DEVELOPMENT OF RAPID, SENSITIVE, HIGH PERFORMANCE LIQUID CHROMATOGRAPHY -TANDEM MASS SPECTROMETRY (HPLC-MS/MS) ASSAY FOR THE SIMULTANEOUS DETERMINATION OF BUPIVACAINE AND PROCAINE IN HUMAN PLASMA

Muni krishnaiah A[1], Ramachandraiah C[1*], Srinivas M[2], Devanna N [3]

[1] Research Scholar, Department of Chemistry, Jawaharlal Nehru Technological University, Anantapur- 515002, Andhra Pradesh, India.

[1*] Department of Chemistry, Sri Kalahastheeswara Institute of Technology, Srikalahasthi-517640, Andhra Pradesh, India.

[2]Acubiosys Pvt.Ltd, Jawaharnagar, BITS Pilani Campus, Hyderabad, India.

[3]Department of chemistry, College of Engineering, Jawaharlal Nehru Technological University, Anantapur-515002, Andhra Pradesh, India.

***Corresponding Author:**

Rama Chandraiah.C

Department of Chemistry, Sri Kalahastheeswara Institute of Technology,

Srikalahasthi-517640, Andhra Pradesh, India.

cramachandraiah@yahoo.com

Mobile No: +919490419750

Abstract- A simple, rapid, sensitive, specific, LC-ESI-MS/MS assay for the simultaneous detection and accurate measurement of Bupivacaine (1-butyl-N-(2,6-dimethylphenyl)piperidine-2-carboxamide) and Procaine (4-aminobenzoic acid 2-diethylaminoethylester) in human plasma. The precipitation method was used to extract the analytes and IS from human plasma. The chromatographic resolution of Bupivacaine, Procaine and corresponding IS was achieved using gradient flow on C18 column. The total chromatographic run-time was 3 min. A linear response function was established for the range of concentration 1-1000ng/ml for Bupivacaine and Procaine. The intra-day and inter-day accuracy and precision values for Bupivacaine and Procaine met the acceptance as per regulatory guidelines.

Keywords: Bupivacaine, Gradient technique, LC-MS/MS, Procaine, Simultaneous estimation.

1.0 INTRODUCTION

Local anaesthetics[1] such as bupivacaine block the generation and the conduction of nerve impulses, presumably by increasing the threshold for electrical excitation in the nerve, by slowing the propagation of the nerve impulse, and by reducing the rate of rise of the action potential. Bupivacaine binds to the intracellular portion of sodium channels and blocks sodium influx into nerve cells, which prevents depolarization. In general, the progression of anaesthesia is related to the diameter, myelination and conduction velocity of affected nerve fibres. Bupivacaine is a widely used local anaesthetic agent. Bupivacaine is often administered by spinal injection[2] prior to total hip arthroplasty. It is also commonly injected into surgical wound sites to reduce pain for up to 20

hours after surgery. In comparison to other local anaesthetics it has a long duration of action. It is also the most toxic to the heart when administered in large doses. This problem has led to the use of other long-acting local anaesthetics: ropivacaine and levobupivacaine. Levobupivacaine is a derivative, specifically an enantiomer, of bupivacaine. Systemic absorption of local anaesthetics produces effects on the cardiovascular[3] and central nervous systems. At blood concentrations achieved with therapeutic doses, changes in cardiac conduction, excitability, refractoriness, contractility, and peripheral vascular resistance are minimal. Production of local or regional anaesthesia is used for surgery, oral surgery procedures, for diagnostic and therapeutic procedures, and for obstetrical procedures.

Procaine (4-aminobenzoic acid 2-diethylaminoethyl ester) is neighbourhood sedatives of the ester compose that has a moderate beginning and a brief term of activity. It is for the most part utilized for invasion anaesthesia, pelvic pain[4], fringe nerve piece, and Spinal cord. Procaine has likewise been examined as an oral passage inhibitor in treatment-experienced HIV patients[3]. Today it is utilized remedially in a few nations because of its sympatholytic, calming, perfusion-improving, and state of mind upgrading impacts. Procaine is utilized in plasma by compound pseudocholinesterase through hydrolysis into para-amino benzoic corrosive (PABA), which is then discharged by the kidneys into the pee. Procaine hydrochloride is a topical soporific, which can obstruct the nerve fibre conduction incidentally and has an opiate impact, solid impact, low lethality, and non-addictive, however on the skin mucosal entrance is frail, inadmissible for surface anaesthesia, patients are utilized for penetration, conduction and spinal anaesthesia. White fine needle precious stone or crystalline powder at room temperature, no scent, taste marginally unpleasant and Ma, dissolvable in water, solvent in ethanol, somewhat dissolvable in chloroform, relatively insoluble in ether.

Combination of anesthetic drug therapy in post operative pain medication is the most interested topic in generic pharmaceutical industry; hence author believes that the simulations estimation method of these drugs in plasma samples would contribute greatly to pharmaceutical industry. In literature survey for estimation of Bupivacaine in plasma, several methods were proposed, such as HPLC[5-7], by GC-MS[8]. For estimation of procaine in plasma several methods were proposed, such as Capillary electrophoresis[9], HPLC[10-11] and by LC-MS[12]. As far as there is no simultaneous UPLC-MS/MS[13] method for estimation of Bupivacaine and procaine in human plasma samples. For measuring Bupivacaine plasma concentrations, especially in the later phase of elimination, a highly sensitive assay is required that is affected by neither plasma components nor other drugs given simultaneously.

Our present work describes a simple, novel, rapid, sensitive and selective method for simultaneous determination of Bupivacaine and Procaine by advanced UPLC-MS/MS equipment. The current method offers many advantages i.e. shorter runtime, less sample volume and can estimate both compounds in a single run. In the current method Bupivacaine and Procaine were used as internal standards, the chemical structure of Bupivacine is shown in Figure 1A and procaine in Figure 1B. This method can be applied to pharmacokinetic study of plasma Bupivacaine and Procaine concentration after oral administration.

2.0 EXPERIMENTAL

2.1 Chemicals and reagents

Bupivacaine and Procaine were purchased from Sigma Aldrich private limited, Bangalore. HPLC grade Methanol (100%), Ammonium acetate and Water used in the entire analysis was prepared from Milli-Q water purifier system procured from Millipore. Blank human plasma was obtained from Supratech Micropath. Ammonium acetate used for the mobile phase preparation was of GR grade and obtained from Merck.

2.2 LC-MS/MS Instrumentation and Chromatographic Conditions

The instrumentation consisted of a modular HPLC (Shimadzu, Kyoto, Japan) coupled to AB Sciex API-4000 mass spectrometer (Applied Bio systems, Ontario, Canada), equipped with an electrospray ion interface operating in positive mode using nitrogen as the nebulizer, auxiliary, collision, and curtain gas. The HPLC system consisted of two LC20AD pumps, online DGU-20A3 solvent degasser, a SIL-HTc auto sampler, and a CTO20A column oven. The high-performance liquid chromatography column was a C18-XBridge column (4.6x50 mm, 3.5 µm) maintained at 400 C with a flow rate of 1.0 mL/min with the split ratio of 60:40. The mobile phase consisted of 0.1 % formic acid (A) and methanol (B). The initial mobile phase composition was 95% A/5% B and continued until 0.8 min, and then it was changed to 5 % A/95% B over 2.2 minutes. After 2.2 min, the mobile phase is slowly shifted to 95% A/5% B by 2.4 min following which it was held at same for 3.5 minutes. The chromatograms were acquired using analyst software (version 1.4.1, Applied Bio systems, Ontario, Canada). Calibration curves were constructed using the peak area ratio of the analyte to the internal standard versus the analyte concentration and by applying the weighted least squares regression algorithm.

