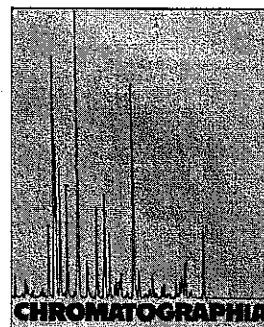


# Development and Validation an LC Method for the Determination of Bendroflumethiazide in Human Plasma and its Pharmacokinetics



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## Abstract

A simple, rapid, sensitive high performance liquid chromatography method with fluorescent detection was developed and validated for the determination of bendroflumethiazide in human plasma. Extraction from the plasma was by liquid-liquid extraction using ethyl acetate. Mosapride citrate was used as the internal standard. The chromatographic separation was performed on reverse phase LiChrosphere C18 column with mobile phase comprising of acetonitrile and phosphate buffer (38:62 v/v). The assay precision ranged from 0.9–12.5 and accuracy between 96.8–108.8%, revealing that the method has good reproducibility over the concentration range of 0.98–100.16 ng mL<sup>-1</sup>. The validated method has been applied to analyze the bendroflumethiazide concentrations for application in pharmacokinetic, bioavailability or bioequivalence studies.

## Keywords

Column liquid chromatography  
Fluorescence detection  
Method validation  
Bendroflumethiazide and bendrofluazide

## Introduction

Bendroflumethiazide often called as bendrofluazide (3-benzyl-3,4-dihydro-6-(trifluoromethyl)-2 H-1,2,4-benzothiazine-7-sulfonamide 1,1-dioxide) is a thiazide diuretic, also known as “water pills” used to lower the amount of water in the body [1]. Actions and uses of bendroflumethiazide is similar to those of hydrochlorothiazide and it has been used in the treatment of edema and urinary tract disorders, kidney problems, cirrhosis, salt, and fluid retention [2,3] as well as

congestive heart failure [4–7], familial hyperkalemia, hypertension [8–10].

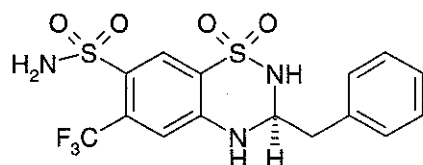
Bendroflumethiazide increases urine production in the kidney by altering the movement of sodium and chloride. Bendroflumethiazide interfere with the renal tubular mechanism of electrolyte reabsorption [11]. Thiazide diuretics like bendroflumethiazide are often taken in combination with other drugs to treat high blood pressure [12–16].

Several methods have been reported for the quantitative analysis of bendroflumethiazide; these include determination in

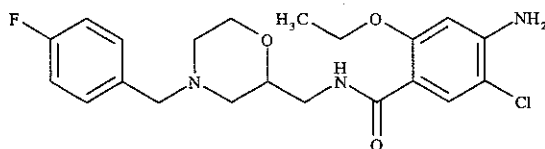
urine samples by liquid chromatography coupled to pneumatically assisted electrospray ionization mass spectrometry [17], GC-MS analysis after derivatization [18,19], capillary zone electrophoresis (CZE) [20,21], chemoluminescent determination by on-line photochemical reaction [22], and colorimetric method [23]. However, these methods are not ideal for pharmacokinetic work, because they have a high detection limit and are time-consuming owing to derivatization, arduous sample preparation, and long chromatographic run times.

Recently, many reports for the determination in urine samples using HPLC with UV detection [24,25] and micellar liquid chromatography using SDS [26–28] have been published. But these methods are applied to the bendroflumethiazide formulations and bulk material and not ideal for pharmacokinetics or bioequivalence work because of high detection limit.

This paper reports a sensitive, specific and simple method for the determination of bendroflumethiazide in human plasma by liquid-liquid extraction and HPLC with fluorescence detection. Validation studies for reproducibility, stability, and recovery were performed. The method is not only more selective and reliable but also faster and simpler than other methods. We have applied this method for the bioequivalence study of two oral dosage forms of bendroflumethiazide (test and reference). The open randomized, cross over study performed on a group of 28



(A) Bendroflumethiazide (mw = 421.42)



(B) Mosapride (mw= 421.90)

Fig. 1. Structures of (A) Bendroflumethiazide and (B) mosapride (internal standard)

healthy, Indian male volunteers confirmed the bioequivalence of both the formulations.

