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Development and validation of a highly sensitive LC-MS/MS method for determination of brain active agent dianhydrogalactitol in mouse plasma and tissues: Application to a pharmacokinetic study

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ABSTRACT

A sensitive and specific liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method was developed and validated for quantitative analysis of 1,2:5,6-dianhydrogalactitol (DAG) in mouse plasma and tissues. Sodium diethyldithiocarbamate (DDTC) was used as the derivatization reagent to improve its LC-MS/MS behavior. Analytes were separated on a Welch Ultimate XB-CN column with a mobile phase consisting of acetonitrile and 0.1% formic acid solution (65:35). The MS analysis was conducted by positive electrospray ionization in multiple-reaction monitoring (MRM) mode. Good linearity ($r^2 > 0.9958$) was observed over the concentration range of 1–1000 ng/mL in plasma and tissue homogenates (brain, liver, heart, spleen, lung and kidney). The intra- and inter-batch precision and accuracy of DAG in plasma and brain samples were all within the acceptable limits. The extraction recovery was stable and no significant matrix effects were observed. The method was successfully applied to study the pharmacokinetic and tissue distribution of DAG in mice after intravenous administration. DAG could cross the blood-brain barrier and had limited liver distribution. Rat primary hepatocytes *in vitro* experiments demonstrated that DAG had a safe profile in liver.

1. Introduction

Glioblastoma, also known as glioblastoma multiforme (GBM), is by far the most common malignant brain tumor [1–3]. Unfortunately, GBM has an extremely poor prognosis as no cure exists for malignant gliomas currently [3]. 1,2:5,6-dianhydrogalactitol (DAG) is a hexitol epoxide (Fig. 1), which has been approved as a chemotherapeutic drug for the treatment of chronic myelogenous leukemia and lung cancer in China. It has also been found that DAG could inhibit human glioma cell growth *in vitro* and *in vivo* by inducing cell cycle arrest at G_2/M phase [4]. Mechanistic studies reveal that DAG may be a potential multitarget agent that can inhibit tumor migration, invasion, and angiogenesis. Recently, it has been tested in phase II trials of glioblastoma in USA [5].

A good understanding of the pharmacokinetics and tissue (especially brain) distribution *in vivo* is crucial for DAG further development and clinical applications. Due to the highly polar nature and lack of chromophores or ionizable groups, development of the analytical method for DAG is quite challenging. The methods reported so far including radioisotope labeling [6–8], GC [9,10] and HPLC [11] were all completed in the 1970s–1980s with no method validation details and could not meet the requirement in current pharmacokinetic research. Briefly, radioisotope-labeled method was nonspecific and couldn't been used in routine preclinical and clinical studies. The limit of detection (LOD) in 1 mL plasma by GC and HPLC methods were 100 ng/mL [9,10] and 50 ng/mL [11], respectively.

To address this challenge, a novel, rapid and sensitive LC–ESI-MS/MS method after derivatization with diethyldithiocarbamate (DDTC) was developed and validated for the determination of DAG in mouse

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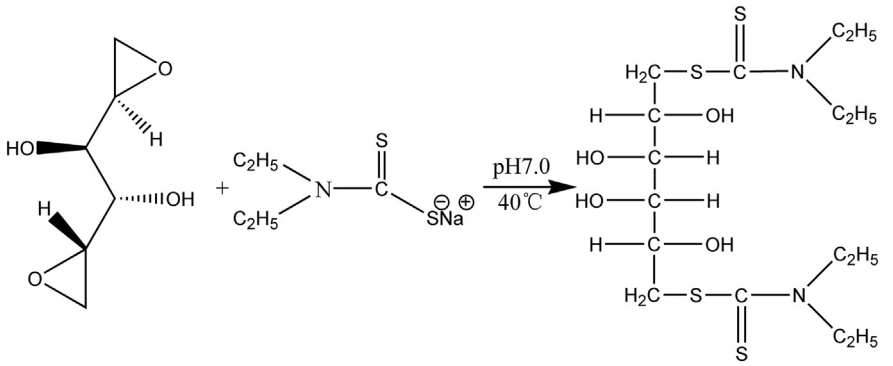

Fig. 1. Reaction pathway of dianhydrogalactitol with diethyldithiocarbamate.

plasma and tissues (brain, liver, heart, spleen, lung and kidney). To the best of our knowledge, this is the first LC-MS method for the quantification of DAG. The lower limit of quantification (LLOQ) was 1 ng/mL in 40 μL plasma or 200 μL tissue homogenate. The method was successfully applied to a pharmacokinetic and tissue distribution study in mice. Furthermore, the hepatic cytotoxicity of DAG was tested using freshly prepared rat primary hepatocytes.

