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High throughput LC-MS/MS method for the quantitation of baclofen in human plasma by solid phase extraction using 96 well plate format

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Abstract

Oral baclofen is indicated for the treatment of spasticity resulting from multiple sclerosis and is very useful for the relief of flexor spasms and concomitant pain, muscular rigidity, and clonus. It can also be used to treat patients with spinal cord injuries and other spinal cord diseases. In present work, a sensitive and high throughput LC-MS/MS method was developed and validated to estimate Baclofen in Human plasma. The developed LC-MS/MS method was sensitive enough to detect Baclofen in human plasma with a high degree of accuracy and precision, using Baclofen-d4 as an internal standard. Using BDS Hypersil C8 5µm, 100 X 4.6mm column and a mobile phase consisting of 2mM Ammonium formate with 0.02% v/v formic acid: Acetonitrile (85:15 v/v) was used to achieve best chromatographic separation. A flow rate of 1 mL/min was used to optimize the run time of 3.2 min. Plasma sample extraction was performed by Solid Phase extraction using 96 well plates. The developed bioanalytical method was validated successfully. This high throughput bioanalytical method developed and validated can be applied to routine sample analysis. this method can accommodate 80 study samples in single run excluding the CC and QC samples. Since the method uses 96 well plate formate it gives flexibility in assigning the CC, QC, and study samples in fixed well before the initiation of the process which in turn avoids the risk of sample mix up during processing. The method validation results were found to be within the acceptance range. It can be concluded that the developed high throughput LC-MS/MS method is sensitive enough to measure Baclofen up to an LOOQ 5.023 ng/mL in Human plasma and cater to high throughput analysis using 96 well plates.

Keywords: Baclofen, Baclofen D4, Solid Phase Extraction LC-MS/Ms method, 96 well plates.

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1. Introduction

Baclofen which is taken orally is indicated for the treatment of spasticity caused due to multiple sclerosis and is particularly useful for the relief of concomitant pain, clonus, flexor spasms and muscular rigidity. It is also used to treat patients suffering with spinal cord diseases and spinal cord injuries. Baclofen cannot be used to treat skeletal muscle spasms caused by rheumatic disorders [1]. Baclofen can be given intrathecally in patients 4 years and above for the management of severe spasticity of the cerebral or spinal original. Intrathecal injection is only reserved for patients unresponsive to oral therapy, or for those who experience intolerable central nervous system side effects at effective doses. Baclofen is also considered for use in spasticity due to traumatic brain injury, only after at least one year of injury [2]. As an antispasmodic agent Baclofen induces muscle relaxation. It stimulates inhibitory neuronal signals in the post-synaptic neurons and inhibits the release of excitatory neurotransmitters in the pre-synaptic neurons [3]. Baclofen as oral formulation is the most used form of medication. Intrathecal baclofen was found to be more effective than oral baclofen in one cross-section study which aimed to relieve spasticity directly at the level of the spinal cord [4]. Baclofen and can cause sedation with tolerance, somnolence, ataxia, respiratory and cardiovascular depression due to its CNS depression properties [5]. Baclofen also stimulates gastric acid secretion and mediates some antinociceptive effects (Fig 1) [6]. Baclofen has an oral bioavailability of 70% to 85%. Following oral administration, it is rapidly absorbed through the gastrointestinal tract with peak plasma concentrations being reached two to three hours after ingestion [3]. Peak effect is observed about four hours after intrathecal administration [4]. The absorption is dose-dependent and increases with higher doses [7-9]. There is intersubject variation in absorption. Jacobson B.M. et al., developed an LC-Ms/Ms method using manual SPE cartridges with an LLOQ 10 nmol/L [10]. Goda R et al., developed an LC-MS/MS method to baclofen in human plasma and

cerebrospinal fluid. The calibration curves for both biologic samples were linear over a concentration range of 0.15-150 ng enantiomer/ml which does not suit our intended calibration range [11,12,13,14]. Kim et al., developed an LC-MS/MS method for the quantification of baclofen in rat plasma, urine, as well as various tissue samples. Protein Precipitation method was used to extract baclofen from various matrices. The assay was linear over a concentration range of 0.25-500ng/mL for rat plasma and brain tissue, and 2-5000ng/mL for rat urine, kidney, and liver [15]. Limon Khatun Nahar et al., developed and fully validated method for the quantification of baclofen in human plasma. using solid-phase extraction (SPE) cartridges. The assay was linear from 25 to 1,000 ng/mL [16]. None of the above-mentioned methods suit our quantification range and high throughput method in human plasma. The aim of the present work was to develop and validate the high throughput LC-MS/MS Method using solid phase extraction technique as per the US FDA guidelines [5,6] to quantify the Baclofen in human plasma using Baclofen D4 as an internal standard.