## Experimental

### Chemicals

Acetonitrile, ethyl acetate, methanol (Merck, India) were of analytical grade. Sodium di-hydrogen ortho phosphate dihydrate, ortho-phosphoric acid was of reagent grade. Water was deionized using a Milli-Q system from Millipore (Bedford, MA, USA). Bendroflumethiazide working standard was from Unichem Laboratories Ltd, India and mosapride citrate dihydrate was obtained from Glenmark, India. The structures of these compounds are shown in Fig. 1.

### Instrumentation and Chromatographic Conditions

HPLC analysis was performed with a Lachrom HPLC system (Merck, Hitachi, Germany) comprising an autosampler (L-7200), a column oven (L-7350), and fluorescence detector (L-7480) with an interface (D-7000). Chromatographic separation was performed on a LiChrosphere HPLC cartridge column packed with RP 18e, (125 × 4 mm, 5 μm, Merck); the isocratic mobile phase was 38:62 (v/v) acetonitrile-10 mM phosphate buffer (pH 3.0). The injection volume was 50 μL and the flow-rate was 1 mL min<sup>-1</sup>. The eluent was monitored by fluorescence detection at 270 nm (excitation) and 400 nm (emission).

### Sample Processing

Plasma specimens (0.5 mL) were pipetted into glass tubes and spiked with 25 μL of internal standard solution (35 μg mL<sup>-1</sup>). After adding 3 mL of ethyl acetate to the glass tubes, the plasma samples were then vortex-mixed for 2 min using Multi-Pulse Vortexer (Glas-Col, Terre haute, USA). The two phases were separated by centrifugation at 3000 rpm for 10 min. The upper organic layer was transferred into another glass tube and completely evaporated to dryness using Turbo Vap LV Evaporator (Zymark, Hopkinton, MA, USA) at 50° C under a stream of nitrogen. The dry residue was reconstituted with 200 μL mobile phase and analysed by HPLC-FLD.

### Bioanalytical Method Validation

#### Calibration and Quality Control Samples

Stock solution of bendroflumethiazide (1 mg mL<sup>-1</sup>) was prepared in methanol. Spiking solution of different concentrations for calibration curve and quality control samples were prepared from this stock solution by an adequate dilution using water/methanol (1/1 v/v). Calibration standards for control plasma were prepared by spiking this stock to obtain the concentration levels of 0.98, 1.97, 4.93, 14.94, 49.80, 76.62, 90.14, 100.16 ng mL<sup>-1</sup> in human plasma. Quality control samples were prepared as bulk, at a concentration of 1.08 ng mL<sup>-1</sup> (LLOQ QC), 2.70 ng mL<sup>-1</sup> (LQC), 40.06 (MQC), and 80.12 ng mL<sup>-1</sup> (HQC).

#### Calibration Curve

A calibration curve of bendroflumethiazide was constructed from a blank sample (plasma sample processed without an IS), a zero sample (plasma processed with IS) and eight non-zero samples covering the total range of 0.98–100.16 ng mL<sup>-1</sup>, including lower limit of quantification. Three samples of each concentration were measured. Linearity was assessed by least squares regression analysis. The calibration curve had to have a correlation coefficient ( $r^2$ ) of 0.98 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

#### Precision and Accuracy

The within-batch precision and accuracy was determined by analyzing eight replicate of quality control samples in a batch. The between-batch precision and accuracy was determined by analyzing eight replicate of each of seeded quality control samples in three different batches. The quality control samples were randomized daily, processed and analyzed in position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria of within- and between-batch precision were 20% or better for LLOQ and 15% or better for the rest of the concentrations and accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the rest of concentrations.