2. Experimental

2.1. Chemicals and reagents

DAG of purity of 99.34% was kindly provided by Guangxi Wuzhou Pharmaceutical (Group) Co., LTD (China). The internal standard (IS) diazepam with purity of 99.9% was purchased from National Institute for the Control of Pharmaceutical and Biological Products (China). Sodium diethyldithiocarbamate (DDTC) was purchased from Aladdin Industrial Corporation. Acetonitrile was HPLC grade and purchased from Tedia Company, Inc. (USA). All other reagents and solvents were analytical grade and obtained from conventional commercial sources. Water was purified with a Millipore Milli Q-Plus system (Millipore, MA, USA).

2.2. Animals

Male C57BL/6 mice (20–27 g) and Sprague-Dawley rats (180–220 g) were used for pharmacokinetic experiments and collection of primary hepatocytes, respectively. Animals were purchased from Shanghai Super-B&K Laboratory Animal Co., Ltd. (Shanghai, China), and were used in accordance with the protocols approved by the animal care committee of China Pharmaceutical University.

Animals were housed under controlled standard conditions with 12/12 h light/dark cycles for at least 1 week prior to the experiments. All animals had free access to a standard diet and water, and were fasted for 12 h but allowed water *ad libitum* before DAG administration.

2.3. Instrumentation and conditions for LC-MS/MS

A 1260 HPLC system (Agilent Technologies) coupled to an Agilent 6420 triple quadrupole mass spectrometer (Agilent Technologies) was used for acquiring LC-MS/MS data. Data processing was performed on the Masshunter software.

The chromatographic separation was carried out on a Welch Ultimate XB-CN column (250 mm × 4.6 mm i.d., 5 μm). The mobile phase consisted of acetonitrile and 0.1% formic acid solution at the ratio of 65:35 was delivered at the flow rate of 1.0 mL/min.

The LC effluent was split at the ratio of 3:7 and the small portion was introduced into the mass spectrometer. The electrospray ionization (ESI) source was set in positive multiple reaction monitoring (MRM) mode monitoring the transition of m/z 445.1 → 116.0 and 285.0 → 193.0 for DAG derivative and IS, respectively. The gas temperature was 350 °C. The gas flow and nebulizer pressure were 11 L/min and 15 psi. The capillary voltage was maintained at 4000 V. The dwell time was 300 ms. The fragmentor was set at 110 V, 135 V, the collision Energy at 20 eV, 35 eV for the DAG derivative and IS, respectively.

2.4. Preparation of calibration standards and quality control samples

The stock solution of DAG, freshly prepared daily in water with an approximate concentration of 1 mg/mL, was serially diluted with water to provide working standard solutions of desired concentrations. The internal standard stock solution with an approximate concentration of 20 mg/mL in DMSO was diluted to approximately 400 ng/mL for plasma and 2000 ng/mL for tissues in acetonitrile as IS working solutions. All the solutions were kept at 4 °C.

40 μL of the working standard solutions of DAG (replaced by water when analyzing unknown samples) were added into clean tubes containing 40 μL blank mouse plasma or 200 μL blank mouse tissue homogenate, resulting in the plasma/tissue homogenate concentrations of 1, 2, 5, 10, 50, 100, 500, 800, and 1000 ng/mL. The quality control (QC) samples were prepared by different person at three concentration levels, namely low (2 ng/mL, LQC), medium (50 ng/mL, MQC) and high (800 ng/mL, HQC). All calibration standards and QC samples were immediately analyzed or stored at −70 °C.

2.5. Preparation of plasma samples

All plasma samples were thawed at room temperature before analysis. An aliquot of 40 μL of plasma was mixed with 20 μL of IS working solution (400 ng/mL) and 40 μL water (replaced by working standard solutions of DAG when analyzing calibration standards and QC samples). After the addition of 40 μL acetonitrile, the mixture was vortexed for 3 min and centrifuged at 14000 rpm for 5 min. A 120 μL aliquot of the supernatant was transferred for '2.7 derivatization'.

2.6. Preparation of tissue samples

Each weighed tissue sample was thawed at room temperature and then homogenized in ice-cold physiological saline (1:3, w/v) before analysis. An aliquot of 200 μL of tissue homogenate was mixed with 20 μL of IS working solution (2000 ng/mL) and 40 μL water (replaced by working standard solutions of DAG when analyzing calibration standards and QC samples). After the addition of 200 μL acetonitrile, the mixture was vortexed for 3 min and centrifuged at 14000 rpm for 5 min. A 420 μL aliquot of the supernatant was transferred for '2.7 derivatization'.

