

Simultaneous Quantitative Determination of Bupropion and its Metabolites by High Performance Liquid Chromatography Tandem Mass Spectrometry Detection: Application to Bioequivalence Study

Pradeep Kumar Shahi^{1,2*}, Haresh Patel¹, Vishal Shah¹, Ashutosh Bhokari¹, Rajamannar Thennati¹, Rakshit Ameta²

¹Pharmacokinetics Department, Sun Pharmaceutical Industries Limited, Tandalja, Vadodara, Gujarat, INDIA.

²Department of Chemistry, Pacific University, Udaipur, Rajasthan, INDIA.

ABSTRACT

Objective: A rapid, selective, sensitive, precise and accurate liquid chromatography in tandem with electro-spray ionization mass spectrometry method has been developed and validated for the simultaneous quantification of bupropion (BPR), hydroxyl bupropion (HBPR), erythrohydrobupropion (EHBPR) and threo hydrobupropion (THBPR) in human plasma using only 100 μ L of human plasma sample. **Methodology:** Multi reaction monitoring detection was performed by electrospray ionization in the positive ion mode, conferring an additional selectivity to the method. The solid phase extraction technique was used for sample preparation. Chromatographic separation of drug and metabolites with better peak shape and resolution was achieved by using an Acquity BEH phenyl column with an isocratic elution of 42 % methanol and 58 % ammonia (0.06%, v/v) aqueous solution at a flow rate of 0.5 ml/min. Methanol was chosen because it enabled good resolution between THBPR and EHBPR as well as good peak symmetry of all the four analytes. Detection was carried out by mass spectrometry using positive electro-spray ionization mode, and the compounds were monitored using multiple reactions monitoring method. Deuterium-labeled isotopes of the compounds were used as internal standards. **Results and Conclusion:** No significant matrix effect was observed in the presented method. The assay method was validated over the concentration range of 1.75-500 ng/ml for BPR; 5-1000 ng/ml for HBPR; 0.5-100 ng/ml for EHBPR; and 2-500 ng/ml for THBPR as per FDA guideline and validated method was successfully applied for estimation of drug and metabolite concentration in the healthy adult volunteers, bioequivalence and pharmacokinetic study of Bupropion hydrochloride 300 mg extended release tablets under fasting condition.

Key words: Simultaneous determination, Liquid chromatography, Mass spectrometry, Bupropion, Metabolites, Plasma, Bioequivalence.

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Correspondence:

Mr. Pradeep Kumar Shahi,
Pharmacokinetics Department,
Sun Pharmaceutical Industries Limited, Tandalja,
Vadodara, Gujarat, INDIA.
Phone: 91-91-9924314799
E-mail: pradeep.shahi@
sunpharma.com

INTRODUCTION

Bupropion hydrochloride is an antidepressant of the aminoketone class, is chemically unrelated to tricyclic, tetracyclic, selective serotonin re-uptake inhibitor. It is designated as (\pm)-1-(3-chlorophenyl)-2-[(1, 1-dimethylethyl) amino]-1- 12 propanone hydrochloride. The molecular weight is 276.2. It

is white, crystalline, and highly soluble in water. It is available in 75 mg and 100 mg IR tablets; and 100, 150, 200 and 300 mg in ER tablets. It appears likely that only a small proportion of any orally administered dose reaches the systemic circulation intact as absolute bioavailability is unknown. Plasma



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bupropion concentrations are dose-proportional following single doses of 100 to 250 mg. Bupropion is extensively metabolized in humans. Three metabolites have been shown to be active: hydroxybupropion, which is formed via hydroxylation of the tert-butyl group of bupropion, and the amino-alcohol isomers threohydrobupropion and erythrohydrobupropion, which are formed via reduction of the carbonyl group. It is indicated for the treatment of depression. Reported C_{max} based on Bupropion hydrochloride 100 mg tablets for BPR, HBPR and THBPR are 136.2 ng/ml, 269.0 ng/ml and 88.8 ng/ml, respectively.

Literature reveals that HPLC methods for plasma,¹⁻⁴ stability method for plasma,⁵ metabolite characterization,⁶⁻⁷ LC-MS methods for human plasma⁸⁻¹³ and rat plasma,¹⁴ pharmacokinetics analysis in human or rat plasma¹⁵⁻²⁴ for bupropion and/its metabolite(s) and LC-MS methods for bupropion with combination²⁵⁻²⁷ are reported. It is noted that reported HPLC methods are not sensitive for the quantification in terminal plasma concentration of bupropion and/or metabolites. The reported methods^{13,15,19,21,22,24} are used for quantification of Bupropion only. While the reported methods^{4,11,14,17,18} are used for quantitation of Bupropion and hydroxyl bupropion from plasma samples and hence are not in accordance to Office of Generic Drug guidance for Bupropion BE study requirement. However, reported LC-MS methods,⁸⁻⁹ are used for estimation of bupropion with other three metabolites for plasma, neither used isotope labelled standards as internal standards nor performed incurred sample reanalysis which are current European regulatory requirement. Addition to this, plasma samples were prepared by precipitation method which could lead to the matrix effect, ion suppression and enhancement of analyte signal. The method reported by Xiaoming Wang *et al.*¹⁰ were used isotope labelled standards as internal standards for estimation of analyte along with 3 other metabolites. However, biological matrix is differing from the normal plasma i.e. umbilical cord plasma and tissue.