#### Recovery

Recovery of the drug and IS was evaluated by comparing the mean responses of eight replicates of extracted low, medium and high quality control samples to the respective aqueous quality control samples with IS.

#### Stability Studies

The bench top stability (at room temperature) of low and high quality control samples were determined by comparing the mean of back-calculated concentrations from the freshly thawed quality control samples with those that were kept on bench top for about 6.0 h.

The freeze thaw stability of low and high quality control samples were tested

with three freezing periods, where the first storage of 24 h at below  $-20^{\circ}\text{C}$  was followed by two additional periods of 12–24 h. The percentage of degradation was determined by comparing the mean of back-calculated concentrations from the three freeze thaw cycles with that of a freshly thawed quality control sample.

Autosampler stability was assessed by storing the low and high quality control samples in an auto sampler ( $10^{\circ}\text{C}$ ) for 47 h followed by re-injecting the same samples and comparing the ration of the mean concentrations.

## Pharmacokinetic Studies

For the pharmacokinetic studies of bendroflumethiazide, a single 5 mg dose of bendroflumethiazide was administered orally to 24 healthy, male human volunteers who were advised about the nature and purpose of the study. The volunteers were selected on predetermined inclusion, exclusion criteria males with a mean age of  $25.81 \pm 4.78$ , mean weight of  $57.88 \pm 5.35$  kg, and mean height of  $167.46 \pm 4.47$  cm. The volunteers had not taken any other medication for at least two weeks before the study. Blood samples were taken by use of vacutainer containing EDTA at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 16 and 24 h after ingestion. Human plasma was obtained by centrifugation at 2000 g for 10 min. Plasma specimens were then stored at  $-20^{\circ}\text{C}$  till analysis and analyzed by the above method. A concentration time curve was plotted and  $AUC$  calculated by trapezoidal rule ( $AUC_{0-24}$ ).  $AUC_{0-\infty}$  was also calculated. Time to achieve the maximum concentration ( $C_{max}$ )  $t_{max}$  was obtained directly from the concentration time curve without interpolation. All the pharmacokinetic data were calculated using Kinetica software.

## Results and Discussion

The aim of our work was to develop a rapid and sensitive method for detecting bendroflumethiazide in human plasma by HPLC for pharmacokinetic studies.

### Method Development

For the chromatographic analysis of bendroflumethiazide we attempted to develop a reversed phase chromato-

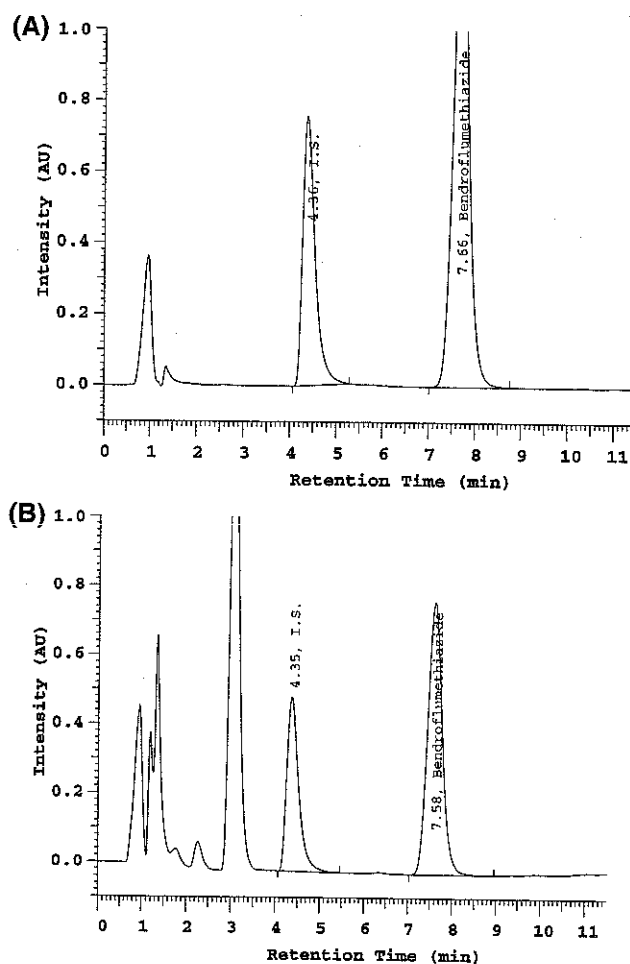


Fig. 2. HPLC-FLD chromatogram of bendroflumethiazide and internal standard (A) in aqueous solution (B) in human plasma after liquid-liquid extraction