2.7. Derivatization

After a 120 μL aliquot of deproteinized plasma supernatant or

420 μL aliquot of deproteinized tissue supernatant was transferred to a 2.0 mL reaction vial, 50 μL 0.1 mol/L potassium phosphate buffer (pH 7.0) and 50 μL 5% (w/v) aqueous solution of DDTC (prepared fresh daily) were added in sequence. After being vortexed vigorously for 30 s, the mixture was incubated at 40 °C for 30 min. The vial was allowed to stand in an ice bath for 5 min to stop the reaction. Newly formed derivative was extracted with 1 mL ethyl acetate for 3 min followed by centrifugation at 14000 rpm for 5 min. A 900 μL aliquot of the supernatant was transferred to a new tube and evaporated to dryness. The residue was reconstituted in 200 μL of acetonitrile-water (50:50), and a 20 μL aliquot of the resulting solution was injected into the LC-MS/MS system.

2.8. Method validation

The present study focused on evaluating the brain penetrating potential of DAG, so a full validation of the developed method was performed for plasma and brain samples in accordance with the guidelines set by the United States Food and Drug Administration (FDA) with respect to specificity, lower limit of quantification (LLOQ), linearity and range, accuracy and precision, extraction recovery, matrix effect, carryover effect, stability and dilution integrity [12].

2.8.1. Specificity

The specificity of the developed method was investigated by comparing the MRM chromatograms of blank mouse plasma or tissue samples from six different sources to see whether there were chromatographic interferences at the retention times of DAG derivative and IS.

2.8.2. Linearity and LLOQ

A calibration curve was used in each run by plotting the peak area ratio of the analyte to IS (*Y*) *versus* plasma or tissue homogenate concentrations (*X*). Least-square linear regression was used for curve fitting with $1/X$ as the weighting factor. The calibration curves were considered acceptable when the correlation coefficient (r^2) was > 0.99. LLOQ was the minimal quantifiable concentration point of the standard curve at which precision (relative standard deviation, RSD) should not exceed 20% and accuracy (relative error, RE) should be within 20% of the nominal value.

2.8.3. Accuracy and precision

Five replicates of QC samples at three concentration levels were analyzed on three consecutive validation batches to evaluate the intra- and inter-batch accuracy (RE) and precision (RSD). The acceptable data for the intra- and inter-batch accuracy was required within 15% of the nominal value and the intra- and inter-batch precision should not exceed 15%.

2.8.4. Extraction recovery and matrix effects

The extraction recovery was evaluated by comparing the mean peak area of the plasma or brain QC sample at three concentration levels in five replicates with that of the reference samples prepared by adding DAG standard working solutions to post-extracted (protein precipitation) blank plasma or brain homogenate at corresponding concentrations. The matrix effect was investigated by comparing the peak area of the samples prepared by adding DAG standard working solutions to post-extracted (protein precipitation) blank plasma or brain homogenate with that of the neat standards at the corresponding concentrations. The extraction recovery and matrix effect of the IS were determined in the same way, but at one concentration level.

2.8.5. Stability

Stability was evaluated by measuring triplicate QC samples at low and high concentration levels exposed to the following conditions: stored at room temperature for 4 h, at −70 °C for two weeks and three freeze/thaw cycles (from −70 °C to room temperature). The samples were considered stable when the assay relative error (RE) was within ± 15% of the nominal concentration. The stability of the ready-to-inject samples in the HPLC autosampler at room temperature for 10 h and the DAG stock solution at 4 °C was also assessed.

2.8.6. Dilution integrity

The level of some plasma samples may exceed the highest concentration of the calibration curve, in which dilution is needed. The dilute integrity experiment was carried out by eight-fold dilution of 5 μg/mL for six replicates. Accuracy within ± 15% and precision < 15% were considered acceptable.

2.9. Pharmacokinetic and distribution study

The validated method was applied to investigate the plasma and tissue profiles of DAG in mice after intravenous administration *via* the tail vein at a dose of 5 mg/kg. The solvent used for administration was saline. Under light ether anesthesia, blood samples (approximately 300 μL) were obtained from the retro-orbital plexus of mouse into heparinized polythene tubes before dosing and subsequently at 5, 15, 30, 60, 90, 120, 180, 240, 300, and 360 min following administration. Five mice were used for each time point. Plasma was separated by centrifugation of the blood samples at 14000 rpm for 5 min and kept at −70 °C until analysis. After the collection of blood at each time point, the mouse was soon sacrificed and tissues including liver, kidney, lung, heart, spleen and brain were collected. Tissue samples were rinsed quickly with physiological saline to remove the blood or content, blotted on filter paper, weighed and then stored at −70 °C. Tissues collected at 5, 30, 60, 120, 240 and 360 min were analyzed, which is enough to plot the tissue distribution profile. The pharmacokinetic parameters including the area under the plasma concentration– time curve during the period of observation (AUC_{0-t}), the area under the plasma concentration–time curve extrapolated to infinity ($AUC_{0-\infty}$), the clearance (*CL*), total body mean residence time (MRT) and elimination half-life ($t_{1/2}$) were calculated by non-compartmental analysis using Drug and Statistics Software (DAS 3.2.7, Shanghai, China).

