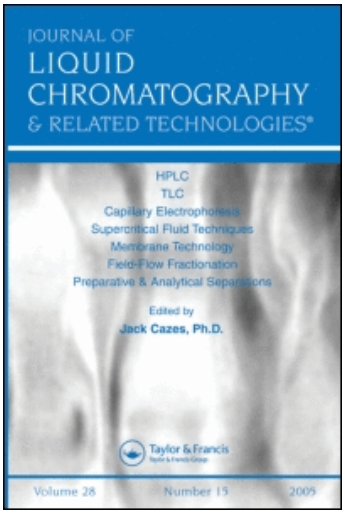

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HPLC ASSAY AND BIOEQUIVALENCE EVALUATION OF BIPHENYL DIMETHYL DICARBOXYLATE (DDB) PRODUCTS

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method was developed for biphenyl dimethyl dicarboxylate (DDB) detection in human serum and bioequivalence (BE) of two commercial DDB tablets was evaluated in 14 normal male subjects. The most suitable extracting solvent for DDB in serum was evaluated among dichloromethane, ether, ethylacetate, hexane, and pentane. Pentane:ether (9:1) mixture showed good extraction recovery of DDB from serum and also excluded serum components, which interfere the peak of DDB when assayed. HPLC conditions were as follows: UV absorbance detector, 280 nm; column, μ-Bondapak C_{18}; mobile phase, 10 mM phosphate buffer (pH 6.0), 33% acetonitrile, and 17% methanol; sensitivity, 0.005 aufs. BE was evaluated by 2x2 Latin square crossover method. DDB tablets (200 mg as DDB) were given orally and the serum concentration was detected by HPLC. The pharmacokinetic parameters, AUC_t, C_{max}, and t_{max} obtained after

dosing were statistically analyzed by ANOVA for crossover design. The results for all parameters were within 20% difference of mean value between reference and test drug. The results of ANOVA showed no significant differences for between group or subject and period. From the results, it is concluded that the bioavailability of test drug is not significantly different from reference drug and the two drugs are bioequivalent.

INTRODUCTION

Biphenyl Dimethyl Dicarboxylate (DDB, Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxy-biphenyl-2,2′-dicarboxylate) is a synthetic mimic of natural product Schizandrin C, an active compound isolated from Schizandriae Fructus.[1] Schizandriae Fructus has been known to have hepatoprotective effect traditionally in Orient.[2,3] DDB was reported to have hepatoprotective effect when hepatic damage was induced by carbon tetrachloride, thioacetamide, or D-galactosamine *in vivo* and *in* vitro.[4-7] DDB is used clinically for treatment of hepatitis and it has been reported that DDB reduces the levels of serum transaminases.[8-11]

DDB is known to be poorly soluble in water and it is poorly absorbed when administered orally.[12] Many studies were done to improve the absorption of DDB.[12-14] Due to its poor absorption, it is important to consider the bioavailability of the pharmaceutical products. To determine the bioavailability, blood concentration of DDB must be determined accurately and precisely. Many detection methods of DDB by HPLC have been performed, but the peak of DDB was hard to detect due to poor absorption and interference of the serum components. In this study, the detection method of DDB in serum by HPLC was established and controlled cross-over study was done to compare the DDB bioavailability of the two products at equivalent single dose of 200mg DDB to identify pharmaceutical equivalents of the two DDB formulations.

EXPERIMENTAL

Materials

The sources of DDB and internal standard(Diazepam) used to make the standards were obtained from Young Poong Pharm. Co. and Sam Jin Pharm. Co., respectively. Acetonitrile and methanol(Merck) were of HPLC grade. Solvents used for the extraction of DDB were an analytical grade.

