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Development of a high-performance liquid chromatographic method for bioequivalence study of flavoxate tablets

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Abstract

An improved HPLC method was developed for the concentration determination of the metabolite of flavoxate, 3-methyl-flavone-8-carboxylic acid (MFCA), in plasma in an attempt to compare two flavoxate tablet formulations. This HPLC method was validated by examining the precision and the accuracy for inter-day and intra-day runs in a linear concentration range of 0.1–24 μg/ml. The coefficients of variation (C.V.) of inter-day and intra-day assays were 0.24–7.18% and 0.06–5.70%, respectively. The standard errors of mean (S.E.M.) were −0.004–8.68% and −2.52–4.86% for inter-day and intra-day assays, respectively. Bioequivalence of the two formulations was determined on 12 normal healthy male volunteers in a single-dose, two-period, two-sequence, two-treatment crossover study. MFCA plasma concentrations were analyzed with this validated HPLC method. The normal pivotal parameters, AUC_{0-last}, AUC_{0-inf} and C_{max}, were calculated and compared using the SAS General Linear Model computer program. The two one-sided *t* distribution test was also performed, as well as the 90% confidence-interval method, for the mean difference of the three pivotal parameters. The results suggest that these two flavoxate tablet formulations are non-bioequivalent when orally administered in a 400-mg dose of two tablets. This result was consistent with the in vitro dissolution of these two formulations.

Keywords: Bioequivalence study; Flavoxate

1. Introduction

Flavoxate or piperidinoethyl-3-methyl-flavone-8-carboxylate, belongs to a series of flavone derivatives synthesized by Da Re et al. [1], which exhibit strong smooth-muscle relaxant activity, especially on the genito-urinary tract [2]. Flavoxate exerts phosphodiesterase (PDE) inhibitory activity about three and five times greater than that of aminophylline in tissue homogenates of guinea-pig ureter and urinary bladder, respectively. It also shows moderate calcium antagonistic activity as does papaverine and the same local anaesthetic activity of lidocaine [3]. Therefore, the mode of action of flavoxate can be related to superimposition of myotropic, calcium antagonistic and local anaesthetic activities.

The recommended dose for adults and children over 12 years of age is 100 or 200 mg three to four times a day. The adverse effects associated with flavoxate include drowsiness, nervousness, headaches, confusion, nausea, vomiting and blurred vi-

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sion. In clinical applications, flavoxate is useful in the symptomatic treatment of dysuria, urgency, nocturia, suprapubic pain, frequency and incontinence due to a variety of urological conditions. Flavoxate was administered to children aged 6–12 years in a dose of 100–200 mg at bedtime for nocturnal enuresis. No adverse reactions were observed and overall response was 33% compared with 17% in the placebo group [4].

Flavoxate is rapidly metabolized in plasma to 3-methyl-flavone-8-carboxylic acid, MFCA [5]. A pharmacokinetic study in humans [6] has shown that flavoxate, when given orally, is rapidly and completely absorbed and metabolized into MFCA, as such was found in the urine. After intravenous administration of the drug, flavoxate disappears from the blood with a half-life of about 5 min. In Bertoli's study with oral administration of 100 mg of flavoxate hydrochloride formulated in sugar-coated tablets, time to maximum concentration (T_{max}) was 112 min, volume of distribution (V_d) was 0.23 kg/l, rate constant (k) was 1.8 h^{-1} and the area under the plasma concentration–time curve (AUC) was 4.02 $mg/l \cdot (mg/kg)^{-1} \cdot h$. The absorption rate is rapid with an absorption half-life of 44 min. On the average, the absorption process starts 55 min after administration. The absorption of flavoxate from the gastrointestinal tract is complete, as shown by the AUC and by the urinary excretion after either I.V. or oral administration of sugar-coated tablets.

