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Development and validation of an HPLC method for the determination of dibenzoylmethane in rat plasma and its application to the pharmacokinetic study

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Abstract

A highly sensitive and simple high-performance liquid chromatographic (HPLC) assay has been developed and validated for the quantification of dibenzoylmethane (DBM) in rat plasma. DBM and internal standard (I.S.) 1-(5-chloro-2-hydroxy-4-methylphenyl)-3-phenyl-1,3-propanedione (CHMPP) were extracted from rat plasma by ethyl acetate/methanol (95:5, v/v) and analyzed using reverse-phase gradient elution with a Phenomenex Gemini C18 5-μm column. A gradient of mobile phase (mobile phase A: water/methanol (80:20, v/v) with 0.1% TFA and mobile phase B: acetonitrile with 0.1% TFA) at a flow rate of 0.2 mL/min, and ultraviolet (UV) detection at 335 nm were utilized. The lower limit of quantification (LLOQ) using 50 μL rat plasma was 0.05 μg/mL. The calibration curve was linear over a concentration range of 0.05–20 μg/mL. The mean recoveries were 80.6 ± 5.7, 83.4 ± 1.6 and 77.1 ± 3.4% with quality control (QC) level of 0.05, 1 and 20 μg/mL, respectively. Intra- and inter-day assay accuracy and precision fulfilled US FDA guidance for industry bioanalytical method validation. Stability studies showed that DBM was stable in rat plasma after 4 h incubation at room temperature, one month storage at −80 °C and three freeze/thaw cycles, as well as in reconstitute buffer for 48 h at 4 °C. The utility of the assay was confirmed by the successful analysis of plasma samples from DBM pharmacokinetics studies in the rats after oral and intravenous administrations.

Keywords: Dibenzoylmethane; HPLC; Pharmacokinetics

1. Introduction

Dibenzoylmethane (DBM) is a natural phytochemical found as a minor constitute in the root extract of Licorice (*Glycyrrhiza glabra* in the family *Leguminosae*), it is a beta-diketone phytochemical (Fig. 1A) with a wide variety of anti-cancer effects. DBM has been shown to prevent the formation of DNA-adducts induced by carcinogen in both *in vitro* [1,2] and *in vivo* [3,4] studies. DBM could induce apoptosis in human prostate and colon cancer cells [5], and induce cycle arrest in prostate cancer cells [6]. DBM has been shown to be able to regulate phase I cytochrome P450 enzymes such as CYP1A1, CYP1A2 and CYP1B1 [7]; and it is also a very strong inducer for phase II detoxification enzymes including NAD(P)H: Quinone reductase [8]. More interestingly, dietary administration of DBM at concentration of 1.0% in diet could strongly inhibit carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumor multiplicity and mammary tumor incidence by 97% in SENCAR mice [3]. The highly potent inhibitory effects of DBM against mutagenesis and tumorigenesis in these previous investigations strongly warrant further studies of its efficacy as a cancer chemopreventive agent.

To our knowledge, no analytical method has been developed and validated to measure the concentration of DBM in those previous studies, nor the pharmacokinetics of DBM has been investigated. Therefore, in this study, we reported the development and validation of a sensitive HPLC assay to quantify DBM in rat plasma, and the use of this assay to characterize

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the basic pharmacokinetic characteristics of DBM in male Sprague-Dawley rats.

2. Experimental methods

2.1. HPLC analysis

2.1.1. Chemicals and reagents

1,3-Diphenyl-1,3-propanedione (dibenzoylmethane, CAS 120-46-7) and internal standard (I.S.) 1-(5-chloro-2-hydroxy-4-methylphenyl)-3-phenyl-1,3-propanedione (CHMPP, CAS 5067-23-2) were purchased from Sigma (St. Louis, MO, USA) at purity of more than 98%, their chemical structures were illustrated in Fig. 1. Acetonitrile and methanol were all HPLC grade from Fisher Scientific. Trifluoroacetic acid (TFA) was of spectrophotometric grade (>99%, Aldrich, WI, USA). Ethyl acetate was purchased from Sigma with purity of 99.9%. Other chemicals used in this study were all in analytical grade unless specified.

