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Simultaneous determination of diallyl trisulfide and diallyl disulfide in rat blood by gas chromatography with electron-capture detection

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A simple, rapid and sensitive method has been developed and validated for the simultaneous quantification of diallyl trisulfide (DATS) and diallyl disulfide (DADS) in rat blood by gas chromatography with electron-capture detection. The analytes were prevented from degradation by addition of acetonitrile and extraction with hexane before gas chromatographic separation. Two calibration curves for DATS were linear over the range of 10–500 ng/mL and 0.2–20 μg/mL, with typical r values of 0.9989 and 0.9993, respectively. Similarly, two calibration curves for DADS were linear in the concentration range of 50–5000 ng/mL and 1–30 μg/mL, with typical r values of 0.9989 and 0.9983, respectively. The limit of detection was less than 10 ng/mL for DATS and 50 ng/mL for DADS, and the assay was highly reproducible, considering the intra-, inter-day relative standard deviations (R.S.D.) below 12%. The developed procedure was successfully applied for the evaluation of the pharmacokinetics of garlic oil following iv administration at a single dose (10 mg) of garlic oil in rats. The results show that the developed method is suitable for pharmacokinetic and therapeutic purposes of DATS and DADS.

1. Introduction

Garlic (*Allium sativum* L.) is widely used as a foodstuff and is known to exhibit a variety of biological activities including hypolipidemic, antithrombotic, antiatherosclerotic, antimutagenic, anticarcinogenic and antibacterial effects (Agarwal KC, 1996). It contains a variety of volatile oils which are pharmacologically active (Lau et al. 1990; Yang et al. 1994; Bordia et al. 1996; Drouin 1999; Salman et al. 1999) and have been identified by means of gas chromatography (Yu et al. 1989). Among these organosulfur compounds (OSCs), diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS) are the three major components. DATS, an oil soluble constituent of garlic (*Allium sativum* L.), has been chemically synthesized (Buckman et al. 1967).

A great deal of effort has been devoted to measuring the levels of some of OSCs *in vitro* and *in vivo*. Lachmann et al. (1994) investigated the pharmacokinetics of the garlic constituents alliin, allicin and vinyldithiine using S^{35} labeling, but this could not distinguish between prototype and its metabolites and was harmful to human volunteers. Arnault et al. (2005) developed a new ion-pair HPLC method to quantify OSCs for evaluation of the garlic therapeutic potential and for validation of a new formulation while Rosen et al. (2001) determined allicin, S-allylcysteine and volatile metabolites of garlic in breath, plasma or simulated gastric fluids by HPLC, or HPLC- and GC-mass spectrometry (MS). However, these methods lack the sensitivity and selectivity required for determining contents of DATS and DADS in a small-volume sample *in vivo* and also are laborious and time-consuming. Mass spectrometry is expensive and hence often not readily accessible in general laboratories.

The aim of the present study was to establish a validated GC-ECD method with a lower limit of quantitation enough to support pharmacokinetic and bioequivalence studies of DATS and DADS and also allow for a modest sample throughput of 50 per day routinely. The method reported in this paper is an accurate and precise GC-ECD method to simultaneously quantify DATS and DADS in rat blood using liquid-liquid extraction. This validation procedure was undertaken strictly in accordance with FDA guidelines.

2. Investigations and results

2.1. Selectivity and chromatography

The separation achieved using the experimental conditions in the present assay for DATS and DADS is shown in Fig. 1. Selectivity was indicated by the absence of any endogenous interference at the retention times of the peaks of interest as evaluated by chromatograms of blank rat blood and blood spiked with the two compounds. The retention times for DADS, DATS and internal standard (I.S.) were 4.5, 9.8 and 12.9 min, respectively.