2.10. Cytotoxicity in rat primary hepatocytes

Rat primary hepatocytes were isolated from Sprague-Dawley male rats by two-step collagenase perfusion as previously described with some modifications [13–15]. Hepatocytes were seeded in 96-well plates at the density of 1×10^4 hepatocytes per well and incubated with 0.1–50 μM DAG for 48 h. The culture medium was Ham's F-12/DMEM (Invitrogen, Carlsbad, CA, USA) (1:1) supplemented with 15% fetal bovine serum (PAA Laboratories GmbH, Linz, Austria). Cells were grown in a humidified incubator with 5% CO_2 at 37 °C. Cell viability was assessed by the CellTiter-Glo™ Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA).

3. Results and discussion

3.1. Method development

3.1.1. Optimization of derivatization

DAG quantitation is particularly difficult because it has neither a chromophore for UV detection nor an ionizable group for MS detection. Considering the epoxide groups or hydroxyl groups in DAG structure, chemically modification by derivatization was tried to achieve the necessary selectivity and sensitivity. After the investigation of n-butaneboronic acid, bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane, and sodium diethyldithiocarbamate (DDTC) as the derivatization reagents, it was found that DAG could quantitatively react with DDTC with good repeatability and sensitivity by both UV and MS detectors. The reaction scheme shown in Fig. 1 was confirmed by the product ions spectra of the precursor ion of DAG derivative and its

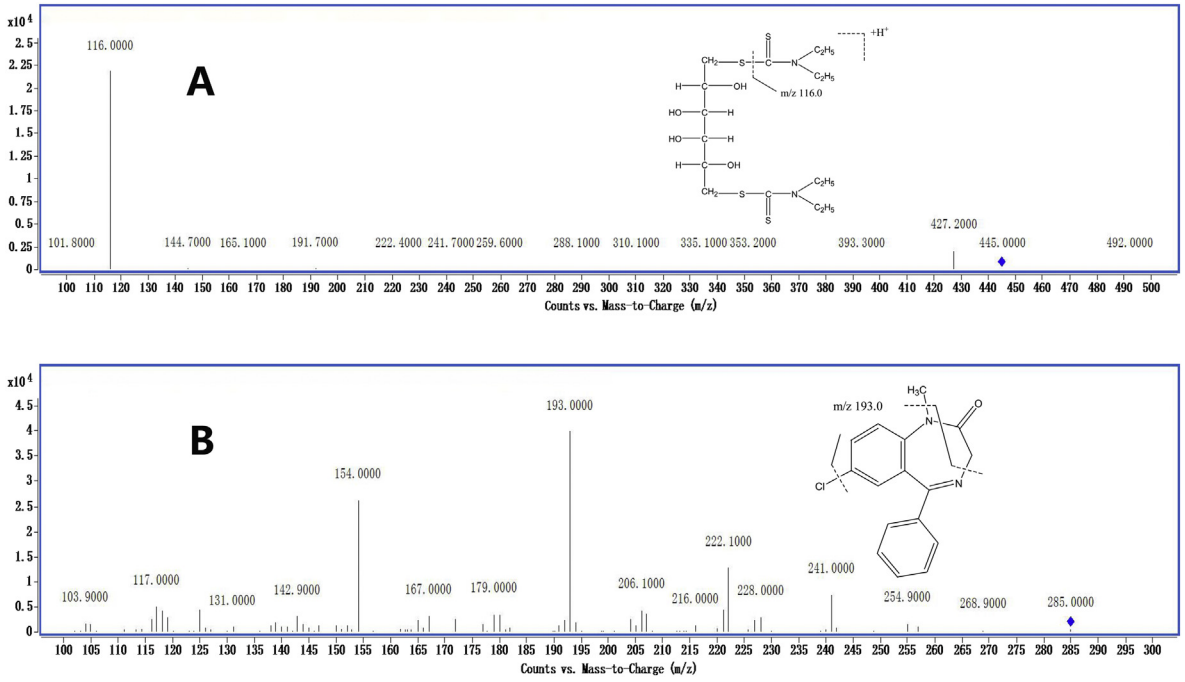

Fig. 2. Positive product ion mass spectra of dianhydrogalactitol derivative (A) and IS (B) and their proposed fragmentation patterns.

fragmentation pattern (Fig. 2).