2. Materials and Methods

2.1. Chemicals and reagents

Working standards of Baclofen and Baclofen d4 were obtained from Clearsynth (Mumbai, India). LC–MS grade methanol and acetonitrile were purchased from Thermo Fisher Scientific India Pvt. Ltd. (Mumbai, India). GR grade ammonium formate was procured from Merck Specialties Pvt. Ltd. (Mumbai, India). GR grade orthophosphoric acid was purchased from Merck. HPLC water was obtained from Milli-Q water purification system (Millipore). Human plasma containing K_2EDTA anticoagulant was obtained from Doctor's pathological lab (Hyderabad, India). Waters Oasis HLB 96 well plates 30 µm (10 mg) were purchased from Waters Corporation (Milford, MA, USA).

2.2. Instrumentation

Agilent 1200 Series equipped with a binary pump for solvent delivery was used for the analysis. Mass spectrometric detection was performed on API-4000 triple quadrupole mass spectrometer (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray inter- face. Quantitation was performed in multiple reaction monitoring (MRM) mode and Analyst software version 1.4.2 (SCIEX) was used for controlling the hardware and data handling.

2.3. Chromatographic conditions

Chromatographic separation was performed on BDS Hypersil C8 5µm, 100 X 4.6mm analytical column. Isocratic mobile phase consisting of 2mM Ammonium formate with 0.02% v/v formic acid: Acetonitrile (85:15 v/v) was delivered at a flow rate of 1.0 mL/min. The auto sampler was set at 5ºC±2ºC and the injection volume was 10 µL. The column oven temperature was set at 35.0 ± 2.0°C. Retention Time of Baclofen was 1.94 and Baclofen D4 was 1.94. The total chromatographic run time was 3.2 min.

2.4. Mass spectrometric conditions

The separation and quantification of Baclofen and Baclofen-D4 were carried out using LC-MS/MS with positive and negative ionization modes, finally fixed to positive ion mode with different mass optimization parameters such as the curtain gas (CUR) and collision energy (CE) for. The final optimized parameters for mass spectrometry are shown in Table 1 (a) and Table (b). Elution of the analyte and ISTD from the same sample is achieved with isocratic mobile phase with good response at low concentrations. The optimized chromatographic conditions are listed in Table 2.

2.5. Preparation of calibration standards and quality control samples

Standard stock solutions of Baclofen and internal standard (Baclofen D4) were prepared by dissolving their accurately weighed amounts in methanol to give a final concentration of 1000 mg/mL. Individual working solutions of analyte were prepared by appropriate dilution of their stock solutions in 50% Methanol. All the solutions were stored in refrigerator at below 2-8◦C and were brought to room temperature before use. Working solution of internal standard (Baclofen, 500 ng/mL) was prepared daily in 50% Methanol and was stored at room temperature. Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with the working solutions (5%) prepared from independent stock weightings. K_2EDTA anticoagulant blank plasma collected from healthy volunteers was screened individually and pooled before use. Calibration standards were prepared at concentrations of 100.462, 201.125, 402.250, 1005.624, 2011.249, 4113.918, 9142.040, 17826.978, 20341.039 ng/mL. Quality control samples were prepared at 100.562 (LLOQ QC), 301.687 (LQC), 1005.624(MQC-I), 10284.795 (MQC-II) and 17141.325 (HQC) ng/mL.

2.6. Sample Preparation

Calibration standards and QCs were processed using Ezypress Positive Pressure SPE Manifold by using 100 µL of Plasma Volume. The steps followed in sample process are described below.