2.3 Preparation of Stock Solutions, Calibration Standards, and Quality Control Samples

Stock solutions of the analytes and internal standards were prepared in methanol and stored between 1 to 10°C. The concentrations were corrected for purity, moisture content, and actual amount weighed as per their certificate of analysis. Working solutions of each analyte for the calibration curve (CC) standards and quality control samples (QC) were prepared in methanol: water (50:50, v/v) separately. The 1% spiking of these working solutions were done individually in plasma to attain the desirable concentration of each analyte. Each calibration curve consisted of one blank sample, one blank sample fortified with IS, and eight calibration points ranging from 1.0–1000 ng/mL for bupivacaine and procaine. The QC samples spiked independently of the CC standard stock comprised of the limit of quantification quality control (LOQQC), low quality control (LQC), middle quality control (MQC), and high quality control (HQC). Aliquots of the CC and QC standards were stored below −50°C.

2.4 Method Validation

2.4.1 Mobile phase optimization

The interaction of mobile phases and LC gradients were optimized to improve LC separation and detection sensitivity for the LC-MS method. Methanol is used as eluent for separation because it promoted π-π interactions between the aromatic rings, but showed poor elution property. Addition of formic acid showed poor resolution, because analyte needs neutral or basic pH hence ammonium acetate is added to aqueous phase to retain for some time.

2.4.2 Internal standard selection:

First Metoprolol was used as IS for positive mode compounds. Metoprolol needs acidic P^H for elution but bupivacaine and procaine needs neutral P^H and dilution with water for supernatant which get after extraction. Hence was fixed finally for positive mode, which showed good elution and resolution property.

2.4.3 Recovery

The recovery is related with the proficiency of the expository detachment technique, inside changeability levels. The recovery of an analyte does not should be of 100%, be that as it may, the amount of analyte recouped and of the inner standard must be predictable, exact and reproducible. The extraction effectiveness of analyte and IS human plasma was resolved from repeat QC samples (n=6) reactions were spiked in post removed human plasma clear example at proportionate fixation. Single focus was utilized to decide recuperation of IS though in the event of analyte (Bupivacaine and procaine) the recuperation was resolved at LQC and also HQC fixations

2.4.4 Carryover:

Within each batch, the specific sample without internal standard is injected immediately after the highest standard to verify that carryover is less than 20% of the LLOQ on a peak area basis.

2.4.5 Selection of HPLC column

Based on previous studies reversed phase C-18 columns were the most frequently used HPLC column for these target analyte. Initially, conventional silica based C-8 analytical columns (i.e., C8, X-bridge) were tested and demonstrated poor separation and bad peak shape. Various factors including the composition of the mobile phase and pH levels were also examined; however, no significant improvement in the chromatographic separation was obtained primarily due to their similar physicochemical properties. C18, X-bridge demonstrated good resolution but showed asymmetrical peaks. Finally Kinetex EVO C18 (5u, 50*4.6mm) showed substantially good resolution, symmetrical peaks and less co-elution between target compounds due to its effectiveness in polarity and aromatic selectivity.

2.4.6 Calibration curve

In order to determine the linearity of response, five replicate sets of quantitation standards, each set prepared on a different day but using the same plasma and dilute stock solution were prepared, each containing 9 different concentration levels (calibration standards of analyte) in human plasma. This will be referred to as inter and intra-day data, and will show that the assay is robust and reproducible on different days. The aim of the experiment was to assess the lowest level which could be quantified by LC-MS/MS. The peak area for each analyte was determined from the extracted ion chromatogram and divided by the peak area of the internal standard obtained in the same way. This was plotted against the nominal concentration of analyte. The simplest appropriate form of regression was chosen as required by the FDA. In this case it was linear $1/x$ or $1/x^2$ where x was the concentration. This gave the calibration curve a stronger weighting towards the lower end of the calibration curve.

The developed method was comprehensively validated as per US Food and Drug Administration (USFDA) guidelines and guidance from the European Medical Agency .The method was validated for selectivity, sensitivity, linearity, precision and accuracy, recovery, dilution integrity, matrix effect, re-injection reproducibility, carryover in the matrix, and stability of the analytes during both short-term sample processing and long-term storage.

2.4.7 Specificity and selectivity

Specificity is a method which produces a response for only a single analyte. Selectivity is the ability of a method to produce a response for the target analyte distinguishing it from all other interferences. The main thing is the response of the LLOQ standards should be greater than the response from the blank biological matrix by a defined factor. Selectivity of the method was assessed using eight different lots of human K3EDTA plasma (which included six normal lots, one hemolyzed, and one lipemic) by screening for the responses of the interfering substances at the retention times of the analytes and internal standards. Interference in each plasma batch was compared to six limit of quantification (LOQ) samples, prepared by pooling two normal blank matrix batches with minimal or no peak area response at the retention time of all peaks. Response of the interfering peaks at the retention times of the analyte and internal standard in the blank matrix must be ≤ 20% of the mean peak area response of the analyte in the LOQ samples for the analyte and must be ≤ 5% of the mean peak area response of the internal standard in the LOQ samples for ISTD. The signal-to-noise ratio of each LOQ sample should be at least five times of that of the blank matrix samples. Four calibration curves were used to demonstrate the linearity of the method. The best-fit curves using weighted linear least square regression analysis were obtained by the peak area ratio of the analyte to internal standard versus analyte concentration. A correlation coefficient $r > 0.99$ was desirable for all the calibration curves.

2.4.8 Precision and accuracy

The precision decides the closeness of contract between progressions of estimations from different examining of the same homogenous test under recommended condition. The coefficient of fluctuation ought not to go beyond 15% with the exception of the LLOQ, where it ought not to exceed 20%. The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The

intra- and inter-day accuracy and precision of the method were determined for the estimation of the analytes in human K3EDTA plasma. They were estimated by replicate analysis of precision and accuracy (PA) batches. Each PA batch consisted of CC standards and six replicates of QC at each level. Intraday precision and accuracy were determined by analyzing two PA batches processed on the same day. Inter-day assay precision and accuracy were determined by analyzing PA batches processed in the duration of 3 days. The analyte peak of the LOQ/LOQQC sample should be identifiable, discrete, and reproducible with a precision (% CV) not greater than 20.0% and accuracy within 20.0%. For standards/QC samples other than the LOQ/LOQQC, accuracy should be within 15% and precision not greater than 15.