The derivatization conditions were optimized in terms of reaction temperature (25, 40 or 60 °C), reaction duration (5, 15, 30, 45, 60, 90 or 120 min), pH (6.0, 7.0 or 8.0) and the derivatization reagent concentration (1%, 2%, 5% or 10%). It turned out when DAG reacted with 5% DDTC at 40 °C for 30 min in the buffer of pH 7.0, the derivative had the strongest and most repeatable response.

After derivatization, the product needs to be extracted from the biological matrix before LC-MS/MS analysis. Four extracting solvents including dichloromethane, chloroform, ether and ethyl acetate were investigated. The signal intensity was relatively higher when the reaction mixture was extracted with ethyl acetate. All plasma samples were analyzed on a single analytical column and there were no significant changes in the chromatographic behavior, confirming that the developed method enabled a good protection for the column.

3.1.2. Optimization of LC-MS/MS conditions

Various columns like Hypersil-CN (Elite, China), Ultimate XB-CN, Ultimate XB-C18 (Welch, China) and Hedera ODS-2 (Hanbon, China) were compared in terms of the chromatographic performance, among which Welch Ultimate XB-CN was chosen due to the appropriate retention time and good separation with the derivatization agent. After the comparison of acetonitrile-water and methanol-water as the mobile phase, acetonitrile-water system was favored for its sharp peak of both DAG derivative and IS. Formic acid (0.1%) was added into the mobile phase to increase the ionization efficiency of the analytes.

A systematic optimization of MS conditions such as detection mode (positive and negative mode), transitions (precursor and product ions) and other instrument or compound parameters for DAG derivative were performed. The target had the strongest response in ESI at positive ion mode with the transition of *m/z* 445.1 → 116.0. To avoid the ionization suppression or enhance caused by the endogenous substances and excess derivatization reagent, the elute between 0–2.0 min and 5.0–6.5 min were switched to waste.

Since isotope-labeled DAG was not available, different chemicals including telmisartan, theophylline, matrine, rhynchophylline, huperzine A, mannitol and diazepam were investigated as the internal standard. Diazepam was selected due to its sharp peak, similar retention time, ionization condition and extraction recovery compared to DAG derivative. In addition, diazepam was stable under the experimental conditions and no matrix effect was observed.

3.2. Method validation

3.2.1. Specificity

Fig. 3 shows the representative MRM chromatograms of blank mouse plasma and brain homogenate, blank plasma and brain homogenate spiked with DAG (1 ng/ml) and IS, and the plasma or brain sample from a mouse at 5 min after intravenous administration of 5 mg/kg DAG. The DAG derivative and IS were well-separated with the retention time of 3.86 min and 4.49 min, respectively. Blank plasma and brain samples yielded clean chromatograms without co-eluting interferences in each MS/MS ion channel, indicating that there were no endogenous interferences in the plasma and brain. Other blank tissues had similar results.

3.2.2. Linearity and LLOQ

The calibration curves demonstrated good linearity ($r^2 > 0.9958$) over the range of 1–1000 ng/mL in mouse plasma and tissue homogenates (brain, liver, heart, spleen, lung and kidney) with a weighting factor of $1/X$ to reduce the effect of large concentrations on the calculation of regression statistics. The typical standard curves and LLOQs are shown in Table 1. During the experiment, no carry-over effects were observed. The LLOQ was 1 ng/ml in plasma (40 μL) and all tissue homogenates (200 μL) which were sensitive enough for the determination of DAG in the following mouse pharmacokinetic and tissue distribution study.

3.2.3. Precision and accuracy

The precision and accuracy were evaluated at three concentration levels of QC samples except LLOQ levels. As listed in Table 2, the intra- and inter-batch precision (RSD) was < 15% and the accuracy (RE) was within 15% of the nominal value for plasma and brain QCs samples. The assay was accurate, reliable and reproducible for the determination of DAG in the biological samples.