- For CC and QC spike 5 µL of each working solutions of Baclofen into 190 µL of human plasma. Aliquot 100 µL of spiked plasma for CC's and QC's preparation.
- Aliquot 100 µL of spiked CC's and QC's samples into polypropylene 96 well plates.
- Add 10 µL of internal standard to each well containing plasma sample except for blank samples.
- Add 10 µL of 50:50v/v MeoH: Water to blank plasma samples.
- Vortex the 96 well plate on mixmate for 60 seconds.
- Condition the Waters Oasis HLB 96 well plate 30 µm (10 mg) with 500µL of methanol followed by 500 µL of Milli Q water.
- Load the mixture of plasma and ISTD samples onto the HLB cartridge.
- Wash the cartridge with 1000 µL (two aliquots of 500 µL each) of extraction buffer and 1000 µL (two aliquots of 500 µL each) of Milli Q Water.
- Elute the sample with 600 µL (two aliquots of 300 µL each) of mobile phase into 96 well collection plate.
- Load the 96 well collection plate into the autosampler.
- Inject 10 □L of the sample onto the LC-MS/MS system.

2.7. Method validation

A complete method validation of Baclofen in human plasma was done following the USFDA and EMEA

guidelines. Validation runs were performed on different days to evaluate selectivity, sensitivity, linearity, precision, accuracy, recovery, matrix effect, dilution integrity and stability. Each validation run was organized with a set of spiked standard samples, blank (with ISTD and without ISTD) and QC samples as per the validation parameter. Standard samples were analyzed at the beginning of the run and QC samples were distributed consistently throughout the validation runs. Selectivity of the method toward endogenous and exogenous components of plasma was evaluated in 6 different human plasma lots. The blank plasma lots were extracted (without addition of ISTD) and injected for LC–MS/MS detection. Later selectivity in each lot was evaluated by comparing the blank peak responses against the mean peak response observed in plasma spiked LLOQ sample (n = 6). Linearity of the method was assessed using three calibration curves analyzed on three different days. Each plot was associated with an eight point non-zero concentration spread over the dynamic range. A linear least squares regression analysis with reciprocate of drug concentration as weighing factor (1/X2) was performed on peak area ratios versus analyte concentrations. Peak area ratios for plasma spiked calibration standards were proportional to the concentration of analytes over the established range. Intra batch (within day) and inter batch (between day) precision and accuracy was evaluated at five distinct concentrations (LLOQ, LQC, MQC, HQC and ULOQ). Precision and accuracy at each concentration level was evaluated in terms of %CV and relative error respectively. The extraction recovery of Baclofen was determined at LQC, MQC-I,MQC and HQC levels. The relative recoveries were evaluated by comparing the peak areas of extracted samples (spiked before extraction) with that of un-extracted samples (blank extracts spiked after extraction). The matrix effect was checked at low and high QC level using six different blank plasma lots (including one hemolytic and one lipemic lot). Matrix factor for analyte and internal standard was calculated in each lot by comparing the peak responses of post extraction samples (blank extracts spiked after extraction) against the peak responses of equivalent aqueous samples prepared in mobile phase. Internal standard normalized matrix factor in each lot was later evaluated by comparing the matrix factor of analyte and internal standard. Stability of analyte in both aqueous solution and in biological matrix were evaluated after subjecting to different conditions and temperatures that could be encountered during regular analysis. Stability in plasma was evaluated in terms of freeze–thaw stability, bench top stability, long-term stability, and extracted sample stability. Freeze–thaw stability was evaluated after seven freeze (at -70ºC) thaw (at room temperature) cycles. Bench top stability was assessed at room temperature and the long-term stability was evaluated at both -20º C and -70º C. Stability of extracted samples was determined after reconstitution (in-injector stability at 5º C). Stability in whole blood was evaluated at room temperature. All the stability assessments were made at LQC and HQC level by comparing the stability samples against freshly prepared samples. Stability of analytes in stock solutions and in working solutions was assessed at room temperature (short-term stability) and at 2-8ºC (long-term stability). All comparisons were made against freshly prepared stock solutions or working solutions. Before each analytical run, system suitability was evaluated by injecting six replicates of MQC sample to check the system precision and chromatography. System suitability was considered acceptable when the coefficient of variation for response ratios was less than 5.0%.

