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Short communication

Determination of bifendate in human plasma by HPLC–MS and bioequivalence on bifendate pills in healthy volunteers

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Abstract

A sensitive and specific method for the determination of bifendate in human plasma was developed, based on high-performance liquid chromatography (HPLC)–mass spectrometry (MS). The samples were extracted from plasma with diethyl ether, followed by separation and evaporation after addition of internal standard diazepam. The residue was reconstituted in methanol and injected into the HPLC–MS. Chromatography was performed on an Inertsil ODS column with a mobile phase consisting of methanol–distilled water (70/30, v/v) at a flow rate of 0.3 mL/min. Quantitative analysis was achieved by MS detection, using a mass spectrometer equipped with an electrospray ionization interface (ESI) and operated in selected ion monitoring (SIM) and positive-ionization mode using target ions at *m/z* 419 for bifendate and *m/z* 285 for internal standard, respectively. The linearity was confirmed in the concentration range of 2–200 ng/mL in human plasma and the precision of this assay was not more than 6.79% over the entire concentration range. The method was sensitive and repeatable enough to be used in pharmacokinetic and bioavailability studies.

Keywords: Bifendate; High-performance liquid chromatography–mass spectrometry; Pharmacokinetic; Bioequivalence

1. Introduction

Bifendate (4,4′-dimethoxy-5,6,5′,6′-bi (methylenedioxy)-2,2′-bicabomethoxybiphenyl, Fig. 1), which was created and widely used in China, is effective in the treatment of chronic hepatitis [1,2] by lowering alanine transaminase (ALT) in patients. It is rapidly absorbed from gastrointestinal tract after oral administration and the plasma concentration is rather low, so it is necessary to establish a simple, sensitive and rapid method to determine the plasma concentration of bifendate and evaluate the relative bioavailability of different formulations.

An RP–high-performance liquid chromatography (HPLC) method was applied to the determination of bifendate in human plasma previously [3]. However, the detection limit was 0.1 μg/mL plasma [3]. Due to its low sensitivity, the dose of medicine taken had to be approximately 200 mg, much more than the therapeutic dose (15 mg). Taking security and reasonableness into consideration, it is not applicable to pharmacokinetics and bioavailability study.

It is well known that HPLC–mass spectrometry (MS) is a very specific and sensitive method when used for the determination of drugs in biological fluids. Sample preparation may be simplified, and the sensitivity is much higher than in HPLC–UV. This paper describes a rapid, selective and sensitive method for the determination of bifendate in human plasma using HPLC–MS, and this method has been successfully used for clinical bifendate pharmacokinetic and bioequivalence studies.

2. Experimental

2.1. Reagents and materials

Test bifendate pill (batch number: 20021115, 1.5 mg/pill) was supplied by Baijia Pharmaceutical Co. Ltd. (Wenling, Zhejiang Province, PR China). Reference pill (batch number: 031209, 1.5 mg/pill) was produced by Zhejiang Medicine Co. Ltd., Xinchang Pharmaceutical Factory (Xinchang, Zhejiang Province, PR China). Bifendate standard (batch number: 10192–0102)was purchased from National Institute For the Control of Pharmaceutical and Biological Products (NICPBP) Beijing, PR China. The internal standard (IS) diazepam (>99.5%) (Fig. 1), was obtained

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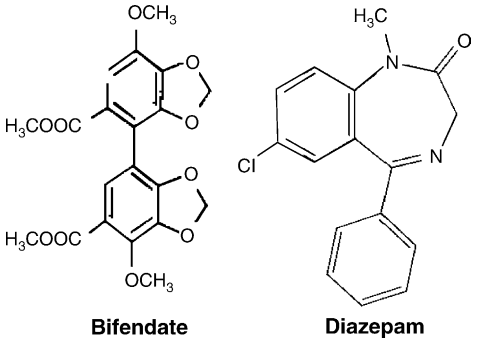

Fig. 1. Chemical structure of bifendate and the internal standard (diazepam).

from Shanghai Institute for Drug Control (Shanghai, PR China). Methanol (HPLC grade reagent) was obtained from Merck Inc. (Darmstadt, Germany). Double distilled water was purified by Millipore Direct-Q^{TM} (Millipore Corporation, Benford, MA, USA). Diethyl ether (analytical grade reagent) was purchased from Yangyuan chemical plant (Changshu, Jiangsu Province, PR China).