Table 1. Precision and accuracy data of back-calculated concentrations of calibration samples for bendroflumethiazide in human plasma

Concentrations Added ( $\text{ng mL}^{-1}$ )	Concentration found (mean $\pm$ S.D, $n=3$ ) $\text{ng mL}^{-1}$	Precision (%)	Accuracy (%)
0.986	$1.072 \pm 0.027$	2.6	108.8
1.972	$1.931 \pm 0.105$	5.5	97.9
4.931	$4.804 \pm 0.223$	4.6	97.4
14.941	$14.461 \pm 0.127$	0.9	96.8
49.805	$48.911 \pm 1.656$	3.4	98.2
76.62	$78.825 \pm 9.847$	12.5	102.9
90.14	$89.611 \pm 5.426$	6.1	99.4
100.16	$99.645 \pm 6.153$	6.2	99.5

Averaged for three individual measurements at each concentration level ( $n=3$ )  
Accuracy = (mean observed concentration) (spiked concentration) $^{-1} \times 100\%$

graphic method with methanol or acetonitrile as the mobile phase. Acetonitrile was used instead of methanol, because acetonitrile gave better sensitivity and resolution. The amount of acetonitrile in the mobile phase was optimized at 38%. Likewise the pH of the mobile phase was optimized at 3.0 by use of orthophos-

phoric acid. We obtained good chromatographic separation under these conditions. Fig. 2A shows the HPLC-FLD chromatograms of bendroflumethiazide and internal standard in aqueous solution and Fig. 2B in human plasma after liquid-liquid extraction. Bendroflumethiazide and mosapride (IS) were

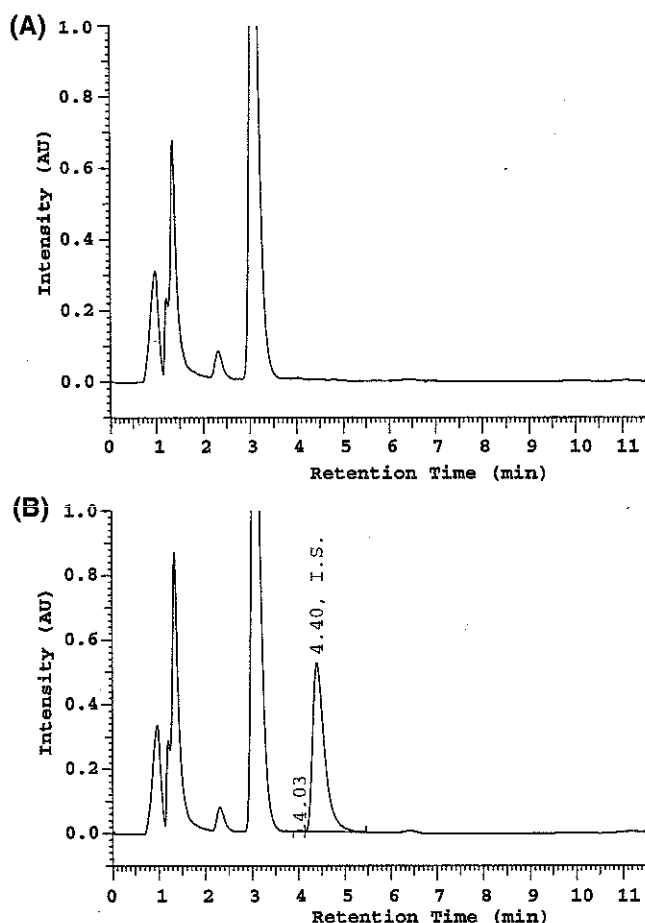


Fig. 3. HPLC-FLD chromatogram (A) Blank human plasma sample (B) Blank human plasma sample with I.S