3.2.4. Extraction recovery and matrix effect

Table 3 summarizes the extraction recovery and matrix effect for

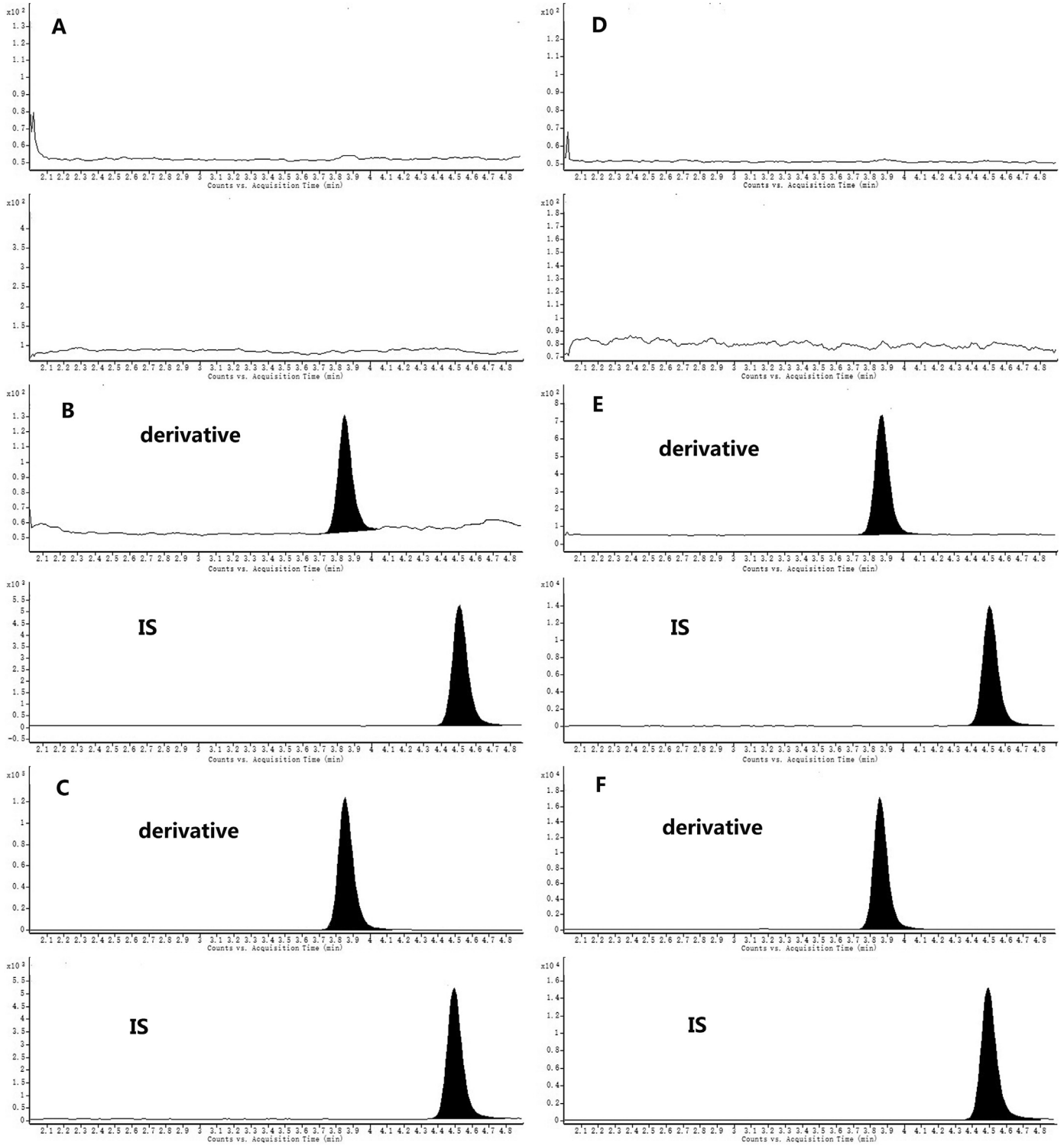

Fig. 3. Representative MRM chromatograms of dianhydrogalactitol in mouse plasma and brain.
(A) blank plasma, (B) blank plasma spiked with dianhydrogalactitol and IS, (C) the plasma sample from a mouse at 5 min after intravenous administration of 5 mg/kg dianhydrogalactitol, (D) blank brain homogenate, (E) blank brain homogenate spiked with dianhydrogalactitol and IS, (F) a brain sample from a mouse at 5 min after intravenous administration of 5 mg/kg dianhydrogalactitol.

DAG and IS in plasma and brain homogenate. The extraction recoveries of DAG in plasma and brain at three examined concentrations were within 95.0%–104.0% and 87.4%–90.5% respectively, indicating the recoveries were high and reproducible. The matrix effects of DAG in plasma and brain were within 98.2–114.1% and 93.0–97.1%, indicating that no endogenous substances significantly suppressed or enhanced the ionization of DAG derivative. The values of extraction recovery and matrix effect for IS in plasma and brain were also acceptable.

3.2.5. Stability

The stabilities of DAG in mouse plasma and brain homogenates

Table 1
Linearity and LLOQ of dianhydrogalactitol in mouse plasma and tissues.

Sample	Standard curves	r^2	Test range	LLOQ
			(ng/mL)	(ng/mL)
Plasma	y = 0.0150x + 0.0773	0.9994	1–1000	1
Brain	y = 0.0167x + 0.0460	0.9999	1–1000	1
Heart	y = 0.0170x + 0.0729	0.9996	1–1000	1
Liver	y = 0.0158x + 0.1169	0.9999	1–1000	1
Spleen	y = 0.0153x + 0.0668	0.9958	1–1000	1
Lung	y = 0.0170x + 0.0539	0.9980	1–1000	1
Kidney	y = 0.0153x + 0.0271	0.9990	1–1000	1

Table 2
Precision and accuracy of dianhydrogalactitol in mouse plasma and brain (n = 5).