3. Results and Discussion

3.1. Method development

For consistent and reliable estimation of analytes it was necessary to give equal importance for optimization of extraction procedure along with chromatographic and mass spectrometric conditions. Analyte and ISTD were tuned in positive polarity mode using electrospray ionization technique. The Q1 and the MSMS scans were made in infusion mode and further compound and gas parameters were optimized in flow injection analysis. The [M+H]+ peaks were observed at m/z of 214.1 and 218.1 for Baclofen and Baclofen D4 respectively. Most abundant product ions were found at m/z of 115.1 and 119.2 for both Baclofen and Baclofen D4 (Fig. 2 and 3) by applying sufficient collision activated dissociation gas and collision energy. Increase in source temperature beyond 450ºC augmented the intensity. A 5% change in ionspray voltage and gas parameters did not affect the signal intensity. In the optimization of chromatographic conditions, isocratic mode was selected as no cross talk was observed between analytes and ISTD. To facilitate protonation formic acid was added to the ammonium formate buffer. Use of acetonitrile over methanol in the mobile phase has shown significant improvement in the signal intensities. Replacement of milli-Q water with 2 mM ammonium formate buffer in mobile phase and addition of 0.02% Formic acid gave good chromatographic peak shapes and further increase in the buffer concentration resulted in loss of response. A flow rate of 1.0 mL/min was used to minimize the run time. In the selection of extraction method, protein precipitation and liquid–liquid extraction techniques were deliberately avoided to reduce base line noise and to get clean samples. Solid phase extraction initiated with individual HLB cartridges. Later the method was adapted in 96 well plate format. Impact of different solutions and their concentration on recovery of analytes was monitored and the final optimized conditions are depicted in Table 2. During the optimization of chromatographic conditions and extraction procedure, more emphasis was given to improve the sensitivity and recovery. No significant matrix effects were observed with the proposed chromatographic and extraction conditions.

3.2. Selectivity

Selectivity of the method in human K2 EDTA plasma was evaluated in six individual matrix lots along with one lipemic and one hemolytic lot. Peak responses in blank lots were compared against the response of spiked LLOQ and negligible interference was observed at the retention time of analytes and ISTD. Fig. 2–4 demonstrate the selectivity of the method with the chromatograms of blank plasma without ISTD, blank plasma with ISTD and LLOQ sample respectively.

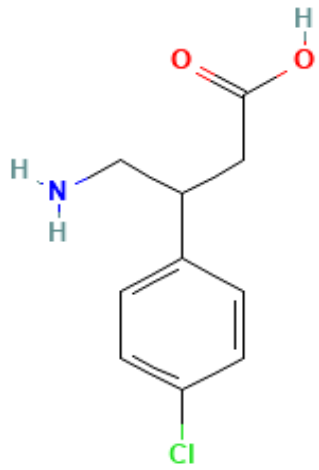

Fig 1: Structure of Baclofen

Table 1(a): Optimized Mass Spectrometry Compound parameters

Parameters	Q1 (amu)	Q3 (amu)	DP (volts)	EP (volts)	CXP (volts)	CE (volts)	Dwell Time (msec)
Baclofen	214.1	115.1	55	12	11	60	150
Baclofen D4	218.1	119.2	55	12	11	60	150

Table 1(b): Optimized Mass Spectrometry Gas and Source parameters

Parameters	Curtain gas (psi)	GAS1 (psi)	GAS2 (psi)	Ionisation voltage (Volts)	Collision associated dissociation gas (psi)	Temperature (°C)
Source or Gas	40	42	50	5000	10	500
Mode of Ionization	Positive					
Resolution	Q1 Unit and Q3 Unit					

Table 2: Optimized Chromatography parameters

Parameter	Condition
HPLC System	Agilent 1200(Make: Agilent, USA)
HPLC Mobile phase	2mM Ammonium formate with 0.02% v/v formic acid: Acetonitrile (85:15 v/v)
Column	BDS Hypersil C8 5µm, 100 X 4.6mm
Flow rate	1.0 ml/min.
Injection volume in µL	10 µL
Column oven temperature in °C	35 ± 2 °C
Autosampler temperature in °C	5 ± 2 °C
Retention time in min	Baclofen: 1.96 min, Baclofen-D4: 1.98 min. (ISTD)
Run time in min	3.2 min.

