ORIGINAL ARTICLE

Development and Validation of a Rapid Liquid Chromatography Tandem Mass Spectrometry Method for Simultaneous Quantification of Bupropion, Hydroxybupropion, Sertraline and Desmethylsertraline in Human Plasma

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Abstract

Objectives: Bupropion and Sertraline are frequently used together in antidepressant treatment; therefore, analytic methods with high sensitivity are needed to provide information for therapeutic drug monitoring (TDM) and drug-drug interaction (DDI) studies. The present research work describes the development and validation of a Liquid chromatography tandem mass spectrometry (LC-MS/MS) assay for simultaneous quantitation of Bupropion, Hydroxybupropion, Sertraline, and Desmethylsertraline in human plasma. **New Approach:** Plasma samples were prepared by protein precipitation with acetonitrile containing 0.1% formic acid. We used an acquity UPLC BEH C18 column with gradient elution and positive electrospray ionization in multiple reaction monitoring mode for chromatographic separation. Method validation was performed in accordance with the bioanalytical method validation guidelines published by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA). **Results:** All analytes were separated within 6 min, and verification of linearity ($r^2 > 0.999$) was obtained for each compound with a lower limit of quantitation (defined as <30% relative standard deviation ranging from 0.2 to 5 ng/mL. The lower limits of quantifications were 1 ng/mL for bupropion, 2 ng/mL for hydroxybupropion, and 0.5 ng/mL for sertraline and desmethylsertraline, respectively. The matrix effects are very low, and the carryover was negligible with over 94% recoveries. The accuracy was from 96.67% to 101.41%, and the precision was all under 5%. All the conditions studied for stability of analyte were confirmed by the two manufacturers. **Conclusion:** The validated LC-MS/MS method is specific, rapid, and sensitive, suitable for pharmacokinetic, TDM, and antidepressant DDI studies.

Key words: Bupropion, human plasma pharmacokinetics, drug–drug interaction, Liquid chromatography tandem mass spectrometry, sertraline

INTRODUCTION

Depression is a prominent health disorder with high psychological, social, and economic costs globally. Antidepressants have been widely prescribed as monotherapy or combination therapy for the treatment of patients with suboptimal response to a single agent.[1] Of the most common antidepressants, Bupropion and Sertraline are clinically relevant due to their different mechanisms of action as well as therapeutic

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efficacy.[2] Bupropion is an atypical antidepressant that inhibits norepinephrine and dopamine reuptake, while Sertraline modulates serotonergic neurotransmission as a selective serotonin reuptake inhibitor. These medications are often used in combination for treatment-refractory depression and augmentation therapy. Bupropion undergoes extensive hepatic metabolism through cytochrome P450 2B6 (CYP2B6) to hydroxybupropion, an active metabolite that accounts for much of bupropion's pharmacological activity and is also a CYP2D6 inhibitor.[3] Sertraline is primarily metabolized in the liver by CYP2C19, CYP2B6, and CYP2D6 into its active metabolite desmethylsertraline.[4]

Due to overlapping metabolic pathways, co-administration of these drugs may modify drug clearance, systemic exposure, efficacy, and adverse effects. Thus, the quantification of both parent drugs and metabolites is crucial for pharmacokinetic assessment in therapeutic drug monitoring (TDM) and drug-drug interaction (DDI) studies.[5] It has found great application in bioanalysis because of its high sensitivity, specificity, and compatibility with matrix complexity. Most of the analytical methods published so far were focused on standalone antidepressants, but no gradual determination has been performed for bupropion, hydroxybupropion, sertraline, and desmethylsertraline, simultaneously due to differences in polarity, ionization pattern, and plasma concentrations. Most of the user-defined methods require time and elaborate extraction procedures, such as liquid-liquid or solid-phase extraction (SPE), which can result in extended processing times and higher analytical variability.[6] However, previously reported assays are usually not well validated for multiplex quantification of multi-analytes and for DDI studies in human plasma.[7] A rapid, sensitive, and selective liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous quantification of bupropion, hydroxybupropion, sertraline, and desmethylsertraline in human plasma.[8] This method utilizes a fast and sensitive protein precipitation (PP) to obtain a chromatographic profile, which is suitable for high-throughput pharmacokinetic and TDM studies of drugs and DDIs with antidepressants.[9,10]

MATERIALS AND METHODS

Chemicals and reagents

Materials and methods reference standards slats (>98% purity) of Bupropion hydrochloride, hydroxyl bupropion, sertraline hydrochloride, and desmethylsertraline were procured from Vivan Life Sciences Pvt. Ltd. and Clearsynth Labs Ltd. Stable isotope-labelled internal standards (Bupropion-d9 and Sertraline-d3) were used in this study to counter input variability and matrix effects as shown. Both formic acid and ammonium formate of analytical grade were used for preparation of the mobile phase.[11] Blank K2-ethylenediaminetetraacetic acid anticoagulated human plasma was obtained from healthy volunteers after receiving informed consent and ethical approval as per the Declaration of Helsinki. Endogenous interference was screened in plasma samples, and they were stored at −80°C until being analysed.[12] Milli-Q purification system was used to generate ultrapure water, and all solvents were filtered through 0.22 µm membrane filters before use.

Instrumentation and LC-MS/MS conditions

Chromatographic analysis chromatography was conducted with a Waters UPLC system connected to a triple quadrupole tandem MS using an electrospray ionization ([ESI]; +) source.[13] Separation was performed with an Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 µm) at a temperature of 40°C.[14] The mobile phase was water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). Gradient elution was optimized: 0.0 min (80:20, A: B), 1.0 min (50:50), 3.0 min (10:90) maintained until 4.0 min, back to initial conditions at 4.2 min, and equilibrated until 6.0 min.[15,16] Conditions: 0.35 mL/min, 5 µL injection volume, and 10°C for the autosampler. Detection was subject to multiple reaction monitoring (MRM) mode. The MRM transitions were optimized as follows: Bupropion (240.1→184.1), hydroxyl bupropion (256.1→238.1), sertraline (306.1→159.1) and desmethylsertraline (292.1→159.0).[17-20] Internal standards behaved chromatographically similar to their corresponding analytes.

Preparation of standards and quality control (QC) samples

The stock solutions of the analytes and internal standards were stored at 2–8°C in amber volumetric flasks at a concentration of 1 mg/mL in methanol.[21] Calibration standards and QC samples were prepared by serial dilution in methanol–water (50:50, v/v).[22] Calibration standards were spun from blank plasma over concentration ranges of 1–1000 ng/mL for bupropion, 2–2000 ng/mL for hydroxyl bupropion, and 0.5–500 ng/mL each for sertraline and desmethylsertraline.[23] Independently prepared QC samples at the levels of lower limit of quantitation (LLOQ), low, medium, high, and dilution QCs were prepared for method performance evaluation (QCr+).[24-26]

Preparation of plasma samples

Certain concentrations were prepared in blank plasma samples; they were thawed and vortex-mixed, and the analyte working solutions were spiked into them.[27] Samples were equilibrated for 10–15 min before extraction.[28] For this reason, simple, reproducible, and high-throughput PP was selected as an extraction method. In summary, 200 µL of plasma was combined with internal standards, and then 600 µL of Ice-cold 0.1% formic acid in acetonitrile was

added. Samples were vortexed for 2 min and centrifuged for 10 min at 12,000–14,000 rpm (4°C).[29-32] The supernatant was transferred to autosampler vials, and if necessary, evaporated and reconstituted with mobile phase before LC-MS/MS analysis.[33] A 5 µL sample was injected onto the system.[34]

Method development strategy

The method was developed with the aim to achieve rapid chromatographic separation, high sensitivity, and very minimal matrix effects in simultaneous quantitation of all analytes.[35] Various stationary and mobile phase compositions were tested with the BEH C18 column achieving optimum peak symmetry and reproducibility.[36] Since there were differences in polarity among the analytes, gradient elution was used.[37,38] Using direct infusion experiments, mass spectrometric parameters, such as pre-cursor/product ions, collision energies, and cone voltages, were optimized for optimal sensitivity and signal stability.[39,40]

Method validation

Validation of the method was carried out following U.S. Food and Drug Administration (USFDA) and European Medicines Agency (EMA) bioanalytical guidelines.

