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Simultaneous Determination of Tolbutamide and Hydroxytolbutamide in Rat Plasma After Acute Hydrogen Sulfide Poisoning by Liquid Chromatography-Mass Spectrometry

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***SUMMARY*. Hydrogen sulfide (H_2S) is a natural decaying product of organic matter, which is highly toxic as a result of environmental and industrial exposure. A sensitive and selective liquid chromatography-mass spectrometry (LC-MS) method for determination of tolbutamide and its metabolite hydroxytolbutamide in rat plasma was developed and validated. We take the method of acetonitrile precipitation to extract the analytes and internal standard carbamazepine from plasma. The chromatographic separation was performed on a Zorbax SB-C18 column (150 × 2.1 mm, 5 μm), using acetonitrile-0.1% formic acid as the mobile phase with gradient elution, delivered at a flow-rate of 0.4 mL/min. Electrospray ionization (ESI) source was applied and operated in positive ion mode, and selected ion monitoring (SIM) mode used to quantify tolbutamide and its metabolite hydroxytolbutamide. Calibration curves were linear in the concentration ranges of 20-5000 ng/mL for tolbutamide and 5–500 ng/mL for hydroxytolbutamide, with a lower limit of quantification (LLOQ) of 20 ng/mL for tolbutamide and 5 ng/mL for hydroxytolbutamide. This developed method was successfully used for determination of tolbutamide and its metabolite hydroxytolbutamide in rat plasma after acute hydrogen sulfide poisoning for pharmacokinetic study. The main pharmacokinetic parameters of tolbutamide and its metabolite hydroxytolbutamide had no significantly different between acute hydrogen sulfide poisoning and control rats. The findings of this study suggest that acute hydrogen sulfide poisoning have no effect on the activity of CYP2C9 enzyme.**

INTRODUCTION

Cytochrome P450s superfamily (CYPs), one kind of biological activities enzyme, which involved in varieties of redox reaction, is widely in organisms are known to play an important role in the biotransformation of many endogenous and exogenous substances [1-3]. CYP2C9 is one of microsomal P450s in subfamily 2C that contributes extensively to the hepatic metabolism of therapeutic drugs, and it is also expressed at a significant level in the human small intestine [4]. Thus, the enzymatic activity of CYP2C9 is clinically important for the drug therapy. For example, warfarin and phenytoin is the substrates of CYP2C9, which have low therapeutic margins, these drugs could become toxic even at the normal therapeutic doses, when the activity of CYP2C9 was inhibited as a result of drug-drug or drug-food interactions [5]. Hence, it is important to evaluate the activity of CYP2C9. Tolbutamide is a first generation oral sulphonylurea hypoglycaemic agent, used in the treatment of non-insulin dependent diabetes since the 1960s; it is particularly important in clinical pharmacological research. The metabolism of tolbutamide in humans has been extensively studied, as it is one of the probe drugs for examination of genetic polymorphism in drug oxidation [6], and it is a probe drug for assessing the degree of CYP2C9 enzyme inhibition or induction that may affect the capacity of an individual to oxidize drugs [7-9].

Hydrogen sulfide (H2S) is a natural decaying product of organic matter, which is highly toxic as a result of environmental and industrial exposure [10]. It is one of the major toxic gases in

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forensic practices and is absorbed by the upper respiratory tract mucosa. Also, it causes histotoxic hypoxemia and respiratory depression by exerting an inhibitory effect on cytochrome oxidase Acute inhalation exposure to low concentrations of hydrogen sulphide may result in irritation to the mucous membranes of the eye and respiratory tract. Acute exposure to high concentrations of hydrogen sulphide results in depression of the nervous system, loss of consciousness and respiratory paralysis. Other health effects have been reported, the most sensitive being the respiratory, neurological and ocular system [11]. However, there has no research about the influence of acute hydrogen sulfide poisoning on the activity of the cytochrome P450 2C9 enzymes.