Table 1

Effects of Extracting Solvent on the Recovery of DDB in Standard Solution

Solvent	Recovery (%)*	
	DDB Solution (1,000 ng/mL)	**DDB Solution (125 ng/mL)**
Dichloromethane	78 ± 5	40 ± 3
Ether	89 ± 3	53 ± 2
Ethylacetate	89 ± 2	27 ± 3
Hexane	63 ± 3	42 ± 2
Pentane	66 ± 2	46 ± 1
Pentane Ether (7:3)	72 ± 3	50 ± 2
Pentane Ether (9:1)	70 ± 2	50 ± 2

* Values reported are the mean ± S.D. of three measurements.

Preparation of Calibration Standard Solution

The calibration standard stock solution of DDB was made by dissolving 50 mg of DDB in 100 mL of acetonitrile. This stock solution was diluted with DDW to concentrations of 10,000, 5,000, 2,500, 1,250, 625, 312.5, and 156.25 ng/mL. The internal standard stock solution was prepared by dissolving 50 mg of diazepam in methanol. This stock solution was further diluted with mobile phase to yield the desired concentration of 5 μg/mL.

Effect of Extracting Solvent on the Recovery

Triplicates of 100 μL of standard solution of concentration of 10,000 ng/mL and 1,250 ng/mL was diluted with 900 μl of DDW to make a concentration of 1,000 and 125 ng/mL. These solutions were mixed with 100 μL of internal standard solution and extracted with 10 mL of extracting solvent, and then centrifuged at 2,500 rpm for 20 min at 4°C. Extracting solvents used in this experiment are shown in Table 1.

Nine mL of the organic phase was transferred into a test tube and evaporated to dryness by Speedvac (Savant AES2000, USA). The residue was reconstituted in 100 μL of the mobile phase and 50 μL of the solution was

injected into the HPLC system. As a 100% recovered DDB control, 50 μL of DDB solution was injected directly to HPLC system. The peak area ratios were used for the assay.

Effect of extracting time on recovery of DDB was also observed. Extracting time was 1, 3, or 5 min, but there was no significant differences between the extracting time (data not shown). So 1 min was decided as the extracting time for the following experiments.

Interference of Serum Components in Detection of DDB

100 μl of standard solution (concentration of 1,000ng/mL) and 100 μL of internal standard solution was mixed and evaporated to dryness by Speedvac. To the residue 1 mL of normal human serum was added and vortexed for 10 sec. 10 mL of extracting solvents shown in Table 1 was added and vortexed for 1 min and then centrifuged at 2,500 rpm for 20 min at 4°C. Nine mL of the organic phase were transferred into a test tube and evaporated to dryness by Speedvac (Savant AES2000, USA). The residue was reconstituted in 100 μL of the mobile phase and 50 μL of the solution was injected into the HPLC system.

Standard Calibration

900 μL of serum was added to 100 μL of each standard solution, to make DDB concentration of 2,000, 1,000, 500, 250, 125, 62.5, 31.25, and 15.625 ng/mL. To these mixtures, 100 μL of internal standard solution, concentration of 5 μg/mL, was added and extracted with 10 mL of pentane:ether (9:1) as described above. Nine mL of the organic phase was transferred into a tube and evaporated by Speedvac. The residue was reconstituted in 100 μL of the mobile phase and 50 μL of the solution was injected into the HPLC system. The peak area ratio of DDB to the internal standard was used for the assay.

Chromatographic Conditions

HPLC conditions are as follows: UV absorbance detector(Spectra-physics), 280 nm; column, μ-Bondapak C_{18}(10 μm, 3.9x300 mm); mobile phase, 10 mM phosphate buffer (pH 6.0), 33% acetonitrile and 17% methanol; flow rate(Waters 510), 1.2 mL/min; sensitivity, 0.005 aufs. The total run time was 16 min. The concentration of DDB in serum were calculated by PC800 integrator(Waters, version 2.0) using peak area ratio to the internal standard.

Intra- and Inter-day Variability

DDB concentrations used to determine the variability were 1,000 ng/mL for the high concentration and 125 ng/mL for the low concentration. DDB extraction was done by pentane:ether (9:1). Other procedures were the same as described above. To determine the inter-day variability, DDB concentration was determined for 3 consecutive days. Intra-day variability was evaluated from three experiments in a day.