2.1.2. HPLC instruments and chromatographic conditions

The Shimadzu HPLC system (SCL-10A vp) consists of a model FCV-10AL vp binary pump, a model SIL-10AD vp autosampler (a 250 μL injector with a 100 μL loop) configured with a 4 °C cooler, and a model SPD-10AV vp UV-Vis detector. The column and autosampler temperatures were kept at room temperature and 4 °C, respectively. The reverse phase chromatography was performed with an analytical Gemini™ C18 column (150 mm × 2.0 mm, 5-μm, Phenomenex, Torrance, CA, USA) protected with a SecurityGuard™ cartridge system (Phenomenex) and a 0.45-μm in-line filter. The optimized method used a binary gradient mobile phase with water/methanol (80:20, v/v) containing 0.1% TFA as mobile phase A and acetonitrile

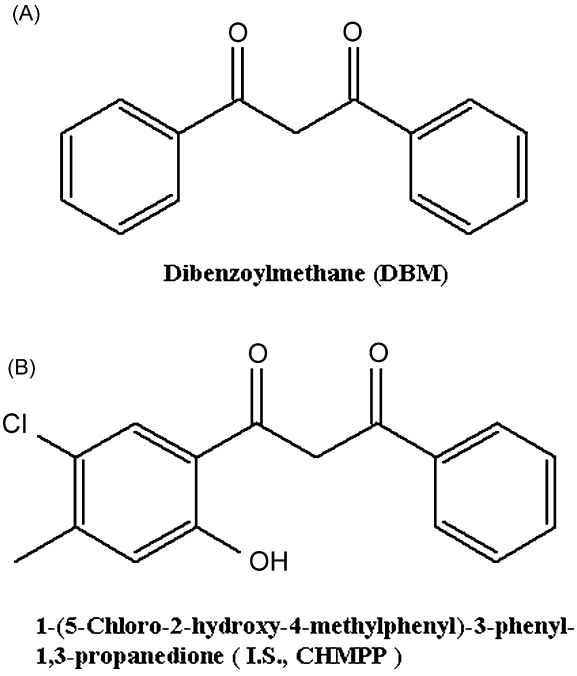

Fig. 1. Chemical structure of DBM (A) and internal standard CHMPP (B).

Table 1
HPLC mobile phase gradient conditions for analysis of DBM

Time (min)	Flow rate (mL/min)	% A	% B
0	0.2	95	5
15	0.2	0	100
20	0.2	0	100
21	0.2	95	5
30	0.2	95	5

Mobile phase A: water/methanol (80:20, v/v) with 0.1% TFA; mobile phase B: acetonitrile with 0.1% TFA.

with 0.1% TFA as mobile phase B, the time program of the gradient was listed in Table 1. The flow rate was 0.2 mL/min and the injection volume was 20 μL. The UV detector was set at a single wavelength of 335 nm. The Class-VP software version 7.1.1 (Shimadzu, MD, USA) was used for instrument control and data analysis.

2.1.3. Stock solutions and standards

Primary stock solutions of DBM (1 mg/mL) were prepared in methanol whereas stock solutions of internal standard CHMPP (1 mg/mL) were prepared in DMSO and stored at −80 °C. Primary stock solution of DBM was firstly diluted quantitatively with methanol to give working solutions with concentrations of 0.5, 1.0, 2.5, 5.0, 10, 50, 100 and 200 μg/mL for the preparation of calibration and quality control (QC) samples. Internal standard CHMPP primary stock solution was diluted 5 times, twice with DMSO to give a working solution with concentration of 40 μg/mL. DBM calibration standards were prepared fresh daily at concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1.0, 5.0, 10 and 20 μg/mL by spiking 50 μL blank rat plasma with 5 μl of methanol (for "zero" standard sample) or DBM working solutions. Each standard sample was also spiked with 2.5 μL of internal standard CHMPP working solution to give a final concentration of 2 μg/mL. In the same manner, QC samples with 2 μg/mL CHMPP and concentration of DBM at low (0.05 μg/mL), medium (1.0 μg/mL) and high (20 μg/mL) were freshly prepared to evaluate accuracy and precision of this HPLC method.

2.1.4. Sample preparation procedures

A 50 μL blank rat plasma, spiked plasma or pharmacokinetics study plasma sample was extracted with 200 μL ethyl acetate/methanol (95:5, v/v) solution by mixing for 2 min on a cyclomix at room temperature, the upper layer was transferred to a clean tube after centrifugation at 10,000 × *g* for 3 min. The extraction procedure was repeated once and the combined organic phase was evaporated to dryness under a stream of nitrogen gas at room temperature. The obtained residue was reconstituted in 100 μL of acetonitrile/water (50:50, v/v) by vortexing for 2 min, filtered through a 0.45 μm Nylon Spin-filter (Analytical Sales, NJ) and transferred into a sample vial for HPLC analysis.

