



RESEARCH ARTICLE

A SIMPLE AND SENSITIVE METHOD FOR THE DETERMINATION OF BACLOFEN IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY TANDEM-MASS SPECTROMETRY

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A simple and sensitive method for determining baclofen in human plasma using ethanol precipitation followed by LC/MS/MS detection is described. Methyl paraben was used as internal standard. This method is more cost effective and simple compared to the existing SPE method. Chromatographic separation was achieved on a C-18 column (100 × 2.1 mm and 3.5 μm) with a gradient elution of mobile phase consisting of 10 mM ammonium formate containing 0.1% formic acid and acetonitrile. The method is linear over a range of 5.0 to 500.0 ng/ml concentration with an extraction efficiency of about 95% and is particularly suitable for pharmacokinetic studies.

Key words: Baclofen, Tandem-mass spectrometry, Method validation.

INTRODUCTION

Baclofen is used as a skeletal muscle relaxant and in the treatment of reversible spasticity resulting from multiple sclerosis (Sweetman, 2002). Baclofen is rapidly absorbed from gastrointestinal tract and the peak plasma concentration is achieved within about 2 h. A sensitive and selective bio-analytical method is required for determining the drug concentration in the plasma. Different methods were proposed for the determination of baclofen in plasma. A number of HPLC methods (Millerioux *et al* 1996; Ban *et al* 2005; Spahn *et al* 1988; Tosunoglu and Ersoy, 1995) and capillary electrophoresis methods (Chiang *et al* 2000; Kowalski *et al* 2004; Chang and Yang, 2003) have been used for separation. UV detection commonly used in HPLC has low sensitivity and also require lengthy extraction, cleanup procedures for analysis of plasma samples. HPLC and capillary electrophoresis techniques with pre-column derivatization followed by fluorescence detection are also used. O-phthalaldehyde-tert-

butanethil (Ban *et al* 2005), 4-chloro-7-nitro-benzofuran (Tosunoglu and Ersoy, 1995), naphthalene-2, 3-dicarboxaldehyde (Chiang *et al* 2000) and anthracene-2, 3-dicarboxaldehyde (Chang and Yang, 2003) are used as derivatizing agents.

Compared to the extensive HPLC and capillary electrophoresis methods, a very few LC-tandem MS detection methods are reported for the determination of baclofen and other drugs in biological fluids (Flardh and Jacobson, 1999; Miksa and Poppenga, 2003; Chhabra *et al* 2012). A solid-phase extraction followed by tandem mass spectrometry is used to determine baclofen in human plasma (Flardh and Jacobson, 1999) and in bovine serum (Miksa and Poppenga, 2003).

The aim of the present work was to develop and validate a simple, sensitive and selective method for determination of baclofen in human plasma using protein precipitation followed by LC/MS/MS detection and its application to pharmacokinetic studies.

MATERIALS AND METHODS

Chemicals and Reagents

The reference standards of baclofen and methyl paraben are obtained from Chemical Division, Natco Pharma Limited, India. Analytical grade acetonitrile, formic acid and ethanol were purchased from Merck, ammonium formate was purchased from Fluka and water used was of double distilled grade.

Liquid chromatography-Mass spectrometry conditions

Waters Quattro-Micro LC/MS/MS equipped with Electron-Spray Ionization probe and a triple Quadrupole analyzer was used. The separation was carried using a C-18 Sunfire column (100 ×

2.1 mm and 3.5 μ m) equipped with an ODS guard column. A gradient elution (**Table 1**) was used with a mobile phase consisting of 10 mM ammonium formate containing 0.1% formic acid-acetonitrile. The voltages, temperatures, desolvation gas and collision gas were optimized to produce maximum intensity of the ions. The optimized mass spectrometric conditions used were as follows - the capillary voltage of 3.6 kV, cone voltage of 25 V, extractor voltage of 2.0 V, RF voltage of 0.2 V, desolvation temperature of 350°C and desolvation gas flow of 650 l/h and collision energy of 18 eV. LC/MS/MS was performed with MRM mode using parent-daughter transition of 214.24>151.0 for baclofen and 151.3>136.0 for methyl paraben.