Table 2. The percent recovery of measurement of bendroflumethiazide and internal standard in human plasma

Nominal concentrations (ng mL <sup>-1</sup> )	% Recovery <sup>a,b</sup>	
	Drug	IS
2.704	63.1	69.3
40.064	67.4	74.9
80.128	74.0	77.5

<sup>a</sup> Averaged for eight measurements at each concentration level ( $n = 8$ )

<sup>b</sup> % Recovery = (response of aqueous) (response of plasma spiked extracted)<sup>-1</sup>

observed at the retention times of 7.66 min and 4.36 min.

### Calibration Curves

Calibration curve was linear over the concentration range of 0.986–100.160 ng mL<sup>-1</sup> of bendroflumethiazide. The eight-point calibration curve gave acceptable results and was used for all the calculations. The mean correlation coefficient of the calibration curves generated during the validation was

0.995. Table 1 shows the measured precision and accuracy of back-calculated concentrations of calibration samples for bendroflumethiazide in human plasma. The precision of the method ranged from 0.9–12.5 and accuracy ranged between 96.8–108.8%, revealing that the method has good reproducibility over a wide concentration range. The calibration curve obtained as described above was suitable for the generation of acceptable data for the concentration of bendroflumethiazide during validation.

### Specificity

HPLC-FLD analysis of the blank human plasma samples showed the separation of bendroflumethiazide and mosapride and no interference with either of these were observed. Hence the specificity of the method was established by comparison with human plasma (control). Representative chromatograms of extracted blank plasma (Fig. 3A), blank plasma fortified with IS (Fig. 3B) are shown indicating no interference in the blank plasma and in drug-free human plasma at the retention time of the analyte and the IS.

### Matrix Effect

The matrix effects in the HPLC-FLD method was evaluated by spiking human plasma with low and high QC samples. Six independent plasma lots were used with six samples from each lot. The percentage of nominal concentrations estimated were well within the acceptable limits. Hence the effect of matrix on estimation of drug is negligible.

### Extraction Recovery

Analyte recovery from a sample matrix (extraction efficiency) is a comparison of the analytical response from an amount of analyte added to that determined from the sample matrix. Because of the basic properties of bendroflumethiazide, extraction was carried out using ethyl acetate as organic solvent. Experiments with spiked compounds resulted in recoveries of 63.1–74% of the drug and 69.3–77.5% for the IS, as summarized in Table 2.

### LLOQ QC and LQC

On the basis of a signal-to-noise ratio (S/N) for 10, the limit of quantitation (LOQ) for bendroflumethiazide was found to be 0.98 ng mL<sup>-1</sup> on injection of 50  $\mu$ L of sample into the HPLC system. As shown in Table 3, the within-batch precision of LLOQ QC and LQC were 8.9 and 9.2 respectively. The between-batch precision was 8.7% and 7.6% respectively. The within-batch accuracy of LLOQ QC and LQC are 104.2% and 102.2% respectively. Whereas between-batch accuracy

**Table 3.** Precision and accuracy of the HPLC-FLD method for determining bendroflumethiazide concentration in plasma samples