Protocol for Bioequivalence Test

Subjects and study design

Fourteen healthy male volunteers (age; 22.4 ± 2.2 years) were selected after passing a screening procedure including a physical examination, blood pathological test, and serum biochemical examination. Volunteers were excluded if they had any history of illness or had taken alcohol or other drugs during the three days proceeding the study. Volunteers were assigned in a random manner into Group 1 (n=7) and Group 2 (n=7). The study design was Latin-square two-period crossover evaluation of the bioavailability of two tablet formulations of DDB, Hepassel® (Young Poong Pharm. Co., test drug) and Nissel® (Pacific Pharm. Co., reference drug). Volunteers, fasted for 12 hours, were assigned to receive either a single dose of the test or control DDB tablet (200 mg as DDB) with 200 mL of water. There was a 7-day washout period between medications. Serial blood samples (5 mL) were withdrawn by venipuncture at 0, 1, 2, 3, 4, 6, 8, and 12 hours after dosing. Light meals were served 4 and 10 hours after the administration. The same standard meals were provided in all periods. The sera was separated from the blood by centrifugation (3,000 rpm, 30 min, 4°C) and kept at -20°C until prepared for analysis by HPLC.

Serum analysis

The serum sample was pretreated and analyzed by HPLC as described above. In brief, 100 μL of internal standard solution (5 μg/mL) was added to 1mL of serum and extracted with 10 mL of pentane:ether (9:1) mixture for 1 min, centrifuged at 2,500 rpm (4°C) for 20 min, then 9 mL of the supernatant was evaporated by Speedvac. The residue was reconstituted in 100 μL of mobile phase and 50 μL was injected on the column. The HPLC condition was the same as described above.