2.2. Instrumentation

The analysis was performed on an Agilent HP1100MSD system (Agilent Corp. Ltd. Palo Alto, CA, USA) equipped with binary pump, on-line vacuum degasser, auto-sampler, column compartment and Agilent ChemStation (Agilent Corp. Ltd. Palo Alto, CA, USA). The samples were separated on an Inertsil ODS (150 mm × 2.1 mm, 5 μm) column (GL. Sciences Inc., Tokyo, Japan). Quantitative analysis was achieved by MS detection using a quadrupole MS equipped with an electrospray ionization interface (ESI) and operated in selected ion monitoring (SIM). Fig. 2 displays the full scan mass spectra of bifendate and diazepam, respectively. Bifendate was detected at the mass/charge ratio 419 while diazepam at 285.

2.3. Stock solutions

Two different stock solutions were prepared by the independent weighing of bifendate. One was prepared for validation and calibration, the other was for quality control samples. Stock solutions of bifendate were prepared at 100 μg/mL in methanol and stored at 4 °C until use. The stock IS solution was prepared as methanol solution (10.1 μg/mL). All stock solutions were proved to be stable for at least two months when stored at 4 °C.

2.4. Preparation of calibration standards (CS) and quality control (QC) samples

Calibration standards samples were prepared by spiking respective stock solutions in blank human plasma at concentrations of 2, 5, 10, 20, 50, 100 and 200 ng/mL. CS samples were prepared from a blank plasma pool. Bifendate stock solution for QC was prepared separately and QC plasma samples