2.2. Linearity

Six point calibration curves on three separate days were linear over the concentration range of 10–500 ng/mL and

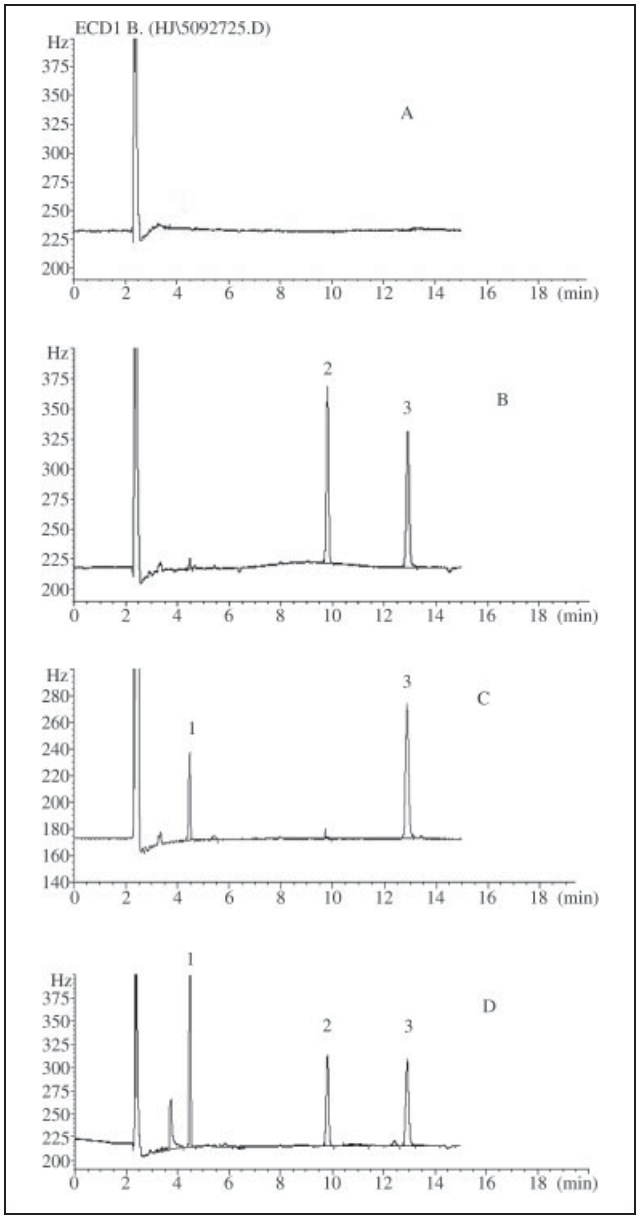

Fig. 1: Chromatograms of (A) blank rat blood, (B) blood spiked with 75 ng/mL of DATS and100 ng/mL internal standard, (C) blood spiked with 300 ng/mL DADS and 100 ng/mL internal standard, (D) blood sample of a rat 15 min after administration of 0.5 mL garlic oil injection (containing about 10 mg garlic oil) via jugular vein cannula. Peak 1. DADS; Peak 2. DATS; Peak 3. I.S

0.2–20 μg/mL for DATS and of 50–5000 ng/mL and 1–30 μg/mL for DADS, respectively. The typical standard curves were: I for DATS, $C = 209.31\ A_i/A_s - 5.684$, $r = 0.9989$; II for DATS, $C = 8.08\ A_i/A_s - 0.094$, $r = 0.9993$; III for DADS, C, $2190.2\ A_i/A_s - 21.95$, $r = 0.9989$; IV:$C = 69.98\ A_i/A_s - 2.005$, $r = 0.9983$, where C, A_i and A_s represent the blood drug concentration, peak area of DATS and I.S., respectively.

2.3. Lower limit of quantitation

Lower limit of quantitation (LLOQ), defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was evaluated by analyzing blood samples which were prepared in five replications, with 10 ng/mL for DATS and 50 ng/mL for DADS.

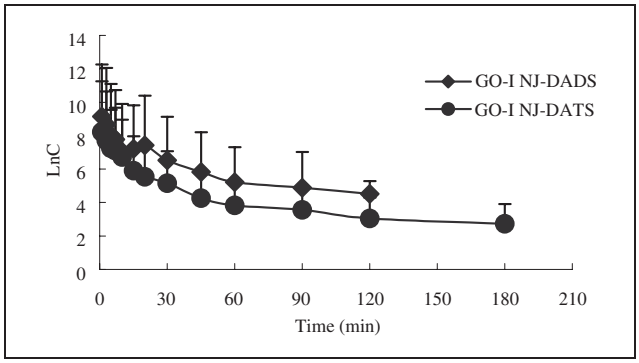

Fig. 2: Mean blood concentration logarithm-time curves of DATS and DADS after iv administration at a single dose of GO injection (containing 10 mg GO) in six rats