After oral administration of flavoxate, traces only of flavoxate were found in blood with the main compound present being either MFCA, conjugated MFCA or bound flavoxate. So bioequivalence studies can be performed only by monitoring MFCA. Furthermore, the validity of using metabolite to test bioequivalence has been described [7–12]. Several assay methods for the determination of MFCA in biological fluids have been reported, including radiometric [4,5] and gas chromatography assays (GC) [2,3]. However, the radiometric method lacks specificity and the specific GC method involves extraction and methylation of samples before injection. A new analytical technique of capillary electrophoresis (CE) has been applied for direct injection of urine samples without extraction [13]. The calibration curve of MFCA in this study was linear in the range of 1–50 μg/ml and the detection limit was 0.2 μg/ml.

With an average maximal concentration around 2 μg/ml for MFCA in plasma after oral administration of flavoxate (given 2.55 mg/kg), it would be better to have a limit of quantitation (LOQ) at about 0.1 μg/ml. The aim of the present study was to improve the assay method of MFCA using a reversed-phase HPLC system. This validated HPLC method was applied to compare the bioequivalence of two tablet formulations by means of analysis of MFCA concentration in plasma.

2. Experimental

2.1. Materials and methods

2.1.1. Drug and reagents

One of the tablet formulations is Genurin (flavoxate 200 mg as HCl salt, R) tablet (lot no. M50076) obtained from the innovative Recordati Industria Chemica E. Farmaceutica Italy. The other one is a test formulation (T) made by a local pharmaceutical company (Taoyuan, Taiwan). The standard, MFCA and internal standard, nifedipine, were both purchased from Sigma Chemical (St. Louis, MO, USA). All other reagents used were reagent grade or better.

2.2. Validation of assay method

Several pre-dose human plasma samples from different subjects were tested for the absence of interfering compounds. The intra- and inter-assay coefficients of variation and standard errors of mean were used to validate the precision and accuracy of the assay by determining standard samples of MFCA in plasma. For inter-day validation, six sets of control samples at six different concentrations (0.1–24 μg/ml) were evaluated on six different days (six standard curves were constructed). The range of the coefficient of variation was reported. For intra-day validation, six sets of controls at six different drug concentrations were assayed with one standard curve on the same run. The range of the coefficients of variation was reported as well. The retention times from injection of pure drug and internal standard, respectively, were used as references for the identifi-

cation of analytes in clinical samples. Fluctuations in retention times could occur due to changes in temperature and column performance.

Examination the daily coefficient of variation for the control samples, as well as the standards, on each analytical run was used to verify reproducibility of the assay method. The limit of quantification (LOQ) was determined from the coefficient of variation. The coefficient of variation for the lower limit of quantification should be less than 20%. Any result below this concentration was reported as ''below assay sensitivity''. To assess the absolute recoveries of the analytes extracted from plasma, the peak heights of extracted plasma samples containing a known amount of each of the analytes with those obtained from a standard solution of each of the respective analytes were compared. Average recovery was evaluated and reported for at least three drug concentrations.

2.3. Physical characterizations

The potency, uniformity and dissolution tests on flavoxate in these two tablet formulations (R and T) were carried out according to the pharmacopoeia specifications. In the preliminary test, results demonstrated that the potency was 99.57% and 96.27% for R and T, respectively. The content uniformity of dosage units were 99.37±0.85% and 96.07±0.86% for R and T, respectively. The results of the potency and uniformity test on these two formulation tablets both meet the criteria of the pharmacopoeia specifications (potency: 90–110%; uniformity: 85–115%). Three dissolution media, including 0.1 N HCl solution, pH 4.5 acetate buffer and pH 6.8 phosphate buffer solutions were employed to compare the dissolution profiles of these two products. The closeness of profiles was statistically determined by the comparison of the f_2 value following the guideline of SUPAC IR.

2.4. Bioequivalence studies

Assay of plasma samples for MFCA concentration was performed by this validated HPLC method under illumination with yellow light in dark rooms. The assay was completed within 2 months or a period with acceptable sample stability following the last day of medication. Quantitative analysis of plasma MFCA concentrations and suitable quality controls was performed as follows.