Table 3: Results of Baclofen Calibration Curve Standards

Analyte	Nominal conc.(ng/mL)	Mean conc. (ng/mL)	%CV	% Nominal
Baclofen	5.023	5.295	9.5	105.4
	10.056	11.087	2.8	110.2
	20.112	20.665	5.8	102.7
	50.281	51.864	5.0	103.1
	100.562	104.257	4.4	103.7
	205.696	209.863	2.7	102.0
	457.102	439.343	3.8	96.1
	891.349	891.919	1.5	100.1
	1017.052	1012.392	1.1	99.5
%CV = percent coefficient of variation				

Table 4: Intra and Inter Batch Precession and Accuracy

QC level	Nominal conc. (ng/mL)	Intra Batch [1]			Inter Batch [2]		
		Mean Conc. observed (ng/mL)	% CV	% Nominal	Mean Conc. observed (ng/mL)	% CV	% Nominal
LLOQQC	5.028	5.105	3.1	101.5	5.125	2.8	102.1
LQC	15.084	15.110	1.1	100.2	15.124	1.5	100.3
MQC-I	50.218	50.875	3.0	101.3	49.995	2.9	99.6
MQC-II	514.240	514.930	1.9	100.1	512.874	2.1	99.7
HQC	857.066	848.055	1.2	98.9	849.927	1.3	99.2
%CV is percent coefficient of variation;							
1 is mean of 6 replicates at each QC concentration							
2 is mean of 18 replicates at each QC concentration							

Table 5: Matrix Effect on Baclofen

Lot #	LQC			HQC		
	MF of Analyte	MF of ISTD	ISTD Normalized Factor	MF of Analyte	MF of ISTD	ISTD Normalized Factor
1	0.98	0.92	1.065	0.859	0.887	0.968
2	0.886	0.908	0.976	0.905	0.873	1.037
3	0.814	0.993	0.820	0.889	0.928	0.958
4	0.967	0.953	1.015	0.968	0.949	1.020
5	0.818	0.946	0.865	0.851	0.879	0.968
6	0.784	0.942	0.832	0.947	0.896	1.057
Mean	-		0.9287	-		1.0014
SD			0.10			0.04
% CV			11.1			4.2
N			6			6
MF: is Matrix Factor						

Table 6: Recovery

Analyte		A	B	% Recovery	Mean Recovery	% CV
Baclofen	LQC	47439.3	72763.8	65.2	63.3	4.4
	MQC-I	141539.8	218848.0	64.7		
	MQC-II	1066775.0	1720609.8	62.0		
	HQC	1601652.2	2614992.4	61.2		
Baclofen d6		255282.4	393548.3	64.9	-	-
A is the mean peak analyte response of Extracted Samples						
B is the mean peak analyte response of un Extracted Samples(aqueous)						

Table 7: Dilution Integrity on Baclofen

Dilution Factor [1]	% Nominal	% CV
1/5	102.2	7.1
1/10	104.7	4.1
1/20	100.8	8.1
1: result is calculated from six replicates at each dilution factor		

Table 8: Stability results

Stability	QC Level	A	% CV	B	% CV	% Change
BT Stability at RT (8 Hrs.)	LQC	14.311	4.7	15.754	3.6	-9.2
	HQC	777.289	3.6	806.398	5.9	-3.6
FT Stability (after 6 FT cycle)	LQC	14.492	3.7	15.106	4.2	-4.1
	HQC	789.678	1.2	798.568	3.6	-1.1
ASS for (57 Hrs.)	LQC	15.266	3.2	15.754	3.6	-3.1
	HQC	831.276	2.4	806.398	5.9	3.1
LTMS for 25 days (≤ -20°C)	LQC	14.228	5.1	15.110	1.1	-5.8
	HQC	789.664	6.2	848.055	1.2	-6.9
LTMS for 25 days (≤ -50°C)	LQC	14.780	2.8	15.110	1.1	-2.2
	HQC	806.041	6.5	848.055	1.2	-5.0
BT: Bench Top Stability ASS: Auto sampler stability LTMS: Long term stability in matrix						
FT: Freeze Thaw stability						
RT: Room temperature						

