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LC–MS/MS assay for baclofen, a derivative of γ –aminobutyric acid (GABA) in human plasma and its clinical application

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Abstract : A high performance liquid chromatography mass spectrometric method for the estimation of baclofen, in human plasma in positive ion mode was developed and validated using baclofen d4 as internal standard (IS). Sample preparation was accomplished by solid phase extraction technique. The reconstituted samples were chromatographed on Kromasil 100-5C8 4.6×150 mm columns using a mobile phase consisting of acetonitrile and 10mM ammonium acetate (80:20, v/v).The method was validated over a concentration range of 20.1 ng/mL to 1000ng/mL for baclofen. This validation report provides the results of selectivity, matrix effect, sensitivity determinations, linearity, precision and accuracy data, the results of recovery, various stabilities, run size evaluation and dilution integrity along with all pertinent documentation.
Keywords : Baclofen; Human plasma; LC–MS/MS; Method validation; Pharmacokinetics.

Introduction

Baclofen is a derivative of the neurotransmitter γ-aminobutyric acid (GABA) and most widely used spasmolytic agent. It is a skeletal muscle relaxant with its prime site of action in the spinal cord, where it binds to the inhibitory GABA-B receptor. After oral administration the drug is quickly absorbed and is widely distributed all over the body, low biotransformation and the baclofen is prevalently excreted by the kidneys without changing the form. The baclofen half-life is two to 2-4days to regulate spasticity accordingly[1,2].

As per literature, only few LC-MS/MS [3-5] have been reported for the determination of baclofen in biological samples. Flärdh *et al.*,1999[3] developed a method for the determination of baclofen in plasma using

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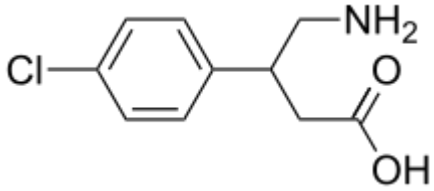
solid phase extraction. Similarly, Kim *et al.*, 2013[4] was also published a LC-MS/MS method for the quantification of baclofen in rat plasma, urine and tissue samples using gabapentin as internal standard. This method employs protein precipitation (PP) for sample preparation. The linearity was established in the range of 0.25–500 ng/mL for plasma and brain tissue, and 2–5000 ng/mL for rat urine, kidney and liver. But the run time was 12 min, which is very high for high throughput bioanalysis of baclofen. The another author Nahar *et al.*,2016[5] quantified baclofen in plasma samples using solid phase extraction technique in the linearity range of 25-1000 ng/mL. However, the run was >5 min, which is not suitable for routine bioanalytical application. The analytical method should satisfy the scientists in terms of simplicity, sensitivity, runtime, time consumption, sample volume and efficient extraction procedure[6,7].

In the present paper, the authors developed and validated a simple and rapid LC-MS/MS method for the determination of baclofen in of human plasma using isotope labeled compound baclofen d4 as internal standard. Use of stable labelled isotope as internal standard helped in obtaining better precision and accuracy and compensate for potential matrix effect. The method was successfully applied to a pharmacokinetic study in healthy male subjects.

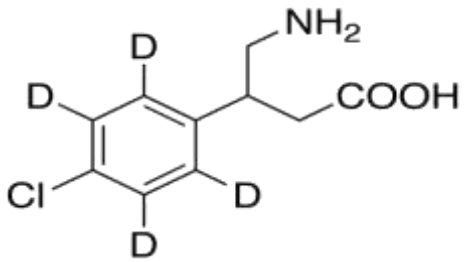
Experimental

Standards and Chemicals

The reference sample of baclofen (99.83%) was obtained from Clearsynth Labs Limited, while baclofen d4 (99.67%) was from Clearsynth Labs Limited (Fig. 1).Milli Q water used for the LC-MS/MS analysis. HPLC grade methanol and acetonitrile were purchased from J.T. Baker, while analytical grade ammonium acetate and hydrochloric acid was from Merck Ltd. The control K_2 human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).



Baclofen



Baclofen d4

Figure 1: Chemical structures of baclofen and baclofen-d4.

LC-MS/MS instrument

A HPLC system (Shimadzu, Kyoto, Japan) equipped with a binary LC-20AD prominence pump, an auto sampler (SIL-HTc) and a solvent degasser (DGU-20A_3) was used for the study. Quantification was achieved with MS-MS detection in positive ion mode for the analyte and the IS using an AB Sciex API-3000 mass spectrometer (Foster City, CA, USA). Analysis data obtained were processed by Analyst software™ (Version 1.4.2).