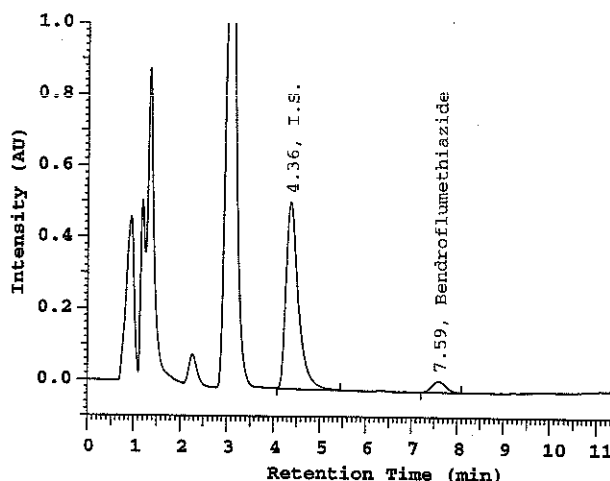
Concentration added (pg mL <sup>-1</sup> )	Within-batch precision (n=8)			Between-batch precision (n=8)		
	Concentration found (mean ± S.D., n=8) pg mL <sup>-1</sup>	Precision (%)	Accuracy (%)	Concentration found (mean ± S.D., n=8) pg mL <sup>-1</sup>	Precision (%)	Accuracy (%)
1.082	1.127 ± 0.100	8.9	104.2	1.101 ± 0.095	8.7	101.8
2.704	2.762 ± 0.255	9.2	102.2	2.743 ± 0.209	7.6	101.5
40.064	37.858 ± 2.300	6.1	94.5	37.245 ± 2.278	6.1	93.0
80.128	80.275 ± 6.388	8.0	100.2	76.928 ± 5.215	6.8	96.0

Averaged for eight measurement at each concentration level (n=8)  
Accuracy = (mean observed concentration) / (spiked concentration) × 100%

was found to be 101.8 and 101.5 respectively. Fig. 4 shows the quantitation limit chromatograms of bendroflumethiazide in human plasma.

### Middle and Upper Concentrations

The middle and upper quantification levels of bendroflumethiazide were 40.06 and 80.12 ng mL<sup>-1</sup>. For the within-batch and between-batch experiment, the precision ranged from 6.1–8.0, whereas the accuracy ranged between 93.0–100.2%.



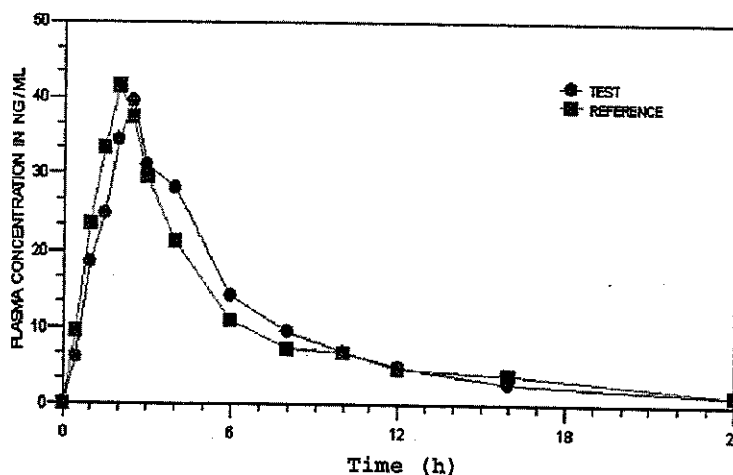
**Fig. 4.** HPLC-FLD chromatograms of quantitation limit of bendroflumethiazide in human plasma after liquid-liquid extraction

### Stability

The stability of the stock solutions were determined by comparing the mean of the area responses obtained from triplicate analysis of aqueous standard (250.4 ng mL<sup>-1</sup>) at 0 h and after 6 h. Ratio of means of area was 103.7% for drug and 98.5% for the IS which is within the acceptable range of 90–110%.

The bench top stability (at room temperature) was determined by comparing the ratio of means of the concentrations for the low and high QCs and was found to be 101.9% and 101.6% as shown in Table 4. This was within the acceptable range of 90–110%.

The freeze-thaw stability was determined by measuring the assay precision and accuracy of the LQC and HQC samples, which underwent three freeze-thaw cycles. The stability data were used to support the repeatability of the analysis. In each freeze-thaw cycle, the frozen plasma samples were thawed at room temperature for 2–3 h and refrozen for 12–24 h. After completion of each cycle the samples were analyzed and results were compared with that of



**Fig. 5.** Overlay graph of time vs. plasma concentration of Bendroflumethiazide for test and reference formulation

zero cycle. The results showed that the analyte was stable in human plasma through freeze-thaw cycles as shown in Table 4. The ratio of means of the concentrations for the low and high QC was 99.7% and 105.1%. This was within the acceptable range of 90–110%. The results demonstrated that human plasma

samples could be thawed and refrozen without compromising the integrity of the samples.