2.4.9 Matrix effect

Matrix effect (relative) was investigated to ensure that the precision and sensitivity of the method were not compromised by the matrix. In order to estimate it, six lots of plasma matrix including lipemic and hemolyzed were chosen and concentrations equivalent to the LOQQC and HQC levels were spiked in duplicate in each lot. Also, freshly spiked CC standards, six LOQQC samples, and six HQC samples were prepared in pooled plasma and processed. The values of the QC samples were back-calculated against the freshly spiked CC standards. For the calculation of the absolute matrix effect (AME), working solutions of the drug and ISTD were prepared at concentrations representing 100% extraction of the QC samples at low, middle, and high concentrations (aqueous samples). Six aliquots from each of six different batches of the screened blank matrix, including one lipemic and one hemolyzed matrix, were taken and processed without the addition of the internal standard. Two aliquots of each post-extracted blank matrix were reconstituted with solution representing the final extracted concentration of the analytes at each QC level. AME is acceptable if %CV at each QC level is ≤ 15% and the %CV between QC levels is ≤ 15%. AME = Mean peak area response in presence of matrix ions Mean peak area response in absence of matrix ions Where AME =1 indicates no matrix effect, AME 1 indicates ion enhancement. Recovery (process efficiency) of the developed extraction method was determined by comparing the mean peak area of the analyte in the processed plasma samples which were pre-spiked with the analytes at the LQC, MQC, HQC levels with the dilution of analytes representing 100% extraction of the QC samples. Similarly, recovery of the ISTD was determined by comparing the mean peak area of ISTD in the extracted MQC samples (n=6) with the dilution representing 100% extraction of the ISTD sample.

2.5 Stability experiments

Stability evaluation is done to show that the concentration of analyte at the time of analysis corresponds to the concentration of the analyte at the time of sampling. Stability of the anlayte is done for the very short period of time.

At each level for LQC and HQC samples, six replicates were used for the following stability parameters:

1. Freeze Thaw stability completed for three cycles
2. Auto-sampler stability
3. Bench top stability

Stability of the analytes was evaluated in plasma under different conditions which occurred during incurred sample handling, and analysis was evaluated during the method validation. Stock solution stability was performed by comparing the area response of the analyte and internal standard in the stability sample, with the area response of the sample prepared from fresh stock solutions. The stability of the working solutions of each analyte was evaluated by comparing the peak area response of the stability working solution kept in ice-cold water under low light, with the area response of the freshly prepared working solution. The stability of the spiked human plasma samples stored in an ice-cold water bath under low light (bench top stability) was evaluated for ~ 7 hr. The auto sampler stability was determined by stored reconstituted stability QC samples for ~ 72 hr under auto sampler conditions (at 10°C) before being analyzed. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at −50°C and thawed at room temperature three times. Stability studies in plasma were performed at the LQC and HQC levels using four replicates at each level. The analyte was considered stable if the % change was less than 15. For sample collection and

handling stability, fresh human K3EDTA whole blood was spiked with the analytes at the LQC and HQC levels and kept in an ice-cold water bath for ~2.5 hr. After the stability period, fresh blood was spiked again and these samples served as comparison samples. The stability and comparison samples were centrifuged at 4°C and the resulting plasma was collected and processed as per the developed analytical method. The stability was calculated as mentioned above. Re-injection reproducibility was performed by injecting all QC samples from an accepted precision-accuracy batch. The calculated concentration of the re-injected QC samples was determined against the CC samples from the same precision and accuracy batch which was analyzed 48 hr before. The % difference between the original and re-injected value was calculated by using formula: % Difference = Original value – Reinjected value Original value X100 Carryover in the matrix was estimated by injecting duplicates of the LOQ and upper limit of quantification (ULOQ) samples, bracketed with the same processed blank sample. Interferences at the retention time of the analytes and ISTD were evaluated by comparing the difference in area response of the first blank matrix to the second blank sample against their respective mean peak area response of the analytes and ISTD in the processed LOQ samples. The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above the ULOQ, which may be encountered during real time incurred sample analysis. The dilution integrity test was performed by preparing samples at a concentration approximately two times the concentration of 90% of the ULOQ. These samples were diluted to two and four times with the blank matrix to bring the concentration within the calibration curve and then analyze against the fresh CC samples.

3.0 RESULTS AND DISCUSSION

3.1 Optimization of MS Parameters and Chromatographic Conditions

Mass spectrometric detection was carried out on an API 4000 triple quadrupole instrument equipped with an ESI source operated in the positive ion mode. The ESI source in positive ion mode was selected, as it increased the signal-to-noise ratio of the analytes, which helped in attaining the lower LOQ. During the optimization of the mass spectrometric parameters, strong and stable signals of analytes and internal standards were noted and the ion transitions *m/z* 289.5 → 140.3, 237.1 → 100.1 were selected for the MRM of bupivacaine and procaine respectively (Fig 2 and Fig 3). The optimization of the source and compound parameters was done by syringe pump infusion of each analyte. The compound parameters were optimized as follows: declustering potential: 70 and 80V, entrance potential: 10V, collision cell exit potential: 14 and 24 V, and collision energy: 39 and 24V for Bupivacaine and Procaine respectively. The source/gas parameters were optimized as follows: curtain gas: 20, collision gas: 4, ion source gas-1: 50, ion source gas-2: 50, ion spray voltage: 5500 V and temperature: 550°C. The product ion scans of bupivacaine and procaine are shown in Table 1.

Important parameters like pH of the mobile phase, concentration and type of the buffer (ammonium acetate and ammonium formate) solution, percentage and type of the organic modifier (acetonitrile and methanol), different columns (reverse phase columns such as Discovery C18, Synergie MAX RP, Kinetix EVOC18, and Phenomenex Luna), and different flow rates (0.4–1.8 mL/min) were attempted for better sensitivity and better chromatographic separation of Bupivacaine and Procaine. The separation was found to be affected by increasing the molarity of the ammonium formate buffer and found to be better with methanol as compared to Acetonitrile. Trials have shown that the mobile phase, acetonitrile: 5 mM ammonium formate buffer at a flow rate of 1 mL/min, with the Kinetix EVOC18 column nullified the problems without compromising on the sensitivity, range, and precision of the method.

3.2 Linearity:

The calibration curve for analyte was generated using a 1✕ linear regression or quadratic regression and performed by the Analyst 1.5 software. The back calculated intra-batch data for standard curves is presented in Table 2 and Table 3. Standards with a back-calculated accuracy outside the range of 85-115% of the nominal concentration were excluded from the regression statistics. The correlation coefficients for all calibration curves were more than 0.99.

3.3 Accuracy & precision:

Intra-batch (n=6/batch, 3 days) and inter-batch (n=18) accuracy and precision for bupivacaine and procaine in Table 4 and Table 5 .The results show acceptable accuracies, especially when taking into account the multistep sample preparation procedure, with most between 90-110% of the nominal concentration. The respective chromatograms of blank, blank+ IS and LLOQ chromatograms of Bupivacaine, Procaine are represented from Figure 4 to 8. The coefficients of variation for each analyte, both intra- and inter-day, are also well below the nominal criteria of less than 15%.

3.4 Recovery:

The recovery for analyte from K_2EDTA human plasma from the LQC, MQC and HQC level was determined by comparing with their respective aqueous quality control samples (Table 6). The average recovery for Bupivacaine and Procaine was found to be 73.47% and 99.85% (n=6) respectively.

3.5 Stability:

3.5.1 Freeze-thaw stability:

Freeze-thaw stability of low, mid and high level quality control samples was validated for three cycles of freezing and thawing. The calculated values for the freeze-thaw stability samples were within the precision and accuracy range determined by the inter-day QC samples (Table 7), leading to the conclusion that the analyte showed no instability due to freezing and thawing over the three cycles.

3.5.2 Auto-sampler stability:

Auto-sampler stability of K_2EDTA human plasma quality control samples was validated by keeping the samples for 24hrs duration in auto-sampler (temperature condition at 4^0C) and then compared with fresh samples. The calculated values for auto-sampler stability (n=6 at low, mid and high QC levels) were within the precision and accuracy range determined by the inter-day QC samples, leading to the conclusion that the analyte showed no instability due to auto-sampler storage of the samples throughout the run time.