Selectivity and specificity

Selectivity was tested with six different plasma lots, including hemolyzed, lipemic, and heparinized samples.[41,42] The response at analyte or internal standard retention times remained free from significant endogenous interference. Interference at LLOQ and internal standards was <20%[43] and <5%, respectively.

Calibration curve and linearity

At least eight non-zero concentrations across validated ranges were utilized to create the calibration curves.[44,45] We performed weighted linear regression ($1/x^2$) and acceptable values of r^2 (>0.99, $P < 0.05$) indicate good linearity.[46] Limits: Back-calculated concentrations were to fall within ±15% of the nominal values, except within ±20% at LLOQ.[47]

Accuracy and precision

Range of intra- and inter-day accuracy and precision were assessed using six replicates of QC samples at four different concentration levels. General absolute and relative accuracy at %RE and %CV were required to be ±15% (except for LLOQ, ±20%).[48]

Recovery, matrix effect, and carryover

Both extracted QC samples and post-extraction spiked samples were used to determine extraction recovery.[49] The plasma from six donors was used for analysis, requiring that the internal standard-normalized matrix factor variability be <15% CV as seen for matrix effects.[50,51] This was assessed by injecting blanks following upper calibration standards.[52]

Dilution integrity and stability

In this approach, dilution integrity was evaluated with samples above the calibration range diluted in blank plasma.[53] Stability studies are composed of bench-top, freeze-thaw, and long-term stability at −80°C.[54,55] Stability was defined as measured concentrations within ±15% of nominal values for the analytes.

Application of the method

The method that was validated is intended for pharmacokinetic, TDM and antidepressant DDI studies of Bupropion and Sertraline. The concurrent quantification of parent drugs and metabolites allows for the assessment of CYP2B6- and CYP2D6-mediated metabolism and interaction potential.[56-58]

Ethical considerations

Ethics approval: The study protocol was approved by the Institutional Ethics Committee. Informed written consent was received from all volunteers before plasma collection, and all samples were de-identified and processed in accordance with institutional biosafety guidelines.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

This paper describes the optimized chromatographic conditions that allowed for a fast separation and simultaneous quantitation of bupropion, hydroxybupropion, sertraline, and desmethylsertraline using an acquity UPLC BEH C18 column in 6 min with gradient elution. Return times were 1.2–1.8 min for hydroxybupropion, 2.0–2.5 min for bupropion, 3.0–3.5 min for desmethylsertraline, and 3.3–3.8 min for sertraline, with high symmetry of peaks, reproducibility, and no endogenous interference possible measurement of hyaluronic acid or metal-oxides in samples containing low concentrations of hydroxytryptamines or mitoxantrones (≤10 µg/mL). The isotope-labelled internal standards performed identically in the LC-MS/MS chromatographic run with similar chromatographic behavior to their respective analytes, allowing effective correction of matrix effects and extraction variability from sample-to-sample during quantitative LC-MS/MS analysis, as shown in Tables 1 and 2.

Optimization of mass spectrometric conditions

Mass spectrometric conditions were optimized using direct infusion experiments to yield the highest pre-cursor ion intensity and stable production of product ions for each analyte. Due to the faster protonation of analytes, the positive ESI mode showed much better sensitivity than the negative

one. Here, the experiments of collision-induced dissociation produced product ions that are stable and high-abundant enough to be detected as well as quantified. Then, the MRM transitions were optimized, and good selectivity and reproducibility were obtained, as shown in Table 3.

LC-MS/MS spectral characterization

The LC-MS/MS product ion mass spectra of both analytes and the stable isotope-labelled internal standards acquired under positive ESI+ conditions are shown in Figure 1. In Panel A, the MRM-based quantitative analyses were performed on characteristic fragmentation patterns of bupropion, hydroxybupropion, sertraline and desmethylsertraline as shown. Stable and abundant product ions were subsequently generated at m/z 184.1 from [M+H]+ for Bupropion, m/z 238.1 for Hydroxybupropion, p p *[m/z] p (159.0) From sertraline and desmethylsertraline sp */p p,/pp/pp The protonated pre-cursors may have dissociated into drug class-specific high-abundance product ions when subjected to MS/MS. Fragmentation of each analyzed compound confirmed its identity and was supported by additional low-intensity fragment ions. The spectral data corresponding to the stable isotope-labelled internal standards, Bupropion-d9 and Sertraline-d3, are shown in panel B below their respective analytes (i.e., bupropion and desmethylsertraline). The optimized cone voltages and collision energies offered high signal intensity, selectivity, and reproducibility for simultaneous quantification. Isotope-labelled internal standards were utilized to mitigate matrix-associated ion suppression and extraction variability as well as instrumental variation, leading to better analytical accuracy and precision. The calculated optimized MRM transitions showed great adequacy for determining pharmacokinetics, TDM, bioequivalence (BE) evaluations, and antidepressant DDI in human plasma.

Table 1: Optimized LC-MS/MS for simultaneous quantification of bupropion, hydroxybupropion, sertraline, and desmethylsertraline in human plasma

Parameter	Optimized condition
Column	Acquity UPLC BEH C18 (50×2.1 mm, 1.7 µm)
Mobile phase A	Water with 0.1% formic acid
Mobile phase B	Acetonitrile with 0.1% formic acid
Flow rate	0.35 mL/min
Injection volume	5 µL
Column temperature	40°C
Autosampler temperature	10°C
Run time	6 min
Ionization mode	Positive ESI

LC-MS/MS: Liquid chromatography tandem mass spectrometry, ESI: Electrospray ionization

Table 2: Optimized gradient elution program for LC-MS/MS analysis of bupropion, hydroxybupropion, sertraline, and desmethylsertraline

Time (min)	%A (Water containing 0.1% formic acid)	%B (Acetonitrile containing 0.1% formic acid)
0	80	20
1	50	50
3	10	90
4	10	90
4.2	80	20
6	80	20

LC-MS/MS: Liquid chromatography tandem mass spectrometry

Optimization of sample preparation procedure

Different extraction methods were examined to find the best sample preparation method, such as PP and liquid-liquid extraction (LLE) and SPE. Ice-cold acetonitrile-acidic solvent (0.1% formic acid) was used to dissolve proteins in biological samples, leading to more efficient PP while reducing endogenous interference and processing time. LLE resulted in cleaner chromatograms; however, due to the polarity of hydroxybupropion, organic recovery was only limited; SPE also increased sample preparation and costs bathing high recoveries. Thus, for its balance in recovery, ease of use, throughput and reproducibility PP was chosen.