Therefore, the purpose of this present study was to mitigate above challenges and to: (a) develop and validate a novel, a selective and sensitive LC-MS/MS assay method that allows the simultaneous quantification of bupropion and all three metabolites with better chromatography separation using solid phase extraction technique and approximately 90% recovery. This method is validated with at the LLOQ of about 1.75 ng/mL for Bupropion, 5.0 ng/mL for Hydroxy Bupropion, 0.5 ng/mL for Erythro Hydro Bupropion and 2.0 ng/mL for Threo Hydro Bupropion (b) applied for pharmacokinetics study for estimation of bupropion and its metabolites

in healthy volunteers administered a single 300 mg oral dose of bupropion tablets and (c) high throughput analysis with low plasma volume, low cost and faster delivery of results. Also reproducibility of method is demonstrated during the samples analysis of healthy volunteer bioequivalence study and through incurred sample analysis data.

MATERIALS AND METHODS

BPR was procured from in-house; HBPR, EHBPR, THBPR, BPR D₉ and HBPR D₆ were procured from Clearsynth Labs Limited, Mumbai, India. LC-MS grade methanol from JT Baker (Centre Valley, PA, USA); LCMS grade Ammonia (25%, v/v) from Merck (Worli, Mumbai, India); LC-MS grade formic acid from Fluka (Germany) and water was produced from Milli-Q water purification system (Millipore Merck, USA) were used. Blank plasma was collected into K₂EDTA tubes from drug-free healthy volunteers, clinical pharmacology unit, Sun Pharmaceutical Industries Limited; Independence Ethics Committee approved these processes.

LC-MS System and Conditions

Chromatographic separation of drug and metabolites with better peak shape and resolution was achieved by using an Acquity BEH phenyl column with an isocratic elution of 42 % methanol and 58 % ammonia (0.06%, v/v) aqueous solution at a flow rate of 0.5 ml/min. Separation and detection was done with a Dionex UHPLC (Thermo Scientific, Germany) integrated to API-5500 triple quadrupole mass spectrometer (A B Sciex, Canada). An electrospray ionization technique was used for better sensitivity for all the analytes. The 21 CFR part 11 approved software, analyst version 1.5.1 (A B Sciex, Canada) was used for method setup, data acquisition, and data processing and reporting. Separation of all the analytes was achieved within 6 min run time. Injection volume of only 1 µl was used to achieve better sensitivity with nil matrix effect and the needle was washed with 100 µl of water: methanol (50:50, v/v) between injections to avoid any carry over. The auto-sampler and column oven temperature was optimized at 6 ± 2°C and 45 ± 2°C, respectively.

The MS was operated in positive ionization mode, to achieve the desired area response over the dynamic range. The electrospray voltage was set at 5 kV, the source temperature at 450°C and dwell time at 0.2 sec. The GS₁ and GS₂ pressures were set of 45 and 60 arbitrary units, respectively. Nitrogen gas was used as the curtain gas which was set at 45 arbitrary units and collision gas was set at 8 arbitrary units. The molecular ion was

Table 1: Mass spectrometry specification for Bupropion and its metabolites with its internal standards.

Analyte name#	Q ₁ mass	Q ₃ mass	DP	EP	CE	CXP
BPR	240.3	184.1	60	10	16	12
HBPR	256.3	130.0	54	10	61	17
EHBPR	242.4	168.1	66	10	23	12
		186.2	66	10	15	14
THBPR	242.4	168.1	65	10	23	12
BPRD ₉	249.3	185.1	62	10	17	13
HBPRD ₆	262.3	244.3	51	10	17	17

#BPR for Bupropion, HBPR for hydroxyl bupropion, EHBPR for Erythrohydrobupropion, THBPR for Threohydrobupropion, D: deuterium standard used as internal standard.

characterized and selected from Q₁ spectra and further fragmented in to Q₂ mode and monitored by Q₃ mode. The most sensitive mass transitions (m/z) were monitored in multiple reaction monitoring. Mass spectrometry specification for bupropion and its metabolites with its internal standards are provided in Table 1.

Stock Solutions, Calibration Standards and Quality Controls (QC)

Stock solutions of all analytes and IS were prepared from their respective reference standards in methanol to achieve the concentration of stock solution of 1mg/ml for BPR, EHBPR, THBPR; 2 mg/ml for HBPR; 0.1 mg/ml for BPRD₉ and HBPRD₆. All stock solutions of analyte and IS were stored at 2-8°C.

Calibration Standards and QC Samples Preparations

The working solutions of calibration standards and quality control samples for all analytes were prepared in human K₂EDTA plasma which was free of significant interference. The BPRD₉ was used as an internal standard for BPR, EHBPR and THBPR however HBPRD₆ was used as an internal standard for HBPR.