3.5.3 Bench-top stability:

Bench-top stability of K_2EDTA human plasma quality control samples was validated by keeping the samples for 4hrs at the room temperature where the sample processing is carried out and then they compared were with fresh samples. The calculated values for bench-top stability(n=6 at low, mid and high QC levels) were within the precision and accuracy range determined by the inter-day QC samples, leading to the conclusion that the analyte showed no instability due to auto-sampler storage of the samples throughout the run time.

4.0 CONCLUSION

In summary, a highly sensitive, specific, reproducible and high-throughput LC-MS/MS was developed and validated to quantify Bupivacaine and Procaine in human plasma as per the regulatory FDA guidelines. The present method involved a simple sample preparation method, which gave consistent as well as reproducible recoveries. The study fulfils the requirement of supporting the possibility to study the full pharmacokinetic profile in individuals. Also, the rationale used for selecting the combination of Bupivacaine and Procaine.

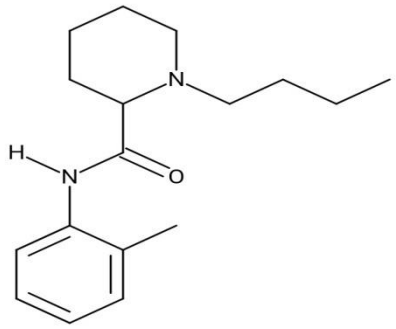

Figure1A: Structure of Bupivacaine

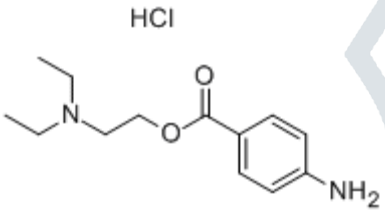

Figure1B: Structural representation of procaine

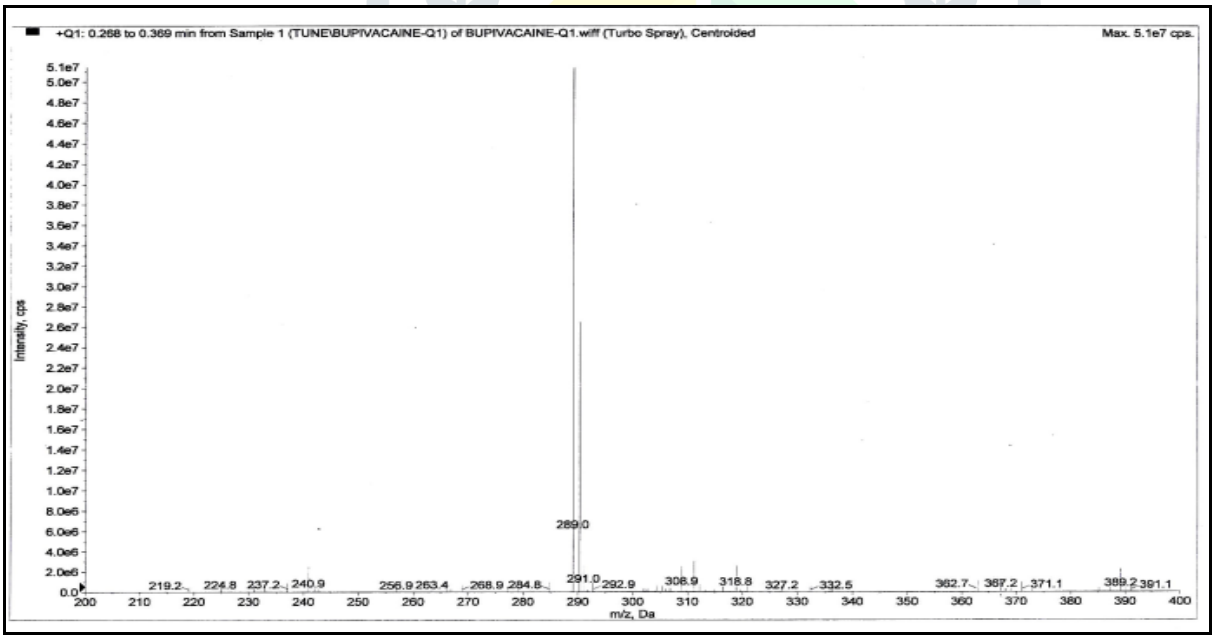

Figure 2 A: Bupivacaine Q1 MS scan tune window showing abundant Parent ion at m/z of 289.0