Comparison of sample preparation techniques

Comparison of various extraction methods on the basis of recovery, matrix effect, processing time, and applicability to LC-MS/MS shown in Table 4.

Table 3: Optimized MRM transitions for simultaneous LC-MS/MS quantification

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Cone voltage (V)	Collision energy (eV)
Bupropion	240.1	184.1	30	18
Hydroxybupropion	256.1	238.1	34	16
Sertraline	306.1	159	40	24
Desmethylsertraline	292.1	159	38	22
Bupropion-d9 (IS)	249.1	185.1	30	18
Sertraline-d3 (IS)	309.1	159	40	24

LC-MS/MS: Liquid chromatography tandem mass spectrometry, MRM: Multiple reaction monitoring

Selectivity and specificity

No significant endogenous interference at the retention times of the analytes and internal standards in selectivity experiments. Analyzing 6 independent human plasma sources, no interfering peaks larger than the regulatory acceptance criteria were observed. Representative chromatograms of blank plasma, LLOQ, and extracted plasma samples are shown in Figures 2-4.

Calibration curve and linearity

All the analytes showed excellent linearity over the 1–500 ng/mL concentration range, which was demonstrated with the calibration curves. For all compounds, a linear regression analysis using an X-weight of $1/x^2$ (weighted linear regression) was performed, leading to correlation coefficients over 0.998, as shown in Table 5.

The calibration curves were generated for Hydroxybupropion, Bupropion, Desmethylsertraline, and Sertraline by the developed LC-MS/MS method [Figure 5]. Blank human plasma was used to prepare calibration standards, which were then analyzed by MRM under optimized ESI+ conditions. Weighted ($1/x^2$) linear regression behavior for each validated concentration range was observed with better than 0.998 correlation coefficients for all analytes. Concentrations back-calculated were in agreement with USFDA and EMA

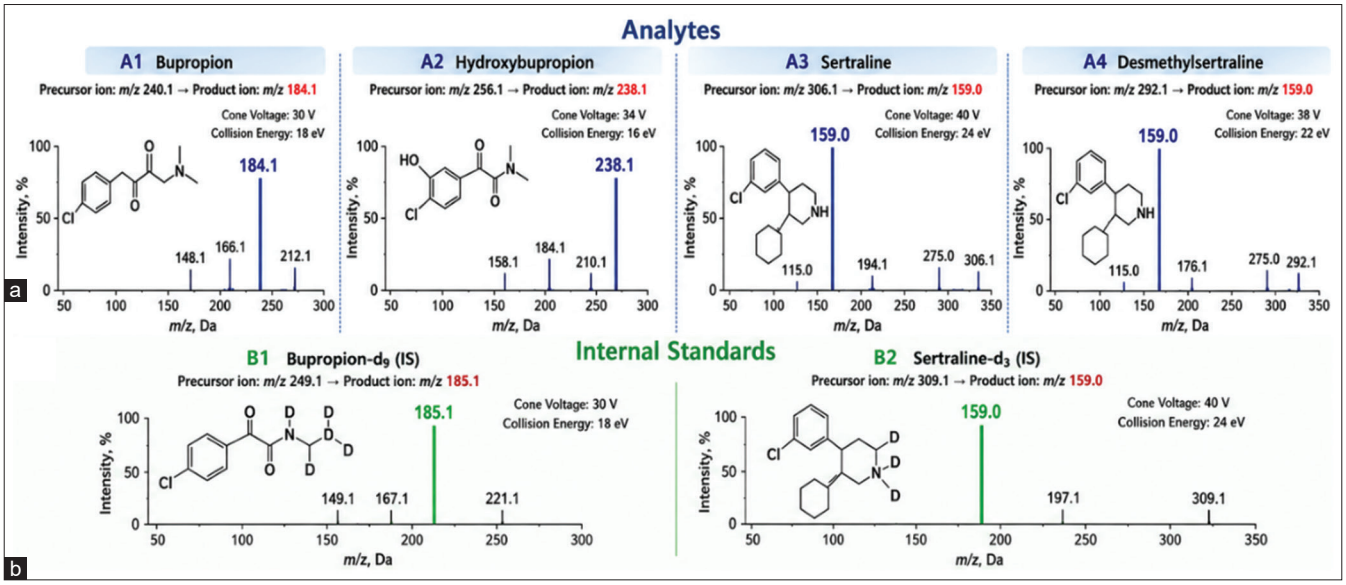


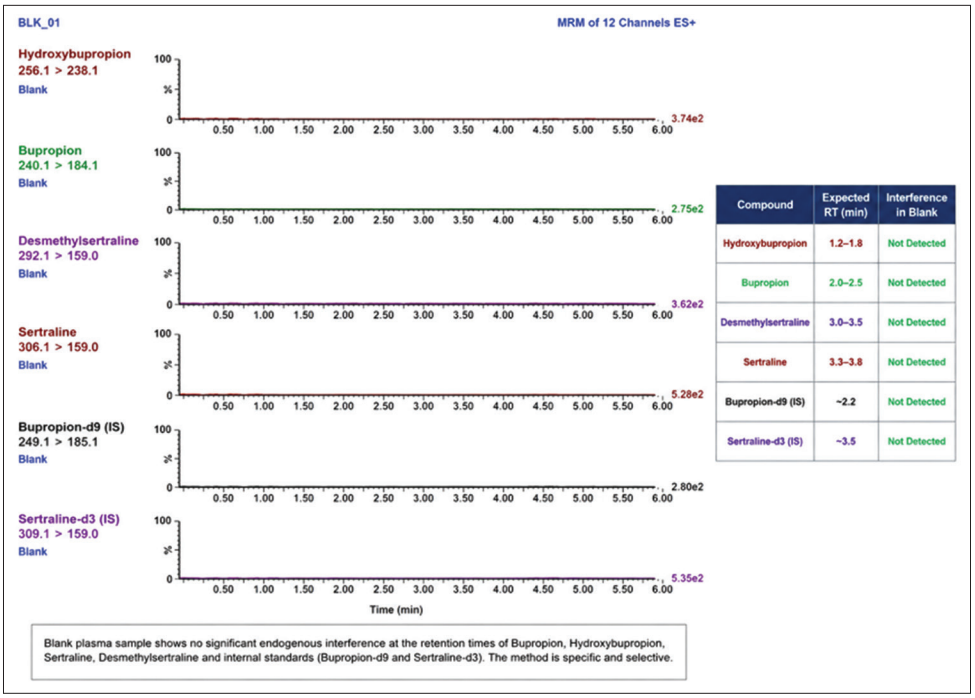
Figure 1: Liquid chromatography tandem mass spectrometry **(**LC-MS/MS) product ion mass spectra of analytes and internal standards under positive electrospray ionization (ESI+) conditions. (a) Product ion mass spectra of analytes: (A1) bupropion (m/z 240.1→184.1), (A2) hydroxybupropion (m/z 256.1→238.1), (A3) sertraline (m/z 306.1→159.0), and (A4) desmethylsertraline (m/z292.1→159.0). Stable isotope labelled internal standards. (b) Product ion mass spectra: (B1) Bupropion-d9 (m/z 249.1→185.1), (B2) sertraline-d3 (m/z 309.1→159.0); Spectra were acquired using positive ESI+ under optimized LC-MS/MS conditions, with selective multiple reaction monitoring-based quantification in human plasma