Table 1. Gradient programme

Time (min)	%A	%B
0	100	0
2	0	100
3	100	0
8	100	0

Preparation of stock solutions

The primary stock solutions of baclofen for preparation of standards and quality controls (QC) were prepared from separate weighing. Acetonitrile and water (1:1) was used as diluent for preparing the primary stock solution. A concentration of 1.0 mg/ml primary stock solution was prepared by weighing appropriate amount of baclofen. This solution was further diluted serially using water as diluent to prepare working stock solutions ranging from 0.1 mg/ml to 0.1 μ g/ml. The primary stock solution of the internal standard was also prepared using acetonitrile and water (1:1) as diluent with a concentration of 1.0 mg/ml by weighing appropriate amount of methyl paraben. The working stock solution was prepared by further diluting the primary stock to prepare 1.0 μ g/ml using acetonitrile and water (1:1) as diluent. All the stock solutions were stored at 4°C.

Preparation of calibration curve samples

The working standard solutions were used to prepare the calibration curve samples. Spiking of calibration curve samples was done using 1 ml of blank plasma. The calibration curve consisted of seven points (5.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0 ng/ml). After spiking, aliquots each of 200 μ l were transferred into eppendorff tubes and

stored at -70°C until further analysis. Three successive validation batches were done and on each validation the aliquots were thawed, 20 μ l of internal standard was added and extracted by precipitation technique.

Preparation of quality control samples

Four different levels of quality control samples (LLOQ, LQC, MQC and HQC) were spiked using the quality control working standard solutions. After spiking for 1 ml of blank plasma again aliquots each of 200 μ l were transferred into eppendorf tubes and stored at -70°C. The concentrations of the quality control samples spiked are 5.0, 15.0, 150.0 and 350.0 ng/ml.

Plasma sample extraction procedure

The calibration curve samples and quality control samples were extracted by precipitation method. To 200 μ l plasma 20 μ l of internal standard (1.0 μ g/ml concentration) was added and vortexed for about 30 sec. Then 800 μ l of absolute ethanol was added, vortexed for 2 min using multipulse vortexer (Glas-Col) and then centrifuged for 10 min at 5°C at 12000 rpm. The upper layer was decanted into a 7.5 ml test tube and evaporated to dryness under a stream of Nitrogen using Speedovap at 55°C for 12 min at 20 psi pressure. The dried extract was

reconstituted with 200 μ l of diluent containing acetonitrile and water (1:1) and an aliquot of 20 μ l was injected into the LC/MS/MS instrument. During each run, a plasma blank sample and a zero standard sample (IS) were also analyzed.

Assay validation

The validation of the method was carried out according to the USFDA Bioanalytical Method Validation Guidance. The validation parameters studied are selectivity, linearity, sensitivity, accuracy, precision, recovery (extraction efficiency), auto sampler stability/post

preparative stability, bench top stability/short term stability and freeze thaw stability.

RESULTS AND DISCUSSION

Selectivity

The selectivity was established by checking six blank plasma samples obtained from six different healthy human donors. In all the blank plasma samples there was no interference detected due to any endogeneous components. A representative chromatogram of blank plasma and blank plasma spiked with analyte and internal standard are shown in **Figures 1-3**.