2.4. Recovery, accuracy and precision

The results for the validation of the method in rat blood are listed in Table 1. The method was accurate and precise and the accuracy at three concentrations ranged from 88.0 to 114.1% for DATS and 92.0 to 112.2% for DADS. The intra- and inter-day precision ranged from 2.8 to 7.5% and 5.2 to 10.8% for DATS, and 1.2 to 7.76% and 3.7 to 11.3% for DADS, respectively. The absolute recoveries ranged from 83.4 to 93.9% for DATS and 89.6 to 96.7 for DADS, respectively.

2.5. Stability of DATS and DADS in rat blood

The stability results showed that DATS and DADS in rat blood stored at room temperature, −20 °C or after freeze-thaw cycles were unstable, while they were stable in the treated blood samples by the modified method (seen item 3.3) and the requirement for analysis was met (Tables 2–4). So the blood samples had to be transferred into acetonitrile immediately after collection from rats in order to stop degradation of DADS and DATS in blood samples.

2.6. Application of the method

The validated method has been successfully used to determine the blood concentrations of DATS and DADS after iv administration to 6 rats at a single dose of 10 mg garlic oil. The mean plasma concentration-time curves of DATS and DADS are shown in Fig. 2. The pharmacokinetic parameters of DATS and DADS calculated by statistical moment analysis using the Drug And Statistics ver 2.0 (DAS ver 2.0), are summarized in Table 5.

3. Discussion

It is difficult to determine DATS and DADS by many usual methods due to their low UV absorption, volatility and instability. It is not possible to determine DATS and DADS in vivo by common HPLC methods using an UV detector, or even by HPLC-MS. A significant amount of DATS and DADS is lost with the organic solvent during evaporating to dryness under a stream of nitrogen. DATS and DADS are also so unstable that it is not possible to obtain plasma by centrifugation. The sample has to be mixed with acetonitrile directly after collecting samples to prevent degradation, followed by extraction into hexane. In addition, the compounds contain a highly electronegative group, –S–, which is very sensitive to ECD. The LLOQ can reach 1 ng/mL or lower if the compound is directly added to hexane or another suitable organic sol-

Table 1: Intra- and inter-day variability, accuracy, and recovery for determination of DATS and DADS

Concentration	Interday variability		Intraday variability		Recovery (n = 3)	
	R.S.D. (%)	Accuracy	R.S.D. (%)	Accuracy	%	R.S.D. (%)
Curve I (ng/mL)						
20	10.8	105.4	6.60	114.1	85.3	10.1
200	6.56	92.5	3.71	90.3	87.5	5.9
400	5.35	91.9	2.83	88.0	83.5	4.8
Curve II (μg/mL)						
0.2	6.74	110.0	7.51	105.0	83.4	6.3
5	5.29	106.8	3.01	104.2	88.6	4.9
20	5.74	112.5	6.02	109.5	93.9	1.7
Curve III (ng/mL)						
50	11.34	112.2	5.78	102.4	95.6	7.2
500	8.32	104.6	7.76	103.4	94.3	4.6
5000	5.75	100.3	1.25	96.5	96.7	3.2
Curve IV (μg/mL)						
1	5.02	98.2	6.90	92.0	90.9	6.1
15	5.40	95.2	4.77	95.5	89.6	5.6
30	3.77	88.7	4.26	92.6	91.8	3.4

Table 2: Stability of DATS and DADS in rat blood stored at room temperature (n = 3)

	Time (h)	Before precipitated by acetonitrile		after precipitated by acetonitrile	
		Concentration (μg/mL)	Degradation (%)	concentration (μg/mL)	Degradation (%)
DATS	0	1.06	–	1.06	–
	3	0.01	99.5	1.05	0.9
	6	0	100	1.04	1.9
	12	0	100	1.03	2.8
DADS	0	5.02	–	5.02	–
	3	1.26	74.9	4.98	0.80
	6	1.03	79.5	5.01	0.20
	12	0.68	86.5	4.99	0.60