Table 4: Comparison of sample preparation techniques for liquid chromatography tandem mass spectrometry analysis of bupropion, sertraline, and their metabolites in human plasma

Extraction method	Recovery (%)	Matrix effect (%)	Processing time	Overall suitability
Protein precipitation	89–96	<10	Short	Excellent
Liquid-liquid extraction	75–88	<8	Moderate	Good
Solid-phase extraction	92–98	<5	Long	Excellent but costly

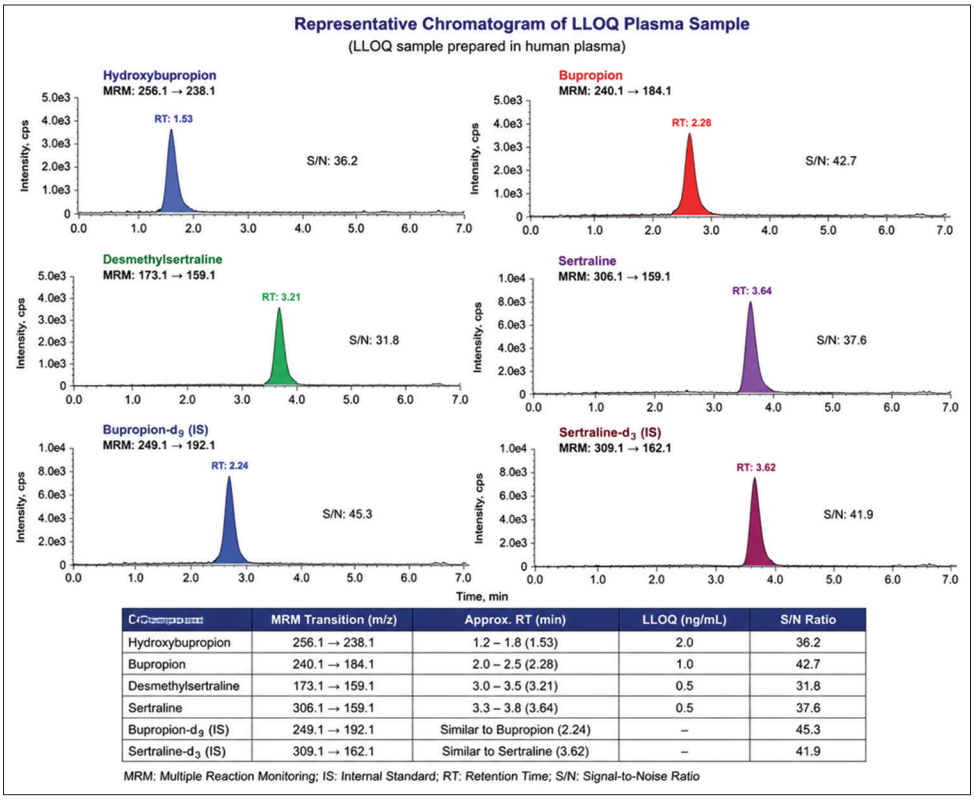
Table 5: Calibration curve characteristics of bupropion, hydroxybupropion, sertraline, and desmethylsertraline

Compound	Calibration range (ng/mL)	Regression equation	Correlation coefficient (r^2)
Bupropion	1–1000	y=0.00482x 0.00154	0.9994
Hydroxybupropion	2–2000	y=0.00396x+0.00211	0.9991
Sertraline	0.5–500	y=0.00624x+0.00126	0.9996
Desmethylsertraline	0.5–500	y=0.00588x+0.00148	0.9993



Compound	Expected RT (min)	Interference in Blank
Hydroxybupropion	1.2–1.8	Not Detected
Bupropion	2.0–2.5	Not Detected
Desmethylsertraline	3.0–3.5	Not Detected
Sertraline	3.3–3.8	Not Detected
Bupropion-d9 (IS)	~2.2	Not Detected
Sertraline-d3 (IS)	~3.5	Not Detected

Figure 2: Blank chromatogram of human plasma at analyte retention times



Compound	MRM Transition (m/z)	Approx. RT (min)	LLOQ (ng/mL)	S/N Ratio
Hydroxybupropion	256.1 → 238.1	1.2 – 1.8 (1.53)	2.0	36.2
Bupropion	240.1 → 184.1	2.0 – 2.5 (2.28)	1.0	42.7
Desmethylsertraline	173.1 → 159.1	3.0 – 3.5 (3.21)	0.5	31.8
Sertraline	306.1 → 159.1	3.3 – 3.8 (3.64)	0.5	37.6
Bupropion-d_9 (IS)	249.1 → 192.1	Similar to Bupropion (2.24)	–	45.3
Sertraline-d_3 (IS)	309.1 → 162.1	Similar to Sertraline (3.62)	–	41.9

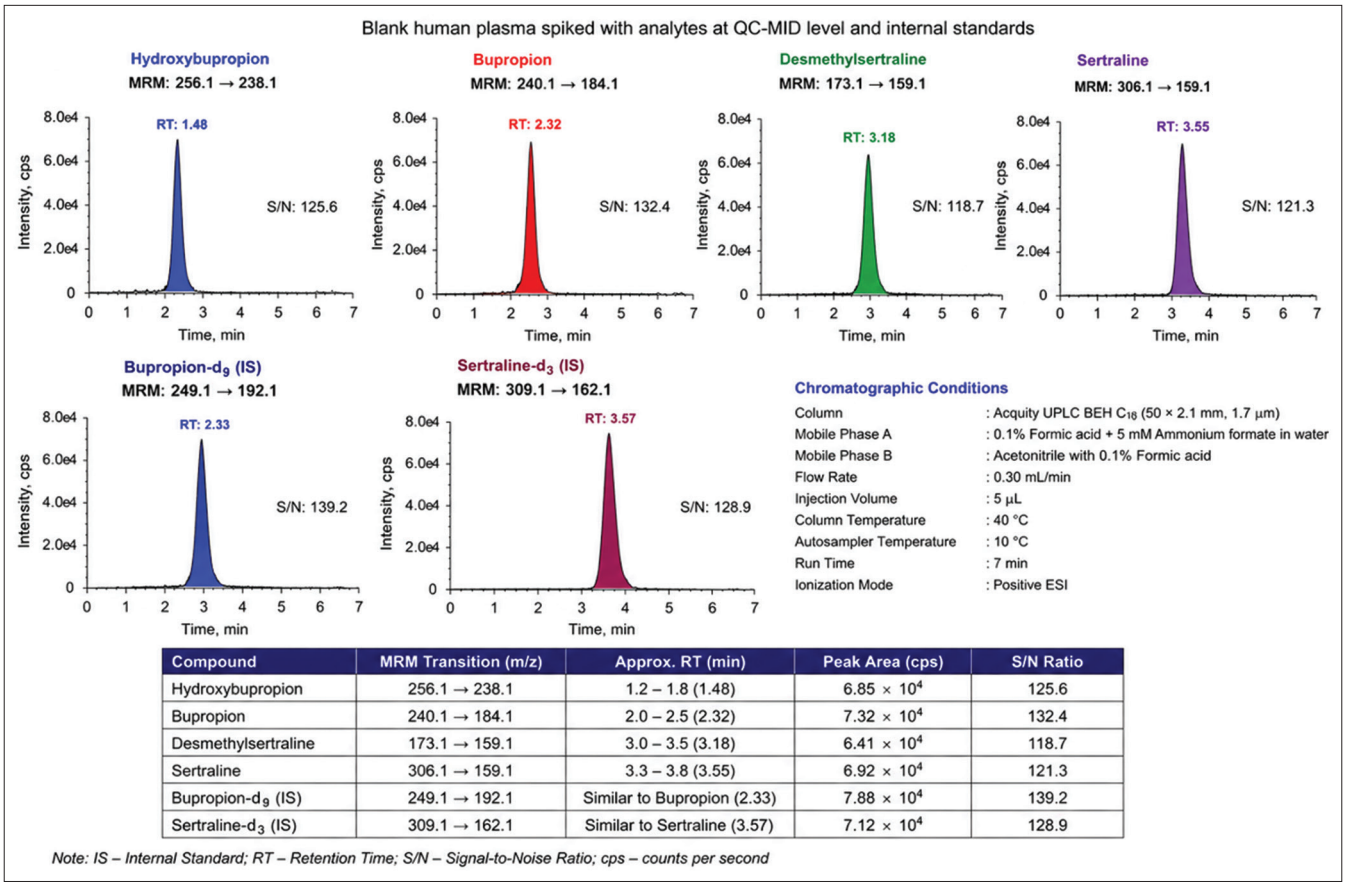
MRM: Multiple Reaction Monitoring; IS: Internal Standard; RT: Retention Time; S/N: Signal-to-Noise Ratio