Sample Pre-Treatment

The 5 µL of working CC/QC solution of analytes were spiked in 95µL of human blank K₂EDTA plasma which were free of significant interference at the retention time (RT) for the transition of analytes and the IS (Q₁/Q₃). The 25 µL of WIS solution (100 ng/ml of each of BPR D₉ and HBPR D₆) were added and 200 µL of 1% v/v of formic acid in water solution were added and followed by solid phase extraction (SPE) sample processing method using HLB (30mg/1cc) which were pre-equilibrated with methanol and water. The cartridges were washed with water followed by elution with 250 µL

Table 2: Linearity range with different QC levels (in ng/ml) for Bupropion and its metabolites.

Levels	Analyte Name#			
	BPR	HBPR	EHBPR	THBPR
CC range	1.75-500.69	4.92-983.13	0.501-100.25	2-500.5
LLOQ	1.75	4.92	0.501	2.00
LQC A	5.25	13.46	1.441	5.75
LQC B	15.74	40.37	4.323	17.25
MQC A	124.96	249.17	26.378	125.00
MQC B	227.42	438.53	44.486	225.00
HQC	387.36	772.42	78.32	387.50
ULOQ	500.69	983.13	100.25	500.5

refer Table 1 for abbreviation of analyte name.

of methanol; two times. The samples were transferred into fresh glass HPLC vial for analysis.

Calibration Curves, Accuracy and Precision and Limit of Quantitation

A calibration curve consisting of a blank (without drug and without IS), zero blank (without drug and with IS), nine standards in the range and QCs (n=7 separate extractions for each level) stated in Table 2, were prepared and analyzed in a single analytical run. Calibration curves were constructed using a linear regression equation of analyte/IS peak area ratios versus nominal concentrations with a 1/ concentration x concentration weighting. Accuracy was defined as a percentage deviation of measured concentration from the nominal value and precision was defined as the percentage coefficient of variation (%CV). Not less than 75% of all standards and 67% of all QCs (50% at each level) in any batch were required to have a percentage deviation within ± 15% except LLOQ where a percentage deviation within ± 20%.

To determine the lower limit of quantitation and upper limit of quantitation 6 replicates of CS₁ and CS₉ were analyzed against calibration curve of precision and accuracy (P and A) batch in same analytical run.

Dilution Integrity

To investigate dilution integrity for clinical samples with concentrations above the reference range, The DQC samples were prepared from 1.5 -3 times of ULOQ concentration by 5 times diluting using interference free blank plasma and these samples were processed and analysed in a single run along with freshly processed calibration standards (CS) and two sets of QC samples. The final concentrations were then derived by back-calculating with the appropriate dilution factor.

Selectivity, Matrix Effect, Recovery and Carry Over

Human K₂EDTA plasma from 6 different sources (4 normal + 1 hemolysed + 1 lipemic) along with LLOQ sample prepared in each lot was analyzed, to determine the interference of an endogenous substance at the RT of all analytes.

To investigate matrix effect, the aqueous samples of LQC-A and HQC samples was prepared by adding 5 μ L of respective working solution and 25 μ L WIS in 470 μ L of methanol, mixed well and transferred to HPLC vials for analysis. The blank human K₂EDTA plasma (4 normal, 1 lipemic and 1 hemolysed) were processed up to elution step as per analytical test procedure. Six vials of each extracted LQC-A and HQC were prepared by adding 5 μ L of respective working solution, 25 μ L of WIS in 470 μ L of processed blank. All these samples were transferred to HPLC vials for analysis.

Relative recovery of all analytes were evaluated by comparing mean analyte responses of six extracted QC samples of LQC-A, MQC-B and HQC level to the six unextracted QC samples at same level. For the IS, mean IS response of eighteen extracted samples was compared to that of the eighteen un-extracted QC samples. Similarly, absolute recovery of all analytes and IS were evaluated by comparing extracted sample to post extracted QC samples at same level. A %CV of $\leq 15\%$ across all QC concentrations was set as the level of acceptance for both matrix effect and recovery in line with the FDA guideline.

Carryover test was performed in the sequence of extracted sample of blank (PB), LLOQ, ULOQ, PB, ULOQ, PB, Un-Diluted Quality Control, PB, DQC, PB. The interference in plasma blank was evaluated against LLOQ as reference sample. The carry-over was insignificant if any blank shows more than 20% response compare to analyte RT and/or more than 5% response compare to IS RT.

Extended Accuracy and Precision, Ruggedness, and Robustness

To evaluate accuracy and precision over extended period to cover actual study sample analysis duration, a CS plus a total of 120 spiked QC samples (24 replicates x 5 QC levels) were processed and analyzed in a single analytical run and evaluated as per P and A criteria.

The P and A experiment was performed with probable changes during study sample analysis like different column with same specification and different analyst. The run consisted of a CS plus a total of 30 spiked QC samples (6 replicates x 5 QC levels) and evaluated as per P and A criteria.

Robustness QCs with change in buffer volume (i.e. 250 μ L of 1%v/v formic acid changed from nominal volume of 200 μ L) comparison QC sets of 6 samples each at LQC-A and HQC level, CS were processed and analysed in a single analytical run. Comparison QCs (i.e. column oven temperature changed from nominal temperature of 45°C to 47°C and auto sampler oven temperature changed from nominal temperature of 6°C to 8°C) were re-injected as robustness QCs with column oven and auto-sampler temperature change.

