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A simple HPLC method for the determination of bifendate: Application to a pharmacokinetic study of bifendate liposome

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Abstract

A rapid, sensitive and simple high-performance liquid chromatographic (HPLC) method with ultraviolet detector (UV) has been developed for the determination of bifendate in 100 μl plasma of rats. Sample preparation was carried out by deproteinization with 100 μl of acetonitrile. A 20 μl of supernatant was directly injected into the HPLC system with methanol–double distilled water (65/35, v/v) as the mobile phase at a flow rate of 1.0 ml/min. Separation was performed with a μBondapak C_{18} column at 30 °C. The peak was detected at 278 nm. The calibration curve was linear ($r^2 = 0.9989$) in the concentration range of 0.028–2.80 μg/ml in plasma. The intra- and inter-day variation coefficients were not more than 6.55% and 6.07%, respectively. The limit of detection was 5 ng/ml. The mean recoveries of bifendate were ranged from 94.53% to 99.36% in plasma. The present method has been successfully applied to the pharmacokinetic study of bifendate liposome in rats.

Keyword: Bifendate

1. Introduction

Bifendate is widely used in China for the treatment of chronic hepatitis by lowering alanine transaminase (ALT) in patients [1–3]. The structure of bifendate (4,4′-dimethoxy-5,6,5′,6′-bi(methylenedioxy)-2,2′-bicarbomethoxybiphenyl, DDB) is shown in Fig. 1. Currently, there are only oral preparations on market because DDB is insoluble in water, which results in low bioavailability. However, for acute hepatitis patients and those with decreased liver functions after surgical operations parenteral dosages would provide the best benefit for them. In order to prepare the DDB solution for intravenous injection, several groups had tried to improve the solubility of DDB in water [4,5]. Recent years, liposome has been introduced as a parenteral drug carrier offering sustained release and organ targeting [6–10]. So far, no study on DDB liposome has been reported yet. In the present study, DDB liposome for intravenous injection was prepared successfully. Then it was necessary to establish a sensitive and simple method for the determination of DDB in biological fluids.

Zhou et al. determined the concentrations of DDB in biological fluids by liquid chromatographic–mass spectrometry (LC–MS) method [11], but the method required MS equipment which might not be available in most of the laboratories. Other researchers had also employed the high-performance liquid chromatographic (HPLC) method with ultraviolet detector (UV) for the assay of DDB [12–14]. But the detection limit was 0.1 μg/ml in plasma and it required complicated sample preparation procedures involving basification of plasma sample, extraction, preconcentration and reconstitution of the analyte. These procedures were time-consuming and quite complicated. Especially, those HPLC methods used 1 ml of plasma for the determination of DDB and this amount of plasma was too much for rats and was not suitable for the assay of DDB in plasma during the pharmacokinetic study.

In the present study, DDB liposome for intravenous injection was prepared, and a sensitive and simple method for the determination of DDB in biological fluids was established. Using

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this method, plasma samples could be applied to HPLC with the simple deproteinization. With UV detector, the analysis of DDB in rat plasma could be achieved using only 100 μl of plasma. It enabled precise and accurate quantitation of DDB in small sample volumes over a wide concentration range. This method was successfully applied to the pharmacokinetic study of DDB in rats after intravenous injection DDB liposome.

2. Experimental

2.1. Materials

Bifendate (DDB, 99.0% purity) was purchased from Zhejiang Hisoar Pharmaceutical Factory. DDB liposome was prepared as following: 10 mg DDB and 300 mg phosphatidylcholine and 10 mg cholesterol were dissolved in 10 ml ethanol and then was dried to a thin film, and the film was hydrated with a 5% glucose solution to make the liposome suspension. The reference formulation was the DDB solution prepared in our laboratory. The other chemical reagents were of analytical grade or better.