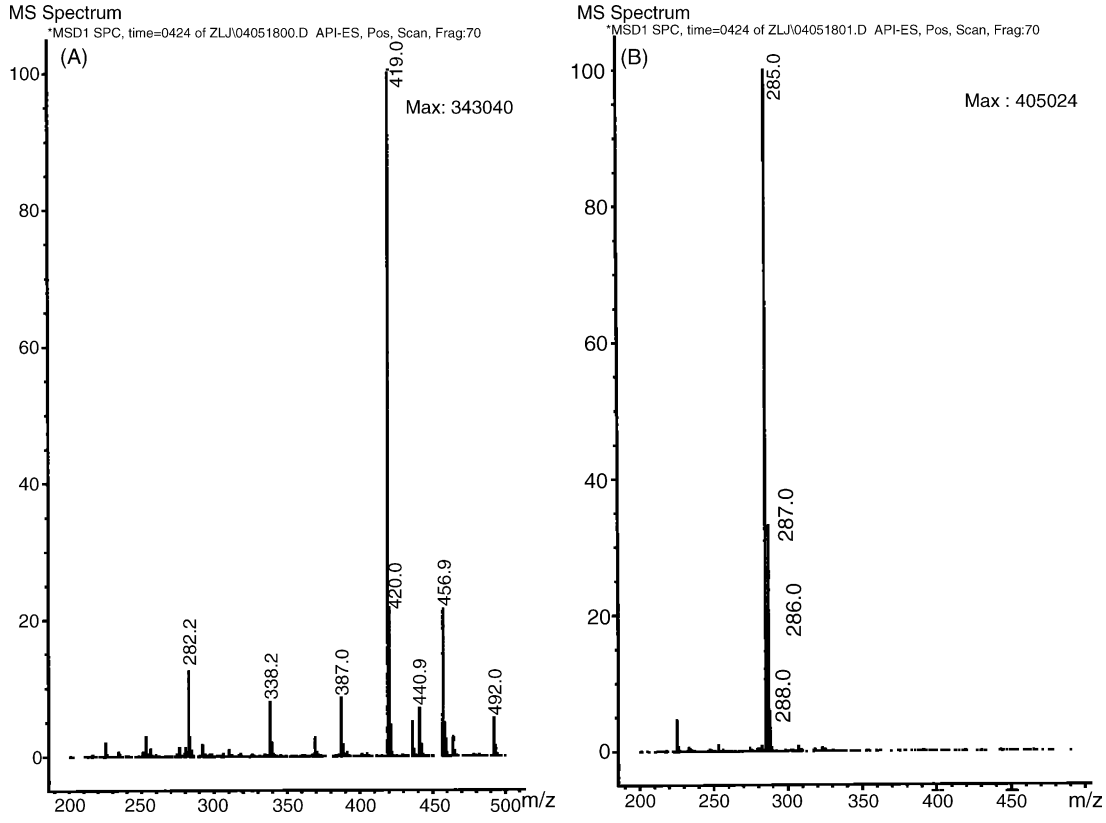

Fig. 2. Full scan mass spectra of bifendate (A) and diazepam (B).

were prepared at 2, 4, 40 and 160 ng/mL in the same manner as for plasma standard. QC samples were prepared from different matrix pools on each day of analysis.

A 500 μL plasma sample was extracted with 5 mL diethyl ether after addition of 100 μL IS solution (10.1 μg/mL). After vortex mixing for 2 min, the supernatant was evaporated in a water bath (40 °C) with nitrogen steam. Then the residue was reconstituted in 100 μL methanol and 5 μL aliquot was injected into the chromatograph.

2.5. HPLC–MS conditions

HPLC separation was performed using an Inertsil ODS (150 mm × 2.1 mm, 5 μm) reversed phase column at a column temperature of 40 °C. Methanol–distilled water (70/30, v/v) was used as mobile phase at a flow rate of 0.3 mL/min. The whole analysis was complete within 7.0 min.

Electrospray ionization was performed in the positive ion mode with nitrogen as the nebulizer and drying gas. The exact conditions were capillary voltage 4000 V, nebulizer pressure 45 p.s.i., drying gas temperature 350 °C, drying gas flow 10 L/min, fragmentor voltage 100 V.

Quantitative analysis was achieved by MS detection in the positive ion mode, using a mass spectrometer (mass range 50–3000) equipped with an electrospray ionization interface. In the selective ion monitoring mode, the m/z 419 was detected for bifendate (MW 418.36), and 285 for the internal standard diazepam (MW 284.74).

2.6. Validation test

2.6.1. Linearity and calibration curve

To make calibration standards, the stock standard solution of bifendate was added to 0.5 mL of drug-free plasma. The spiked concentrations of the calibration standard were 2, 5, 10, 20, 50, 100 and 200 ng/mL. The samples were then processed as described in Section 2.4. Each concentration of analysis was based on triplicates. The calibration curves were constructed by plotting the peak-area ratio of bifendate to internal standard versus spiked concentration. The calibration curves were calculated by least-squares regression.

2.6.2. Specificity and interference

Chromatograms of the sample prepared with human blank plasma were visually inspected for peaks from endogenous sources which might correspond to the bifendate and IS peaks. The standard plasma sample extract was dissolved in 100 μL methanol and 5 μL aliquot was injected into the chromatograph to determine the detection limit ($S/N = 3$).

2.6.3. Accuracy and precision

Five replicate samples at three concentration levels (5, 20, 100 ng/mL, $n = 5$) were prepared and assayed to determine the intra-day accuracy expressed as the relative error (RE) and the intra-day precision expressed as the relative standard deviation (R.S.D.). The same method was used for five days to assess inter-day precision.

2.6.4. Extraction recovery

The extraction recovery of bifendate was determined at concentrations of 5, 20 and 100 ng/mL separately. Recovery was calculated by comparison of the peak areas of bifendate extracted from plasma samples with those of standards diluted in the mobile phase.