Preparation of stock and working solutions

Stock solutions of baclofen and the IS (1mg/mL) were separately prepared in methanol. Working solutions for stock concentration and quality concentrations were prepared in acetonitrile and water (80:20, v/v).The IS working solution (10µg/mL) was also prepared with diluent.

Preparation of calibration curve standards and quality control samples in human plasma

Calibration curve contains a set of 8 non-zero concentrations ranging from 20.1, 40.2, 100,201, 402, 600, 800and 1000ng/mL of baclofen were prepared. The QCs prepared for baclofen are 20.8 ng/mL (LLOQ QC), 60.2 ng/mL (LQC), 110 ng/mL (MQC1), 506 ng/mL (MQC2), and 750ng/mL (HQC). These samples were stored at –70°C until use.

Chromatographic conditions

Chromatographic separation was carried out on a reversed phaseKromasil100-5C8, 4.6×150mm column. A mobile phase comprising of acetonitrileand10mM ammonium acetate buffer (80:20, v/v) at a flow rate of 0.8 mL/min. Retention time of baclofen and the IS was found to be approximately 1.85 ± 0.3min.

Mass spectrometry conditions

An API-3000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with Turboionspray ™ (ESI) interface was used for the study. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM) with the transition pairs of *m/z* 214.0 precursor ion to the *m/z* 115.2 for baclofen and *m/z* 218.0 precursor ion to the *m/z* 119.3 product ion for the IS. The source parameters viz. the nebulizer gas (GS1), collision gas (CAD), and curtain gas were set at 3, 8 and 8 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP), collision cell exit potential (CXP) and focusing potential (FP) were 30, 53, 10, 4, 310 V for baclofen and for the IS. The chromatographic data was processed by Analyst Software™ (version 1.4.2).

Preparation of sample

A 200 µL of the plasma sample was pipetted in to 5mL polypropylene tubes,20 µL of IS dilution (baclofen d4 at a concentration of 10µg/mL) was added to it and vortexed. Strata-X 33µm polymeric sorbent cartridge (30 mg/1 mL) was used for sample mixture loading. Cartridges were pre-conditioned with 1.0 mL of methanol followed by 1 mL water. The extraction cartridge was washed with 0.5 mL of 0.25% hydrochloric acid followed by 1 mL water after applying the pressure maximum. Then the samples were eluted with 1 mL of methanol and evaporate to dryness under nitrogen gas at 55°C. The samples were reconstituted in 250 µL of mobile phase and injected.

Method validation

The present method was validated for carryover test, selectivity, matrix effect, sensitivity, linearity, precision and accuracy, recovery, dilution integrity, and stability as per US FDA guidelines[8].

Pharmacokinetic study

A pharmacokinetic study was performed in healthy Indian male subjects (n = 6) under fasting condition. All the volunteers provided with written informed consent. Blood samples were collected after oral administration of 10 mg baclofen tablet at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 16 and 24 h and collected in K_2 EDTA vacutainer (5 mL) collection tubes (BD, Franklin, NJ, USA). A predose sample was also collected before administration of each drug formulation. The tubes were centrifuged at 4000 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –70 ± 10 °C. Plasma samples were spiked with the IS and processed as per the proposed extraction procedure. WinNonlin (Version 5.2) software package was used to calculate the pharmacokinetic parameters by non–compartmental model.

Results and discussions

Method development

The detected full scan mass spectra in positive mode recognized the addition of a proton to molecular ions $[M+H]^+$ of m/z 214 to 115for baclofen and from m/z 218 to 119 for the IS, respectively. Initially, analyte and the IS were tuned in positive and negative ionization modes using ESI source. The high intense signals were obtained in positive ion mode than the negative mode. Data in the multiple reaction monitoring (MRM) mode was considered, which showed better selectivity. The compound and source dependent parameters were suitably altered to get most intense signals and reproducible response. Data in the multiple reaction monitoring (MRM) mode was considered, which showed better selectivity[9,10].

Once the mass spectrometer conditions were set, the mobile phase composition was optimized with acetonitrile and methanol by varying its proportion with volatile buffers like ammonium formate, ammonium acetate, as well as acid additives like formic acid and acetic acid in varying strength. A mobile phase comprising of acetonitrile and 10mM ammonium acetate buffer (80:20, v/v) was found to be best for the present purpose. Kromasil, 100-5C8, 4.6×150 mm gave a sensible peak shape and response even at LLOQ quantity the analyte at retention time of 1.85 ± 0.3min.The flow rate of 0.8 mL/min allowed a runtime to 3.0 min. Reported methods were used SPE [3,5] and PP [4] to extract baclofen from human plasma samples. For LC–MS analysis, there should be a proper extraction procedure which can yields good recovery with no or minimal matrix effect. SPE techniques gives high recovery and clean sample cleanup without matrix effect.