Table 3: Stability of DATS and DADS in rat blood stored at −20 °C (n = 3)

Time (d)	DATS		DADS	
	Concentration (μg/mL)	Degradation (%)	concentration (μg/mL)	Degradation (%)
0	1.06	–	5.02	–
5	0.13	87.7	0.26	94.82
10	0.12	95.3	0.22	95.62
30	0.03	97.2	0.05	99.00

Table 4: Stability of DATS and DADS after three freeze-thaw cycles (n = 3)

freeze-thaw cycles	DATS		DADS	
	concentration (μg/mL)	Degradation (%)	concentration (μg/mL)	Degradation (%)
0	1.06	–	5.02	–
1	0.11	89.6	0.64	87.25
2	0.03	97.2	0.32	93.63
3	0.01	99.1	0.12	97.61

Table 5: Pharmacokinetic parameters of DATS and DADS after iv administration at a single dose of GO injection (containing 10 mg GO) in six rats

Parameters	T_{max} (min)	C_{max} (ng/mL)	$AUC_{0\text{-}t}$ (mg/L*min)	$MRT_{0\text{-}t}$ (min)
DATS	1.0	3692.8	31945.0	7.3
DADS	1.0	9335.8	91087.9	19.5

vent for DATS. Accordingly, it is possible to measure it even after several dilutions during the extraction procedure.

Since GO contained multi-constituents volatile oils, only one indicator cannot reflect its whole characteristics in the *in vivo* study. Therefore DATS and DADS, which are the major active components and the content of which in total GO are higher than 70%, were selected as indicators and simultaneously determined by GC-ECD in the pharmacokinetic study. Fig. 2 indicates that DATS and DADS rapidly decreased *in vivo*. It was noted that the DADS profile of GO-SLN descended first, and then rose slightly and decreased again, implying that DATS was transformed into DADS and so the concentrations of DADS were controlled by the rate of elimination and biotransformation.

The described method was established as a rapid analytical tool for GO pharmacokinetic study, requiring a short retention time, high precision, sensitivity and small-volume blood for analysis. The parameters of the assay validation are satisfactory and suitable for the standard biological analysis. This simple preparation procedure based on a one-step extraction and a total run time of 15.0 min, allows for a modest sample throughput of 50 per day routinely.

4. Experimental

4.1. Materials

Standard drug of diallyl trisulfide (DATS) was purchased from Shangdong Jinxiang Food and Medicine Co.Ltd. (the original purity is about 90%, but this can be increased to 97% after refining). Diallyl disulfide (DADS, purity 80%, remainder composed of other allyl sulfides but it can reach higher than 99% after refining) was obtained from Sigma-Aldrich, Steinheim (Belgium), and the garlic oil Injection was prepared in our lab. In addition, 1,4-dichloronitrobenzene (I.S.) and the other organic solvents used were all of analytical-reagent grade (Shenyang Chemical Reagents Co. Ltd., China.)

4.2. Equipment and method development

Analyses were performed on a Agilent 6890N gas chromatograph equipped with an ^{63}Ni electron-capture detector. A fused-silica capillary column (30 m × 0.25 mm I.D.) was used coated with a 0.25 μm thick film of 5% phenyl methyl siloxane (Agilent, USA) as the stationary phase. The conditions for gas chromatographic separation were described as follows. The oven temperature was set at 120 °C and maintained for 15 min. Temperatures at the injection port and detector were 180 °C and 250 °C, respectively. Ultra-pure nitrogen (purity > 99.999%, Shenyang Kerui Special Gases Co. Ltd., China) was used as a carrier gas and make-up gas at flow-rates of 1 mL/min and 60 mL/min, respectively. All injections were carried out in the split injection mode with a split ratio of 1 : 10.