Stability and Re-Injection Reproducibility

All the matrix stability were performed using the bulk spiked samples which were pre-checked (immediately after preparation) for accuracy. Six replicates of bulk spiked LQC-A and HQC samples were subjected for different stability conditions to mimic the study sample analysis condition. These stability samples were processed and analyzed in a single run along with freshly processed calibration standards (CS) and two sets of QC samples. The short term and long term analyte and IS stock as well as working solution for both LLOQ and ULOQ level were evaluated for required storage conditions and durations against freshly prepared stock samples and analyzed in a single run by six replicates injection at each level.

To evaluate the reinjection reproducibility experiment, quality control samples of one P and A batch were kept into auto sampler after analysis at $6 \pm 2^\circ\text{C}$ and reinjected after 8 h and evaluated against the acceptance limits of accuracy ($\pm 15\%$ of their respective nominal concentration) and precision (%CV ≤ 15).

Application of Method

The validated method has been employed for estimation of BPR, HBPR, EHBPR and THBPR concentrations in human volunteer's bioequivalence study under fasting condition, after administration of a single dose ER tablet containing 300 mg bupropion HCl. The study was conducted according to ethic committee and current GCP guideline. There were a total of 27 blood collection time points including the pre-dose samples at 0.0 and post dose samples at 2, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10, 11, 12, 16, 24, 36, 48, 72, 96, 120, 144 and 168 h time intervals in separate vacationers containing K₂EDTA as an anticoagulant. The plasma from these samples was separated by centrifugation at 3300 rpm at $4 \pm 2^\circ\text{C}$ for 15 m within 1.5 h after blood sampling collection. These obtained plasma samples were stored at $-20 \pm 5^\circ\text{C}$ or colder condition until analysis. The pharmacokinetic parameters were computed using Win-Nonlin® (Pharsight Corporation, version 5.3) using

non compartmental analyses and 90% confidence interval was computed using SAS software (SAS® Institute Inc., USA and version 9.2)

RESULTS

LC-MS Specification

During the method development, mass parameters, chromatography conditions, mobile phase compositions, extraction conditions were optimized through several trials to achieve better signal to noise level at lower limit of quantitation for all the analytes.

The electrospray ionization (ESI) detection technique has been provided a maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain better sensitivity and signal stability in positive polarity. Maximum response was obtained in positive ion mode as compared to the negative ion mode. The parent ion peaks and product ions in the ESI mode of all analytes with IS were optimized to get maximum sensitivity. The isotopes labeled internal standards were used to compensate loss during sample preparation and avoid the matrix effect during the analysis. The optimized mass transitions and compound dependent parameters of all analytes along with IS were provided in Table 1. The scan width was set at 0.01 m/z and the dwell time at 0.2 sec.

Chromatography Optimization

Initially, a different composition of mobile phase with varying combinations was tried with aim to develop the method with better chromatography resolution, better signal and peak shape. Maximum response was achieved with mobile phase containing methanol and ammonia (0.06%, v/v) solution in water. Isocratic mode with different flow rate and composition were tried. The best signal along with a marked improvement in the peak shape was observed for all the analytes using a methanol and the buffer with proportion of 42:58 %, v/v as mobile phase and flow rate of 0.5 ml/minute (m). Different column i.e. Acquity BEH C18 (100 x 2.1 mm, 1.7 μ and 150 x 2.1 mm, 1.7 μ), Acquity BEH C8 (100 x 2.1 mm, 1.7 μ), Acquity BEH phenyl (100 x 2.1 mm, 1.7 μ), Hypurity C18 (100 x 2.1 mm, 3 μ) and Zorbax XDB C18 (150 x 4.6mm, 3.5 μ) were evaluated during the method development but better signal and resolution was achieved with short length column of Acquity BEH phenyl (100 x 2.1 mm, 1.7 μ). All analytes were eluted within 6.5 m. Also utilization of stable isotope-labeled or suitable analog drugs as an IS was helpful to attain better accuracy and precision over the dynamic range.

Sample Pre-Treatment Optimization

Start up with different extraction procedures like protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE) were tried to obtain better recovery and low matrix effect but ion suppression effect was encountered with protein precipitation method for both the analyte and IS. Hence further method was optimized with SPE and LLE technique and finally concluded that SPE technique was more suitable for extraction of the drug and IS with better recovery and low matrix effect.

Auto sampler wash solution was optimized from 50% methanol to avoid any carry over effect. The sample volume was selected as 100 μ L to attain higher level sensitivity for intended application. These optimized detection parameters, chromatographic conditions and extraction procedure resulted in accurate and precise detection of all analytes in human plasma.

Representative chromatograms are shown in Figure 1, with a RT of 3.08 m for BPR, 1.65 m for HBPR, 4.23 m for EHBPR, 4.75 m for THBPR, 3.01 m for BPR D₆, and 1.65 m for HBPR D₆.