3. Results

3.1. Selectivity and specificity

High selectivity was found for the determination of drugs in plasma samples. The SIM chromatograms of blank plasma and spiked plasma samples (2 ng/mL) are shown in Fig. 3. No endogenous sources of interference were observed at the retention time of the analyte. A sample from a subject after intake of bifendate was also shown in Fig. 3. The detection limit was 1.0 ng/mL ($S/N = 3$).

3.2. Linearity and calibration curves

Acceptable linearity was observed over the concentration range of 2–200 ng/mL plasma ($r = 0.9992$–0.9999). The R.S.D. ($n = 5$) of the slope calculated with calibration curve data was 6.47%, showing a good repeatability (Table 1).

3.3. Accuracy and precision

The intra- and inter-day precision and accuracy values are listed in Table 2. The R.S.D. of bifendate ranged from 2.44 to 6.34% for intra-day and from 5.18 to 6.79% for inter-day, respectively. The RE of bifendate ranged from −3.6 to 1.3%.

3.4. Extraction recovery

The mean extraction recoveries of bifendate determined at low, medium and high concentrations in plasma are shown in Table 3. The R.S.D. of the recoveries were 7.88, 6.63 and 6.46% in 5, 20, 100 ng/mL plasma standard, respectively. No matrix effect for bifendate was observed to influence the ionization of analytes for these different plasmas. This indicated that the method yielded good extraction and high recovery values, and the extracts were clean with little or no detectable co-eluting compounds that could influence the ionization of the analytes.

Table 1
Inter-day precision of the slope and intercept of standard curves ($r = 0.9992$–0.9999)

Days	Slope	Intercept	Correlation coefficient
1	0.0270	−0.0336	0.9994
2	0.0293	−0.0331	0.9998
3	0.0302	−0.0329	0.9999
4	0.0311	−0.0289	0.9994
5	0.0320	−0.0345	0.9992
Mean ± S.D.	0.0299 ± 0.0019	−0.0326 ± 0.0022	0.9995 ± 0.0003
R.S.D. (%)	6.47	6.60	0.03