2.2. Apparatus and operating conditions

The analysis was performed on a Shimadzu model 20AT LC system (Chiyoda-Ku, Tokyo, Japan), equipped with one pump (LC-20AT), a 20 μl injection loop, column oven (CTO-10A), and UV detector (SPD-20A). The wavelength of this detector was set to 278 nm. The system was controlled through a system controller (SCL-10A) and a personal computer using a CLASS-VP 5.0 workstation with a data processing system (Shimazu, Kyoto, Japan) installed on it. The sample separation was carried out on a μBondapak C_{18} reversed-phase column (250 mm × 4.6 mm, 5 μm) protected by a guard column (10 mm × 2.5 mm, 5 μm) which was packed with the same μBondapak C_{18} material. The mobile phase was a mixture of methanol and water (65:35, v/v). Elution was performed isocratically at 30 °C at a flow rate of 1.0 ml/min.

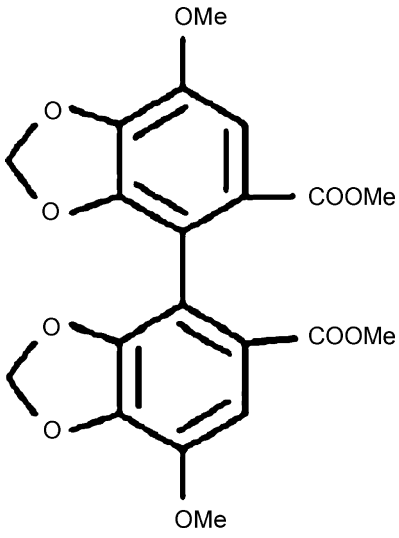

Fig. 1. The chemical structure of DDB.

2.3. Preparation of stock solution and calibration standard solutions

A stock standard solution of DDB (560 μg/ml) was prepared in methanol and further diluted to 140 μg/ml with methanol. A series of standard solutions were obtained by further dilution of the stock solution with methanol. All solutions were stored at 4 °C and were brought to room temperature before use. A 2 μl of the standard solution was transferred into 98 μl of blank rat plasma to obtain a calibration standard concentration at 0.028, 0.056, 0.14, 0.28, 0.70, 1.40 and 2.80 μg/ml of DDB, respectively. Quality control (QC) samples containing 0.028, 1.40 and 2.80 μg/ml of DDB were prepared in the same way.

2.4. Sample preparation

A 100 μl of each plasma sample was transferred into a 1.5 ml polyethylene centrifuge tube. A 100 μl of acetonitrile

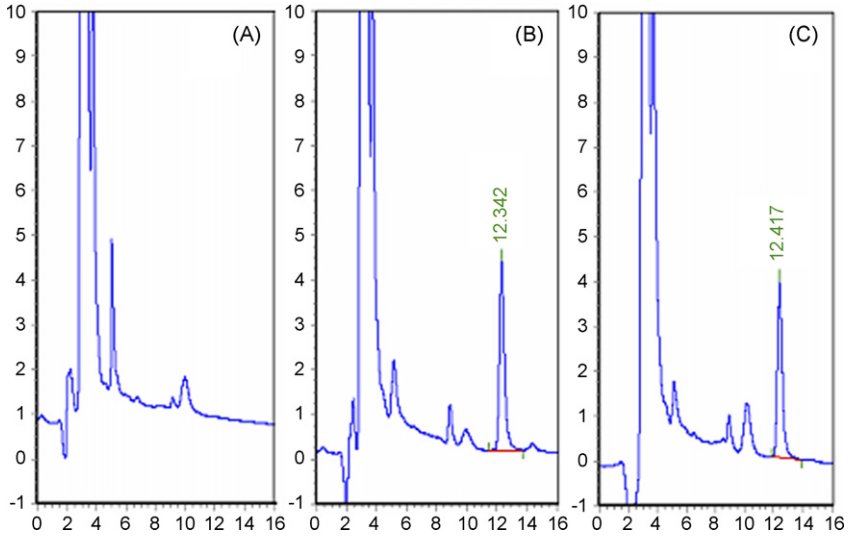

Fig. 2. Typical chromatograms of blank rat plasma (A), blank rat plasma spiked with 0.30 μg/ml DDB (B) and rat plasma sample containing 0.28 μg/ml of DDB (C).