Calibration Curves, Accuracy and Precision and Limit of Quantitation

The method was linear with weighing factor (1/x²) in the range of 1.75-500.69 ng/ml for BPR; 4.92-983.13

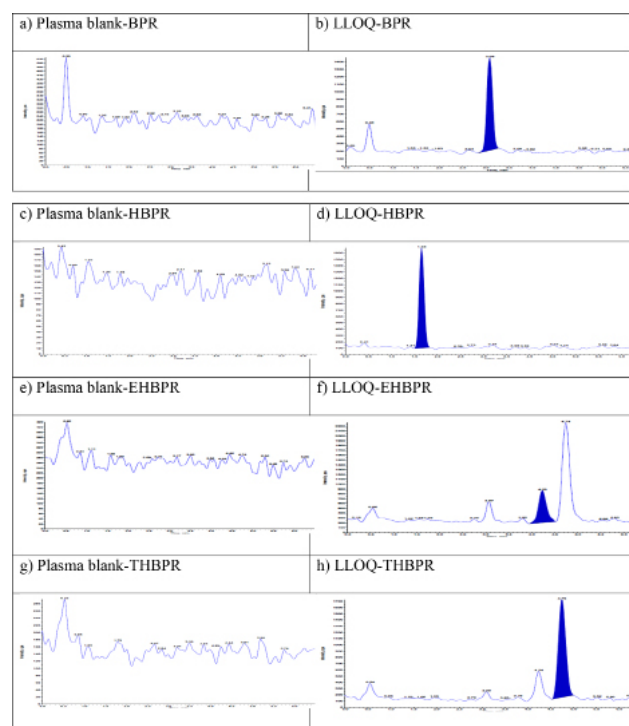


Figure 1: Chromatograms of blank plasma and LLOQ samples of Bupropion and its metabolites.

Table 3: Intraday and Interday accuracy and precision of Bupropion and its metabolites.

Mean (Precision in %CV) [%Accuracy]				
QC levels (ng/ml)	BPR	HBPR	EHBPR	THBPR
Intraday accuracy and precision				
LLOQ	1.51(6.6)[86.5]	4.47(6.1)[90.8]	0.47(7.3)[93.9]	1.69(5.7)[84.3]
LQC-A	4.92(5.8)[93.7]	12.79(6.1)[95]	1.46(6.9)[101.4]	5.55(8.2)[96.4]
LQC-B	17.2(5)[109.3]	37.68(5.4)[93.3]	4.34(4.7)[100.5]	16.78(5.7)[97.3]
MQC-A	131.64(4.1)[105.3]	253.45(3)[101.7]	27.63(3)[104.8]	129.74(3.1)[103.8]
MQC-B	209.34(2.3)[92]	405.05(2.4)[92.4]	44.47(10.9)[100]	217.26(10.6)[96.6]
HQC	371.12(2.6)[95.8]	712.84(3)[92.3]	75.17(4.1)[96]	365.8(3.8)[94.4]
ULOQ	489.48(5.4)[97.8]	946.58(5.6)[96.3]	98.65(5.9)[98.4]	486.23(5.6)[97.1]
Interday accuracy and precision				
LLOQ	1.68(9.9)[95.7]	4.71(7.2)[95.8]	0.48(7.8)[95.9]	1.87(8.3)[93.4]
LQC-A	5.25(5.7)[99.9]	13.26(5.2)[98.5]	1.5(6.4)[104]	5.88(6.6)[102.2]
LQC-B	17.07(4.1)[108.4]	37.66(4.3)[93.3]	4.28(3.7)[99.1]	16.69(4.1)[96.8]
MQC-A	137.97(9.4)[110.4]	266.61(10.8)[107]	28.59(10.5)[108.4]	135.59(11)[108.5]
MQC-B	209.62(2.5)[92.2]	408.03(2.2)[93]	42.56(6.4)[95.7]	210.14(6.2)[93.4]
HQC	371.63(2.7)[95.9]	722.06(2.9)[93.5]	74.2(3.6)[94.7]	364.44(3.6)[94]
ULOQ	488.77(4.8)[97.6]	953.31(5.1)[97]	96.83(5.1)[96.6]	482.09(5)[96.3]

Table 4: Matrix effect and recovery for Bupropion and its metabolites.

Analyte Name	Level	%CV of IS-normalized matrix factor	Absolute recovery (%)	Relative recovery (%)
BPR	LQC-A	2.1	95.8	91.0
	MQC-B	NA	95.2	90.4
	HQC	1.3	95.5	90.0
HBPR	LQC-A	2.8	100.3	98.3
	MQC-B	NA	100.1	95.3
	HQC	1.5	99.7	94.4
EHBPR	LQC-A	3.8	97.1	96.0
	MQC-B	NA	100.6	96.6
	HQC	1.3	99.5	95.5
THBPR	LQC-A	1.6	98.1	96.1
	MQC-B	NA	100.0	96.4
	HQC	2.0	98.9	95.4

ng/ml for HBPR; 0.501-100.25 ng/ml for EHBPR; and 2-500.5 ng/ml for THBPR Intra and inter-day accuracy and precision was well within the acceptance criteria as per FDA and EMA guidelines (Table 3). The mean regression coefficient was > 0.99 for all analytical run for all analytes.

Dilution Integrity

The precision and accuracy of DQC samples were found within acceptance criteria. 67% of QCs samples were in range of 85-115% with %CV ≤15%.