Several methods have been published for simultaneous determination of tolbutamide and its metabolites in different biological matrices such as plasma or urine for monitoring or toxicological purposes, such as high-performance liquid chromatography (HPLC) [12-17], gas chromatography-mass spectrometry (GC-MS) [18,19] and high-performance liquid chromatography-mass spectrometry (LC–MS) [20,21]. In recent years, LC-MS technique has been widely applied in the analysis of biological samples [22-24]. In this present study, we developed a sensitive and selective liquid chromatography–mass spectrometry (LC–MS) method for determination of tolbutamide and its metabolite hydroxytolbutamide in rat plasma and to investigate the effects of acute hydrogen sulfide poisoning on cytochrome P450 2C9 activities.

MATERIAL AND METHODS

Chemicals and Reagents

Tolbutamide (purity > 98.0%), hydroxytolbutamide (purity > 98.0%) and carbamazepine (IS, purity > 98.0%) were purchased from Sigma-Aldrich Company (St. Louis, USA). LC-grade acetonitrile and methanol were purchased from Merck Company (Darmstadt, Germany). While LC-grade formic acid was obtained from Tedia Company (Cincinnati, USA). Ultra-pure water was obtained in the laboratory using a Milli-Q purification system from Millipore (Bedford, USA).

Preparation of Standard Solutions

The primary standard stock solutions of tolbutamide (100 µg/mL), hydroxytolbutamide (100 µg/mL) and carbamazepine (50 µg/mL) were separately prepared in 50 mL volumetric flasks with methanol-water (50:50, *v/v*) and stored at 4 °C. Working solutions for calibration and controls were prepared from the stock solution by dilution using methanol-water (50:50, *v/v*). The IS working solution (2.0 µg/mL) was prepared by diluting its stock solution with methanol-water (50:50, *v/v*).

Calibration curves were prepared using blank plasma spiked at concentrations of 20, 50, 100, 500, 1000, 2000 and 5000 ng/mL for tolbutamide and 5, 10, 20, 50, 100, 200, and 500 ng/mL for hydroxytolbutamide. Low, medium, and high quality control (QC) samples at concentrations of 50, 500, and 4000 ng/mL for tolbutamide and 10, 50, and 400 ng/mL for hydroxytolbutamide were prepared by in a same way as the calibration standards.

Instrumentation and Conditions

The LC-MS system consisted of a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) and a Bruker Esquire HCT ion-trap mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ionization source and controlled by ChemStation software (Version *B.01.03* [204], Agilent Technologies, Waldbronn, Germany).

Chromatographic separation was achieved on a Agilent Zorbax SB-C18 (150 × 2.1 mm, 5 µm) column at 40 °C. The flow rate was 0.4 mL/min. A gradient elution programme was conducted for chromatographic separation with mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile) as follows: 0-4.0 min (10-80% B), 4.0–8.0 min (80-80% B), 8.0-9.0 min (80-10% B), 9.0-13.0 min (10-10% B).

Analyses were performed with the ESI source operated in positive mode with a drying gas (N_2) flow of 6 L/min, nebulizer pressure of 30 psi, drying gas temperature of 350 °C, capillary voltage of 1.5 kV. SIM mode was applied to quantify analyzes using target ions at *m/z* 271 for tolbutamide, *m/z* 287 for hydroxytolbutamide, *m/z* 237 for carbamazepine (IS).

Sample preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 10 µL of the internal standard working solution (2.0 µg/mL) was added to 100 µL of collected plasma sample followed by the addition of 200 µL acetonitrile. The tubes were vortex mixed for 0.5 min. After centrifugation at 14900 g for 10 min, the supernatant (2 µL) was injected into the LC-MS system for analysis.