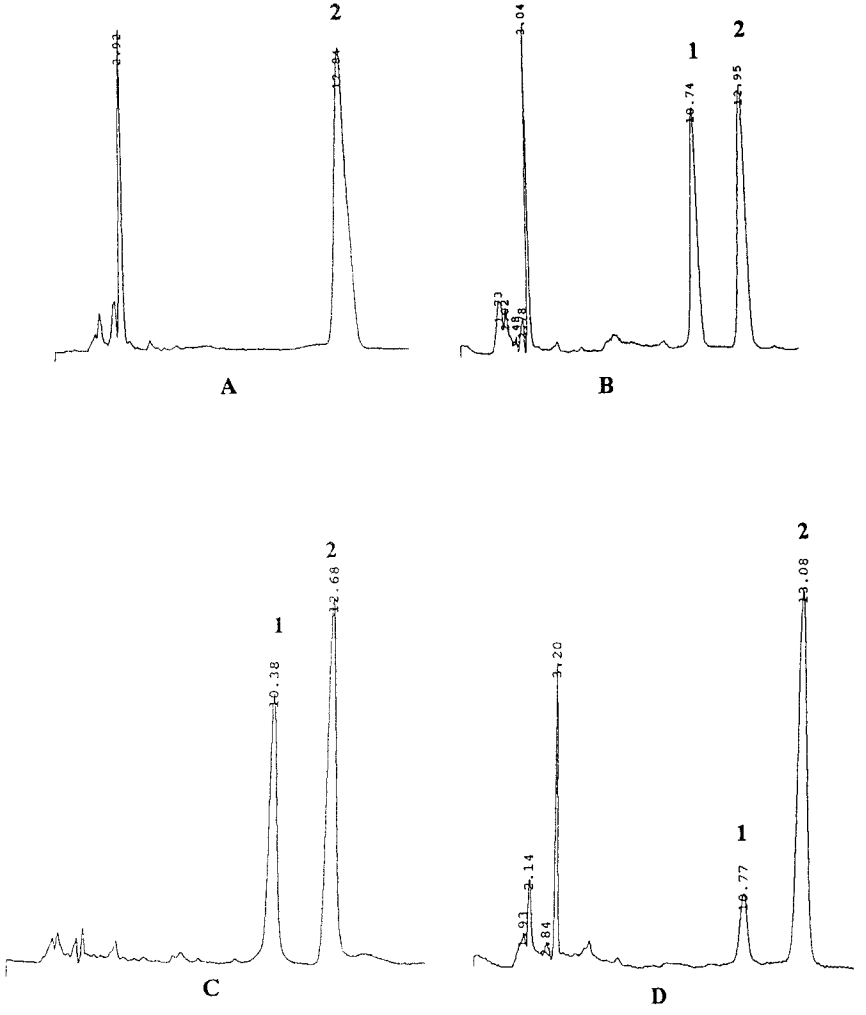

Figure 1. Chromatograms of (A) human blank serum spiked with internal standard; (B) human blank serum spiked with DDB and internal standard; (C) serum from a volunteer 3 hour after dosing; (D) serum from a volunteer 12 hour after dosing. Peaks: 1 = DDB; 2 = Internal Standard (Diazepam).

Pharmacokinetics and Statistical Analysis

The bioequivalence evaluation of the two tablet formulations of DDB was based on the pharmacokinetic parameters such as the area under the serum concentration-time curve (AUC_t), the peak serum concentration (C_{max}), and the time of peak serum concentration (t_{max}). AUC_t was calculated by trapezoidal rule. C_{max} and t_{max} were directly obtained from DDB concentration-time curves of each volunteers. Analysis of variance was performed to evaluate the statistical significance of the mean values of pharmacokinetic parameters (AUC_t, C_{max}, t_{max}).

RESULTS AND DISCUSSION

Effect of Extracting Solvent on Detection of DDB

DDB in standard solution was extracted with solvents such as dichloromethane, ether, ethylacetate, hexane, and pentane. The extraction recovery of DDB is shown in Table 1. Ether and ethylacetate showed the highest extraction recovery of 89%. But ether and ethylacetate also extracted various components in serum so that it was impossible to detect the peak of DDB. Dichloromethane, which has a recovery of 78%, also extracted various components of serum so that the peak of DDB was overlaid with the peak of serum components. Pentane and hexane which have a recovery of 66% and 63%, respectively, excluded the interfering components of serum but the recovery of DDB was low. Therefore, ether, which has the highest recovery of DDB, was mixed with pentane and used as the extracting solvent. Two kinds of mixture were used as shown in Table 1. The DDB chromatogram extracted with pentane:ether (7:3) mixture showed analyte peaks slightly interfered by serum components (chromatogram not shown) while that of the pentane:ether (9:1) extracted sample did not. Therefore the pentane:ether (9:1) mixture was used as the extracting solvent throughout the experiment.

Chromatograms of blank serum and post dose serum of the volunteer extracted by pentane:ether (9:1) shows the specificity within this detection method by absence of interfering peaks from serum components (Fig.1). The analytes, DDB and the internal standard (diazepam) were detected at 10.7 and 12.9 min, respectively The calibration curve between DDB concentration and peak area ratios was linear within the ranges of 15.625 to 1,000 ng/mL ($y=0.536x-0.03443$, $r^2=0.999$) (Fig. 2).

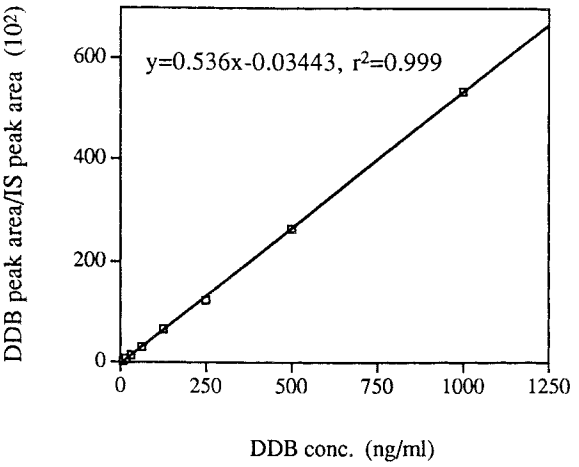

Figure 2. The relationship between peak area ratio of DDB to internal standard (Diazepam) and serum concentration of DDB in human.