2.4.1. Instrumentation

A HPLC system equipped with a pump (Jasco PU-980, Jasco, Tokyo, Japan) and a Jasco AS-950-10 autosampler was used. A reversed-phase C_8 column (Hypersil® MOS, 250×4.6-mm I.D., Life Sciences International, London, UK) with a particle size of 5 μm was employed. The mobile phase consisted of a v/v ratio of methanol: acetonitrile: triethylamine solution (1.5%, pH 3.0 adjusted with phosphoric acid) of 10:30:60. This solution was filtered and degassed ultrasonically before use. The flow-rate was set at 1.2 ml/min. The eluent was detected with a JASCO UV-975 UV–Vis detector at 290 nm. The HPLC system was controlled by a PC workstation with Chromatography Data Station software (SISC, Taiwan) installed.

2.4.2. Internal standard solution and sample preparation

The plasma sample preparation and extraction method was performed under the illumination of yellow light in dark rooms and elucidated step by step as follows. All containers used were amber colored or well wrapped with aluminum foil. Plasma (1 ml) was spiked with 0.1 ml of internal standard solution (nifedipine, 3 μg/ml in methanol). After vortex mixing for 10 s, the mixture (0.2 ml) was applied to another clean tube and spiked with 1 ml acetonitrile. The mixture was vortexed for 3 min and centrifuged at 4000 rpm (1500 *g*) for 10 min. The upper layer was collected and evaporated under a stream of nitrogen gas until completely dry. Then, 200 μl of mobile phase was added to dissolve the residue and 100 μl was injected automatically into the HPLC system.

2.4.3. Quantification

A calibration curve for MFCA at concentrations ranging from 0.1 to 24 μg/ml in plasma was prepared. Standard samples were prepared by adding the analyte to drug-free plasma and these were extracted and analyzed as described above. Peak height ratios of each analyte to the internal standard were measured and the calibration curve was ob-

tained from the least-squares linear regression. The regression line was used to calculate the concentrations of the respective analytes in the unknown samples.

2.4.4. Subjects

The protocol of the bioequivalence study was first approved by the Internal Review Board of Taipei Medical College Hospital. A total of 12 healthy male subjects participated in this study after signing a consent form. The subjects had a mean±SD age of 22±2 years (20–25 years), body weight of 66±6 kg (55–78 kg) and height of 172±5 cm (165–184 cm). Subjects with a history of drug allergies or idiosyncrasies, renal or hepatic impairment or drug or alcohol abuse were excluded. Subjects who used medications of any kind within 2 weeks of the start or during the study were also excluded.

2.4.5. Drug administration

Subjects were advised not to take any medication 2 weeks before the study and were requested to fast for at least 10 h overnight the day before each treatment. A single dose (200 mg) consisting of one R (Genurin) or T tablet according to the randomization plan was given to each subject in a fasting state for each treatment period. Fasting continued for a further 4 h after drug administration. The drug was administered with 200 ml of water. Subjects were provided a standard meal 4 h (lunch) and 10 h (supper) after drug administration in each treatment. The washout period between the two treatment periods was 1 week, which is longer than 10 times the elimination half-life of this drug.

2.4.6. Blood samples

Heparized venous blood samples, 5–10 ml, were collected by means of an indwelling venous cannula of the cubital vein on profile days according to the time schedule, which included a blank before-drug sample just prior to dosing and then at 0.17, 0.33 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 12 h after drug administration. Any deviation from the stated sampling times was recorded on the form. Plasma was immediately separated by centrifugation at 3000–4000 rpm for 10 min, then was transferred to suitably labeled tubes and stored at −20°C or a temperature with an optimal sample stability until assay.