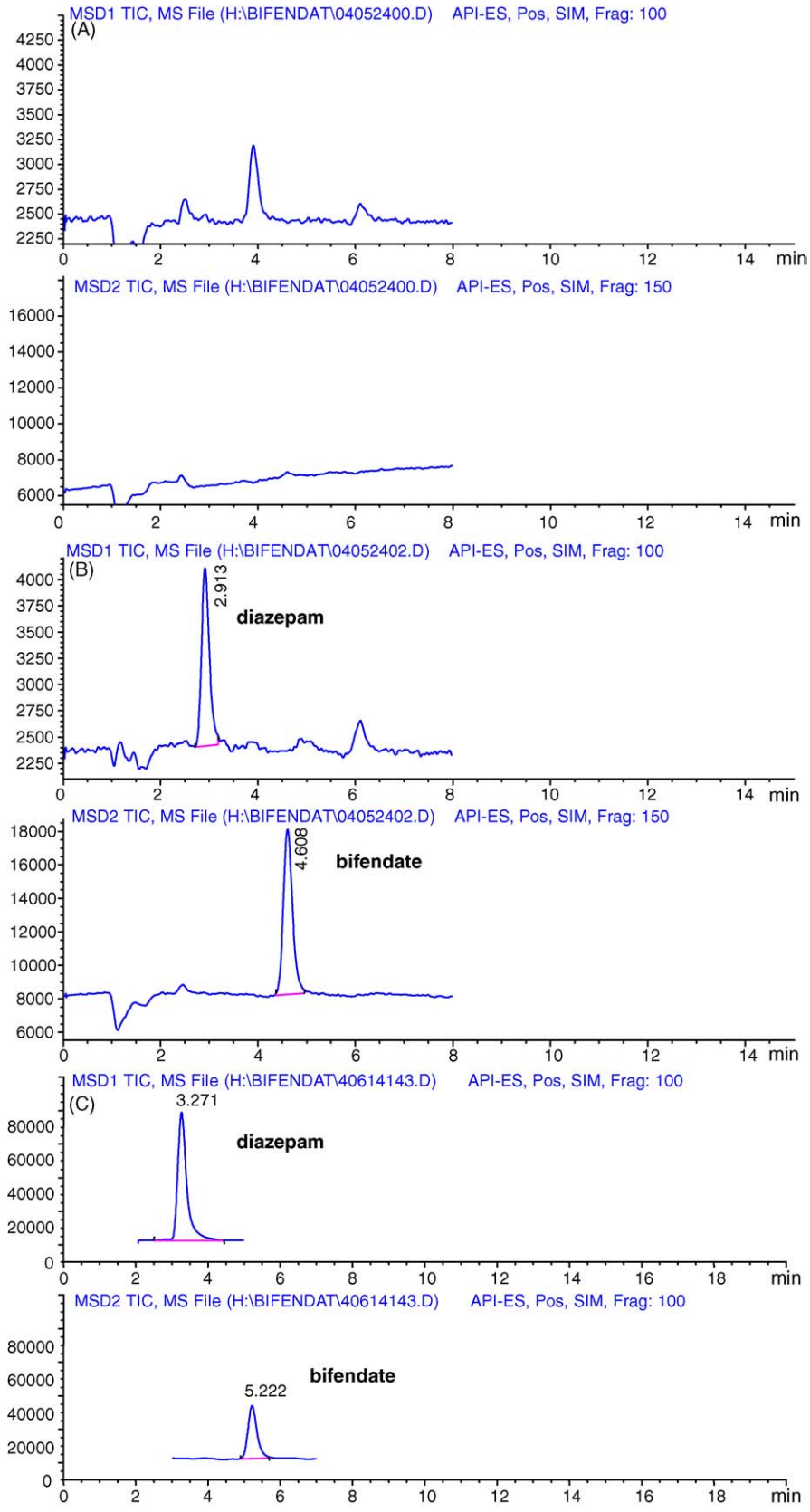

Fig. 3. Representative HPLC–MS chromatograms of blank plasma (A) and spiked plasma with IS and 2 ng/mL (LLOQ concentration) of bifendate (B) and a subject after intake of bifendate (C).

Table 2
Precision and accuracy of bifendate spiked in human plasma by LC–MS ($n = 5$)

Actual concentration (ng/mL)	Detected concentration (mean ± S.D.) (ng/mL)	R.S.D. (%) (intra-day)	R.S.D. (%) (inter-day)	RE (%)
5.0	4.82 ± 0.302	6.34	6.79	−3.6
20.0	20.3 ± 0.397	2.44	5.63	1.3
100.0	101 ± 8.14	2.66	5.18	0.7

Table 3
Extraction recoveries of LC–MS method ($n = 5$)

Actual concentration (ng/mL)	Recovery (mean ± S.D.)	R.S.D. (%)
5.0	83.45 ± 0.07	7.88
20.0	73.60 ± 0.05	6.63
100.0	67.21 ± 0.04	6.46

3.5. Stability

The stability of bifendate in plasma samples placed on the auto-sampler was first determined. After injection of spiked samples at the concentration level of 50 ng/mL in triplicate, no significant degradation of bifendate was observed. The stability was also assessed after storage at −20 °C for one month of plasma samples spiked at 50 ng/mL. Bifendate was determined at the 1st, 10th and 30th day ($n = 3$). The results obtained were between 95 and 105% of the initial value. Freeze and thaw stability was evaluated by examining the plasma samples (50 ng/mL) after repeated thawing and freezing for three times. No significant degradation of bifendate was observed.

4. Application

The present HPLC–MS method was employed to study the bioequivalence on two formulations of bifendate pills. Twenty healthy male volunteers (mean age 22.2 years, mean body mass index 22.3) were entered and the study was completed. Each participant received a single dose of test and reference pills in a balance 2 × 2 Latin square design experiment, separated by one week washout period. The volunteers fasted overnight before the administration of drug (15 mg) and 4 h post-dosing. Any medication, cigarette, wine and drinks containing caffeine were not allowed at least two weeks prior to and during the periods of the test. Blood samples (3 mL) were drawn and transferred into heparinized tubes before dosing and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 16, 24 h post-dosing. The plasma samples were separated and frozen at −20 °C before analysis.

