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Short communication

Rapid microbore liquid chromatographic analysis of biphenyldimethyl dicarboxylate in human plasma with on-line column switching

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Abstract

A fully automated method including microbore liquid chromatography and column switching was developed for the analysis of biphenyldimethyl dicarboxylate (DDB) from human plasma samples. After direct injection of plasma samples (100 μl) into the system, deproteinization and analyte fractionation occurred on a Capcell Pak MF Ph-1 column (20×4 mm I.D.) and the DDB fraction was transferred from the MF Ph-1 column to an intermediate column (35×2 mm I.D.) using 15% acetonitrile in phosphate buffer (50 m*M*, pH 7.0). The main separation was performed on a microbore C_{18} column (150×1.5 mm I.D.) using 45% acetonitrile in water. The method showed excellent sensitivity (detection limit of 5 ng/ml) and good precision (C.V.≤3.0%), and shortened total analysis time (20 min). In the concentration range of 5–200 ng/ml, the mean recovery was 90.7±1.8% and the response was linear (r^2≥0.999).

Keywords: Biphenyldimethyl dicarboxylate

1. Introduction

Biphenyldimethyl dicarboxylate (dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxy-biphenyl-2,2′-dicarboxylate, DDB), a synthetic derivative of the naturally occurring schizandrin, has been used as a hepato-protective drug in the treatment of chronic hepatitis. The bioavailability of the poorly-water soluble DDB varies with its solubilization, and therefore, the studies have been needed to design a new DDB formulation that is more effectively absorbed [1].

High performance liquid chromatography (HPLC) [1,2] methods have been reported for the determination of DDB in biological samples. These methods involve time-consuming liquid–liquid extraction using a relatively large sample volume (1 ml) and do not provide good assay sensitivity for the therapeutic monitoring of DDB. A rapid and sensitive method for the measurement of DDB in plasma samples over the concentration range of 5–200 ng/ml is required.

Column switching is multidimensional technique that can directly analyze biological samples in conventional-size [3,4] and microbore LC [5–9]. Dual- [5,6] and triple-column systems [6–9] have

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been used for the direct analysis of hundreds of microliters of biological fluids without any loss in the sensitivity increase and chromatographic efficiency obtained by the microcolumns. In particular, a triple-column system using an intermediate column is designed to save the analysis time in a 1.5 mm and smaller main column and protect the precolumn from high pressure compared to the dual-column system [6].

The purpose of this study was to develop an automated microbore HPLC method with a triple-column system for the direct analysis of DDB from plasma over the range of 5–200 ng/ml. The present method requires only 100 μl of plasma sample volume and provides good assay sensitivity (5 ng/ml) and short total analysis time (20 min) including the sample clean-up. This method was successfully applied to the study of the pharmacokinetics of DDB in human volunteers.

2. Experimental

2.1. Materials and reagents

DDB was obtained from Young Poong Pharm. Co. (Incheon, South Korea). HPLC grade methanol and acetonitrile were purchased from Burdick and Jackson, Inc. (Muskegon, MI, USA). A stock solution of DDB was prepared by dissolving DDB in 100 μg/ml of acetonitrile and aliquots were spiked with drug-free human blank plasma to obtain the calibration plasma standards at five concentrations of 5, 10, 50, 100, and 200 ng/ml. Plasma samples were filtered with a low protein binding membrane syringe filter (0.22 μm, PVDF, Millipore, Bedford, MA, USA).

2.2. Column switching system

The schematic diagram of the column switching system for a semi-micro LC is shown in Fig. 1. The system consisted of the Nanospace SI-1 series (Shiseido, Tokyo, Japan), i.e., two 2001 pumps, 2002 UV–VIS detector, 2003 autosampler, 2004 column oven, 2012 high pressure switching valve, and 2009 degassing unit. The system was operated by Syscon (Shiseido, Tokyo, Japan) and the signals were processed by S-MicroChrom (Shiseido, Tokyo, Japan).