Pharmacokinetics

Male Sprague-Dawley rats (200-220 g) were obtained from Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China) used to study the pharmacokinetics of tolbutamide and its metabolite hydroxytolbutamide after acute hydrogen sulfide poisoning. All 24 rats were housed at Wenzhou Medical University Laboratory Animal Research Center. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical University and were in accordance with the Guide for the Care and Use of Laboratory Animals. Animals were housed under controlled conditions (25 ± 1 °C, RH 55 ± 10%) with a natural light-dark cycle. They were allowed to adapt to the housing environment for at least 1 week before the study. Animals were randomly assigned to the three groups (8 rats/ group: the 0 ppm, 100 ppm and 200 ppm exposure group) at the end of the acclimation period by means of their prestudy body weight. Animals were exposed in HOPE-MED 8050-1 stainless steel and glass toxicant exposure cabinet contained within permanent 3 m^3 stainless steel and glass inhalation exposure chambers for 30 min. The target H2S exposure concentrations were 0, 100 ppm and 200 ppm. Diet was prohibited for 12 h before the experiment but water was freely available. Blood samples (0.3 mL) were collected from the tail vein into heparinized 1.5 mL polythene tubes at 0, 0.8333, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, and 48 h after intravenous administration of tolbutamide (0.75 mg/kg). The samples were immediately centrifuged at 3000 g for 10 min. The plasma obtained (100 µL) was stored at -20 °C until analysis.

Statistical analysis

Pharmacokinetic parameters were expressed as means ± SD and ANOVA with a post hoc test (differences between groups) were used. Statistical analyses were performed using SPSS statistical software, version 16.0.

RESULTS AND DISCUSSION

Method Development

The mobile phase played a critical role in achieving good chromatographic behavior (including peak symmetry and analysis time) and appropriate ionization. Acetonitrile was chosen as the organic solvent because it offers sharper peak shape and lower pressure than methanol. The introduction of formic acid into the mobile phase could improve the sensitivity, therefore acetonitrile-0.1% formic acid was chosen as mobile phase. Gradient elution provided better peak symmetry, proper retention time, and avoided the matrix effects for the analyte and IS compared to isocratic elution [23,24].

Sample preparation is a key step for the determination of drugs in biological samples. The simple and effective protein precipitation was employed in our work; it was much easier than literatures. Acetonitrile was chosen as the protein precipitation solvent because it exhibited better effect than methanol or trichloroacetic acid (10%), which could provide acceptable recoveries.

Specificity and Matrix effect

No interfering endogenous substances were observed at the retention times of the analytes and IS. The matrix effect of the assay was evaluated at 50, 500, and 4000 ng/mL for tolbutamide and 10, 50, and 400 ng/mL for hydroxytolbutamide. Three samples at each level were analyzed. The percent nominal concentrations determined were 94.1 ± 7.1%, 95.5 ± 7.1% and 93.5 ± 4.2% at each concentration level (n=3). The same evaluation was performed for hydroxytolbutamide, the percent nominal concentrations determined were 94.5 ± 8.3%, 98.4 ± 3.3% and 95.2 ± 3.1% at each concentration level (n = 3). The evaluation was also performed for IS and the percent nominal concentration was 94.3 ± 5.4% (n = 3). The results indicate that ion suppression or enhancement from the plasma matrix was negligible for this analytical method.

Linearity and Lower Limit of Quantification

Calibration curves for tolbutamide and hydroxytolbutamide were generated by linear regression of peak area ratios against concentrations, respectively. The regression equation for the calibration plot were $Y = 0.000576C + 0.012733$ with $r = 0.9954$ for tolbutamide and $Y = 0.001229C + 0.015036$ with $r = 0.9971$ for hydroxytolbutamide (Y is the peak ratio of analyte to IS, and C is the concentration of analyte in rat plasma), for concentrations in the range 20-5000 ng/mL for tolbutamide and 5-500 ng/mL for hydroxytolbutamide, respectively.