Sample	Nominal concentration	Precision RSD (%)		Accuracy RE (%)	
	(ng/mL)	Intra-batch	Inter-batch	Intra-batch	Inter-batch
Plasma	1	10.8	12.4	17.2	15.1
	2	6.3	6.7	−7.2	−6.9
	50	4.1	12.3	−8.6	−9.3
	800	2.2	6.3	−2.9	3.5
Brain	1	11	13.5	18.9	16.8
	2	1.2	5.4	12.8	11.4
	50	1.8	4.8	−14.7	−12.3
	800	3.4	9.3	12.6	13.1

Table 3
Extraction recovery and matrix effect of dianhydrogalactitol and IS in mouse plasma and brain (n = 5).

Sample	Compound	QC(ng/ml)	Extraction recovery (%)	Matrix effect (%)
Plasma	DAG	2	99.5 ± 10.6	98.2 ± 11.3
		50	104.0 ± 6.4	114.1 ± 0.4
		800	95.0 ± 4.0	102.1 ± 5.0
	IS	400	94.7 ± 5.2	98.9 ± 4.1
Brain	DAG	2	90.2 ± 6.8	93.0 ± 4.9
		50	87.4 ± 6.8	93.4 ± 1.7
		800	90.5 ± 3.6	97.1 ± 3.7
	IS	400	82.0 ± 3.0	96.2 ± 2.5

under various storage conditions were evaluated at low and high concentrations. The results in Table 4 demonstrated that DAG was stable after stored at −70 °C for two weeks, three freeze-thaw cycles and 4 h at room temperature. The DAG derivative in the ready-to-inject samples was stable in the HPLC autosampler at room temperature for 10 h. In addition, the stock solutions of DAG (freshly prepared daily) were stable after storage at 4 °C for at least 12 h.

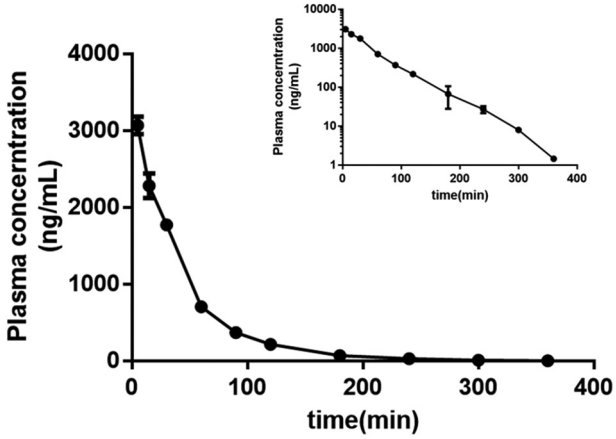

Fig. 4. Mean plasma concentration-time curve of dianhydrogalactitol in mice after intravenous administration 5 mg/kg dianhydrogalactitol. The insert shows a logarithm scale for concentration.

3.2.6. Dilution effect

To demonstrate the ability to dilute and analyze samples at concentration above the upper LOQ, plasma samples containing DAG at a concentration of 5 μg/mL was diluted eight times with the blank plasma. The dilution integrity was (107.6 ± 5.5)% of the nominal concentrations, which were within the limit of 85–115%. Since the tissue samples were homogenized with 3-fold saline, the tissue homogenate didn't need elution before analysis during the tissue distribution experiment.

3.3. Pharmacokinetic study

The proposed LC-MS/MS method was successfully applied to a pharmacokinetic study after intravenous administration of DAG at dose of 5 mg/kg to mice. The mean plasma concentration *versus* time is shown in Fig. 4 and the main pharmacokinetic parameters calculated by non-compartment model are listed in Table 5. The plasma concentrations of DAG were detected at all time points from 5 min to 6 h, demonstrating that the analytical method was sensitive enough.

After intravenous administration, DAG plasma concentrations declined rapidly with $t_{1/2}$ of mean value of 43.5 min. Meanwhile, the mean value of the apparent volume of distribution (V_d) was 2.143 L/kg implying that DAG exhibited an obvious tissue uptake after intravenous administration.

3.4. Tissue distribution

The tissue distributions of DAG in mice after intravenous administration were shown in Fig. 5. DAG was distributed *in vivo* rapidly and

Table 4
Stability of dianhydrogalactitol in mouse plasma and brain under various conditions (n = 3).