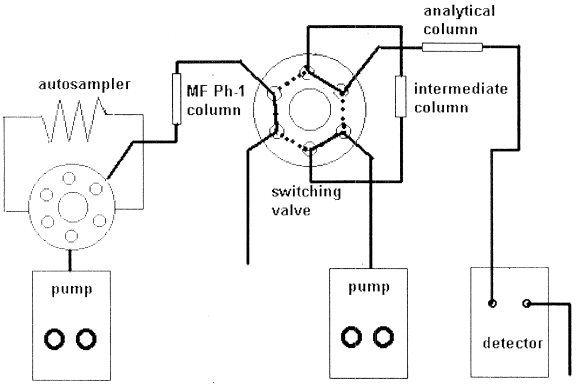

Fig. 1. Schematic diagram of a column switching system for a semi-microcolumn LC. Position A, ———; position B, ----------.

2.3. HPLC conditions

For the pre-separation and concentration of DDB from the plasma samples, Capcell Pak MF Ph-1 cartridge (20×4 mm I.D., 5 μm, Shiseido, Tokyo, Japan) and Capcell Pak C_{18} UG 120 (35×2 mm I.D., 5 μm) were used as the pre-column and the intermediate column, respectively, with 15% acetonitrile in 50 m*M* phosphate buffer (pH 7.0). The main separation was performed on Capcell Pak C_{18} UG 120 (150×1.5 mm I.D., 5 μm) by 45% acetonitrile in water at a flow-rate of 0.1 ml/min. The column temperature was 30°C and the effluent was monitored at 280 nm.

2.4. Analytical procedure

2.4.1. Step 1 (0–4.2 min, valve position A)

After injection of the plasma sample (100 μl) on a Capcell Pak MF Ph-1 column, plasma proteins were removed and the target compound was fractionated using 15% acetonitrile in 50 m*M* phosphate buffer (pH 7.0) at a flow-rate of 0.5 ml/min. The intermediate column and analytical column were equilibrated using the mobile phase.

2.4.2. Step 2 (4.2–6.1 min, valve position B)

The DDB fraction was transferred from Capcell Pak MF Ph-1 pre-column and refocused onto an intermediate C_{18} column by the washing solvent at a flow-rate of 0.5 ml/min.

2.4.3. Step 3 (6.1–20.0 min, valve position A)

The analytes, enriched on an intermediate C_{18} column, were separated on a microbore C_{18} column with 0.1 ml/min of 45% acetonitrile in water. In the meanwhile, the MF Ph-1 column was equilibrated with a washing solvent.

2.5. Method validation

The limit of detection (LOD) for DDB was determined as the concentration of drug giving a signal-to-noise ratio greater than 5:1. The recovery of DDB from plasma was determined by the analysis of fixed amounts of DDB in plasma, followed by a replicate injection of the same amount of a standard in 5 μl of mobile phase directly onto the microbore column providing the 100% value. Five DDB-spiked plasma standard samples over the concentration range of 5–200 ng/ml were quantified to evaluate the linearity, precision (the coefficient of variation (C.V.) of replicate analysis) and accuracy (the bias between theoretical and actual concentration). Intra- and inter-day reproducibility was evaluated from six experiments in a day and six consecutive days, respectively.

2.6. Pharmacokinetics of DDB in human volunteers

Three healthy male volunteers received a single oral dose of a DDB tablet (100 mg). Blood samples (1 ml) were withdrawn from the forearm vein at 0.67, 1.3, 2.0, 2.7, 3.3, 4.0, 6.0, 8.0, and 10.0 h post-dosing, transferred to Vacutainer tubes, and centrifuged. Following centrifugation (3000 *g*, 15 min, 4°C), plasma samples were transferred to eppendorf tubes and stored at −70°C prior to analysis. Drug concentrations were determined as the mean of duplicate samples. The peak concentration (C_{max}) and the time to peak concentration (T_{max}) were determined by visual inspection from each volunteer's plasma concentration–time plots for DDB. The area under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal method from 0 to 10 h. Plasma elimination half-life ($t_{1/2}$) was determined from the descending slope of the concentration–time profile after logarithmic transformation of the concentration data.