Table 1
Intra-day ($n = 5$) and inter-day ($n = 3$) precision and accuracy of the method for determination of DDB in rat plasma samples

Added concentration (μg/ml)	Intra-day ($n = 5$)			Inter-day ($n = 3$)		
	Concentration found (mean ± S.D.) (μg/ml)	Precision[a] (%)	Accuracy[b] (%)	Concentration found (mean ± S.D.) (μg/ml)	Precision[a] (%)	Accuracy[b] (%)
0.028	0.029 ± 0.0019	6.55	103.57	0.028 ± 0.0017	6.07	99.81
0.14	0.13 ± 0.0036	2.77	92.86	0.14 ± 0.0018	1.29	99.75
2.80	2.79 ± 0.046	1.65	99.64	2.78 ± 0.039	1.40	99.29

[a] Expressed as relative standard deviation.
[b] Expressed as [(mean observed concentrations/nominal concentrations) × 100].

was added to each plasma sample and vortex-mixed (SW-80A vortex shaker, Shanghai Medical University Instrument Plant, Shanghai, China) for 3 min. The denatured protein precipitation was separated by centrifugation at 10,000 × *g* for 10 min at 4 °C (Refrigerated Centrifuge 3K30, Sigma, Germany). A 20 μl of supernatant was directly injected into the HPLC system for analysis.

2.5. Validation of assay method

The selectivity of the method was investigated by comparing the UV chromatograms of blank plasma, standard plasma sample spiked with DDB and plasma sample at 10 min after intravenous injection of DDB liposome (equivalent to 0.9 mg/kg of DDB).

The linearity of the calibration curve was determined by plotting the peak area (y) of the analyte versus the nominal concentration (x) of DDB.

To determine the within-day accuracy and precision of the method, three samples of plasma (0.028, 1.40 and 2.80 μg/ml) were analyzed five times on the same day. To determine the between-day accuracy and precision, three samples of plasma (0.028, 1.40 and 2.80 μg/ml) were run at three different days. Assay precision was expressed as the relative standard deviation (R.S.D.) (coefficient of variation). Accuracy (expressed as percent of nominal values) was determined by comparing the calculated concentration from the calibration curve to the known concentration.

The recoveries of DDB were determined at low (0.028 μg/ml), medium (1.40 μg/ml) and high (2.80 μg/ml) concentrations. A 2 μl of the standard solution was transferred into 98 μl of blank rat plasma. Then, the samples were analyzed according to Section 2.4. For the reference material, the same concentration standard solution was injected directly to the HPLC system. The recoveries were obtained by comparing the areas of those two samples. The experiments were repeated three times at 0.028, 1.40 and 2.80 μg/ml of DDB, respectively.

Sample stability was determined by analyzing QC samples after three freeze–thaw cycles and exposed to ambient temperature or −20 °C over a time period of 24 h.

Table 2
Recoveries data of DDB in rat plasma samples ($n = 3$)

Added concentration (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Recoveries[a] (%)	R.S.D.[b] (%)
0.028	0.027 ± 0.0012	99.18	4.56
0.14	0.13 ± 0.0043	94.53	3.31
2.80	2.79 ± 0.026	99.36	1.12

[a] Expressed as [(mean observed concentrations/nominal concentrations) × 100].
[b] Expressed as relative standard deviation.

2.6. Animal experiment

Rats (male, 180–220 g) were purchased from the Experimental Animal Center of China Pharmaceutical University. Twelve rats divided randomly into two groups were fasted for 24 h with free access of water. The DDB solution equivalent to 0.9 mg/kg DDB (PEG400:distilled water = 20:80, v/v) was injected intravenously into the tail vein of one group of rats (the control group). DDB liposome suspension equivalent to 0.9 mg/kg DDB was injected intravenously into the tail vein of another group of rats (the test group).

After intravenous injection, the rats were anaesthetized with aether and a heparinized capillary was then inserted into the eyeground veins to get 0.5 ml blood at the time intervals of 5, 10, 15, 30, 45, 60, 90, 120, 240 and 480 min, respectively. The plasma samples were collected after centrifugation at 1500 × *g* for 5 min and then stored immediately at −20 °C.

Pharmacokinetic parameters of clearance data (CL) and half-life ($t_{1/2\beta}$) were estimated according to a two-compartment model using the 3p87 program (The Chinese Society of Mathematical Pharmacology, Beijing, China). The area under the plasma concentration–time curve (AUC), up to the last measured time, was calculated using a linear trapezoidal method. The mean residence time (MRT) and elimination rate constant (k_{el}) were computed by statistic moment analysis. Differences between the two formulations in each parameter were statistically evaluated by a one-way analysis of variance test. All these pharmacokinetic parameters were reported as the mean ± standard deviation as demonstrated in Section 3.