2.4.7. Pharmacokinetic data analysis

The following parameters were assessed for the period of 0–12 h: the area under the plasma concentration–time curves from time zero to the last measurable MFCA sample time and to infinity [AUC_{0-last} and AUC_{0-inf}, Eq. (1)]; maximum concentration (C_{max}); time to maximum concentration (T_{max}); and relative bioavailability and relative total clearance [Cl/F, Eq. (2)]. All pharmacokinetic variables were calculated by non-compartmental methods. C_{max} and T_{max} were obtained directly from the concentration–time-curve data. The area under the concentration–time curve from time zero (predose) to time of last quantifiable concentration (AUC_{0-last}) was calculated using the linear trapezoidal method and Cl/F is equal to (dose/AUC_{0-inf}). The terminal rate constant, K_{el}, was calculated by applying a log-linear regression analysis to at least the last three time points. $T_{1/2}$ is the terminal half-life calculated by Eq. (3). MRT is the mean residence time of the drug and is calculated by Eq. (4), where $AUMC_{0-last}$ is the area under the moment-versus-time curve to the last sample point and is determined using the linear trapezoidal method.

$$AUC_{0-inf} = AUC_{0-last} + \frac{C_{last}}{K_{el}} \quad (1)$$

$$\frac{Cl}{F} = \frac{K_{el} \cdot V_d}{F} = \frac{Dose}{AUC_{0-inf}} \quad (2)$$

$$T_{1/2} = \frac{\ln 2}{K_{el}} \quad (3)$$

$$MRT = \frac{AUMC_{0-inf}}{AUC_{0-inf}} = \frac{AUMC_{0-last} + \left[C_{last} \cdot t_{last}/K_{el} + C_{last}/K_{el}^2\right]}{AUC_{0-inf}} \quad (4)$$

2.4.8. Statistical analysis

A two-way ANOVA performed with the SAS General Linear Models Procedure at a significance level of 0.05 was carried out. The test (T) and reference (R) treatments of each study were com-

pared with respect to relevant pharmacokinetic variables using an analysis of variance with subject, treatment and period effects with the raw data. Point estimates and 90% confidence intervals for the ''T/R'' mean ratios of these raw data were calculated. Whenever there was no statistically significant difference, statistic power to detect at least a 20% difference between products was checked using Eqs. (5) and (6), where n is the number of subjects and MSE is the mean square error of the error term with the degree of freedom, df. MSE and df are obtained from ANOVA tables of SAS output. Delta (δ) is 20% of the least square mean from the reference. Bioequivalence of the test treatment to the reference treatment was assessed on the basis of the confidence intervals for the ''T/R'' mean ratios of these raw variables in relation to the bioequivalence range of 80–120% for the raw data.

$$t_{\beta,\,\mathrm{df}} = \frac{\delta}{\sqrt{\mathrm{MSE}\cdot\frac{2}{n}}} - t_{0.975,\,\mathrm{df}} \quad (5)$$

$$\text{Power} = 1-\beta \quad (6)$$

3. Results and discussion

Fig. 1 shows typical HPLC chromatograms of sample analysis. No interfering peaks were observed for drug-free human plasma. The retention times of MFCA and nifedipine were around 8 and 13 min, respectively. Good separation and baselines with low background were observed. The peaks of interest were well resolved and there was no interference from endogenous plasma substances. The inter-day