3. Results and discussion

3.1. Triple column system

The use of a microcolumn (1.5 mm I.D.) for the determination of DDB from plasma samples resulted in many advantages, such as small plasma volume (100 μl vs. 1 ml), increased sensitivity (500 pg vs. 5 ng of DDB), high column efficiency, and lower solvent consumption over the conventional-size HPLC [1,2]. As shown in Fig. 2, there was no interference peak at the retention time of DDB (15.6 min).

The triple column system [6–9] was also a useful on-line sample preparation technique for the rapid analysis of the low levels of DDB in plasma samples without any loss in high sensitivity provided by microbore LC. The pre-column packing, washing solvent, and valve-switching time must be chosen in order to obtain good recovery and a clean chromatogram using column switching.

Capcell Pak MF Ph-1, bonded hydrophilic polyoxyethylene groups and hydrophobic phenyl groups to silicone polymer-coated silica [10], was the appropriate pre-column for the deproteinization and DDB concentration because plasma proteins pass through the column quickly due to restricted access to the surface of packing while retaining DDB on the bonded phenyl phase.

To choose the washing solvent and the appropriate switching time, the retention behavior of DDB on the MF Ph-1 pre-column was examined using different mixtures of acetonitrile and 50 m*M* phosphate buffer (pH 7.0). By using 15% acetonitrile in 50 m*M* phosphate buffer (pH 7.0) as the washing solvent, DDB was separated from plasma proteins on the MF Ph-1 precolumn and DDB was quantitatively recovered from MF Ph-1 to an intermediate column in proper time.

The intermediate column (35×2 mm I.D.) was used to protect a main column and save the analysis time, because without an intermediate column it takes 9.5 min to transfer the DDB fraction from the MF Ph-1 pre-column to the analytical column at a flow-rate of 0.1 ml/min.

The MF Ph-1 precolumn was exchanged after injection of 40 plasma samples (equivalent to 4.0 ml plasma). The intermediate and main columns showed