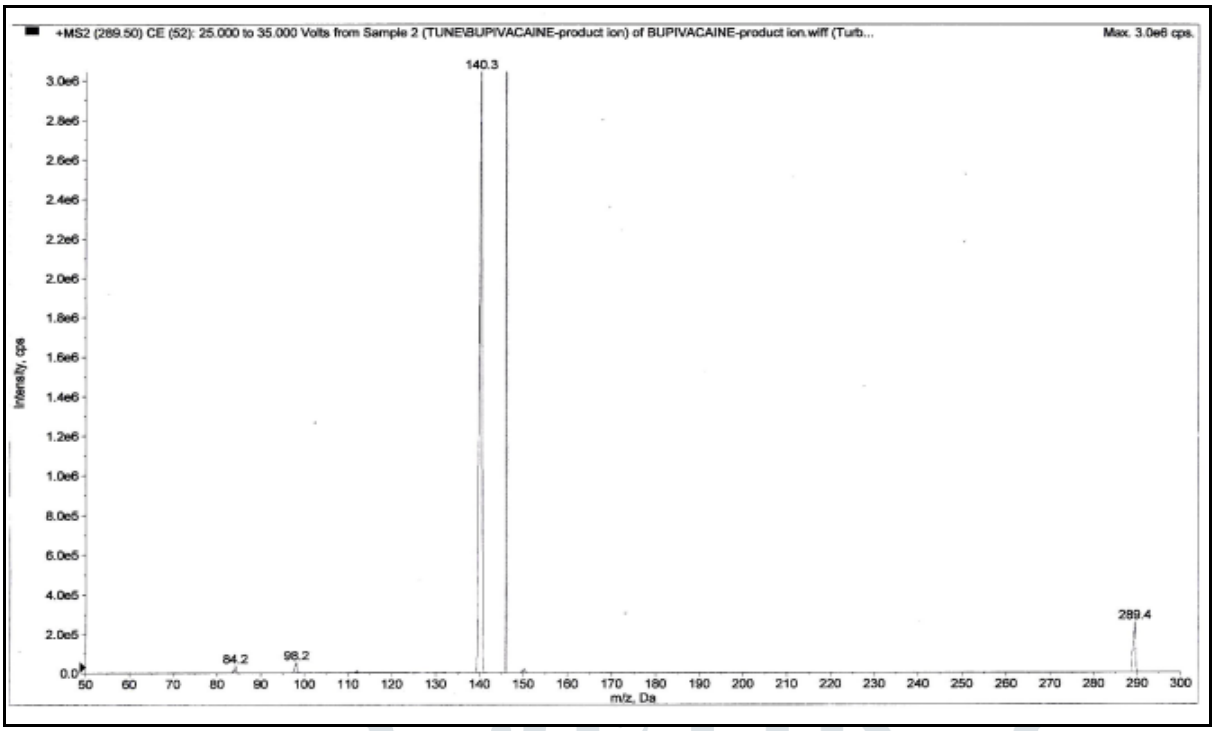

Figure 2B: Product ion mass spectra of Bupivacaine in positive ionization mode

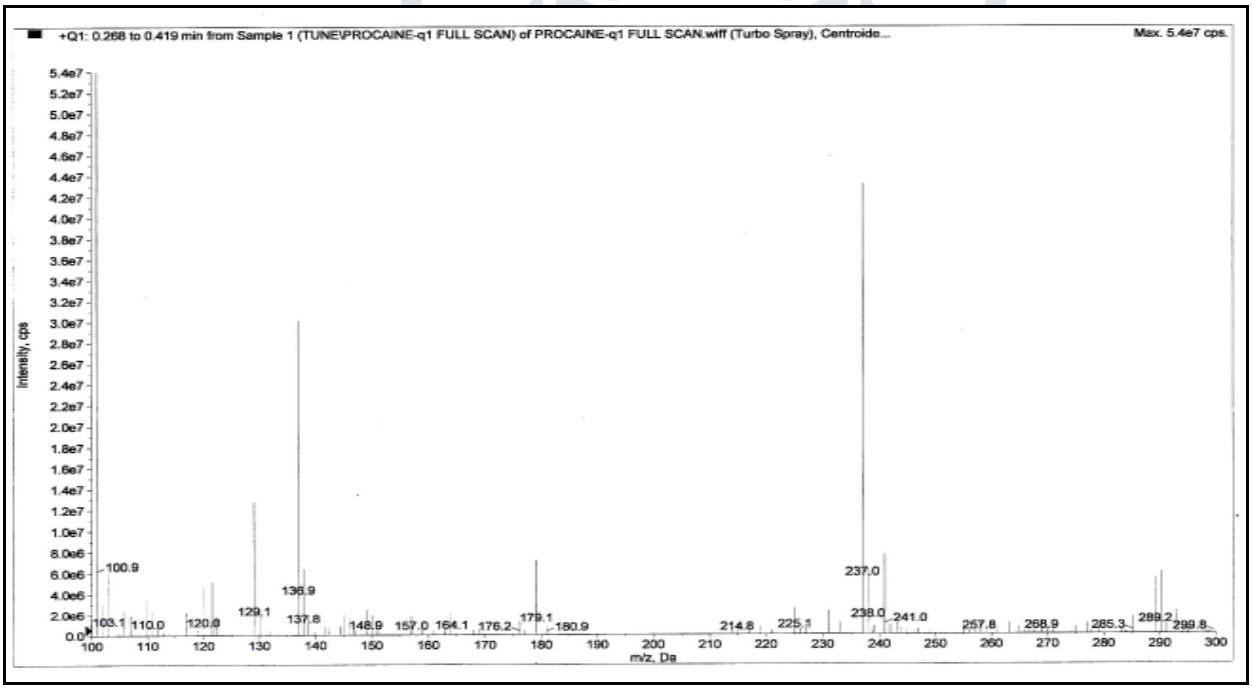

Figure 3A: Procaine Q1 MS scan tune window showing abundant Parent ion at m/z of 237.0