Selectivity, Matrix Effect, Recovery and Carry Over

No significant interference was observed in any lots of plasma samples (normal, lipemic, hemolysed). In addition, zero standards samples were prepared in duplicate from single lot of plasma and were analyzed to determine the interference at RT of respective analyte due to respective IS. No significant interference was observed at the RT of any analytes. % CV of IS normalized matrix factor for all analytes was found within acceptance criteria and it was below 4% for both LQC-A and HQC level (Table 4).

Mean recovery (absolute and relative) values were approximately $\geq 90\%$ for all analytes and IS, respectively. The difference in %CV of recoveries (absolute and relative) across each QC level was within 15% for all analytes (Table 4). There was no significant injector carry over observed for any of the analyte and IS.

Extended Accuracy and Precision, Ruggedness, and Robustness

The mean % nominal value and %CV were in range of 85-115% and less than 15%, respectively across all QC levels for all analytes. This indicates that method was accurate and precise over extended period and to cover actual study samples analysis time. The % CV, % nominal and % change of robustness and comparison samples were within acceptance limit with predefined changes in method. The mean % nominal value and %CV were in range of 85-115% and less than 15%, respectively across all QC levels for all analytes for Robustness experiment.

Stability and Re-Injection Reproducibility

The concentrations were calculated by the slope and intercept of calibration curve. The mean concentration of plasma stability samples was evaluated in relation with the nominal values and %CV were also calculated by $\text{mean} \times 100 / \text{SD}$. %Accuracy for all analytes was found within 85-115%. Hence all analytes were considered to be stable in plasma with defined storage condition and duration. For BT stability in blood, area ratio of stability sample versus freshly prepared samples was compared and % change was found within 15% for said storage condition and duration. Similarly for stock solution stability, area response of stability sample versus freshly prepared samples was compared and % change was found within 10%. The results of matrix stability are provided in Table 5.

For re-injection reproducibility, the % change of more than 67% of reinjected QC samples was within $\pm 15\%$ of back calculated concentration of previously analyzed QC's of P and A batch after 8 h. Hence it was concluded

Table 5: Stability studies for bupropion and its metabolites.

Stability	Level	Mean (%CV) [% accuracy] , (mean concentration in ng/ml)			
		BPR	HBPR	EHBPB	THBPB
Bench-top stability plasma (RT, 6h)	LQC-A	5.16(5.2)[98.3]	13.3(3.3)[98.8]	1.45(3.4)[96.6]	5.9(2.7)[102.1]
	HQC	378.61(2.8)[97.7]	720.8(2.8)[93.3]	73.37(2.8)[94]	366.98(2.9)[94.1]
Post extraction stability (RT, 7 h)	LQC-A	5.27(4)[100.3]	13.26(3.9)[98.5]	1.58(6.6)[105.8]	6.09(4.4)[105.4]
	HQC	372.83(2.7)[96.2]	722.65(2.5)[93.6]	78.87(4.1)[101.1]	373.8(3.3)[95.9]
Auto sampler stability (6°C, 96 h)	LQC-A	4.89(4.7)[93.2]	12.98(3)[96.4]	1.52(12.2)[101.4]	6.04(10.8)[104.4]
	HQC	392.39(2.7)[101.3]	744.01(2.9)[96.3]	74.19(5.2)[95.1]	365.52(4.8)[93.8]
Freeze-thaw stability (-20°C, 4 cycles in water bath)	LQC-A	5.04(9.2)[95.9]	12.63(9.1)[93.8]	1.48(9)[98.6]	5.72(9.2)[98.9]
	HQC	388.17(2.7)[100.2]	750.32(1.9)[97.1]	79.02(2.5)[101.3]	392.35(2.6)[100.6]
Freeze-thaw stability (-35°C, 4 cycles in water bath)	LQC-A	5.24(1.9)[99.8]	13.19(2.6)[98]	1.51(1.5)[101]	5.89(4.1)[101.9]
	HQC	392.83(1.2)[101.4]	758.11(2.4)[98.1]	79.21(1.8)[101.5]	390.29(2.1)[100.1]
Freeze-thaw stability (-65°C, 4 cycles in water bath)	LQC-A	5.41(1.7)[103]	13.16(3)[97.7]	1.52(4.3)[101.8]	5.94(4.7)[102.7]
	HQC	386.43(2.2)[99.8]	747.76(2.1)[96.8]	78(2.9)[99.9]	386.23(3.4)[99.1]
Long Term plasma stability (-20°C, 65 Days)	LQC-A	5.07(5.1)[96.6]	13.67(4.2)[101.5]	1.48(2.5)[98.8]	5.92(4.2)[102.5]
	HQC	386.85(2.9)[99.9]	752.13(2.7)[97.4]	75.17(6.2)[96.3]	367.13(5.4)[94.2]
Long Term plasma stability (-35°C, 65 Days)	LQC-A	5.09(5.7)[97]	13.64(3)[101.3]	1.42(6.4)[95.1]	5.8(4.4)[100.3]
	HQC	396.39(4.9)[102.3]	772.98(4.7)[100.1]	75.56(3.8)[96.8]	371.72(4.6)[95.4]
Long Term plasma stability (-65°C, 65 Days)	LQC-A	4.97(2.3)[94.6]	13.66(6)[101.5]	1.42(6.4)[95.1]	5.75(4.1)[99.5]
	HQC	391.48(3.3)[101.1]	756.26(2.9)[97.9]	75.56(3.8)[96.8]	363.88(3.5)[93.3]

Table 6: Mean \pm SD pharmacokinetic parameters of Bupropion and its metabolites in 67 healthy volunteers after oral administration of Bupropion HCl 300 mg ER tablets test and reference products under fasting condition.