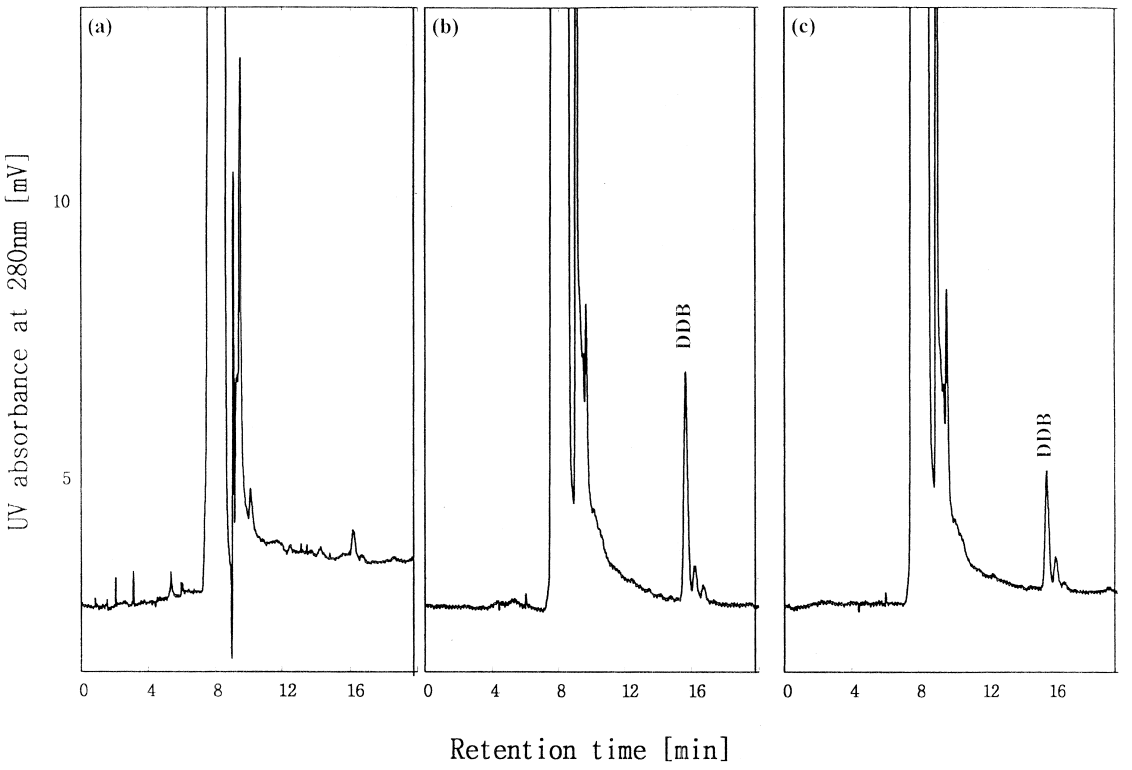

Fig. 2. Chromatograms of (a) blank plasma, (b) blank plasma spiked with DDB (50 ng/ml), and (c) plasma sample at 6 h after an oral dosing of 100 mg DDB to a healthy male volunteer.

no decrease in efficiency after more than 200 injections of plasma samples.

3.2. Method validation

The mean absolute recovery of DDB from plasma samples was 90.7±1.8%. The calibration curve of peak area vs. the concentration of DDB in plasma was linear giving a correlation coefficient of 0.999 in the range of 5–200 ng/ml. The LOD of DDB was 5 ng/ml using 100 μl plasma and sufficient for the therapeutic monitoring of DDB compared to the previous method [4] (15 ng/ml using 1 ml plasma). The intra- and inter-day precision and the accuracy of the assay are shown in Table 1. The actual amount in the spiked plasma samples deviated from −0.2% to 0.4% of the theoretical amount, and the assay was precise because C.V. was less than 3.0%.

Table 1
Reproducibility of biphenyldimethyl dicarboxylate in plasma samples (N=6)

Theoretical concentration (ng/ml)	Concentration found (ng/ml)		C.V. (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
5	5.0	5.1	2.8	3.0
10	10.2	9.9	2.4	2.7
50	49.9	50.2	0.6	1.8
100	99.8	102.0	0.6	2.0
200	200.6	200.1	1.2	1.4

Table 2
Plasma concentrations of DDB in human after an single oral dose of 100 mg of DDB

Time (h)	Plasma concentration of DDB (ng/ml)			
	1	2	3	Mean±SD
0.67	22.8	5.4	6.6	11.6±9.7
1.33	49.6	28.5	32.9	37.0±11.1
2.00	80.4	50.5	53.5	61.5±16.5
2.67	86.8	35.2	43.1	55.0±27.8
3.33	79.6	34.8	75.5	63.3±24.8
4.0	71.1	30.6	85.5	62.4±28.5
6.0	34.6	23.8	36.3	31.6±6.8
8.0	33.9	17.3	23.9	25.0±8.4
10.0	26.3	13.0	20.4	19.9±6.7

3.3. Pharmacokinetics of DDB in human volunteers

The applicability of this method was proved in the study of the pharmacokinetic disposition of DDB in humans. Table 2 shows the plasma concentrations of DDB after a single oral administration of a commercial DDB tablet (100 mg DDB) by three male volunteers and the pharmacokinetic parameters, such as AUC, C_{max}, T_{max}, and $t_{1/2}$ of DDB, were 369.6±117.3 ng×h/ml, 74.3±20.6 ng/ml, 2.89±1.02 h, and 6.31±2.02 h, respectively. These values are comparable to the pharmacokinetic parameters (AUC=746.1±136.9 ng×h/ml, C_{max}= 129.6±20.4 ng/ml, T_{max}=3.14±0.53 h) obtained by an single oral dose of 200 mg DDB [2].

4. Conclusion

For the direct analysis of DDB from human plasma samples, an automated microbore HPLC method using column switching has been developed without a time-consuming sample clean-up. Many advantages such as excellent sensitivity (5 ng/ml using 100 μl plasma), reproducibility, specificity, and speed (20 min per sample) were demonstrated. The applicability of the method was demonstrated in the pharmacokinetic study of DDB in man.

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