4.3. Blood sample preparation

A 0.2-mL of rat blood was transferred to a 5-mL of glass test tube, and then 20 μL of 10% (v/v) dilute hydrochloric acid was added followed by brief mixing on a YKH- vortex-mixer (Jiangxi Medical Appliance. Co. Ltd., China). Next, 0.2 mL of acetonitrile containing I.S. (100 ng/mL or 2 μg/mL) was added and the tube vortexed immediately. Hexane (0.4 mL or 1 mL) was added, and the tube was capped and shaken vigorously for 0.5 min then centrifuged at 2000 × g for 5 min in a bench-top centrifuge (TDL-5, Shanghai Anting. Medical Appliance Co. Ltd., China). Following this, 1 μL of the organic layer was used for gas chromatographic analysis.

4.4. Preparation of calibration standards

Stock solutions of DATS (1 mg/mL), DADS (10 mg/mL) and I.S. (100 ng/mL or 2 μg/mL) were prepared in methanol. Working solutions were further diluted daily with methanol and stored at 4 °C. The standard curves for DATS (10–500 ng/mL, or 0.2–20 μg/mL) and DADS (50–5000 ng/mL and 1–30 μg/mL) were freshly prepared on the day of analysis by adding 10 μL of the appropriate working solutions to 0.2 mL of drug-free rat blood. Calibration curve I was obtained with standard DATS of final concentrations of 10, 20, 40, 100, 200 and 500 ng/mL in rat blood and calibration curve with those of 0.2, 0.5, 1, 5, 10, and 20 μg/mL. Accordingly, Calibration curve was performed with standard DADS of the final concentrations of 50, 100, 200, 500, 1000, 5000 ng/mL and Calibration curve II with those of 1, 5, 10, 15, 20, 30 μg/mL. Quality control (QC) samples were prepared in bulk at concentrations of 20, 100 and 400 and 0.2, 5, 20 μg/mL DATS and 50, 500, 5000 ng/mL and 1, 15, 30 μg/mL DADS stored at 4 °C.

4.5. Accuracy, precision, lower limit of quantitation (LLOQ) and recovery

Accuracy, inter- and intra-day precisions of the method were determined for each compound according to FDA guidance for industry bioanalytical method validation. Three replicate spiked blood samples were subjected to inter- and intra-day assay at different low, medium, and high concentrations (20, 100, 400 ng/mL and 0.2, 1, 10 μg/mL for DATS, 50, 500, 5000 ng/mL and 1, 15, 30 μg/mL for DADS) of each analyte. The concentrations were calculated using calibration curves prepared and analyzed in the same run. Accuracy was calculated as the deviation of the mean from the nominal concentration. Inter- and intra-day precision was expressed as the relative standard deviation of each calculated concentration. For the concentration to be accepted as the LLOQ the percentage deviation from the nominal concentration (accuracy) and the relative standard deviation were less than or equal to 20%, respectively, under the condition of at least five times the response compared with the blank response. Average recovery of each compound was determined by comparing peak area obtained after injection of the processed QC samples with those achieved by direct injection of the same amount of drug in distilled water at different concentrations (three samples for each concentration level).

4.6. Stability of DATS and DADS in rat blood

Blood samples of the final concentration 1 μg/mL of DATS and 5 μg/mL of DADS were prepared for being quantitated stored in different conditions, such as room temperature, −20 °C and three freeze-thaw cycles. The same samples after precipitated by acetonitrile and extracted by hexane were quantitated by the above-mentioned method.

4.7. Application of the method

Six Wistar rats (260–300 g) were used in the experiments. The animals were housed in hanging wire cages with free access to food and water and a 12 h light/dark cycle (lights on at 6 a.m.) at a temperature of 19–20 °C. The experimental protocol was designed according with the guidelines of the Chinese Council on Animal Care and approved by General Hospital of Shenyang Military Region Animal Care Committee. For the experiments, 0.5 mL of garlic oil solution (containing 10 mg garlic oil) was administered via a jugular vein cannula. Blood samples were taken from the rats at predetermined intervals and processed immediately.

4.8. Calculation of pharmacokinetic parameters

Blood drug concentration-time curves of DATS and DADS were evaluated by statistical moment analysis using the Drug And Statistics ver2.0 (DAS ver2.0). The maximum blood concentration (C_{max})and the time to C_{max} (T_{max}) were obtained directly from the individual blood concentration versus time curves. The area under blood concentration-time curve up to the last quantifiable blood concentration (AUClqc) was determined by the linear trapezoidal method.

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