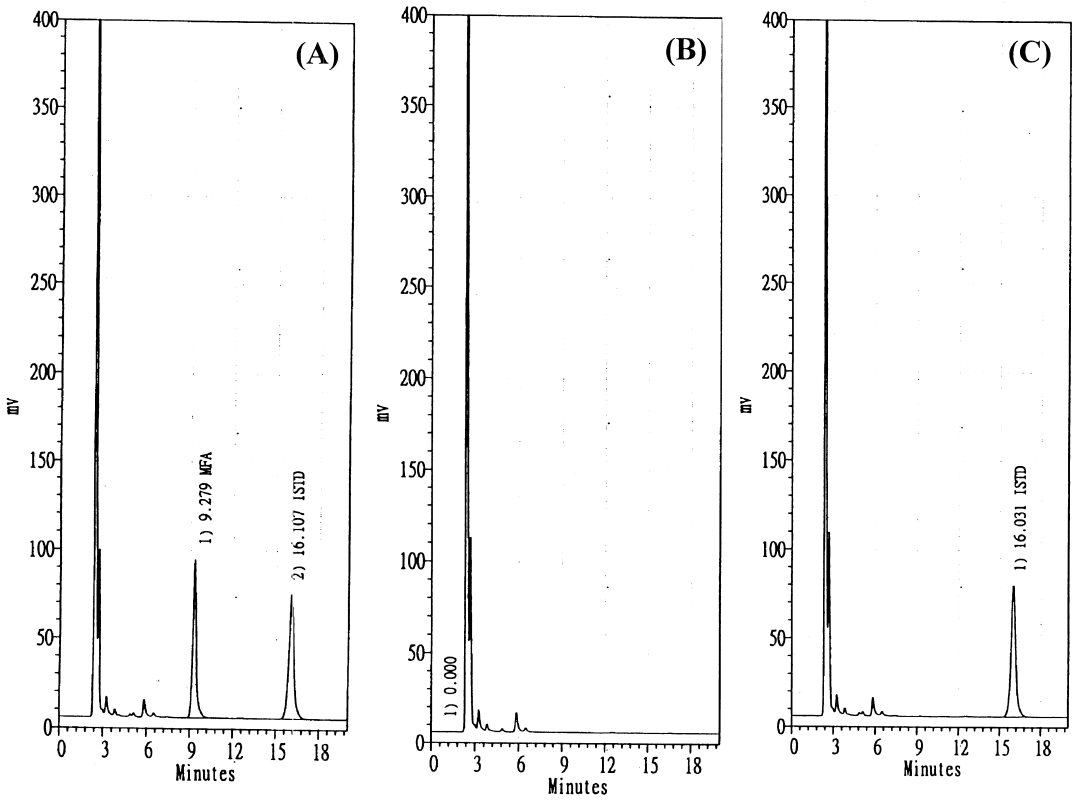

Fig. 1. HPLC chromatograms: (A) MFCA and nifedipine (ITSD); (B) blank plasma; (C) internal standard, nifedipine (ITSD).

Table 1
Intra-day and Inter-day validation of MFCA assay study

Amount (μg/ml)	0.1	0.5	1	5	10	24
Intra-day						
Mean ($n=6$)	0.0975	0.5157	1.0486	5.1060	9.7601	24.0721
SD[a]	0.0056	0.0088	0.0206	0.0492	0.0574	0.0153
C.V.[b] (%)	5.70	1.71	1.97	0.96	0.59	0.06
Relative error (%)	−2.52	3.13	4.86	2.12	−2.40	0.30
Inter-day						
Mean ($n=6$)	0.0913	0.5215	1.0480	4.9482	10.0128	24.0010
SD	0.0066	0.0130	0.0177	0.1137	0.1777	0.0565
C.V. (%)	7.18	2.49	1.69	2.30	1.77	0.24
Relative error (%)	−8.68	4.29	4.80	−1.04	0.13	0.0044

[a] Standard deviation.
[b] Coefficients of variation.

and intra-day validations are shown in Table 1. The coefficients of variation (C.V.) of inter-day and intra-day assays were 0.24–7.18% and 0.06–5.70%, respectively, indicating that the analysis has good precision. The standard errors of mean (S.E.M.) were −8.68–0.004% and −2.52–4.86% for inter-day and intra-day assays, respectively, it depicting the high accuracy of the analysis.

The linearity of the calibration curve of MFCA (Fig. 2) was well correlated ($r^2>0.999$) within the range of 0.1–24 ng/ml. All data show very good reproducibility of sample analysis. The absolute standard errors of mean in each sample analysis run for QC samples were between −4.57% and 4.99%, indicating that the stability of the drug in plasma during storage periods was acceptable.