3. Results and discussion

3.1. Sample preparation

Initially, a liquid–liquid extraction process was used by adding 5 ml diethyl ether into the plasma, and vortex mixing for 5 min. After centrifugation at 1500 × *g* for 15 min, the supernatant was quantitatively decanted into a clear tapered centrifuging tube and was evaporated under nitrogen at 40 °C.

Table 3
Stability data for DDB ($n = 3$ per test and each concentration, ±S.D.)

	Added concentration (μg/ml)		
	0.028	0.14	2.80
Freeze–thaw stability (%)	96.96 ± 3.93	98.86 ± 2.79	99.65 ± 1.21
At room temperature, 24 h (%)	99.54 ± 6.07	102.33 ± 3.78	98.78 ± 0.86
At −20 °C, 24 h	95.98 ± 5.56	98.67 ± 2.36	98.84 ± 0.89

Then the residue was resuspended in 100 μl of mobile phase and centrifugated at 10,000 × g for 5 min. Aliquot of 20 μl was injected into the HPLC system. However, the method was very complicated and time-consuming. After several trials, a protein precipitation procedure using acetonitrile was finally adopted and proved to be simple and reliable for the sample preparation in this work. The results showed that deproteinization had a good resolution and high recovery.

3.2. Specificity

The chromatograms were free of interference from other compounds after precipitation of protein from plasma. The peak of DDB was symmetrical and the baseline separation was obtained in plasma (Fig. 2). The retention time was at about 12.3 min for DDB. These observations indicated that the assay had adequate specificity.

3.3. Sensitivity

Under the experimental conditions described above, the lowest limit of detection (LOD) of DDB in plasma was 5 ng/ml at signal to noise ratio of 3 and the lowest limit of quantification (LOQ) of DDB in plasma was 20 ng/ml.

3.4. Linearity

The calibration curve of DDB was linear in the range of 0.028–2.80 μg/ml in plasma. Using the linear least squares regression, the calibration line of DDB was $y = (91.74 \pm 15.46)x$ (μg/ml) + (1843.58 ± 324.78) ($n = 3$) with $r^2 = 0.9989$.

3.5. Precision and accuracy

The summaries of intra- and inter-day precision/accuracy at low, medium and high concentrations of DDB in plasma are listed in Table 1. The intra- and inter-day precisions of DDB are within 10.0% in plasma. The intra- and inter-day accuracy are ranged from 92.86% to 103.57% in plasma. And we also determined the precision and accuracy at the LOQ level, the data are 11.35% and 93.02%, respectively.

Based on the "Guidance for Industry: Bioanalytical Method Validation (FDA, May 2001)", a precision of less than 20% (relative standard deviation, R.S.D.) is required, and an accuracy between 80% and 120% of the theoretical value and the reproducibility of the assay is adequate. Our results indicate that the accuracy and precision of the current assay are within the recommendations.

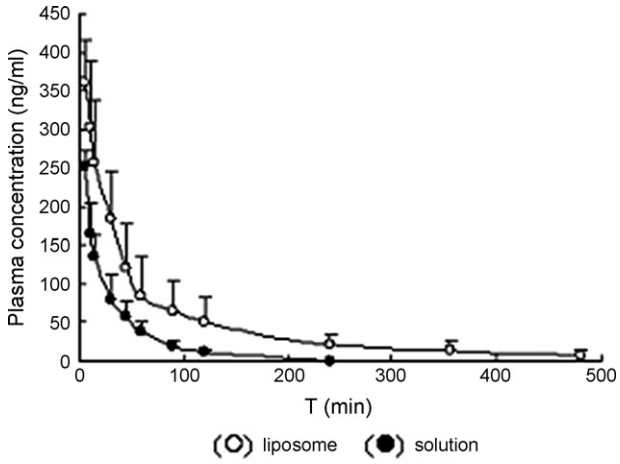

Fig. 3. Mean plasma concentration profile of DDB liposomes and solution after intravenous injection into rats ($n = 6$).