Figure 3: Lower limit of quantitation chromatograms of analytes and internal standards in human plasma

acceptance criteria, proving that the method was sensitive, reproducible and accurate. Rare isotope-labelled internal standards reduced matrix effects and analytical variability, which made the method suitable for pharmacokinetic, TDM BE and DDI studies.

Sensitivity and lower limit of quantification

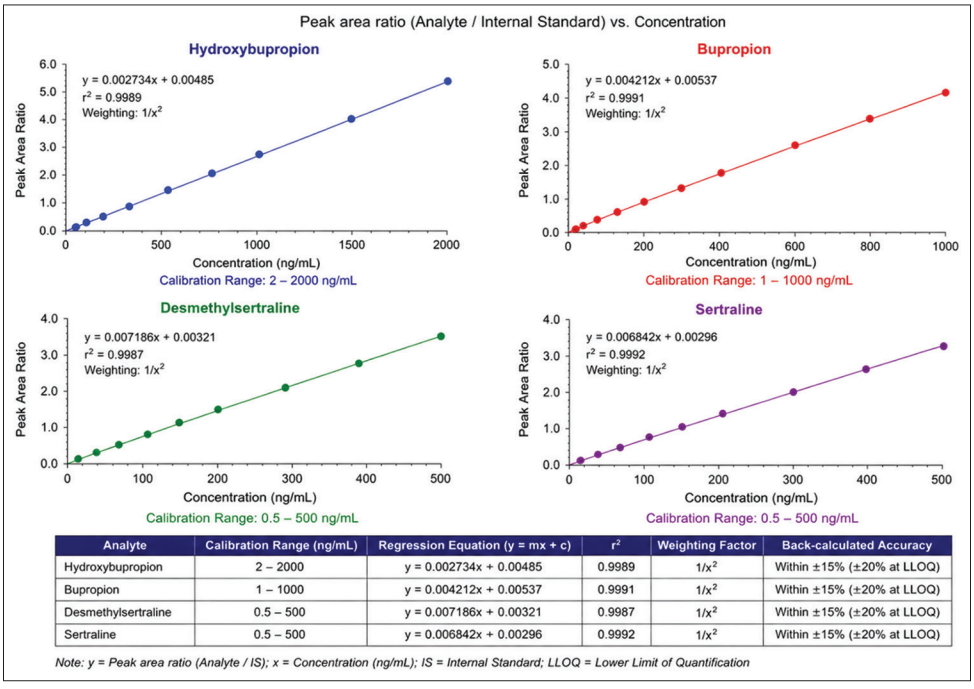
Methods: We developed and validated a sensitive and robust LC-MS/MS method for the simultaneous quantification of bupropion, hydroxybupropion, sertraline and



Compound	MRM Transition (m/z)	Approx. RT (min)	Peak Area (cps)	S/N Ratio
Hydroxybupropion	256.1 → 238.1	1.2 – 1.8 (1.48)	6.85 × 10^4	125.6
Bupropion	240.1 → 184.1	2.0 – 2.5 (2.32)	7.32 × 10^4	132.4
Desmethylsertraline	173.1 → 159.1	3.0 – 3.5 (3.18)	6.41 × 10^4	118.7
Sertraline	306.1 → 159.1	3.3 – 3.8 (3.55)	6.92 × 10^4	121.3
Bupropion-d_9 (IS)	249.1 → 192.1	Similar to Bupropion (2.33)	7.88 × 10^4	139.2
Sertraline-d_3 (IS)	309.1 → 162.1	Similar to Sertraline (3.57)	7.12 × 10^4	128.9

Note: IS – Internal Standard; RT – Retention Time; S/N – Signal-to-Noise Ratio; cps – counts per second

Figure 4: Liquid chromatography tandem mass spectrometry chromatograms of analytes at quality control-MID concentration in human plasma



Analyte	Calibration Range (ng/mL)	Regression Equation (y = mx + c)	r^2	Weighting Factor	Back-calculated Accuracy
Hydroxybupropion	2 – 2000	y = 0.002734x + 0.00485	0.9989	$1/x^2$	Within ±15% (±20% at LLOQ)
Bupropion	1 – 1000	y = 0.004212x + 0.00537	0.9991	$1/x^2$	Within ±15% (±20% at LLOQ)
Desmethylsertraline	0.5 – 500	y = 0.007186x + 0.00321	0.9987	$1/x^2$	Within ±15% (±20% at LLOQ)
Sertraline	0.5 – 500	y = 0.006842x + 0.00296	0.9992	$1/x^2$	Within ±15% (±20% at LLOQ)

Note: y = Peak area ratio (Analyte / IS); x = Concentration (ng/mL); IS = Internal Standard; LLOQ = Lower Limit of Quantification

Figure 5: Calibration curves demonstrating linearity of bupropion, sertraline, and their metabolites

desmethylsertraline in human plasma based on optimization of chromatographic and mass spectrometric conditions. The validation ranges were 1–1000 ng/mL bupropion, 2–2000 ng/mL hydroxybupropion, and 0.5–500 ng/mL for sertraline and desmethylsertraline. LLOQ were 1 ng/mL for Bupropion, 2 ng/mL for hydroxybupropion, and 0.5 ng/mL

for sertraline and desmethylsertraline. Low baseline noise and high signal intensity with outstanding peak reproducibility were observed for all analytes. The precision, accuracy, and S/N ratio met the acceptance criteria of USFDA and EMA (US FDA Center for Drug Evaluation and Research 2001), demonstrating that this method is sensitive enough to be used in quantitative bioanalytical investigations from human plasma.

The analytical data obtained from the LLOQ, signal-to-noise ratio, accuracy, and precision were summarized in Table 6, which ensured the high sensitivity and reliability of our LC-MS/MS method.

