arsenic in human is 50–250 ng/mL [30]. However, to the best of our knowledge, there has been no information concerning minimal toxic plasma concentration of As(III) and MMA in human or other mammals. In a clinical pharmacokinetic study of arsenic trioxide administered intravenously at a daily dose of 0.08 mg/kg in a patient, the serum concentration ranges of 5.8–13.1 ng/mL for As(III), 5.5–13.2 ng/mL for MMA and 5.1–14.4 ng/mL for DMA [12]. In patients at the recommended arsenic trioxide intravenously dose of 0.15 mg/kg per day, the plasma concentration range of As(III) was 5–28 ng/mL [11]. Therefore, it is quite possible that the proposed method might be sensitive enough for monitoring the plasma levels of the arsenic species in pharmacokinetic and toxicological studies.

3.4. Pharmacokinetic parameters

After oral administration of Realgar or NHJDP, only DMA and As(V) were detected in the dog plasma. DMA was found to be the predominant species; with plasma levels detectable from the first sampling time point (0.17 h after dosing) onwards up to 36 h after dosing. The plasma concentration–time profile of DMA is illustrated in Fig. 4. The main pharmacokinetic parameters of DMA estimated are shown in Table 4. However, As(V) was detectable in plasma only from time points 0.5 h to 2.5 h after dosing. It was not possible to generate a full concentration–time profile to estimate the pharmacokinetic parameters for As(V), because of its relatively low concentrations and the fast eliminating features.

The majority of mammals have been found to metabolize inorganic arsenic into methylarsonic acid (MMA) and dimethylarsinic acid (DMA) via methylation [31]. It has been suggested that the methylation of arsenic in the body is the main detoxification pathway, since relative acute toxicity (rat, oral) decreases from inorganic arsenite (LD_{50}, 15 mg/kg) and arsenate (LD_{50}, 112–175 mg/kg) to MMA (LD_{50}, 700 mg/kg) and DMA (LD_{50}, 2600 mg/kg) [32–34]. In the present study, no traces of MMA or more toxic As(III) were detected at any sampling time points. DMA and As(V) with much less toxicity were the species that appeared in the systematic circulation of beagle dogs following Realgar and NHJDP oral administration.

DMA reached the maximum plasma concentrations of 57.0 ± 32.0 and 14.7 ± 4.2 ng/mL following NHJDP and Realgar administration with the AUC_{0-36} as (635.9 ± 418.2) and (151.1 ± 12.9) ng h/mL, respectively. By taking the doses (Table 4) into account, C_{max} and AUC values were normalized by the equivalent As doses, i.e. milligrams of total arsenic per kilogram of body weight. For Realgar, the equivalent As dose was 11.31 mg As/kg and that for NHJDP was 28.45 mg As/kg. The normalized peak plasma concentration of DMA (C_{max}/dose) for NHJDP administration was increased in comparison with Realgar, since they were (2.1 ± 1.1) and (1.4 ± 0.4) (ng/mL)/(mg/kg), respectively, while the AUC_{0-36} of DMA did not have significant statistical differences. However, the elimination of DMA was faster with NHJDP administration than with Realgar alone since a decreased $t_{1/2}$ was observed for NHJDP. The time to maximum concentration of DMA was similar between Realgar and NHJDP administration.

Realgar contains 90% arsenic disulfide (As_2S_2) or tetra-arsenic tetra-sulfide (As_4S_4), which is poorly soluble in water and most organic solvents, and is often contaminated with small amounts of soluble arsenics such as As_2O_3, which is toxic. Therefore, as specified in the Chinese Pharmacopeia before it was put in pharmaceutical use, Realgar must be processed by a traditional grinding-in-water procedure known as "*Shui Fei*" to produce ultrafine particles and to remove the water soluble toxic contaminants [35]. The Realgar used for the preparation of NHJDP and for the present study were all processed with water-ground, and the total soluble arsenic (As(III) + As(V)) (Table 1) in the extract were all with the pharmacopeia limits (0.53% for Realgar and 0.066% for NHJDP) and thus would not be likely to cause toxic effects under the dose recommended by the Pharmacopeia.

In mammals, inorganic arsenic administered is metabolized by the consecutive reduction and methylation reactions. As(V) is first reduced to As(III), which is methylated to MMA; MMA is reduced to monomethylarsonous acid (MMA(III)), which is methylated to DMA, and DMA is finally reduced to dimethylarsinous acid (DMA(III)). The lifetime of DMA(III) is short because it is efficiently taken up by red blood cells [36]. Although the metabolism pathway of As(III) and As(V) has been studied in depth, the mechanisms of absorption and transportation of Realgar particles are quite different from As(III) and As(V) [37]. Little is known at present on the metabolism of Realgar and it is ambiguously described based on the knowledge of soluble arsenic. The total soluble arsenic (As(III) + As(V)) in the extract of Realgar and NHJDP come mainly from the small fraction of soluble contaminants, accounting for less than 0.2% were not the major active components. Although the solubility of Realgar is very poor, small fractions of the sulfide can still be leached by the body fluid in the presence of large amount of enzymes and the transformation of As_4S_4 in vivo is a complex process. Therefore, the mechanism of Realgar's action could not be simply regarded as the action of soluble arsenic in Realgar. Linear pharmacokinetic of total arsenic following oral administration of Realgar has been demonstrated in rats over a wide range of doses (clinical equivalent dose to toxic dose) [38,39], which would, in a sense, allow normalization of PK parameters of DMA by total As for a rough comparison of the values, but still have drawbacks and

thus there is a need for further investigation into the metabolism of Realgar.

The observed changes in the pharmacokinetic parameters suggested that the extent of absorption and elimination of As from Realgar might be altered by other ingredients in the compound prescription of NHJDP. Since Realgar is commonly prescribed in compound Chinese medicines, the amount of arsenic leached from the Realgar-containing compound might be increased or reduced by the coexisting components and therefore the arsenic absorption and metabolism might be altered. In vitro dissolution studies showed that the leaching of the arsenic from a Realgar-containing compound *Liu Shen Wan* was enhanced [40], while from a compound *An Gong Niu Huang Wan* less arsenic was leached than that from Realgar [17]. However, in vitro studies do not necessarily reflect the events taking place in vivo. For better understanding of how the components exert their effect on Realgar a great deal of work remains to be done.

4. Conclusion

An HPLC–HG–AFS method for the determination of arsenic species of As(III), DMA, MMA and As(V) in dog plasma was established and validated, and successfully applied to the pharmacokinetic study of the arsenicals after single oral administration of Realgar and NHJDP in beagle dogs. DMA and As(V) were detected in dog plasma following ingestion of Realgar and NHJDP. The different pharmacokinetic behavior of DMA between Realgar and NHJDP administration might be attributed to the drug–drug interactions among the chemical constituents in the compound preparation of NHJDP. The obtained information might be useful for the understanding of the compatibility mechanisms of arsenic-containing compound TCMs.

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