3.6. Extraction recovery and stability

The extraction recoveries of DDB in plasma samples were determined by comparing peak areas from plasma samples spiked before extraction with those from standard solutions at the same DDB level. The summaries of the mean recoveries are listed in Table 2. The results show that the extraction recoveries of DDB are ranged from 94.53% to 99.36% in plasma.

The stability experiments were aimed at testing samples under all possible conditions that the samples might experience after collecting and prior to analysis. The summaries are listed in Table 3. The study results demonstrated that no significant degradation of DDB in plasma occurred under different experimental conditions. The mean concentrations following the storage period were ranged from 95.98% to 102.33% in plasma.

Table 4
Pharmacokinetic parameters of DDB after intravenous injection of DDB solutions or liposomes in rats ($n = 6$)

Compartmental parameters	Parameters values ± S.D.		ANOVA (P)
	Liposome	Solution	
MRT_{0-T} (min)	127.11 ± 18.93	41.78 ± 4.13	<0.01
AUC_{0-T} (min ng/ml)	27210.81 ± 241.56	8576.59 ± 321.12	<0.05
k_{el} (min^{-1})	0.018 ± 0.0016	0.057 ± 0.011	<0.05
CL (mg/(min (ng/ml)))	3.88E−5 ± 2.58E−6	1.16E−4 ± 1.02E−5	<0.05
$T_{1/2\beta}$ (min)	120.74 ± 10.23	39.04 ± 5.62	<0.01

3.7. Application of HPLC method

The method described here was applied in a preclinical study of DDB to compare the pharmacokinetics of the DDB solution and DDB liposomes. The mean concentration–time profiles of DDB in plasma were shown in Fig. 3. The main pharmacokinetic parameters were listed in Table 4. The MRT and k_{el} value in the test group were 127.11 ± 18.93 min and 0.018 ± 0.0016 min^{-1}, respectively. In the control group, the MRT and k_{el} value were 41.78 ± 4.13 min and 0.057 ± 0.011 min^{-1}, respectively ($P < 0.01$). The value of AUC of DDB liposome in plasma was about three folds higher than that of the DDB solution. The pharmacokinetic parameters of DDB solution indicated that DDB was rapidly cleared in bloodstream. However, the pharmacokinetic parameters of DDB liposome indicated that the liposome did increase the concentrations of DDB in blood, retard the clearance and exhibit the property of sustained release. All these results implied that DDB liposome could remain a significantly longer time period than DDB solution in vivo.

4. Conclusions

Sample preparation plays an important role in accurately measuring the concentration of drugs in biological samples. Several different approaches were carried out to measure the concentration of DDB in rat plasma. Initially, we chose methanol and ethanol as the protein-precipitating solvent and the recovery was less than 60% and 65%, respectively. However, when we used acetonitrile as the protein-precipitating solvent, the recovery was more than 94%. Meanwhile, further experiments demonstrated that acetonitrile as protein precipitation solvent was proved a simple and reliable method for sample preparation. So acetonitrile rather than methanol or ethanol was selected as protein-precipitating solvent to achieve the expected peak shapes and high recovery of analyte.

The accumulation of liposomes in the mononuclear phagocyte system (MPS), primarily uptaked by liver Kupffer cells and spleen fixed macrophages, is a well-known phenomenon which may be useful for the treatment of liver diseases, however it can also cause toxic effect [15,16]. The DDB liposome could increase the concentration of DDB in rat plasma, and higher amounts of DDB in the plasma maybe helpful for the treatment of chronic hepatitis. It is still unknown whether the DDB liposome could increase the concentration of DDB in other tissues such as spleen and kidney, and might cause toxic effect.

In summary, a rapid, sensitive and simple HPLC method has been developed for the analysis of DDB in rat plasma using UV detector. Because of the relatively short chromatographic run time and straightforward sample preparation procedure, a sample throughput of about 100 per day was achieved. This method proved to be effective in measuring the concentration of DDB in rat plasma without any significant loss of resolution. Importantly, this method has been successfully applied to pharmacokinetic studies of DDB liposome in rats. Giving the LOQ (20 ng/ml), high accuracy and precision, the method reported here should be suitable for in vivo study of other DDB preparations in rats.

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