Pharmacokinetic parameters	BPR		HBPR		EHBPR		THBPR	
	Test	Reference	Test	Reference	Test	Reference	Test	Reference
AUC _{0-t} (ng.h/ml)	1621.99 \pm 562.83	1750.9 \pm 595.3	16468.18 \pm 9057.5	19071.95 \pm 9955.56	1687.2 \pm 818.29	1877.35 \pm 765.96	10228.47 \pm 6658.73	11422.39 \pm 6956.23
AUC _{0-inf} (ng.h/ml)	1691.5 \pm 578.49	1817.89 \pm 607.17	16792.65 \pm 9118.41	19464.76 \pm 10102.21	1736.78 \pm 840.6	1933.65 \pm 794.73	10674.9 \pm 6938.68	11974.32 \pm 7376.18
C _{max} (ng/ml)	136.74 \pm 48.95	166.73 \pm 59.52	392.52 \pm 187.62	457.35 \pm 198.49	33.72 \pm 13.19	36.79 \pm 12.4	228.99 \pm 112.89	249.85 \pm 114.43
T _{max} (h)	8.5 \pm 3.12	4.5 \pm 1.76	12 \pm 4.94	11 \pm 5.38	16 \pm 5.97	12 \pm 5.33	12 \pm 3.92	9 \pm 4.38
K _{el} (h ⁻¹)	17.71 \pm 6.52	17.39 \pm 6.05	18.46 \pm 3.58	18.95 \pm 3.73	25.62 \pm 5.82	25.86 \pm 6.67	34.88 \pm 7.19	35.89 \pm 8.65
t _{1/2} (h)	0.05 \pm 0.03	0.05 \pm 0.03	0.04 \pm 0.01	0.04 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01	0.02 \pm 0	0.02 \pm 0.01

Table 7: Statistical summary of Bupropion and its metabolites in 67 healthy volunteers after oral administration of Bupropion HCl 300 mg ER tablets test and reference products under fasting condition.

PK Variables	BPR		HBPR		EHBPR		THBPR	
	Ratio of LSM ¹ (%)	90% Geometric C.I. ²	Ratio of LSM ¹ (%)	90% Geometric C.I. ²	Ratio of LSM ¹ (%)	90% Geometric C.I. ²	Ratio of LSM ¹ (%)	90% Geometric C.I. ²
AUC _{0-t}	92.50	79.75-107.30	86.16	74.62-99.49	89.71	76.77-104.85	89.41	76.85-104.01
AUC _{0-inf}	92.91	80.33-107.47	86.10	75.33-98.40	89.68	77.48-103.80	89.02	76.86-103.10
C _{max}	81.92	73.35-91.49	85.66	76.29-96.18	91.50	80.88-103.52	91.54	80.59-103.97

¹Calculated using least square means according to the formula: e(LSM Treatment (A) – LSM Treatment (B)) X 100

²90% Geometric Confidence Interval using ln-transformed data.

that any reinjection can be performed during study sample analysis if there is a case of instrument failure.

The validated method has been successfully applied, to quantify analyte and metabolites concentrations in human bioequivalence study under fasting condition, after administration of Bupropion HCl 300 mg ER Tablets as an oral dose. The pharmacokinetic parameters evaluated were C_{max} (maximum observed drug concentration), AUC_{0-t} and AUC_{0-inf} (area under the plasma concentration–time curve measured t time and infinite time, using the trapezoidal rule), t_{max} (time to observe maximum drug concentration), K_{el} (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of the least square regression) and t_{1/2} (terminal half-life as determined by the quotient 0.693/K_{el}). Results of pharmacokinetics parameters of bupropion and its metabolites are provided in Table 6.

The plasma concentrations of all analyte and metabolites were used for all pharmacokinetic calculations. Any value below quantification level was considered as zero (0) prior to pharmacokinetics analysis and any subject with pre-dose concentration more than 5% of their

C_{max} was excluded from BE statistical analysis and the 90% confidence intervals was calculated based on the remaining subjects.

The Test/Reference ratios for C_{max}, AUC_{0-t}, and AUC_{0-inf} were within 80-125% for all analytes. The 90 % confidence interval of C_{max}, AUC_{0-t} and AUC_{0-inf} for BPR and HBPR are included in Table 7. The mean concentration versus time profile of bupropion and metabolites in human plasma from 67 subjects that are receiving Bupropion HCl 300 mg ER Tablets as test and reference is shown in Figure 2. The metabolites data was considered as supportive evidence for comparability of therapeutic outcome of test and reference product.