Hence, we also employed SPE for sample preparation. SPE was tried with Oasis HLB, Starata X polymeric sorbent, Orochem celerity deluxe, Bond ElutPlexa and Orpheus C_{18} extraction cartridges with/without acidic buffer addition. Of all the above, promising results were obtained with Starata X polymeric sorbent (30 mg/1mL), which can produce a clean chromatogram for a blank sample and yields the highest recovery for the analyte and the IS from the plasma. Stable labeled isotopes as internal standard increases the bioanalytical assay precision and accuracy. Hence, in the present work baclofen stable labeled isotope baclofen d4 was employed as an internal standard.

Selectivity and chromatography

The degree of interference by endogenous plasma components with the analyte and the internal standard was assessed by analysis of blank plasma lots obtained from 11 individual lots. As showed in Fig. 2A no significant interference in the blank plasma traces was observed from endogenous components in drug–free plasma at the retention time of analyte and the IS. Moreover, no significant interference was found from the IS to the MRM channel of the analyte (Fig. 2B). Fig. 2C shows an LLOQ sample of baclofen along with the IS.

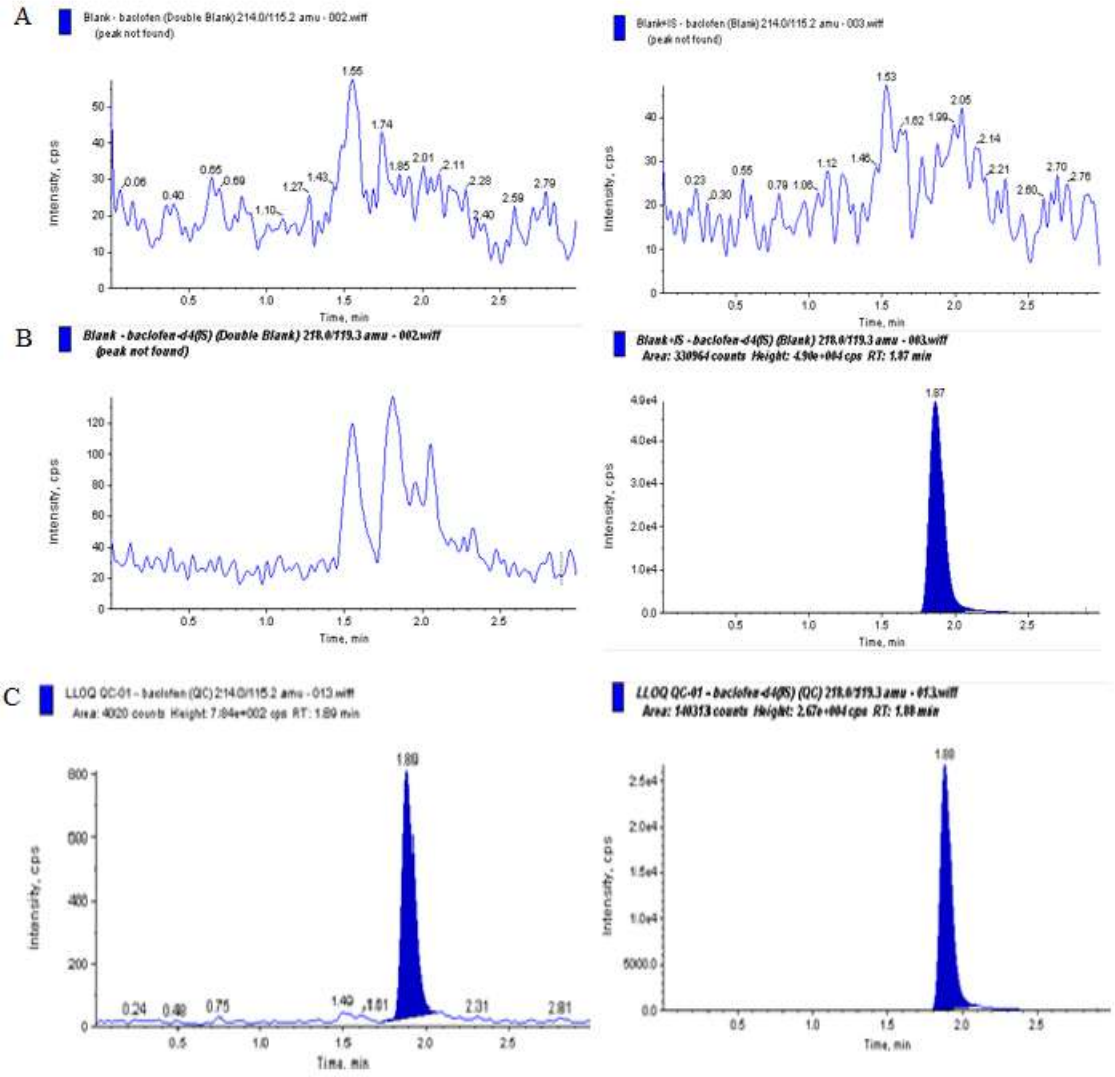


Figure 2: Typical MRM chromatograms of baclofen (left panel) and IS (right panel) in human blank plasma (A) and human plasma spiked with IS (B) and an LLOQ sample along with IS (C).

Sensitivity and matrix effect

The signal–to–noise ratio (S/N) was measured at a concentration of 20.1 ng/mL, which is set as a lowest limit of reliable quantification (LLOQ) for the analyte. The S/N ratio at this concentration was found to be ≥10. The precision and accuracy at LLOQ concentration were found to be 2.86% and 99.81%, respectively. Matrix effect assessed by at LQC and HQC levels comparing the mean area response of post–extraction spiked samples with mean area of aqueous samples (neat samples). These samples were prepared in mobile phase and injected. The IS normalized matrix factor was 0.983 for LQC and 0.982 for HQC. The results indicate no significant matrix effect was found in all the plasma lots tested.

Linearity, Precision and Accuracy

A total of five successful calibration curves run during the validation in the concentration range of 20.1–1000ng/mL for baclofen with a mean correlation coefficient of ≥ 0.99. After comparing the two weighting models (1/x and $1/x^2$),a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship. Intra–day and inter–day precision and accuracy results of baclofen for five analytical runs in spiked quality control samples are summarized in Table 1.