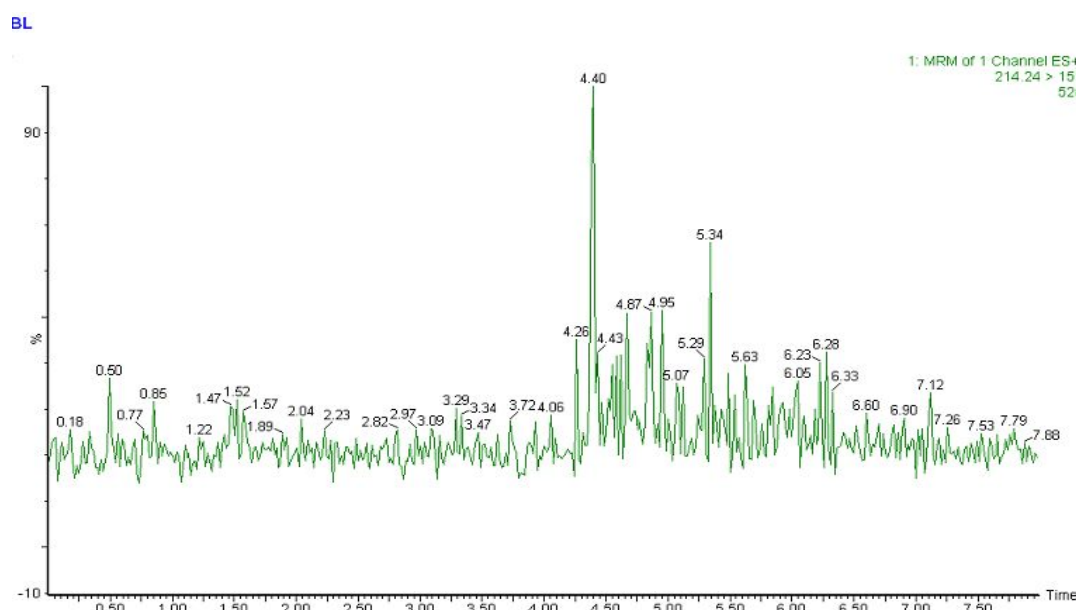


Fig. 1. Representative chromatogram of blank plasma

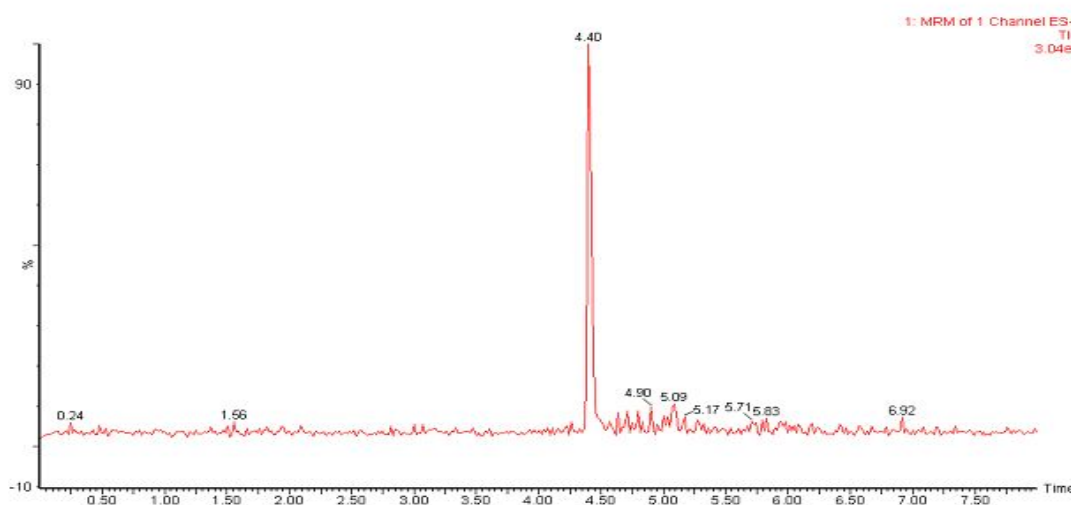


Fig. 2. Representative chromatogram of blank plasma spiked with baclofen at LOQ level

Linearity

To establish the range of baclofen concentrations that can be assayed by using the present method,

seven different concentrations ranging from 5.0 to 500.0 ng/ml are taken and analyzed. The calibration curve is represented in **Figure 4**. The

area ratio obtained against each concentration is plotted against the amount of baclofen. A straight line fit was made through the data points by linear regression analysis and a constant proportionality was observed with minimal data scattering. Five different

calibration curves were analyzed and the correlation coefficients were greater than 0.99. This confirmed that the calibration curves were linear over the range of 5.0 to 500.0 ng/ml. The curve parameter summary of five calibration curves are given in **Table 2**.