2.2. HPLC method validation

2.2.1. Specificity and selectivity

The chromatographic interference from endogenous compounds was assessed by comparing chromatograms of blank rat

plasma, plasma spiked with DBM and internal standard CHMPP, and plasma samples obtained from DBM pharmacokinetic studies in the rats.

2.2.2. Sensitivity

The lower limit of quantification (LLOQ) was determined during the evaluation of the linear range of calibration curve. LLOQ was defined as the lowest concentration yielding a precision with CV less than 20% and accuracy within 15% of the theoretical value (i.e. accuracy between 85 and 115%) for both intra- and inter-day analysis.

2.2.3. Linearity of calibration curve

Calibration curve was obtained by plotting the peak area ratios of DBM/CHMPP to the spiked DBM theoretical concentrations in blank plasma. The linearity of calibration curve was evaluated by linear regression analysis. The minimally acceptable correlation coefficient (r^2) for the calibration curve was 0.99 or greater.

2.2.4. Precision and accuracy

In order to assess the intra- and inter-day precision and accuracy of the assay, DBM QC samples at low, medium and high concentrations were prepared as described above. The intra-day precision of the assay was assessed by calculating the coefficients of variation (CV) for the analysis of QC samples in three replicates; and inter-day precision was determined by the analysis of QC samples on three consecutive days. Accuracy was calculated by comparing the averaged measurements and the nominal values, and was expressed in percent. The criteria for acceptability of precision were that the relative standard deviation (RSD) for each concentration level should not exceed ±15% with the exception of the LLOQ, for which it should not exceed ±20%. Similarly, for accuracy, the averaged value should be within ±15% of the nominal concentration except for the LLOQ, where the limit was ±20%.

2.2.5. Recovery

The extraction recovery of DBM was determined with QC samples by comparing peak area ratio of DBM/CHMPP to those of standards in acetonitrile at equivalent concentrations and expressed in percentage.

2.2.6. Stability

Stability of DBM in rat plasma at room temperature for 4 h was evaluated using QC samples in triplicates. Three freeze–thaw cycles (−80 °C/room temperature) were applied to QC samples to assess the stability of DBM. Freezing stability of DBM in rat plasma was assessed by analyzing QC samples stored at −80 °C for 1 month. The in-autosampler (4 °C) stability of DBM in the reconstitute solvent was evaluated by reinjecting QC samples 48 h after the initial injection. The peak area of DBM in different QC levels at initial condition was used as reference to determine the relative stability of DBM in the experiments described above.

2.3. Pharmacokinetics of DBM in the rats

Male Sprague-Dawley rats with weight between 250 and 300 g and jugular vein cannulae were purchased from Hilltop Lab. Animals Inc. (Scottdale, PA, USA). Rats were housed at Animal Care Facility of Rutgers University under 12 h light–dark cycles with free access to food and water. Upon arrival, rats were given AIN-76A diet (Research Diets, NJ, USA) free of antioxidant and acclimatized for 3 days. Rats ($n = 3$) were fasted overnight and given DBM at dose of 50 mg/kg in a vehicle of Cremophor EL/tween-80/ethanol/water (2:1:1:6, v/v/v/v) by oral gavages (p.o.). Rats ($n = 3$) were also given DBM at dose of 10 mg/kg in the same vehicle as an intravenous (i.v.) bolus through the jugular vein cannulae. Blood samples (200 μL) were collected at 2 (i.v. only), 7.5, 15, 30 min, 1, 1.5, 2, 4, 6, 8, 12, 24 and 36 h following DBM administration. Plasma was separated immediately by centrifugation and stored at −80 °C until analysis.

The obtained DBM plasma concentration data was analyzed using WinNonlin 4.0 software (Pharsight, CA, USA) to obtain the appropriate pharmacokinetic parameters.