Fig. 3 displays the bioavailability with the mean of MFCA plasma concentration–time profile in 12 volunteers for the T and R products. The pivotal pharmacokinetic parameters were calculated correspondingly and statistical analysis results for two formulations of MFCA tablets are delineated in Table 2. The mean±SD ratios of AUC_{0-last}, AUC_{0-inf} and C_{max} of the T formulation to the R formulation (Genurin) are 1.23± 0.29, 1.24± 0.29 and 1.56± 0.66, respectively. There was significant difference ($P>0.05$) in bioavailability between the two products as indicated by these three parameters. The 90% confidence intervals of the mean difference were in a range of 107.8–134.2%, 107.8–135.4% and 115.9–167.4% for AUC_{0-last}, AUC_{0-inf} and C_{max}, respectively. The 90% confidence interval of the mean difference for these three pivotal parameters fell outside the range of 80–120%. The same results of statistical analysis were obtained using the two one-sided t distribution methods. The values of statistical power to compare mean ratios of AUC_{0-last}, AUC_{0-inf} and C_{max} between the two products were close to 1.0000. The results of ANOVA of three pivotal parameters show that the

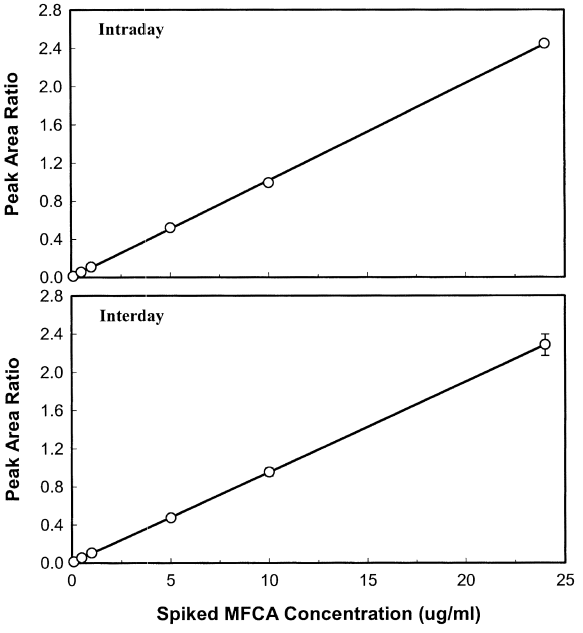

Fig. 2. A typical calibration curve for the assay of MFCA plasma concentrations.

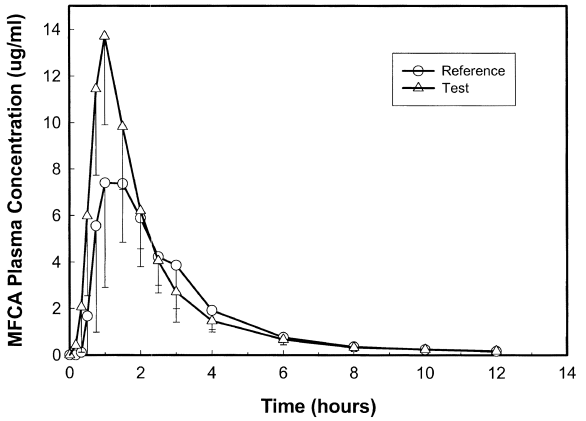

Fig. 3. MFCA mean plasma concentration–time profile in 12 volunteers for the test and reference products.

treatment was the only one determined to be significant. There was no group, period or treatment effect on these three pivotal parameters in this crossover design.

Other pharmacokinetic parameters, such as K_{el}, T_{max}, $T_{1/2}$, MRT_{0-inf} and Cl/F, between the two products are also shown in Table 2. No significant difference was found for these parameters between the two products. The mean $AUC_{last-inf}$ was less than 5.59% and 10.71% for Genurin and Laxurin tablets, respectively, indicating that the average absorption period of flavoxate were very close to completeness as a consequence of the designed sampling time.