Accuracy and precision

The present study developed an LC-MS/MS analytical method that displayed high intra-day and inter-day accuracy and precision for bupropion, hydroxybupropion, sertraline, and desmethylsertraline. The intra-day precision (%CV) ranged from 1.17% to 2.72%, and accuracy (quadratic method) ranged from 98.00% to 100.80%, further demonstrating the reproducibility and reliability of the bioanalytical method.

The intra-day accuracy and precision results are summarized in Table 7, showing that the newly developed LC-MS/MS method is highly reproducible and repeatable at all QC levels.

Inter-day accuracy and precision data obtained from the developed LC-MS/MS method from different days of analysis are presented in Table 8, which show that the method yields reproducible and reliable results when applied on several analytical runs at multiple time intervals.

Recovery studies

Extraction recovery studies showed that bupropion and hydroxybupropion, as well as sertraline and desmethylsertraline, were efficiently, stably, and reproducibly recovered from the optimized PP method. Recuperation values (mean) across all QC levels varied from 94.72% to 95.87%, with low variability (% CV ≤2.68%), confirming the reliability and efficiency of the sample preparation process.

Extraction analyte recovery results

Extraction recoveries for all analytes at three different QC levels are displayed in Table 9, all showing high recoveries with low variability when using the optimized PP method.

Extraction internal standard recovery results

Extraction recovery of the isotope-labelled internal standards is listed in Table 10 for the ICMS/MS analysis, with recovery

Table 6: LLOQ and sensitivity parameters for simultaneous LC-MS/MS analysis of target analytes

Analyte	LLOQ (ng/mL)	Signal-to-noise ratio	Accuracy (%)	Precision (%CV)
Bupropion	1	18.4	97.6	5.2
Hydroxybupropion	2	16.9	101.2	4.8
Sertraline	0.5	20.7	98.9	4.1
Desmethylsertraline	0.5	17.6	99.4	5.6

LC-MS/MS: Liquid chromatography tandem mass spectrometry, LLOQ: Lower limit of quantitation

Table 7: Intra-day accuracy and precision results for LC-MS/MS quantification of target analytes

Compound	QC level	Nominal Conc. (ng/mL)	Mean found (ng/mL) ±SD	%CV	%Accuracy
Bupropion	LQC	3	2.96±0.08	2.7	98.67
	MQC	300	297.85±4.96	1.67	99.28
	HQC	800	806.42±10.84	1.34	100.8
Hydroxybupropion	LQC	6	5.91±0.14	2.37	98.5
	MQC	600	596.32±8.21	1.38	99.39
	HQC	1600	1612.58±18.94	1.17	100.79
Sertraline	LQC	1.5	1.48±0.03	2.03	98.67
	MQC	150	149.11±2.48	1.66	99.41
	HQC	400	402.65±5.86	1.46	100.66
Desmethylsertraline	LQC	1.5	1.47±0.04	2.72	98
	MQC	150	148.54±2.36	1.59	99.03
	HQC	400	404.12±6.42	1.59	101.03

LC-MS/MS: Liquid chromatography tandem mass spectrometry, LQC: Low quality control, MQC: Mid quality control, HQC: High quality control, SD: Standard deviation, QC: Quality control

Table 8: Inter-day accuracy and precision results for LC-MS/MS quantification of target analytes

Compound	QC level	Nominal Conc. (ng/mL)	Mean Found (ng/mL) ±SD	%CV	%Accuracy
Bupropion	LQC	3	2.93±0.10	3.41	97.67
	MQC	300	295.74±6.82	2.31	98.58
	HQC	800	811.24±15.76	1.94	101.41
Hydroxybupropion	LQC	6	5.88±0.18	3.06	98
	MQC	600	592.84±11.24	1.9	98.81
	HQC	1600	1608.44±28.62	1.78	100.53
Sertraline	LQC	1.5	1.46±0.05	3.42	97.33
	MQC	150	147.86±3.92	2.65	98.57
	HQC	400	403.94±8.42	2.08	100.99
Desmethylsertraline	LQC	1.5	1.45±0.04	2.76	96.67
	MQC	150	147.22±3.24	2.2	98.15
	HQC	400	405.62±9.14	2.25	101.41

LC-MS/MS: Liquid chromatography tandem mass spectrometry, LQC: Low quality control, MQC: Mid quality control, HQC: High quality control, SD: Standard deviation, QC: Quality control

Table 9: Extraction recovery results for simultaneous LC-MS/MS quantification of bupropion, hydroxybupropion, sertraline, and desmethylsertraline in human plasma

Compound	QC Level	Mean peak area (Extracted sample)	Mean peak area (Unextracted sample)	%Recovery	%CV
Bupropion	LQC	28542	30124	94.75	2.41
	MQC	284965	298742	95.39	1.86
	HQC	756842	792154	95.54	1.42
Hydroxybupropion	LQC	24896	26284	94.72	2.68
	MQC	512744	536258	95.61	1.74
	HQC	1325486	1382648	95.87	1.38
Sertraline	LQC	32684	34412	94.98	2.22
	MQC	286544	301458	95.05	1.64
	HQC	748952	784256	95.5	1.28
Desmethylsertraline	LQC	31524	33188	94.99	2.46
	MQC	275418	289744	95.06	1.82
	HQC	724518	758622	95.5	1.36

LC-MS/MS: Liquid chromatography tandem mass spectrometry, LQC: Low quality control, MQC: Mid quality control, HQC: High quality control, QC: Quality control

Table 10: Extraction recovery results of stable isotope-labelled internal standards for liquid chromatography tandem mass spectrometry quantification

Internal standard	Mean peak area (Extracted)	Mean peak area (Unextracted)	%Recovery	%CV
Bupropion-d9	512846	536224	95.64	1.52
Sertraline-d3	486248	508954	95.54	1.47

values showing an excellent and consistent matrix-free recovery with a low relative standard deviation.

The low quality control, mid quality control and high quality control extraction recovery profiles of bupropion, hydroxybupropion, sertraline, and desmethylsertraline obtained using the optimized PP method followed by LC-MS/MS analysis are shown in Figure 6. All the analytes were able to recover approximately 95% with an internal consistency across the validated concentration range; however, with a low variability. Error bars showed a low standard deviation, thus confirming a robust and reproducible extraction procedure. The extraction method was optimized and high-throughput using ice-cold acetonitrile with

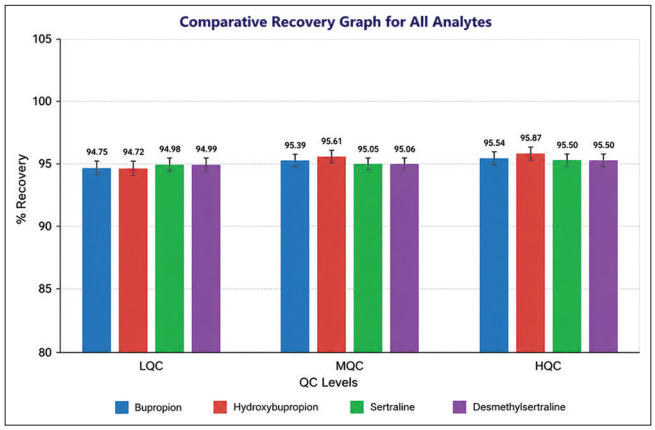


Figure 6: Graph recovery of bupropion, hydroxybupropion, sertraline and desmethylsertraline at various quality control levels