The LLOQ for tolbutamide in plasma was 20 ng/mL, the precision and accuracy at LLOQ were 10.4 and -9.5%, respectively. The LLOQ for hydroxytolbutamide in rat plasma was 5 ng/mL, the precision and accuracy at LLOQ were 11.3 and 9.1%, respectively. The detection limit, de-

fined the concentration giving a signal with signal to noise ratio of 3, was 6 ng/mL for tolbutamide and 2 ng/mL for hydroxytolbutamide in rat plasma.

Precision, Accuracy and Extraction Recovery

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three validation days. Intra-day precision and the inter-day precision were both better than 11% at each QC level. The accuracy of the method ranged from -9.3 to 7.8% at each QC level. Mean recoveries of tolbutamide and hydroxytolbutamide were better than 89.1%. The recovery of the IS (200 ng/mL) was 95.7%. Assay performance data are presented in Table 1. The above results demonstrate that the values are within the acceptable range and the method is accurate and precise.

Stability

All the stability studies were conducted at 50, 500, and 4000 ng/mL for tolbutamide and 10, 50, and 400 ng/mL for hydroxytolbutamide in rat plasma with three replicates for each concentration under different storage conditions. The RSDs of the mean test responses were within 15% in all stability tests of tolbutamide and hydroxytolbutamide in rat plasma.

Application

The validated method has been successfully used to quantify tolbutamide and its main metabolite hydroxytolbutamide concentration in the rat plasma samples after intravenous administration of 0.75 mg/kg tolbutamide. The pharmacokinetic parameters were computed by Drug and Statistics Software (DAS) 2.0. The pharmacokinetic profile of tolbutamide and hydroxytolbutamide was best described by an open non-compartment model, Table 2.

The representative tolbutamide and its metabolite hydroxytolbutamide concentration vs. time profiles of 24 rats were presented in Figs. 1 and 2. The main pharmacokinetic parameters of tolbutamide and its metabolite hydroxytolbutamide had no significantly different between acute hydrogen sulfide poisoning and control rats. The findings of this study suggest

Compound	Concentration (ng/mL)	RSD (%)		RE (%)		Recovery (%)
		Intra-day	Inter-day	Intra-day	Inter-day	
Tolbutamide	50	9.1	10.5	-8.2	3.4	89.1 ± 7.8
	500	6.5	5.4	0.8	-9.3	93.8 ± 5.6
	4000	4.5	6.3	5.3	-6.4	94.1 ± 4.7
Hydroxytolbutamide	10	10.3	5.6	7.8	5.6	89.1 ± 7.8
	50	5.4	3.4	-6.7	-7.8	93.8 ± 5.6
	400	3.8	6.8	-4.5	4.5	94.1 ± 4.7

Table 1. Precision, accuracy and recovery for tolbutamide and hydroxytolbutamide in rat plasma (n = 6).

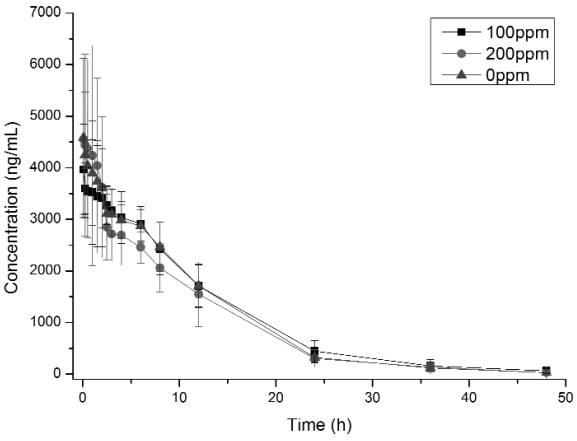

Figure 1. Mean plasma tolbutamide concentration time profile after intravenous administration of tolbutamide (0.75 mg/kg) in 24 rats.