3. Results and discussion

3.1. Method development

The UV–vis absorbance of DBM was scanned from wavelength of 200–800 nm on a Beckman DU530 UV-Vis spectrophotometer. As shown in Fig. 2, DBM (25 μg/mL) has maximum UV absorption at wavelength of 335 nm in acetonitrile; therefore, wavelength of 335 nm was chosen for UV detection in this assay. Chromatograms of DBM standard (1 μg/mL in acetonitrile) analyzed by reverse phase HPLC analytical columns including Luna Phenyl-Hexyl, Gemini C18, Synergi Max-RP from Phenomenex (150 mm × 2.0 mm, 5-μm), Waters Xterra C18 and Shimadzu Premier C18 were compared. The Gemini C18 column was selected for the assay based on its given DBM retention time, peak shape/symmetry and selectivity (data not shown). The mobile phase used for the assay was of very simple composition and achieved optimal separation of DBM and the I.S. CHMPP without interference from other components in rat plasma (Fig. 3). The final flow rate and gradient of

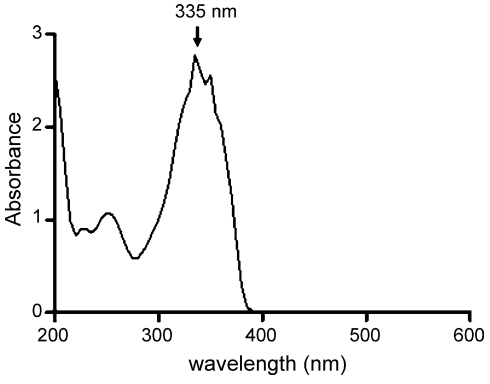

Fig. 2. UV–vis absorption spectra (200–500 nm) of DBM in acetonitrile (25 μg/mL).

Table 2
The intra- and inter-day accuracy and precision of QC samples ($n = 3$)

	Added concentration (μg/mL)	Measured concentration (μg/mL)	Accuracy (%)	CV (%)
Intra-day	0.05	0.0528 ± 0.0054	105.6	5.6
	1	1.0085 ± 0.0326	100.9	0.9
	20	20.1070 ± 0.8835	100.5	0.5
Inter-day	0.05	0.0496 ± 0.0053	99.2	10.7
	1	1.0288 ± 0.0639	102.9	6.2
	20	20.5771 ± 1.0851	102.9	5.3

mobile phase condition were chosen to achieve balanced results in terms of peak shape, resolution and sensitivity of DBM as well as I.S. CHMPP (Table 1). Carry over in the assay was evaluated by checking blank plasma sample after injection of QC samples at high concentration; no significant carry over (less than 0.3%) was found.

Liquid–liquid extraction method was used for sample preparation; the extraction solution ethyl acetate/methanol (95:5, v/v) was chosen based on previous study in which the same solvent was used to extract the β-diketon analogue curcumin [9]. For the reconstitution buffer, initially a solution of starting mobile phase (A:B, 95:5, v/v) was used, but the peak area of both DBM and CHMPP was much less than using the acetonitrile/water (50:50) as reconstitution buffer, therefore the latter was chosen for the assay. These selected conditions of sample preparation and liquid chromatographic conditions enabled the establishment of the LLOQ of DBM as low as 0.05 μg/mL by using 50 μL of rat plasma.