3.3. Linearity and sensitivity

The linearity of each calibration curve was determined by plotting the peak area ratio (y) of analytes to ISTD versus the nominal concentration (x) of analyte. Calibration curves were linear from 5.023 to 1017.052 ng/mL with r values more than 0.9992. The r values, slopes and intercepts were calculated from three intra and inter day calibration curves using weighted (1/X2) linear regression analysis. The observed mean back calculated concentrations with accuracy (% Nominal) and precision (%CV) are presented in Table 3. The lower limit of quantitation (LLOQ) for determination of analytes was found to be 5.023 ng/mL. At LLOQ (n = 6) accuracy (% Nominal) was 101.5% with a %CV of 3.1%.

3.4. Precision and accuracy

Precision and accuracy was evaluated using three intra and inter day precision and accuracy runs, with each batch consisting of six replicates of quality control samples at four concentration levels (LLOQ, LQC, MQC and HQC). The intra batch precision was between 1.1 to 3.1 % with % Nominal between 98.9 to 100.4. The inter batch precision was between 2.9 to 5.3 % with % Nominal between 95.2 to 101.5 Results of precision and accuracy are presented in Table 4.

3.5. Matrix effect

Co-eluting matrix components can suppress or enhance the ionization but might not result in a detectable response in matrix blanks due to selectivity of the MS detection, however they can affect the precision and accuracy of the assay. Therefore, the potential for variable matrix related ion suppression was evaluated in six independent sources (containing one hemolytic and one lipemic lot) of human plasma, by calculating the IS normalized matrix factor. The mean IS normalized matrix factor ranged between 0.9288 and 1.0012 with a %CV of 4.2 to 11.2 as shown in Table 5.

3.6. Extraction recovery and dilution integrity

The extraction recovery of analytes from EDTA plasma was determined by comparing the peak responses of plasma samples (n = 6) spiked before extraction with that of plasma samples spiked after extraction. The recovery was found to be 65.2%, 64.79%,62.0% and 61.2% at LQC, MQC_I, MQC-II and HQC levels respectively. The mean recovery was found to be 63.3% with %CV of 4.9%, as shown in Table 6. For Internal standard the recovery was found to be 64.9%. The Dilution integrity experiment was carried out at 3 times the ULOQ concentration. After 1/5, 1/10 and 1/20 dilution the mean back calculated concentration for dilution QC samples was within 85–115% of nominal value with a %CV of ≤8.1 as shown in Table 7.

3.7. Stability

Stability evaluations were performed in both aqueous and matrix-based samples. The stock solutions were stable for a period of 8 h at room temperature and for 16 days at 2-8ºC. Stock dilutions in 50% methanol were stable up to 8 h at room temperature. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). The analyte was stable up to 6 h on bench top at room temperature and over 6 freeze-thaw cycles. The processed samples were stable up to 57 h in autosampler at 5ºC. Reinjection reproducibility is done for 60 h. The long-term matrix stability was evaluated at both -20ºC and -70ºC over a period of 25 days. No significant degradation of analytes was observed over the stability duration and

conditions. The stability results presented in **Table 8** were within 85-115%.

4. Conclusions

A rapid, sensitive, high throughput and accurate liquid chromatography with electrospray ionization tandem mass spectrometry method was developed for determination of Baclofen in human plasma with short chromatographic run time of 3.2 min. The method offers high selectivity with a LOQ of 5.023 ng/mL. The extraction method utilizes a low sample volume of 100µL and shown consistent and reproducible recoveries for analyte and ISTD with minimum plasma interference and matrix effect. The validated method can be successfully used to a clinical and tox studies. Use of Baclofen d4 as an ISTD will not compromise the accuracy of analytical results as this is the deuterated compound of analyte. The high throughput method can reduce overall processing time and allowing to process and analyse more than 80 samples in single time.

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Conflict of interest

The authors declare that there is no conflict of interest.

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