Stability of low and high quality control samples, after processing and its internal standard in the autosampler provide advantage for determining a large number of plasma samples. Eight

**Table 4.** Stability of bendroflumethiazide in human plasma samples

Sample concentration (ng mL <sup>-1</sup> ) (n=8)	Concentration found (mean ± S.D) (ng mL <sup>-1</sup> )	Precision (%)	Accuracy (%)
Bench top stability (6 h)			
2.704	2.86 ± 0.07	2.6	101.9
80.128	78.66 ± 2.42	3.1	101.6
Freeze thaw stability (after 3 cycles)			
2.704	2.67 ± 0.20	7.8	99.7
80.128	76.00 ± 2.07	2.7	105.1
Autosampler stability (47 h)			
2.704	2.81 ± 0.04	1.6	100.2
80.128	78.48 ± 9.23	11.8	101.3

Averaged for eight measurement at each concentration level (n=8)  
Accuracy = (mean observed concentration) (spiked concentration)<sup>-1</sup> × 100%

**Table 5.** Pharmacokinetic data of bendroflumethiazide in human plasma samples

Parameter	Test (mean ± S.D.)	Reference (mean ± S.D.)
AUC <sub>(0-24)</sub> (ng h mL <sup>-1</sup> )	238.860 ± 53.11	231.509 ± 44.73
AUC <sub>0-∞</sub> (ng h mL <sup>-1</sup> )	250.320 ± 52.75	244.529 ± 44.21
C <sub>max</sub> (ng mL <sup>-1</sup> )	36.694 ± 7.51	37.315 ± 6.68
t <sub>max</sub> (h)	2.563 ± 0.63	2.500 ± 0.96
K <sub>el</sub> (1/h)	0.138 ± 0.02	0.125 ± 0.03
t <sub>1/2</sub> (h)	5.102 ± 0.69	5.930 ± 2.08

sets of quality control samples (low and high) were placed into the autosampler at 15° C. They were analyzed at once and three sets 47 h later. The ratios of means of the concentrations for the low and high QC were 100.2 and 101.3%. This was within the acceptance range of 90–110%. A difference in response between 0 and 47 h for bendroflumethiazide signifies the percent change. Stability of the extracted dry residues was also established to be over 28 h (deviation observed < 10%).

### Pharmacokinetics

Overlay graph of mean concentration vs. time of the two formulations (Test and Reference) is shown in Fig. 5. The area under the curve from 0–24 h was determined with the help of the linear trapezoidal rule. The extrapolation to infinity that is necessary for AUC<sub>0-∞</sub> evaluation was calculated using a linear regression model from the last three data points in the elimination phase that has been log transformed. Maximum concentration achieved (C<sub>max</sub>) was obtained directly from the measured concentration without interpolation. Assuming the multiplicative models expected mean of these parameters of the test and reference formulations are computed in Table 5. The

parametric point estimates for the mean of test medication/the mean of reference medication were found within the commonly accepted bioequivalence range of 0.80–1.25. Therefore, the results indicate that the proposed method is suitable for pharmacokinetic studies to determine the concentration of bendroflumethiazide in human plasma.

### Conclusions

A sensitive and specific HPLC-FLD method for the determination of bendroflumethiazide in human plasma has been developed. The developed method was validated and found that the assay has good precision and accuracy over a wide concentration range, and no interference caused by endogenous compounds was observed. The limit of quantification of bendroflumethiazide was 0.98 ng mL<sup>-1</sup>. This simple, rapid and robust assay will enable the complete processing of large numbers of samples for pharmacokinetic studies of bendroflumethiazide in human plasma.

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