Sample	Nominal concentration	Room temperature for 4 h		Three freeze-thaw cycles		−70 °C For two weeks		Autosampler For 10 h	
	(ng/ml)	RSD(%)	RE (%)	RSD(%)	RE (%)	RSD(%)	RE (%)	RSD(%)	RE (%)
Plasma	2	0.4	13.0	6.7	4.9	6.5	14.7	7.5	10.9
	800	4.6	8.3	2.9	9.0	4.4	6.5	3.6	5.9
Brain	2	0.9	11.7	3.9	14.8	4.5	11.3	6.8	12.7
	800	3.5	4.7	0.8	4.4	5.7	8.2	5.0	7.6

Table 5
Non-compartmental pharmacokinetic parameters of dianhydrogalactitol after a single intravenous administration of 5 mg/kg dianhydrogalactitol in mice (n = 5).

Parameters	Unit	Value
AUC_{0-t}	ng/mL * min	148504 ± 4920
$AUC_{0-\infty}$	ng/mL * min	148735 ± 4676
MRT_{0-t}	min	43.3 ± 5.1
$MRT_{0-\infty}$	min	43.9 ± 4.4
$t_{1/2}$	min	43.5 ± 32.7
V_d	L/kg	2.143 ± 1.667
CL	L/min/kg	0.034 ± 0.001

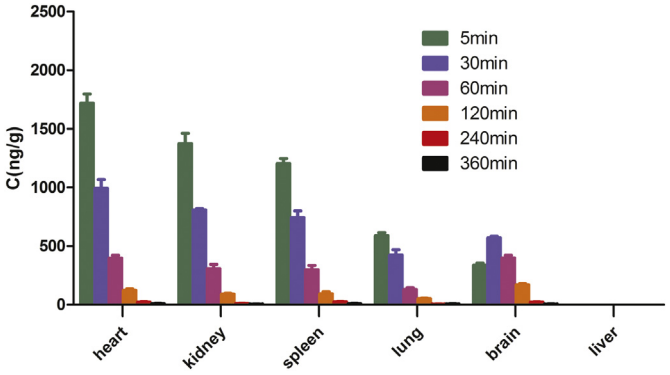

Fig. 5. Tissue distribution of dianhydrogalactitol in mice after intravenous administration of 5 mg/kg DAG.

extensively, and the tissue area under the curve from 5 min to 360 min was in the following order: heart > kidney > spleen > brain > lung > > liver. The DAG was eliminated rapidly in all tissues except brain. The present study demonstrated that DAG could cross the blood-brain barrier and exist for relatively long time, supporting its potential application against brain tumors. In addition, the antitumor activity in kidney is worthy of further investigation considering its high exposure in the kidney.

Interestingly, DAG was almost invisible in mouse liver, possibly due to a rapid metabolism and/or poor penetration into the hepatocytes. It has been reported that liver injury is the main adverse effects of chemotherapy drugs [16]. Thus, the limited liver distribution of DAG may be advantageous for its clinical applications.

3.5. Cytotoxicity in rat primary hepatocytes

To test the above hypothesis that DAG could avoid drug-induced liver injury due to its limited liver distribution, the freshly isolated rat primary hepatocytes were used to investigate the hepatic toxicity of DAG. Hepatocytes have been considered as gold standard *in vitro* model for the assessment of drug metabolism and toxicity due to the expression of multiple important metabolizing enzymes and drug-transporting proteins [17,18]. After a 48 h-treatment, DAG (0.1–50 μM) didn't decrease the cell viability (Fig. 6), suggesting that DAG and its possible metabolites may be safe to the liver.

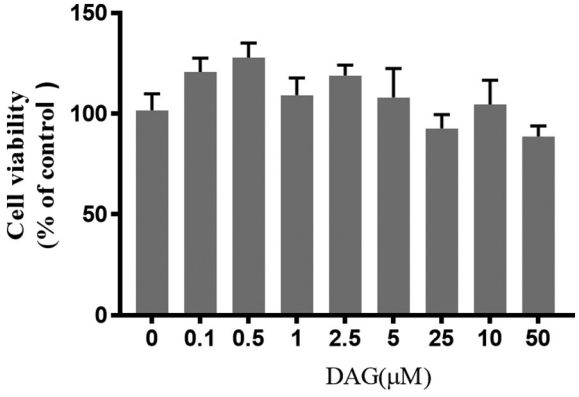

Fig. 6. Cytotoxicity of dianhydrogalactitol in rat primary hepatocytes. Data are representative of 3 independent experiments.

4. Conclusion

A sensitive, specific and reproducible LC-MS/MS method was developed and validated to determine DAG concentration in mouse plasma and tissues after derivatization with DDTC. The method was successfully applied to a pharmacokinetic and tissue distribution study. After intravenous administration, DAG could cross the blood-brain barrier and had limited liver distribution. *In vitro* testing using freshly isolated rat primary hepatocytes suggested a safe profile of DAG in liver.

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