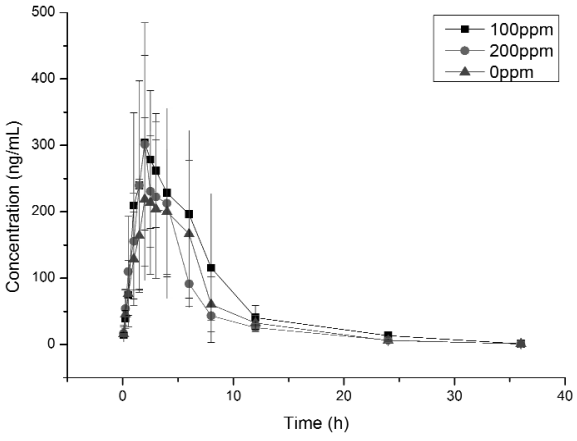

Figure 2. Mean plasma hydroxytolbutamide concentration time profile after intravenous administration of tolbutamide (0.75 mg/kg) in 24 rats.

Pharmacokinetic parameters		Tolbutamide	Hydroxytolbutamide
$t_{1/2z}$ (h)	100 ppm	6.858 ± 1.954	4.085 ± 0.481
	200 ppm	6.108 ± 1.153	4.502 ± 0.527
	0 ppm	6.336 ± 0.714	4.218 ± 0.924
t_{max} (h)	100 ppm	0.083	3.857 ± 1.215
	200 ppm	0.083	3.071 ± 0.932
	0 ppm	0.083	2.750 ± 1.414
$AUC_{(0-t)}$ (ng/mL*h)	100 ppm	51015.1 ± 7643.6	2430.2 ± 922.2
	200 ppm	45987.5 ± 10715.9	1623.9 ± 425.0
	0 ppm	49479.9 ± 6760.4	1725.1 ± 1097.1
$AUC_{(0-\infty)}$ (ng/mL*h)	100 ppm	51578.8 ± 7788.9	2436.1 ± 921.0
	200 ppm	46266.8 ± 10705.6	1629.0 ± 424.6
	0 ppm	49775.8 ± 6705.6	1730.1 ± 1099.2
$MRT_{(0-t)}$ (h)	100 ppm	9.921 ± 1.747	7.023 ± 1.244
	200 ppm	9.134 ± 1.868	5.833 ± 0.555
	0 ppm	9.139 ± 0.993	6.506 ± 1.203
$MRT_{(0-\infty)}$ (h)	100 ppm	10.442 ± 2.284	7.121 ± 1.324
	200 ppm	9.434 ± 2.125	5.960 ± 0.587
	0 ppm	9.437 ± 1.097	6.620 ± 1.259
CL (L/h/kg)	100 ppm	0.015 ± 0.003	0.340 ± 0.102
	200 ppm	0.017 ± 0.004	0.496 ± 0.164
	0 ppm	0.015 ± 0.003	0.562 ± 0.282
V (L/kg)	100 ppm	0.145 ± 0.038	2.049 ± 0.758
	200 ppm	0.150 ± 0.046	3.259 ± 1.259
	0 ppm	0.142 ± 0.037	3.314 ± 1.485
C_{max} (ng/mL)	100 ppm	4351.3 ± 1000.5	388.8 ± 231.7
	200 ppm	4996.6 ± 2096.0	364.2 ± 180.4
	0 ppm	4667.7 ± 1427.1	286.7 ± 216.1

Table 2. The main pharmacokinetic parameters after intravenous administration of 0.75 mg/kg tolbutamide in 8 rats (Mean ± SD).

that acute hydrogen sulfide poisoning have no effect on the activity of CYP2C9 enzyme. This may be related to hydrogen sulfide poisoning time is too short; therefore it did not affect the function of CYP2C9 enzyme.

CONCLUSIONS

A stable, selective and sensitive LC–MS method with positive mode ESI has been developed for the simultaneous determination of tolbutamide and its metabolite hydroxytolbutamide in rat plasma. This developed method with simple protein precipitation for sample preparation was successfully applied for determination of tolbutamide and metabolite hydroxytolbutamide in rat plasma for pharmacokinetic study. The findings of this study suggest that acute hydrogen sulfide poisoning have no effect on the activity of CYP2C9 enzyme.

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