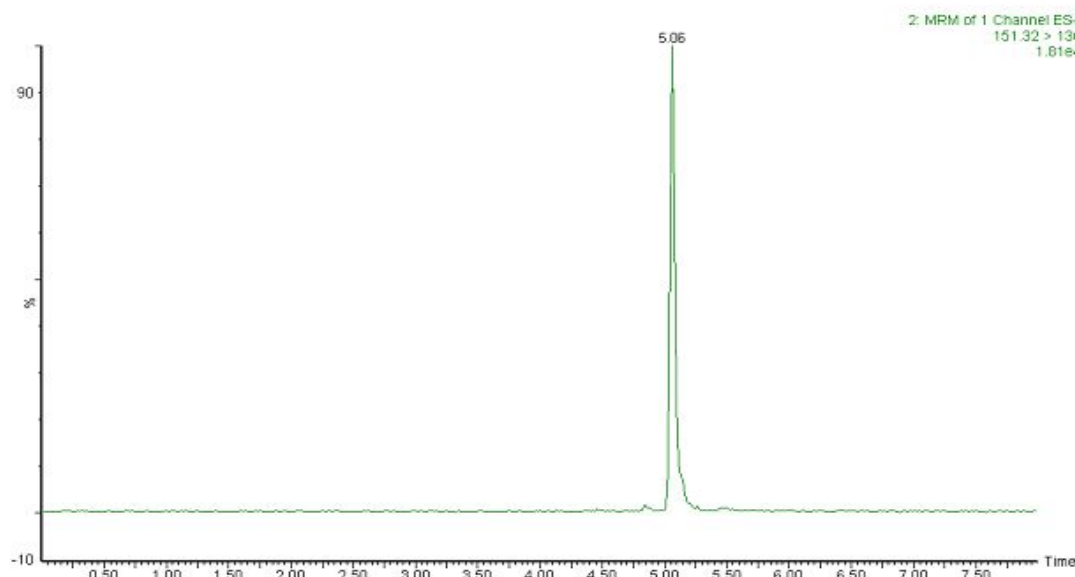


Fig. 3. Representative chromatogram of blank plasma spiked with internal standard

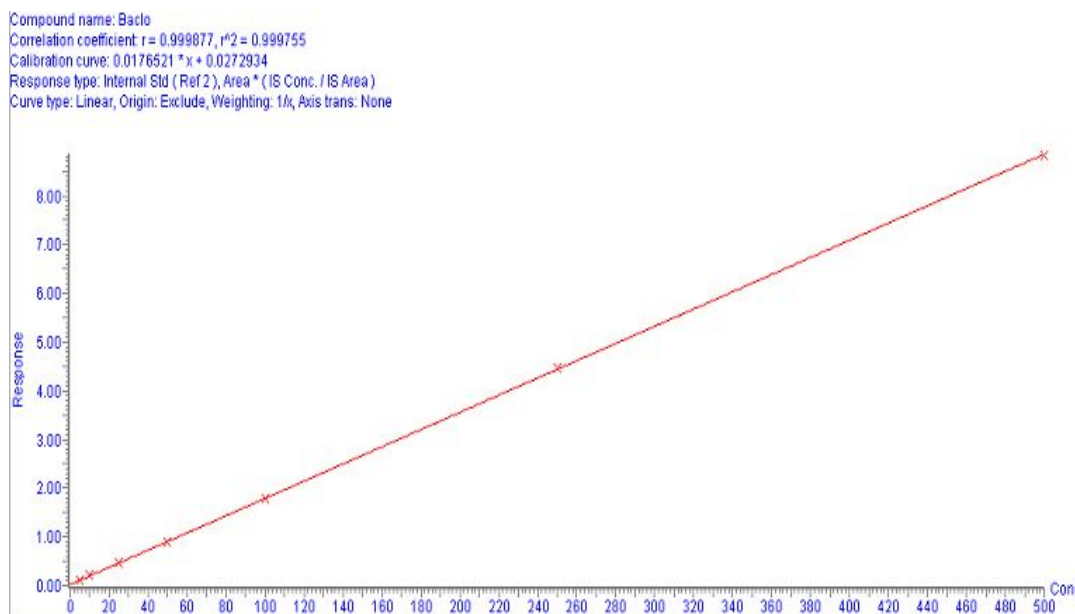


Fig. 4. Calibration curve

Lower limit of quantification

The lower limit of quantification was determined as 5.0 ng/ml. The response was greater than 5 times compared to the response of blank plasma sample. The accuracy obtained was 98.03% and precision 9.06%.

Accuracy and Precision

The accuracy of the assay is defined as the ratio of the mean of the assay values to the actual values expressed in percentage. The accuracy and precision was checked by analyzing six replicates of all three quality control samples.

Table 2. Curve parameter summary for baclofen

Curve code	Slope (a)	Y-intercept (b)	Coefficient of determination (r)
1	0.024	0.079	0.994
2	0.037	0.036	0.995
3	0.013	0.066	0.995
4	0.015	0.002	0.998
5	0.018	0.027	0.999

The intra-day/within run accuracy ranged from 94.80% to 103.7% and the intra-day/within run precision ranged from 5.72% to 7.53%. The inter-day/between run accuracy ranged from

94.96% to 102.12% and the inter-day/between run precision ranged from 5.87% to 7.61%. The results intra-day and inter-day accuracy and precision are tabulated in **Table 3**.