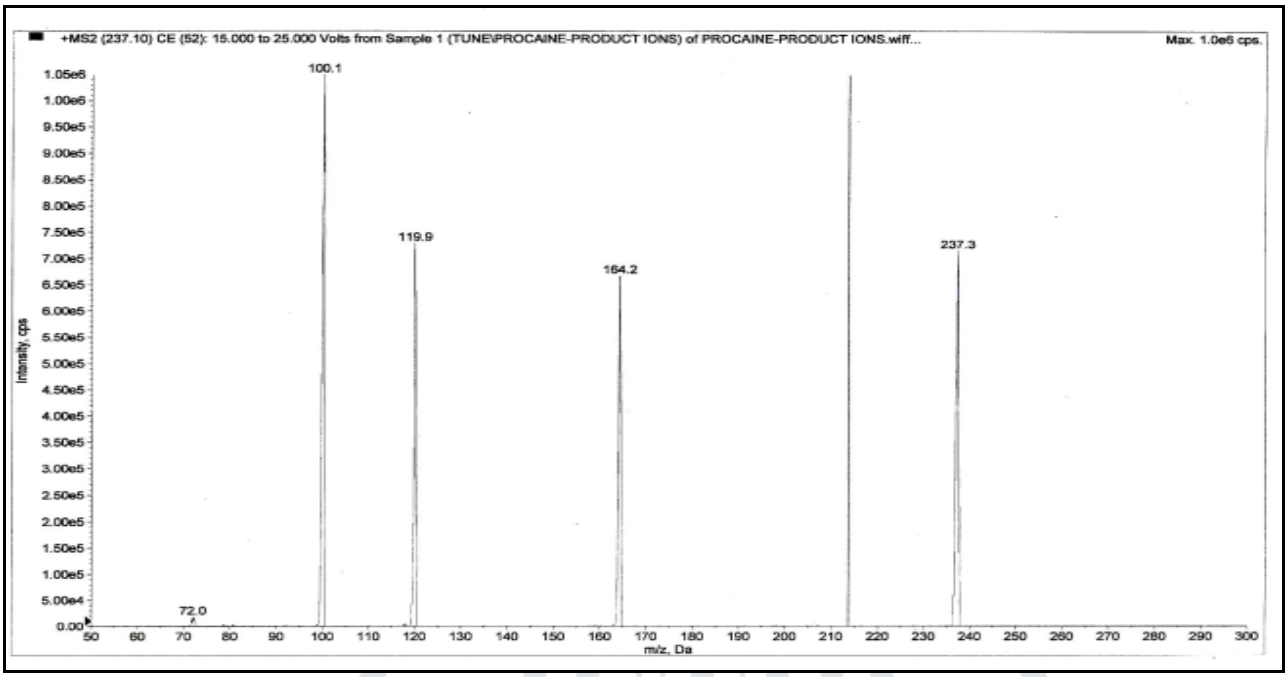

Figure 3B: Product ion mass spectra of Procaine in Positive ionization mode

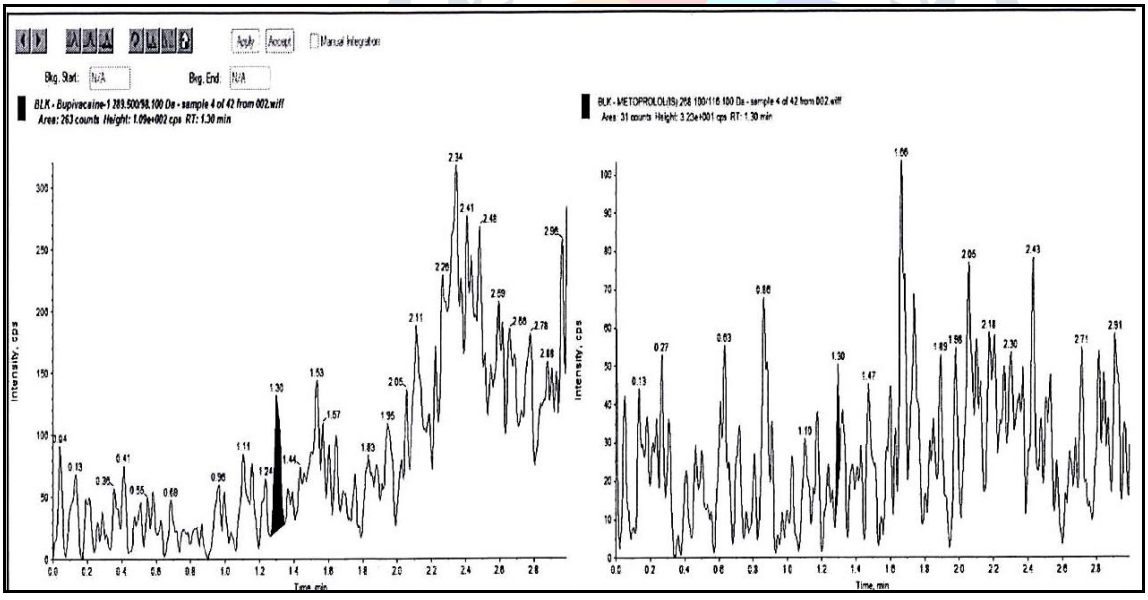

Figure 4A: Representative example of blank chromatogram for Bupivacaine

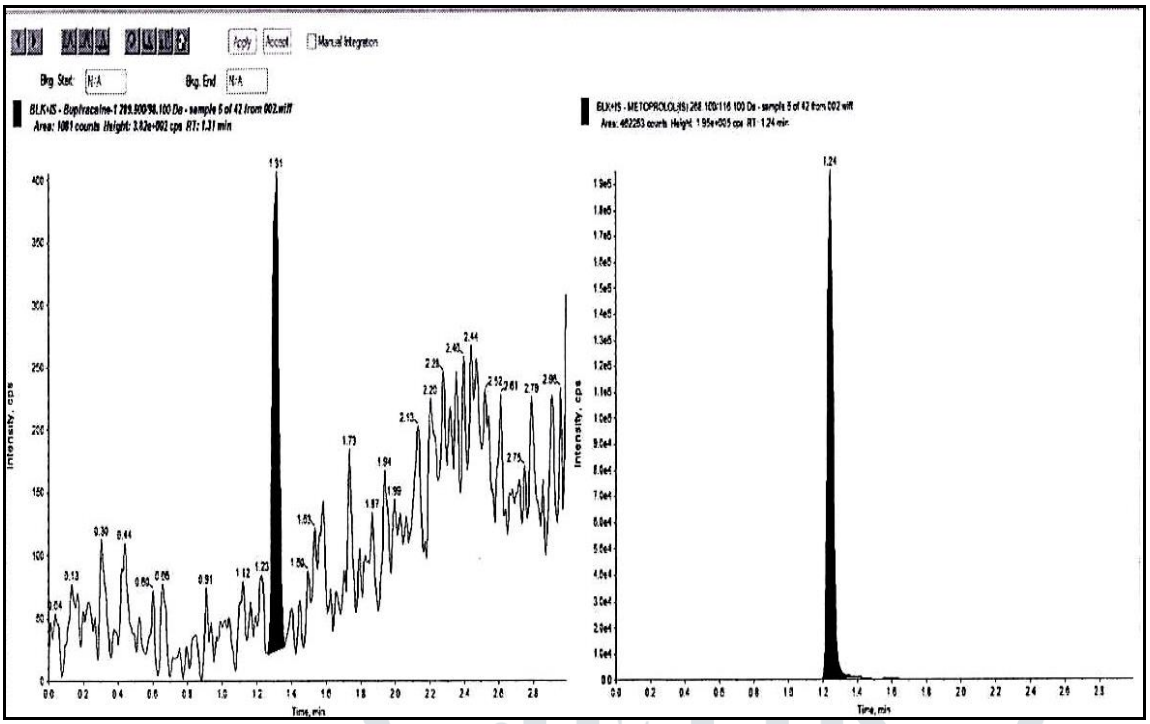

Figure 4B: Representative example of blank +IS chromatogram for Bupivacaine

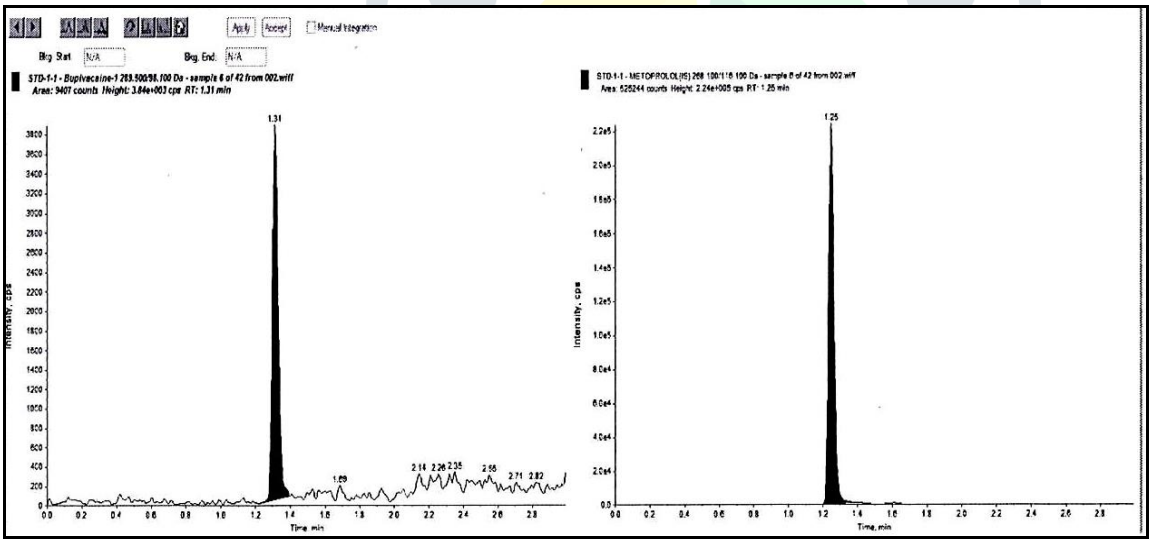

Figure 5: Representative example of LLOQ chromatogram for Bupivacaine

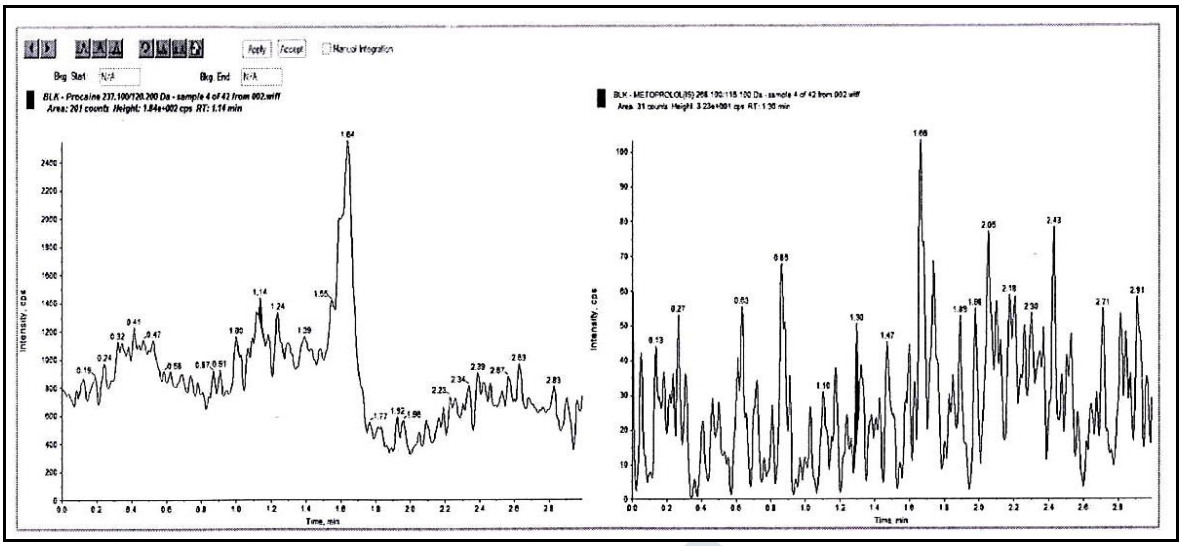

Figure 6: Representative example of blank chromatogram for Procaine

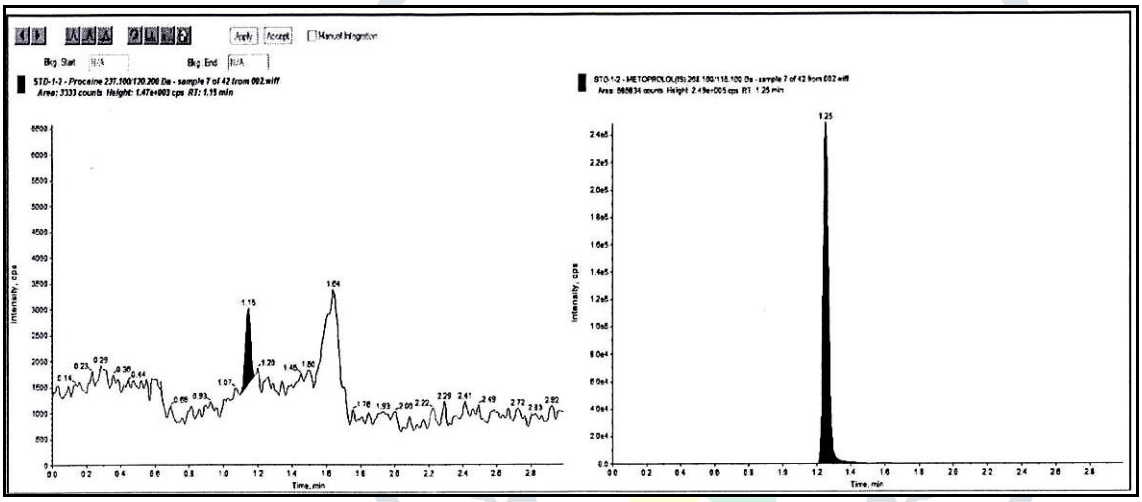

Figure 7: Representative example of LLOQ chromatogram for Procaine

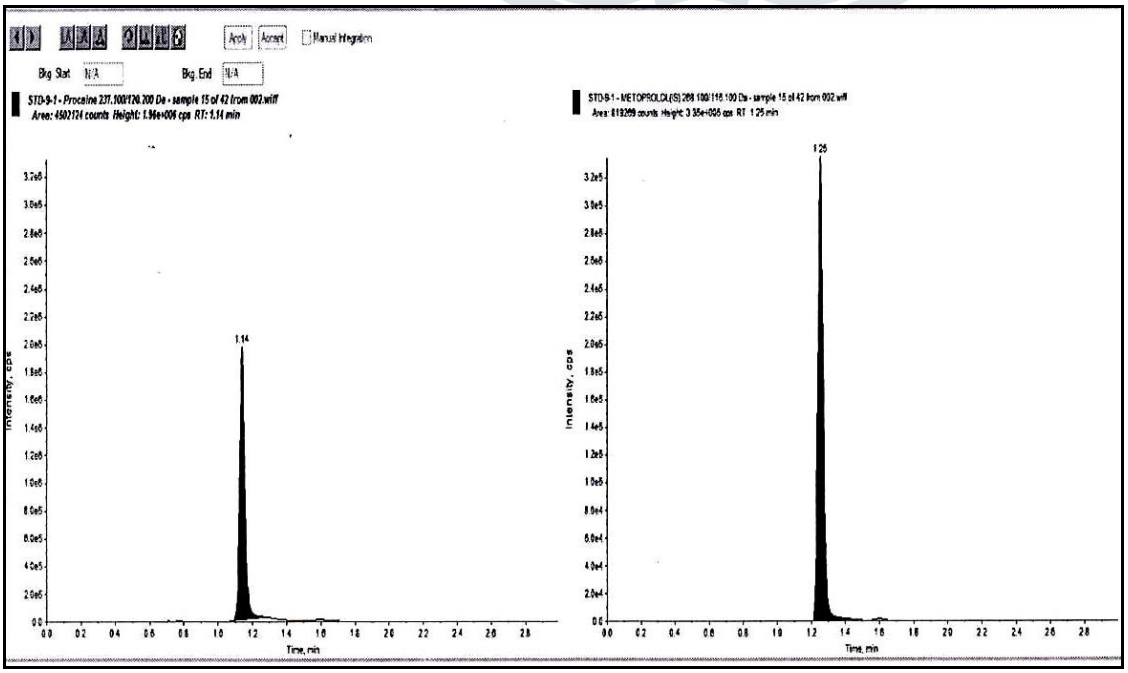

Figure 8: Representative example of ULOQ chromatogram for Procaine

Table 1: Summary of MRM transition conditions:

Compound	Mode of ionization	Q_1 mass (m/z)	Q_3 mass (m/z)	DP	EP	CE	CXP
Bupivacaine	Positive	289.5	140.3	70	10	39	14
Procaine	Positive	237.1	100.1	80	10	24	10

Table 2: Back-calculated standard curve data for Bupivacaine in human plasma

Concentration (ng/mL)							