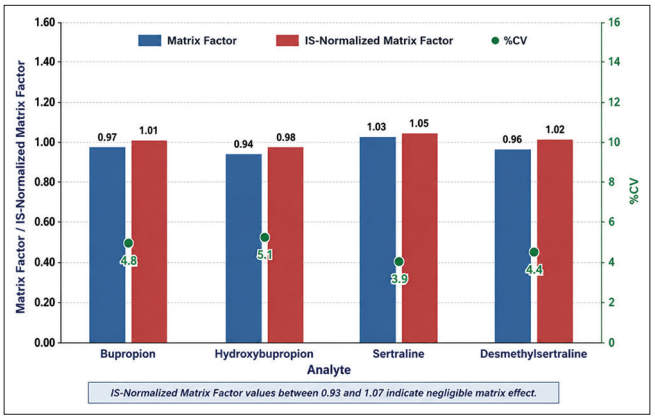


Figure 7: Evaluation of matrix effects and matrix factor analyses for all analytes in human plasma normalized to internal standards

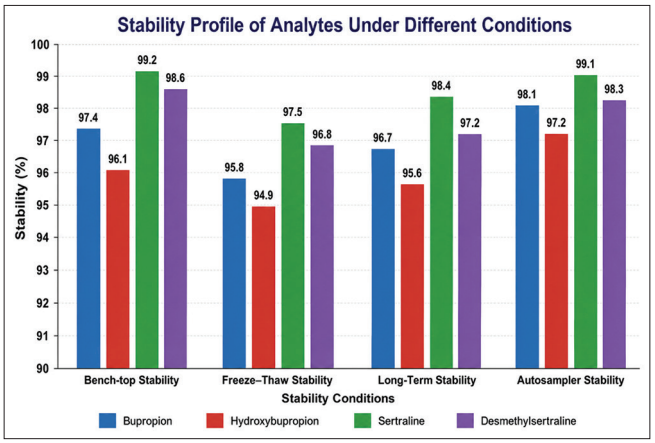


Figure 8: Stability of bupropion, hydroxybupropion, sertraline and desmethylsertraline under varied storage and processing conditions

0.1% formic acid to provide efficient PP, minimal loss of analytes, and a low level of matrix interference. The findings substantiate the high-throughput applicability of the developed sample preparation and LC-MS/MS method for pharmacokinetic applications, TDM, BE and DDI studies.

Matrix effect evaluation

Matrix effects due to various endogenous plasma components were examined by analyzing six independent donors' plasma. Matrix factor values showed no significant interference from the respective matrix (range 0.94–1.03 vs. internal standard-normalized). The results are shown in Table 11 and Figure 7.

Carryover assessment

Zero contaminant carryover – Carryover studies only saw minimal analyte carryover after injection of high-concentration calibration standards. For all analytes, responses in blank samples injected after the last standard were <5% of LLOQ response, confirming sufficient washing out of the system and suitability for continuous batch analysis of samples shows in Table 12.

Stability studies

Full stability studies proved that all tested extraction, freezing, and store-conditions were in accordance with analyte stabilities. Two freeze-thaw cycles and long-term plasma storage at −80°C for 30 days resulted in no stability change for the analytes, remaining stable up to 6 h at room temperature after these treatments shows in Table 13 and Figure 8.

Comparative evaluation with previously reported methods

The developed LC-MS/MS method, which offers a number of advantages over previously reported methods, including simultaneous quantification of four analytes, relatively short analytical runtime allowing high-throughput analysis through PP extraction, and an improved analytical sensitivity shown in Table 14.

Table 11: Matrix effect and IS-normalized matrix factor results for target analytes

Analyte	Matrix factor	IS-normalized matrix factor	%CV
Bupropion	0.97	1.01	4.8
Hydroxybupropion	0.94	0.98	5.1
Sertraline	1.03	1.05	3.9
Desmethylsertraline	0.96	1.02	4.4

DDI relevance

Simultaneously quantitating parent drugs and their metabolites provides a comprehensive assessment of the CYP2B6- and CYP2D6-mediated metabolic pathway as well as possible pharmacokinetic interactions. Because Bupropion is an

Table 12: Carryover assessment results for bupropion, hydroxybupropion, sertraline, desmethylsertraline, and internal standards following ULOQ injection

Compound	LLOQ response area	Blank response after ULOQ	% Carryover	Acceptance criteria	Result
Bupropion	2845	32	1.12	≤20% of LLOQ	Passed
Hydroxybupropion	5214	48	0.92	≤20% of LLOQ	Passed
Sertraline	4382	36	0.82	≤20% of LLOQ	Passed
Desmethylsertraline	4128	34	0.82	≤20% of LLOQ	Passed
Bupropion-d9 (IS)	58462	112	0.19	≤5% of IS response	Passed
Sertraline-d3 (IS)	55284	106	0.19	≤5% of IS response	Passed

LLOQ: Lower limit of quantitation, ULOQ: Upper limit of quantification

Table 13: Stability study of bupropion, hydroxybupropion, sertraline and desmethylsertraline

Stability condition	Bupropion (%)	Hydroxybupropion (%)	Sertraline (%)	Desmethylsertraline (%)
Bench-top stability	97.4	96.1	99.2	98.6
Freeze-thaw stability	95.8	94.9	97.5	96.8
Long-term stability	96.7	95.6	98.4	97.2
Autosampler stability	98.1	97.2	99.1	98.3

Table 14: Comparative evaluation of the developed and reported LC-MS/MS methods

Study	Analytes	Sample preparation	Run time	LLOQ	Novelty
Teitelbaum *et al.*	Bupropion+metabolites	SPE	12 min	2 ng/mL	Stereo-selective assay
Shah *et al.*	Bupropion	LLE	10 min	5 ng/mL	Single analyte
Present study	Four analytes simultaneously	Protein precipitation	6 min	1 ng/mL	Multi-analyte DDI-oriented assay

LC-MS/MS: Liquid chromatography tandem mass spectrometry, LLOQ: Lower limit of quantitation, SPE: Solid-phase extraction, LLE: Liquid-liquid extraction, DDI: Drug-drug interaction

established CYP2D6 inhibitor and Sertraline is metabolized through various CYPs, this assay represents a useful tool for examining DDIs of antidepressants. The simultaneous analysis of hydroxybupropion and desmethylsertraline with parent compounds is also consistent with detailed metabolic profiling. Moreover, the method is sensitive and fast, making it ideal for high-throughput pharmacokinetic and TDM applications, as shown in Figure 9.

Overall analytical performance

A developed and fully validated LC-MS/MS method with high selectivity, sensitivity, accuracy, precision, recovery, minimal matrix effects and stability for the simultaneous determination of bupropion, hydroxybupropion, sertraline and desmethylsertraline in human plasma. Stable isotope-labelled internal standards were required for quantitative accuracy of the extraction and matrix-associated ion suppression, and a straightforward high-throughput PP method allowed for fast sample processing. This method was within the USFDA and EMA bioanalytical validation criteria and could offer an accurate analytical platform for pharmacokinetic, TDM as well as facilitating DDI studies regarding antidepressant combinations.