DISCUSSION

The proposed bio-analytical method for simultaneous determination of parent drug and its metabolites is sensitive, selective, precise, accurate, rugged and reproducible. This method was successfully applied in bio-equivalence study to evaluate the plasma concentrations of parent drug and metabolites in study of healthy human volunteers and study was completed without any

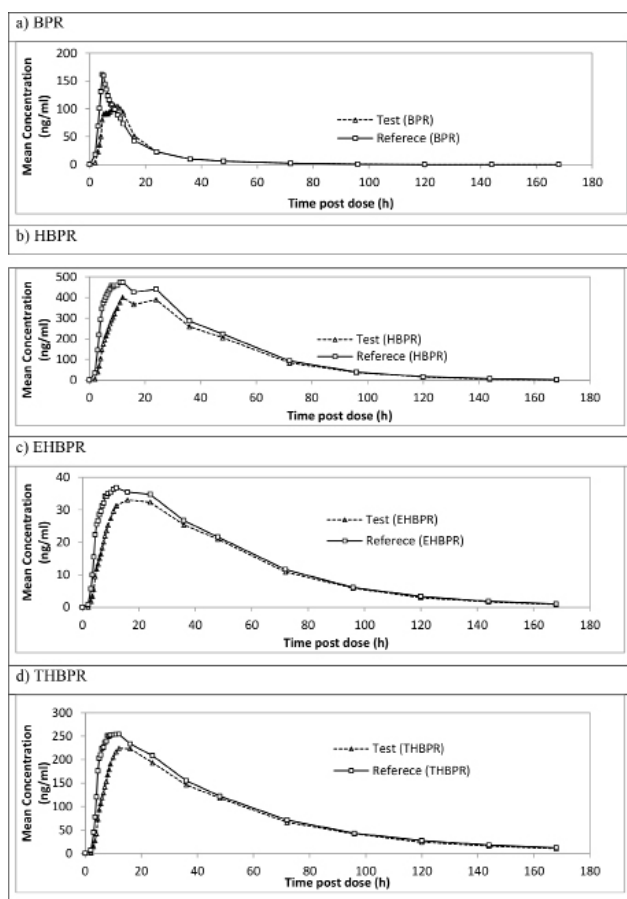


Figure 2: Mean bupropion and its metabolites plasma concentrations of test versus reference after oral administration of 300 mg bupropion HCl tablets in 67 healthy volunteers.

batch failure. Also incurred sample analysis result was within acceptance for more than 99 % of samples.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

BEH: Bridged ethoxy hybrid; **FDA:** Food and Drug Administration; **IR:** Immediate Release; **ER:** Extended Release; **C_{max}:** Maximum plasma concentration; **HPLC:** High Performance Liquid Chromatography;

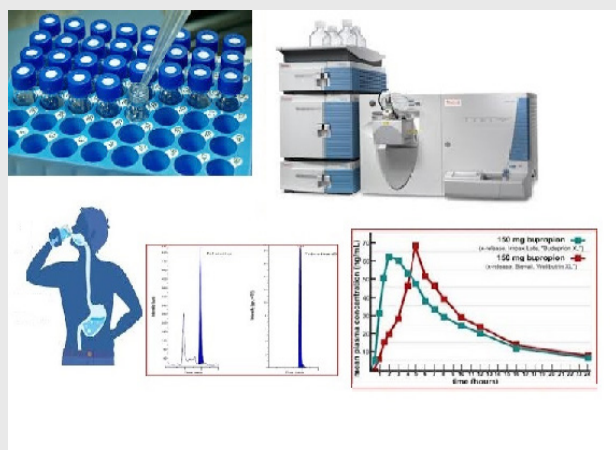
USA: United State on America; **LCMS:** Liquid Chromatography Mass Spectrometry; **K2EDTA:** Dipotassium Ethylene Diamine Tetra Acetate; **BE:** Bioequivalence; **CFR:** Code Federal Regulation; **LLOQ:** Lower Limit of Quantification; **MS:** Mass Spectrometry; **GS:** Gas; **DP:** Clustering Potential; **EP:** Entrance Potential; **CE:** Collision Energy; **CXP:** Cell Exit Potential; **QC:** Quality Control, **CS:** Calibration Standard; **WIS:** Working Internal Standard; **HLB:** Hydrophilic Lipophilic Balance; **EMA:** European Medicines Agency; **LQC:** Low QC; **MQC:** Med QC; **HQC:** High QC; **DQC:** Diluted QC; **ULOQ:** Upper Limit of Quantification; **PB:** Plasma Blank; **GCP:** Good Clinical Practice, **CV:** Co-efficient of Variation; **SD:** Standard Deviation.

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PICTORIAL ABSTRACT



SUMMARY

- The current study proved that how to develop and to validate a novel, a selective and sensitive LC-MS/MS assay method which allows the simultaneous quantification of bupropion and all three metabolites with better chromatography separation using solid phase extraction technique and required sensitivity.
- The proposed method was satisfied current challenges and regulatory requirements with respect to high throughput analysis with low plasma volume, low cost, faster delivery of results, isotope labelled internal standard, metabolite measurement, and incurred sample analysis data.
- The proposed method was applied for pharmacokinetics study for estimation of bupropion and its metabolites in healthy volunteers administered a single 300 mg oral dose of bupropion tablets.

About Authors



Pradeep Kumar Shahi has industrial experience of 17 years in Bio-analytical and Pharmacokinetics discipline. His research is based on bio-analytical method development for simultaneous determination, Validation and application of a method to bio-equivalence study.

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