Std conc.	**Batch-1**	**Batch-2**	**Batch-3**	**Mean**	**SD**	**% CV**	**% Accuracy**
1	0.94	1.00	1.00	0.98	0.035	3.53	98.0
2	1.96	2.01	2.03	2.00	0.036	1.80	100.0
10	9.78	9.76	9.77	9.77	0.010	0.10	97.7
50.01	51.05	50.67	50.86	50.86	0.190	0.37	101.7
200.03	217.39	215.51	215.10	216.00	1.221	0.57	108.0
500.06	491.02	486.71	485.74	487.82	2.811	0.58	97.6
800.1	862.49	854.86	853.13	856.83	4.980	0.58	107.1
900	851.03	888.39	841.80	860.41	24.670	2.87	95.6
1000	959.14	950.65	948.72	952.84	5.543	0.58	95.3

Table 3: Back-calculated standard curve data for procaine in human plasma

Concentration (ng/mL)							
Std conc.	**Batch-1**	**Batch-2**	**Batch-3**	**Mean**	**SD**	**% CV**	**% Accuracy**
1	1.00	0.96	0.97	0.98	0.021	2.13	97.7
2	1.90	2.06	2.05	2.00	0.090	4.47	100.2
10	11.77	11.74	11.55	11.69	0.119	1.02	116.9
50.01	57.62	56.75	55.71	56.69	0.956	1.69	113.4
200.03	225.88	221.88	217.77	221.84	4.055	1.83	110.9
500.06	474.81	466.19	457.54	466.18	8.635	1.85	93.2

800.1	794.61	793.17	778.33	788.70	9.012	1.14	98.6
900	882.34	858.19	876.21	872.25	12.553	1.44	96.9
1000	936.18	970.12	960.75	955.68	17.528	1.83	95.6