The dissolution profiles in different media of the two formulation tablets are shown in Fig. 4, which

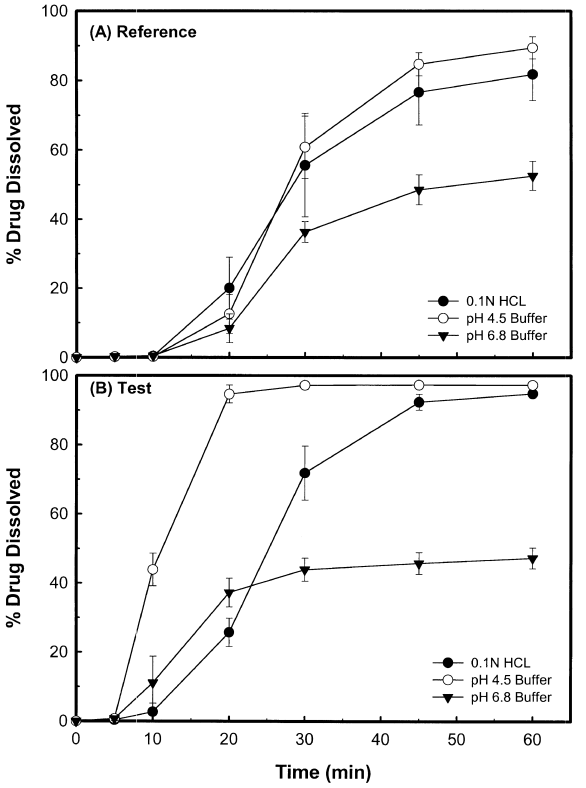

Fig. 4. Dissolution profiles in different media of the two formulation tablets.

demonstrates that f_2 values were 33.24, 6.35 and 39.75 for dissolution in the media of 0.1 N HCl, pH 4.5 acetate buffer and pH 6.8 phosphate buffer solutions, respectively. A f_2 value outside the range of 50–100 suggests that the two dissolution profiles

Table 2
Pharmacokinetic parameters of MFCA for BE study

Parameters	Reference		Test	
	Mean (SD)[a]	C.V.[b] (%)	Mean (SD)	C.V. (%)
AUC_{0-last} (μg/ml*h)	21.9463 (3.4848)	15.88	26.5546 (5.3108)	20.00
AUC_{0-inf} (μg/ml*h)	22.9019 (3.9072)	17.06	27.8491 (6.0781)	21.83
C_{max} (μg/ml)	10.1518 (3.1881)	31.40	14.3855 (3.3022)	22.96
K_{el} (h^{-1})	0.2440 (0.1037)	42.50	0.2206 (0.1176)	53.32
T_{max} (h)	1.50 (0.79)	52.70	1.00 (0.26)	26.11
$T_{1/2}$ (h)	3.30 (1.27)	38.42	4.06 (2.13)	52.57
MRT_{0-inf} (h)	2.94 (0.59)	19.93	2.55 (0.47)	18.35
CL/F (l/h)	18.01 (3.58)	19.75	14.93 (2.95)	19.77

[a] Standard deviation.
[b] Coefficients of variation.

are dissimilar. A good correlation between in vitro dissolution and in vivo bioequivalence was apparent.

4. Conclusions

In conclusion, the HPLC assay method using a reversed-phase system was improved to be acceptable for assaying the major metabolite of flavoxate, MFCA, in plasma. The bioequivalence of two 200-mg flavoxate tablet formulations with oral administration of a 400-mg dose of two tablets in 12 healthy, normal male volunteers was examined by monitoring its metabolite. However, the statistical analysis results based on comparisons of three pivotal parameters (AUC_{0-last}, AUC_{0-inf} and C_{max}) showed that these two tablet products appear to be non-bioequivalent.

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