The mean (20 volunteers) plasma concentration–time curves of bifendate were similar for test pills and reference ones (Fig. 4).

The main pharmacokinetic parameters (C_{max}, T_{max}, $AUC_{0–24\,h}$, $AUC_{0–\infty}$, $t_{1/2}$) of bifendate are shown in Table 4, and the relative bioavailability of the test pill was 98.76 ± 14.27%. A Wilcoxon test was established for T_{max} and no statistical difference was shown between the two formulations ($P > 0.05$). The variance analysis of $AUC_{0–24\,h}$, $AUC_{0–\infty}$, and C_{max} of bifendate was calculated using the Program 3P97 authorized by Chinese Pharmacologic Society (Beijing, PR China). The results

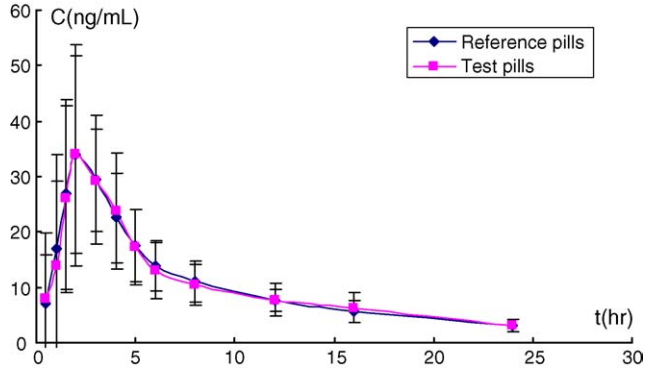

Fig. 4. The mean plasma concentration curves after oral administration of 15 mg bifendate pills ($n = 20$).

Table 4
Pharmacokinetic parameters of bifendate after oral administration (15 mg) of two formulations to 20 subjects (mean ± S.D.)

Parameter	Test pills	Reference pills
$AUC_{0–t}$ (ng h mL^{-1})	250.5825 ± 72.2464	253.2185 ± 57.3126
$AUC_{0–\infty}$ (ng h mL^{-1})	294.2227 ± 74.0768	296.8089 ± 60.9255
C_{max} (ng mL^{-1})	42.5901 ± 14.9431	40.6349 ± 14.8643
T_{max} (h)	2.225 ± 0.8025	2.275 ± 0.7159
$t_{1/2}$ (h)	9.4311 ± 2.6386	9.0928 ± 2.3733

Table 5
Results of the analysis of variance and the two one-side t tests for pharmacokinetic parameters (C_{max}, $AUC_{0–t}$ and $AUC_{0–\infty}$)

Parameter	t_1	t_2	$t_{1–2\alpha}$	90% confidential interval (%)
lg C_{max}	6.858	4.487	1.734	97.87–112.17
lg $AUC_{0–t}$	6.914	8.413	1.734	93.02–102.91
lg $AUC_{0–\infty}$	6.582	7.717	1.734	93.07–103.71

(Table 5) indicated that the two formulations of bifendate were bioequivalent in 90% confidential limit.

5. Discussion and conclusion

A highly sensitive and specific method for the determination of bifendate was here developed by using high-performance liquid chromatographic separation with mass spectrometric detector. The whole analysis was completed within 7 min. Besides, only 0.5 mL of plasma was required for each determination of bifendate, and thus the stress to volunteers or patients in clinical studies was greatly reduced. This method is very suitable and

convenient for pharmacokinetics and bioavailability study of the drug bifendate.

The results of statistical analysis showed that the two formulations of bifendate were bioequivalent in 90% confidential limit, and the relative bioavailability of the test pill was $98.76 \pm 14.27\%$.

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