Table 4: Intra- and inter-run precision and accuracy for bupivacaine in human plasma

		Concentration (ng/mL)		
		LQC (4.2)	**MQC (500)**	**HQC (900)**
BATCH-1 (N=6)	Intra-run mean	4.06	449.58	830.53
	Intra-run SD	0.31	24.38	33.03
	Intra-run % CV	7.55	5.42	3.98
	Intra-run % Accuracy	97	90	92
BATCH-2 (N=6)	Intra-run mean	4.49	544.04	957.31
	Intra-run SD	0.17	28.91	35.83
	Intra-run % CV	3.79	5.31	3.74
	Intra-run % Accuracy	107	109	106
BATCH-3 (N=6)	Intra-run mean	4.47	539.21	990.40
	Intra-run SD	0.18	28.60	47.94
	Intra-run % CV	4.02	5.30	4.84
	Intra-run % Accuracy	106	108	110
INTER-BATCH (N=18)	Inter-run mean	4.30	487.27	878.80
	Inter-run SD	0.12	22.6	53.45
	Inter-run % CV	2.79	4.63	6.0
	Inter-run % Accuracy	102	97	98

Table 5: Intra- and inter-run precision and accuracy for procaine in human plasma

		Concentration (ng/mL)		
		LQC (4.2)	**MQC (500)**	**HQC (900)**
BATCH-1 (N=6)	Intra-run mean	4.55	538.13	992.91
	Intra-run SD	0.17	28.54	51.29
	Intra-run % CV	3.70	5.30	5.17
	Intra-run % Accuracy	108	107	110
BATCH-2 (N=6)	Intra-run mean	4.17	441.74	888.24
	Intra-run SD	0.31	23.76	38.04
	Intra-run % CV	7.43	5.38	4.28
	Intra-run % Accuracy	99	88	89
BATCH-3 (N=6)	Intra-run mean	4.11	433.53	842.53
	Intra-run SD	0.30	23.32	33.78
	Intra-run % CV	7.41	5.38	4.01

	Intra-run % Accuracy	98	87	94
INTER-BATCH (N=18)	Inter-run mean	3.97	458.91	885.63
	Inter-run SD	0.26	28.93	42.67
	Inter-run % CV	6.5	6.3	4.81
	Inter-run % Accuracy	95	92	98

Table 6: Recovery data of Bupivacaine and Procaine

Analyte	Concentration			AVG. RECOVERY	SD	% CV
	LQC	MQC	HQC			
Bupivacaine	73.36	74.74	72.32	73.47	1.22	1.66
Procaine	98.81	100.70	100.04	99.85	0.96	0.96

Table 7: Stability data showing the % CV of analyte:

Analyte name	Fresh samples	Freeze-thaw stability samples	Auto-sampler stability samples	Bench-top stability samples
Bupivacaine				
LQC	8.77	13.28	12.26	5.39
MQC	4.87	11.81	4.36	8.97
HQC	4.76	5.99	2.96	11.21
Procaine				
LQC	5.75	12.26	11.98	6.93
MQC	6.05	5.21	5.48	7.52
HQC	4.48	2.57	3.08	3.02

ACKNOWLEDGMENTS

The authors are mostly thankful to the principal, Sree Kalahastheeswara Institute of Technology, Chittoor, Andhra Pradesh for providing necessary facilities to carry out the work.

5.0 REFERENCES.

1. Daryanavard, S.M.; Jeppsson-Dadoun, A.; Andersson, L.I.; Hashemi, M.; Colmsjo, A.; Abdel-Rehim, M.; Molecularly imprinted polymer in microextraction by packed sorbent for the simultaneous determination of local anesthetics: lidocaine, ropivacaine, mepivacaine and bupivacaine in plasma and urine samples. *Biomedical Chromatography.* **2013**, *27*, 1481-1488.
2. Kondo, Y.; Hirose, N.; Sakatani, K.; Maeda, T.; Kato, J.; Ogawa, S.; Katayama, Y.; Effect of Spinal Anesthesia for Elective Cesarean Section on Cerebral Blood Oxygenation Changes: Comparison of Hyperbaric and Isobaric Bupivacaine. *Advances in Experimental Medicine and Biology.* **2012**, *765*,109-114.
3. Yilmaz, M.; Celebi, H.; Akcali, D.; Gurel, N.; Pre-treatment of bupivacaine-induced cardiovascular depression using different lipid formulations of propofol. *An International Journal of anaesthesiology, Intensive care, pain, and critical emergency medicine.* **2014**, *58*, 298-302.

4. Kronenberg, R.M.; Ludin, S.M.; Fischer, L.; Severe case of Chronic Pelvic Pain Syndrome: Recovery after Injection of Procaine into the Vesicoprostatic Plexus- Case Report and Discussion of Pathophysiology and Mechanisms of Action. *Hindawi*, **2018,** 1-5.
5. Murello, I.; Costa, J.; Salva, P.; Determination of Bupivacaine in Human Plasma by HPLC, *Journal of liquid chromatography*. **2007**,*16*,3509-3514.
6. Qin, W.W.; Jiao, Z.; Zhong, M.K.; Shi, X.J.; Zhang, J.; Li, Z.D.; Cui, X.Y.; Simultaneous determination of procaine, lidocaine, ropivacaine, tetracaine and bupivacaine in human plasma by high-performance liquid chromatography. *Journal of Chromatography B*. **2010**, *878*, 1185-1189.
7. Wael Abu Dayyih; Enas Alkhader; Eyad Mallah; Tawiq Arafat; Development and validation of a stability- Indicating HPLC method for Determination of Bupivacaine in Human Plasma. *International journal of pharmaceutical analysis*. **2013**, *38*, 1134-1135.
8. Hans Onnerud; Fatma Bassiouny; Mohamed Abdel-Rehi; Chromatographic Behavior of Bupivacaine and five of its Major Metabolites in Human Plasma, Utilizing Solid-Phase Extraction and Capillary Gas Chromatography. *Journal of Chromatographic Science*. **2010**, *48*, 294-298.
9. Jipei Yuan, Jianyuan Yin, Erkang Wang. Characterization of procaine metabolism as probe for the butyrylcholinesterase enzyme investigation by simultaneous determination of procaine and its metabolite using capillary electrophoresis with electrochemiluminescence detection. Journal of chromatography A. 2007; 1154(1-2):368-372.
10. Dhananjeyan, M.R.; Trendel, J.A.; Bykowski, C.; Sarver, J.G.; Ando, H.; Erhardt P.W.; Rapid and sensitive HPLC assay for simultaneous determination of procaine and para-aminobenzoic acid from human and rat liver tissue extracts. *Journal of Chromatography B*. **2008**, *867*, 247-252.
11. Yu Ting He, Jing Dong Peng, Jin Xia Tang and Cheng Zhang. Incorporation of high performance liquid chromatography with resonance Rayleigh scattering detection for determination of procaine and lidocaine in human plasma. *Analytical Methods*. **2013,** *5*, 7110-7116.
12. Dhananjeyan, M.R.; Bykowski, C.; Trendel, J.A.; Sarver, J.G.; Ando, H.; Erhardt, P.W.; Simultaneous determination of procaine and para-aminobenzoic acid by LC-MS/MS method. *Journal of chromatography B*. **2007**,*847*,224-230.
13. Willard Merrits, Dean and wittle, "Instrumental Methods of Analysis" 8th Edition.