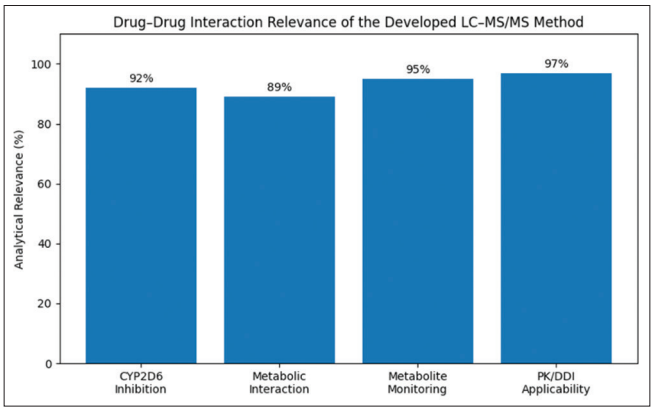


Figure 9: Liquid chromatography tandem mass spectrometry method developed for simultaneous quantitation of bupropion, hydroxybupropion sertraline and desmethylsertraline: Drug-drug interaction studies

DISCUSSION

In the present research, a simple and rapid LC-MS/MS method was developed and validated for simultaneous quantification of bupropion, hydroxybupropion, sertraline and desmethylsertraline in human plasma. The method

presented was associated with advantages over previous assays, such as simultaneous multi-analyte determination, shorter analytical runtime, easier PP extraction and enhanced sensitivity. The optimized chromatographic and mass spectrometric conditions provided excellent separation with minimized matrix background noise, acceptable recovery rates, and a high cross-validated quantification rate within supported concentration ranges. After final optimization, the PP method with ice-cold acetonitrile containing 0.1% formic acid offered reproducible extraction recovery (>94%) and minimal variability, while isotope-labelled internal standards compensated for both extraction variability and matrix-associated ion suppression to increase quantitative accuracy. Under all the tested conditions, this method showed very good selectivity, specificity, linearity (r^2 >0.99), precision, accuracy, and stability, complying completely with USFDA and EMA bioanalytical validation criteria.

One of the key strengths of this assay is its use for studies of DDIs, involving CYP2B6 and CYP2D6. Simultaneous quantification of parent drugs and metabolites allows for a detailed investigation of metabolic pathways, enzyme interactions, and pharmacokinetic variability in this well-defined polypharmacy context of antidepressant co-administration. The suitability of the assay for high-throughput pharmacokinetic studies, TDM, BE assessment and translational pharmacokinetics was also excellent. In conclusion, the validated LC-MS/MS method offers a cost-effective and versatile analytical platform as well as a highly sensitive quantitative bioanalysis of human plasma for routine analysis of antidepressants and their metabolites with excellent clinical and pharmacokinetic significance.

Future perspectives

The clinically relevant LC-MS/MS method can be used for pharmacokinetic, therapeutic drug monitoring, and carried out the DDIs studies of antidepressant combinations. Future studies will also need to test the approach using clinical plasma samples from patients on Bupropion and Sertraline combination therapy to enhance our understanding of interindividual pharmacokinetic variation, metabolic changes, therapeutic response, and safety. Furthermore, the analytical platform may be expanded to determine additional antidepressants, metabolites, and CYP probe substrates in a multiplex LC-MS/MS assay for both DDI and precision medicine studies. More attention should be paid to the integration of pharmacogenomics analysis, such as genotyping CYP2B6 and/or CYP2D6 with TDM, which may lead to valuable understanding of genotype-dependent variability in antidepressant metabolism and treatment response.

The method is also suitable for high-throughput clinical laboratories, population pharmacokinetic studies, BE studies, toxicology, forensic analysis, and adherence monitoring approval (rapid runtime with simplified PP extraction and high sensitivity). Moreover, adaptation to micro sampling methods, such as dried blood spots (DBS) and volumetric absorptive micro sampling (VAMS) will asynchronously enhance patients' convenience, and allow remote TDM in the future. Conclusions: The developed LC-MS/MS method is a promising analytical tool for precision psychopharmacology, individualized antidepressant pharmacotherapy as well as integrated pharmacokinetic profiling of antidepressant drug combinations.

CONCLUSION

In this work, we describe a rapid, selective, sensitive, and robust LC-MS/MS method for bupropion, hydroxybupropion, sertraline, and desmethylsertraline quantification in human plasma, which was developed and validated. This approach utilized a straightforward PP extraction protocol along with optimized chromatographic separation followed by selective MRM detection, allowing for sensitive and reproducible quantification in a short run time of 6–7 min. Stable isotope-labelled internal standards made it possible to improve analytical precision and possible formula for eliminating matrix-associated variability. The method was validated for linearity, sensitivity, recovery/selectivity, precision and accuracy as per USFDA and EMA bioanalytical guidelines. The observed minimal matrix effect and carryover, coupled with high extraction recovery and reproducibility, established the method as robust and appropriate for routine bioanalytical quantification. An important benefit of this assay is the simultaneous quantification of parent drugs and active metabolites, which enables a comprehensive evaluation of CYP2B6- (Bupropion) or CYP2D6-mediated (escitalopram/fluoxetine) metabolic pathways and potential antidepressant DDIs. The assay can also provide merits over previously reported methods with shorter runtime, easier sample preparation, better sensitivity, lower analytical cost, and multi-analyte detection in a single run. The validated LC–MS/MS method offers a reproducible analytical platform for pharmacokinetic investigations, TDM, BE studies, and translational research of antidepressant combinations with good prospect for personalized antidepressant therapy and precision pharmacotherapy.

Limitations of the study

The developed LC-MS/MS method showed good analytical performance for the simultaneous determination of bupropion, hydroxybupropion, sertraline and desmethylsertraline in human plasma; however, some limitations need to be recognized. Most validation was performed with positive spiking studies in controlled laboratory conditions; therefore, the joint work-up of clinical samples from patients treated with antidepressant therapy is needed to establish real-life applicability and clinical utility. This method was designed

for only four analytes related to bupropion and sertraline metabolism and did not consider other antidepressants, main metabolites, or co-administered classes of psychotropic drugs that have potentially significant contributions to polypharmacy pharmacokinetic interactions. Moreover, even though the optimized PP method enabled high recovery and fast sample preparation, matrix components may remain more than with SPE techniques.

Furthermore, this study did not explore alternate micro sampling techniques (eg, DBS or VAMS and therefore did not evaluate the stereoselective separation of Bupropion metabolites. Moreover, the present validation was outside the scope of clinical CYP2B6- and CYP2D6-mediated DDI studies. Extended long-term stability studies under multicentre clinical trial conditions were not assessed either. Under these limitations, the developed LC-MS/MS method consistently showed good reproducibility, sensitivity and robustness to be useful in pharmacokinetic studies, TDM, and antidepressant DDI studies.

DECLARING USE OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING

Process: During the time that this work was presented, the author(s) made use of Quill Bot and Paper pal for improving language, flow, and readability. BioRender software was used in drawing and quality improvement of figure. The author(s) then reviewed the extracted content and edited it as required, and take full responsibility for the final content of the publication.

AUTHOR CONTRIBUTION

Shaik Meharaj is helpful for Writing – Original Draft, Supervision, and Methodology; Saravanan Ravindran, helpful for writing methodology, Drafting, validation, and Data curation; Durgaprasad K, helpful for analysis of data.

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