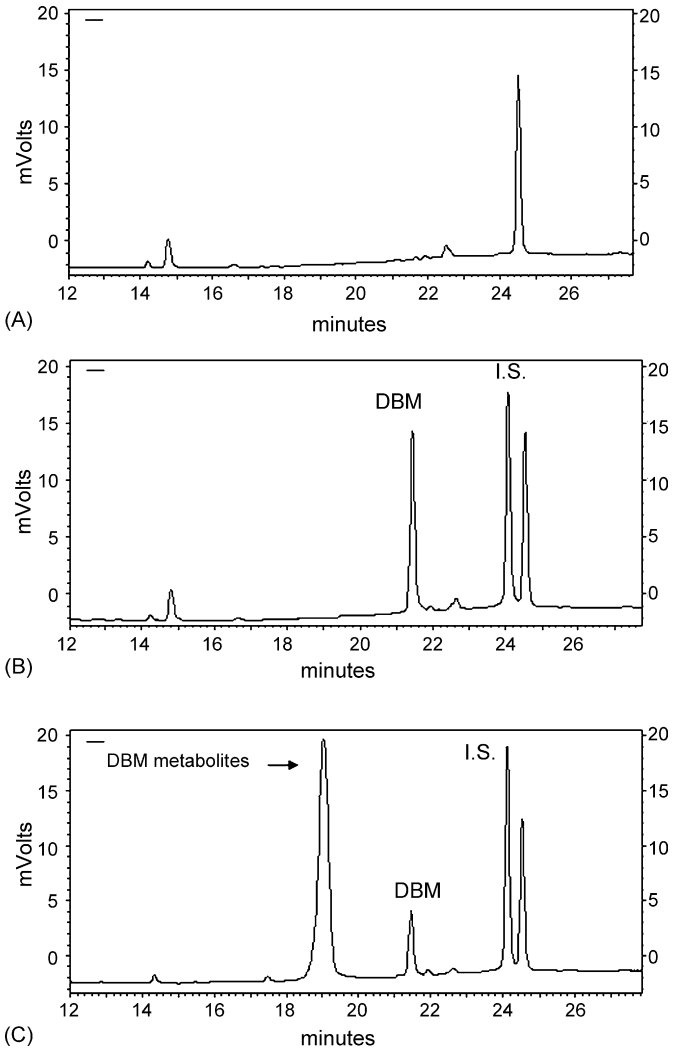

Fig. 3. Representative chromatograms of: (A) blank rat plasma; (B) blank rat plasma spiked with DBM (0.5 μg/mL) and internal standard (I.S.) CHMPP (2 μg/mL); and (C) rat plasma sample at 8 h after oral administration of DBM at dose of 50 mg/kg and spiked with 2 μg/mL I.S. CHMPP.

3.2. HPLC method validation

3.2.1. Specificity and selectivity

Fig. 3 represents chromatograms of DBM and I.S. CHMPP from rat plasma after liquid–liquid extraction along with blank plasma sample. No interference of endogenous peaks with DBM or CHMPP at their respective retention times (DBM $t_R = 21.417$ min; CHMPP $t_R = 24.050$ min) in blank rat plasma was observed. The capacity factor (k) for analyte DBM and I.S. CHPMPP were 8.18 and 9.32, respectively. Fig. 3C showed that there were no *in vivo* DBM metabolites interfering with the parent compound DBM and I.S.

3.2.2. Sensitivity

The lower limit of quantification was defined as those quantities that were 10-fold above the background noise, with precision errors of less than 20% (CV) and inaccuracy between ±20% (bias). The LLOQ of DBM extracted from 50 μL rat plasma was found to be 0.05 μg/mL after injection of 20 μL of the 100 μL reconstitutes. The mean percent accuracy value for plasma samples was 98% and precision coefficient of variation was below 10% at the LLOQ (Table 2).

3.2.3. Linearity of calibration curve

The calibration curves for DBM were linear over the concentration range of 0.05–20 μg/mL in rat plasma. The mean (±SD) regression equation from three replicate calibration curves on different days was $y = (0.0015489 \pm 0.000022)x + (0.0141 \pm 0.0099)$ with correlation coefficient $r^2 = 0.9995 \pm 0.0006$.

3.2.4. Precision and accuracy

Table 2 shows a summary of intra- and inter-day precision and accuracy. In the range of 0.05–20 μg/mL, intra- and inter-day accuracy ranged from 100.5 to 105.6 and 99.2 to 102.9%,

Table 3
Stability of DBM at various experimental conditions

QC sample (μg/ml)	Stability condition	% Remaining ± SD
0.05	4 h at room temperature	98.9 ± 10.5
	3 freeze–thaw cycles	95.7 ± 11.5
	30 days storage at 80 °C	98.5 ± 5.9
	48 h in autosampler at 4 °C	98.6 ± 7.5
1	4 h at room temperature	95.1 ± 4.5
	3 freeze–thaw cycles	99.4 ± 1.2
	30 days storage at 80 °C	107.9 ± 3.0
	48 h in autosampler at 4 °C	100.5 ± 2.6
20	4 h at room temperature	96.3 ± 1.3
	3 freeze–thaw cycles	102.4 ± 1.2
	30 days storage at 80 °C	109.8 ± 2.1
	48 h in autosampler at 4 °C	100.7 ± 3.5

respectively. Therefore, the intra- and inter-day accuracies (% deviation) were within ±20% for the LLOQ and ±15% for other QC samples. The intra- and inter-day assay precision (CV) ranged from 0.5 to 5.6 and 5.3 to 10.7%, respectively, which were also within the acceptable range of 20% at LLOQ and 15% at other QC samples. The relative higher %CV in the inter-day analysis compared to the intra-day analysis (Table 2) is probably due to slightly different composition of the reconstitute buffer acetonitrile/water (50:50, v/v) used at different days. These results indicated that the present assay has very good accuracy and precision.