Table 1 : Precision and accuracy data for baclofen.

Quality control	Run	Concentration found Mean±SD (ng/mL)	Precision (%)	Accuracy (%)
Intra–day variations (*n*=12 at each concentration)				
LLOQ		23.5±0.79	3.36	113.12
LQC		60.1±1.13	1.88	99.96
MQC1		108±1.81	1.67	97.81
MQC2		474±5.56	1.17	93.62
HQC		720±9.82	1.36	95.91
Inter–day variations (*n*=30 at each concentration)				
LLOQ		22.2±1.55	6.98	106.99
LQC		60.3±1.40	2.32	100.19
MQC1		108±1.89	1.74	98.11
MQC2		483±15.1	3.12	95.34
HQC		729±11.1	1.52	97.16
Nominal concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 20.8, 60.2, 110, 506and 750ng/mL, respectively.				

Recovery and dilution integrity

The recovery of baclofen at LQC, MQC2 and HQC levels were found to be 74.81%, 63.48% and 68.64%, respectively with mean recovery of 68.98±5.67%. Similarly, the recovery of IS was 69.31% with the precision range of 3.17–7.79%. Sample dilution was performed to extent the ULOQ suitable of higher dose of baclofen. The ULOQ can be extended up to 1650 ng/mL by 1/2 and 1/4 dilutions with screened human blank plasma. The precision (%CV) an accuracy for two-fold dilution was 0.78% to 95.50%, respectively. Similarly, the precision (%CV) an accuracy for four-fold dilution was 1.90% to 93.58%, respectively.

Table 2 : Stability data for baclofen in plasma (n=6).

Stability test	QC (spiked concentration, ng/mL)	Mean ± SD (ng/mL)	Accuracy/ Stability (%)	Precision (%)
Aautosampler stability (at 5°C for 55 h)	60.2 750	60.0 ± 0.96 7523 ± 10.8	99.78 100.36	1.59 1.44
Wet extract stability (at 2–8°C for 50 h)	60.2 750	61.9 ±1.40 749± 16.5	102.97 99.89	2.27 2.20
Bench top stability (15 h at room temperature)	60.2 750	59.63±0.83 740±9.56	99.10 98.64	1.40 1.29
Freeze–thaw stability (four cycles)	60.2 750	61.5±1.87 746± 16.4	102.26 99.55	3.05 2.19
Reinjection stability (26 h)	60.2 750	62.7 ± 1.00 738± 8.24	104.2 98.43	1.60 1.12
Long–term Stability (at –70°C for 80 days)	60.2 750	57.6±1.07 732±10.1	95.77 97.59	1.87 1.39

Stability studies

The mean % nominal values were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels and the precision (% CV) values were within 15% (Table 2) for all the stability tests carried out during the entire course of method validation. All the above stability results were found to be within the acceptable limits during the entire validation.