Table 3. Intra-day and Inter-day accuracy (% nominal) and precision (% CV)

	ng/ml	% Nominal	% CV
intraday	15.0	97.91	7.53
	150.0	103.7	5.72
	350.0	94.80	6.57
interday	15.0	102.12	7.61
	150.0	101.55	5.87
	350.0	94.96	6.54

Recovery (Extraction efficiency)

The percentage recoveries were determined by measuring the peak area of the extracted plasma quality control and compared to the peak area of extracted blank plasma spiked with standards containing the same concentrations. The recovery of baclofen and internal standard were 92.7% and 95.6% respectively.

Stability

Autosampler stability/post preparative stability was determined for ~24 h to cover the anticipated run time for analytical batch and also to allow for delayed injection owing to unforeseen circumstances like instrument malfunction. The extracted six replicate QC samples at three different concentrations were kept at autosampler temperature of 5°C for ~24 h and analyzed against fresh standards. The concentrations of the stability samples and fresh samples were determined from a calibration curve assayed on the same day. The results indicated that the autosampler stability was not compromised even after 24 h of storage. Bench-top stability/short-term stability was measured to cover the duration of the time taken to extract the samples. Bench-top stability was checked by analyzing six replicate QC samples at three different concentrations. The spiked QC samples

were kept for 6 h at ambient temperature and processed thereafter. The concentration of the stability samples were compared against freshly spiked and processed standards. Baclofen was found to be stable even after 6 h. Freeze thaw stability of the spiked quality control samples was determined during three freeze thaw cycles. Low, medium and high QC samples were analyzed in six sets. The percentage degradation was determined by comparing the concentration of baclofen from the freshly prepared plasma validation samples at the same concentrations. It was found that even after three freeze thaw cycles the concentrations of baclofen are nearly the same with the original concentrations and the percentage remained at the end of the three cycles is from 94.59 to 101.04%.

Application of the method

The validated method was applied to determine the concentrations of baclofen in real plasma samples collected periodically up to 36 h after oral administration of 20 mg tablet to 12 healthy male volunteers during the development of a conventional formulation. The mean plasma concentration curves of test and reference are represented in **Figure 5**. The AUC measured from 0 h to last collection point was greater than 90% to the AUC extrapolated from 0 h to infinity.

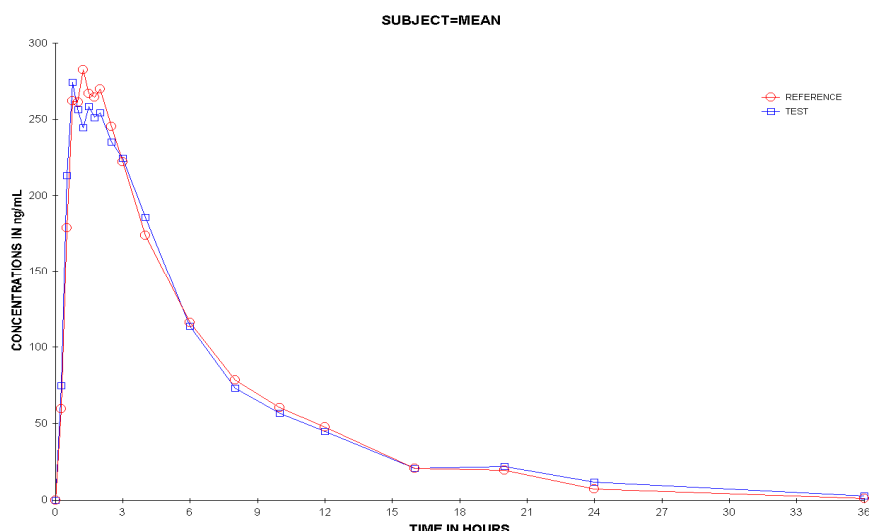


Fig. 5. Mean plasma concentration curves of baclofen test and reference

CONCLUSION

It can be concluded that the proposed method was validated for the estimation of baclofen in human plasma over a concentration range of 5.0 to 500.0 ng/ml. The precision and accuracy were very much within the prescribed limits in this concentration range. Expected recoveries were observed in the present processing technique for LQC, MQC and HQC. The drug was found to be stable to the effect of three freeze thaw cycles up

to 6 h delay on the bench top. The above data indicates the suitability of the method for pharmacokinetic studies.

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