3.2.5. Recovery

Recovery was evaluated by comparison of the DBM/CHMPP peak area ratios of the extracted samples at the three QC levels with the standard solutions of equivalent concentrations. The mean extraction recovery of DBM was 80.6, 83.4 and 77.1% for low, medium and high QC samples, respectively.

3.2.6. Stability

DBM primary stock solution (1 mg/mL in methanol) was stable for at least 3 months (data not shown) at −80 °C. The stability study results of DBM under various conditions were summarized in Table 3. DBM at all QC levels was stable in rat plasma for 4 h at ambient temperature, after three freeze/thaw cycles, as well as after storage at −80 °C for 1 month. DBM was also stable in the reconstituted buffer for 48 h in the autosampler at 4 °C. The high stable property of DBM in rat plasma suggested that no special care was needed during sample preparation. The high stability of DBM in reconstituted buffer at 4 °C also suggested that a large batch of samples could be processed at one time within 48 h, which would compensate for the shortcoming of relative long analyze time of this assay.

3.3. Application of the developed HPLC method to pharmacokinetics study

With LLOQ of 0.05 μg/mL, plasma concentrations of DBM in pharmacokinetics study were successfully quantified by the assay up to 24 h (concentrations in 36 h samples were below LLOQ); the mean plasma concentration–time profiles after oral (50 mg/kg) and intravenous (10 mg/kg) administration of DBM in the rats were shown in Fig. 4. The basic pharmacokinetic parameters of DBM in the rats were determined using non-compartmental analysis of WinNonlin program and listed in Table 4. DBM has long terminal half life (6.9–9.6 h); the clearance was about 0.7 L/h/kg after the i.v. administration, and the steady state volume of distribution was about 5.0 L/kg. These parameters suggested that DBM is a low clearance compound with high volume of distribution in SD rats. DBM also showed a relative low absolute bioavailability (*F*) of 11.5% in rat, suggesting that the mechanism of low absorption in the intestine or extensive gut/liver metabolism may be involved.

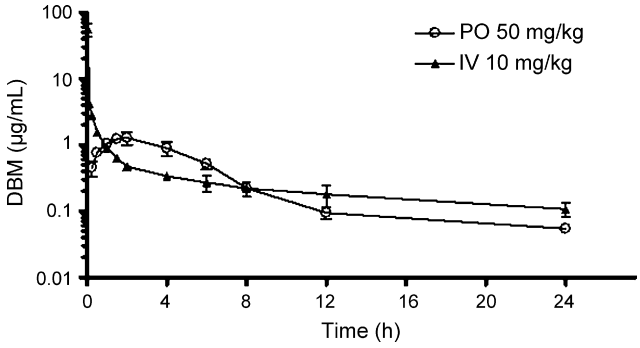

Fig. 4. Mean plasma concentration–time profiles of DBM following single oral (50 mg/kg, $n = 3$) and IV (10 mg/kg, $n = 3$) administration in male Sprague-Dawley rats.

Table 4
Pharmacokinetic parameters of DBM in male SD rats generated by non-compartmental analysis

Parameters	Oral ($n = 3$)	IV ($n = 3$)
Dose (mg/kg)	50	10
C_{max} (μg/mL)	1.5 ± 0.4	–
T_{max} (h)	2.0 ± 0.0	–
AUC_{∞} (h μg/mL)	9.3 ± 1.9	16.2 ± 5.5
$T_{1/2}$ (h)	6.9 ± 4.3	9.6 ± 2.9
CL (L/h/kg)	–	0.7 ± 0.3
Vdss (L/kg)	–	5.0 ± 1.2
MRT (h)	5.1 ± 0.1	3.8 ± 0.2
F (%)	11.5 ± 2.4	–

Data are presented as mean ± SD.

4. Conclusion

A simple, sensitive, accurate and precise HPLC method was developed and validated for the first time to quantify DBM in rat plasma. Simple liquid–liquid extraction method was used to prepare the samples and a Gemini C18 column was used to analyze the samples. The present method was applied successfully to a pharmacokinetic study of DBM in the rats, in which basic pharmacokinetic parameters such as absolute bioavailability, clearance, terminal half life, steady state volume of distribution, etc. were determined. The chromatographic condition as well as sample preparation method of the current assay will likely facilitate the development and validation of HPLC-UV analyti-

cal assays to analyze DBM in other biological matrixes such as urine and tissue homogenates, which will be our future studies.

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