Table 2

Intra-Day Variability of the Assay When DDB in Serum Was Extracted with Pentane:Ether Mixture (9:1)*

	Concentration of DDB (ng/mL)	Concentration Detected (ng/mL)	Coefficient of Variation (%)
Day 1	1,000	702.17 ± 22.29	3.17
	125	62.78 ± 3.08	4.90
Day 2	1,000	734.54 ± 47.80	6.51
	125	64.86 ± 3.37	5.19
Day 3	1,000	708.39 ± 38.94	5.50
	125	60.85 ± 2.77	4.56

* Values reported are the mean ± S.D. of three measurements.

The coefficients of variation (CV) of intra-day assay were ranged from 3.17% to 6.51%, and those of inter-day assay was 2.40% to 3.20% (Table 2, 3). From the results, it is concluded that application of this method is convenient and accurate in pharmacokinetic study of DDB products.

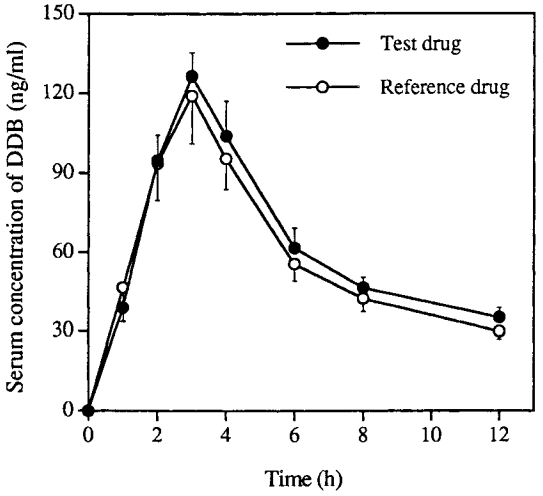

Figure 3. DDB concentration in human serum-time curves of volunteers after oral administration of two DDB products. Each point represents the mean concentration (± S.D.) of 14 normal volunteers.

Table 3

Inter-Day Variability of the Assay When DDB in Serum Was Extracted with Pentane:Ether Mixture (9:1)*

Concentration of DDB (ng/mL)	Concentration Detected (ng/mL)	Coefficient of Variation (%)
1,000	715.03 ± 17.18	2.40
125	62.83 ± 2.01	3.20

* Values reported are the mean ± S.D. of three measurements.

Bioequivalence Test of DDB Products

Figure 3 shows the mean serum concentration-time curves of DDB of volunteers after a single oral dose of Hepassel® (test drug) and Nissel® (reference drug), 200 mg as DDB, by 2x2 Latin square crossover method. The

Table 4

The Mean Values and Confidence Limit of AUC_{to} C_{max} and t_{max} of Test and Reference Drug*

Products	AUC_t (ng.hr/mL)	C_{max} (ng/mL)	t_{max} (hr)
Test drug	746.09 ± 136.94	129.57 ± 20.40	3.14 ± 0.53
Reference drug	698.46 ± 176.95	124.02 ± 38.52	3.21 ± 0.43
Confidence limit	6.189%	4.475%	2.222%

* Values reported are the mean ± S.D. of 14 volunteers.

mean concentration at times were not significantly different and both drugs showed maximum serum concentration at 3 hour. Statistical analysis for each pharmacokinetic parameters, such as AUC_t, C_{max} and t_{max} were estimated by ANOVA (2x2 Latin square crossover method). The means of C_{max} , T_{max}, and AUC_t of the test drug were within ± 20% of those of the reference drug as shown in Table 4, which shows that the two products are bioequivalent according to the principle of the bioequivalence test. The results of ANOVA showed that the two products were not significantly different. From the results above it is concluded that the bioavailability of Hepassel® (test drug) is significantly not different from that of Nissel® (reference drug) and that the two DDB products are bioequivalent.

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