Pharmacokinetic study results

The proposed method was successfully used to estimate the real time plasma samples of baclofen obtained from the pharmacokinetic study (n=6). The mean concentration (C_{max}) in plasma (407±69.3ng/mL) for baclofen was attained at 1.08±0.56 h (t_{max}) with a half–life ($t_{½}$) of 5.13±0.70 h. The area under the plasma concentration–time curve from time zero to last measurable time point (AUC_{0-t}) and area under the plasma concentration time curve from time zero to infinity time point (AUC_{0-inf}) for baclofen were 2173±317 and 3377±332ng*h/mL, respectively. Fig. 3 depicts the mean plasma concentration *vs* time profile of baclofen in healthy subjects.

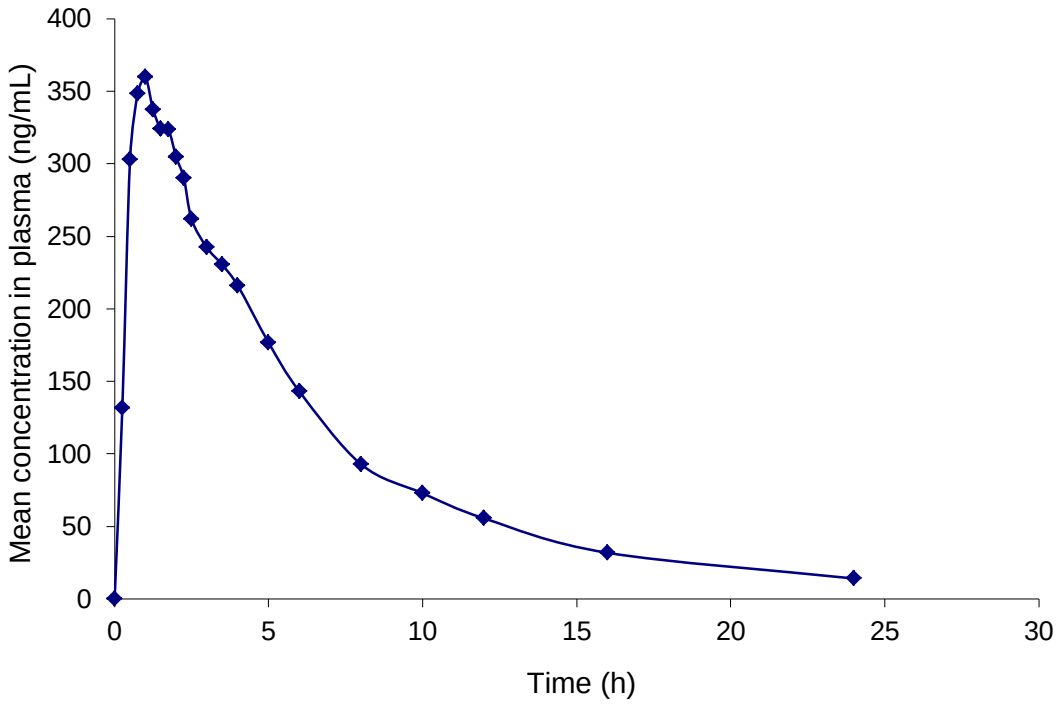


Figure 3:Mean plasma concentration–time profile of baclofen in human plasma following oral dosing of baclofen (10 mg tablet) to healthy volunteers (n=6).

Conclusions

The proposed LC–MS/MS assay method is straightforward, rapid, specific and sensitive for the quantification of baclofen in human plasma. This method is highly sensitive and utilizes 200 µL plasma volumes for sample processing. The SPE method gave reliable and reproducible recoveries for the analyte and the IS from plasma. Additionally, the total analysis time is the shortest compared to all these methods. Thus, the advantage of this method is that a relatively large number of samples can be analyzed in short time, thus increasing the output. The stability of the analyte in human plasma and in aqueous samples under various conditions has been broadly studied and is meeting acceptance criteria. The method was found to be reliable and reproducible to support pharmacokinetic study in humans. From the results of all the validation parameters, developed method can be effective for bioavailability and bioequivalence investigation and therapeutic drug monitoring with the desired precision and accuracy.

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