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NOVEL METHOD FOR QUANTIFICATION OF MORPHINE IN HUMAN PLASMA USING LC-MS/MS

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Abstract
Liquid chromatography - tandem mass spectrometry method was developed for the determination of morphine in human plasma. The drug was extracted from plasma by using protein precipitation method and subjected to LC-MS/MS analysis. The separation was performed on a HILIC column (Zwitterions hydrophilic interaction liquid chromatography) in acetonitrile, ammonium acetate buffer, pH 3.5, using a flow rate of 0.5 ml/min. The chromatographic run time was 5 min per injection, with retention time was 1.8min for morphine and 1.5 min for IS. The standard curve shows linearity for plasma of concentration which ranges from 3.75 – 125 ng/ml. The limit of quantification was: 0.93 ng/ml for morphine. The recovery ranged from 95 to 100% for analyte. The developed method may be very selective and can be used for the routine determination of opiates in body fluid.

Introduction

Morphine is one of the purified constituent of the opium poppy coming under the family of papaver somniferum. Morphine is mainly used for the treatment of cancer pain. Cancer pain related to the pharmacological measures. There are several types of opioid receptors such as delta; kappa and mu have been identified at various sites in the nervous system and other tissue. Morphine is mainly combining with mu (μ) receptor and reducing the perception of pain. Commonly drug metabolized by two ways that is phase I and phase II reaction. In phase I reaction the function group is added or generated in molecules through hydrolysis, oxidation and reduction. The phase II reactions are detoxification reaction; in this the molecule is conjugated with some endogenous species by glucuronic acid conjugation. The other phase II reactions are sulphation, methylation and acetylations.The metabolism of morphine has been studied in animals and humans. The latter is considered to the analgesic effect of morphine, especially when repeated doses are given by mouth (1, 2, and 3). It binds to opioid receptors in the brain and central nervous system (CNS), reducing the perception of pain as well as the emotional response to pain.Among opioids, morphine is relatively hydrophilic and its glucuronide metabolites are even more so (4). Some animal experiments show that M6G is a potent opioid agonist with the potential to contribute to the analgesic response after morphine administration (5, 6, 7). M3G, on the other hand, has been found to not elicit antinociceptive effects (6,7) because M3G does not have significant affinity for opioid receptor and has generally been considered to be an inactive metabolite and also this metabolite has been shown to functionally antagonize the antinociceptive effects of morphine and M6G (8,9). It was recently suggested that the analgesic response to morphine in pain patient would depend1 on the M3G/M6G ratio obtained and that paradoxical pain would appear when the metabolites were the wrong ratio (10). Morphine produces a number of well characterized undesirable side effects that include respiratory depression, sedation, dysphoria, decreased gastric motility, nausea, vomiting. Some clinical reports indicate that large doses of morphine and other opioid analgesic may produce altered pain behaviors such as hyper analgesia and allodynia (10).Morphine is routinely used as a reference compound in drug discovery programs targeting new analgesics. In order to draw pharmacokinetic/pharmacodynamic (PK/PD) relationships and/ or understand the impact of various disease states on PK, it is of interest to measure plasma concentrations of both M and its metabolites. Consequently fast, easy and reliable analytical methods are required to ensure adequate turnaround times and high sensitivity. Various methods have been developed for the simultaneous determination of M and its metabolites in biological fluids. Opioid analgesics have been successfully analyzed by immunoassay (11).High performance liquid chromatography (HPLC) methods are widely used, and allow simultaneous analysis of MOR and its glucuronides detected by ultraviolet (UV) detection (16), fluorescence (FD) detection (12,13), combined electrochemical detection (ECD)-UV detection (19), and combined ECD-FD detection (14,15), UV and FD detection were not sensitive enough for Morphine and its glucuronide in biological samples, and these compounds could be detected by ECD but combined ECD chromatographic consume long time to determine.HPLC with pre-or-post column derivatization of the analytes has also been used (16,17). Such method can require long incubation times and do not detect any metabolites without the phenolic hydroxyl group or primary amine, for example M3G and C. More complex methods, such as gas chromatography-mass spectrometry (GC-MS) (18) have also been used. Such techniques may give unequivocal results, but are extremely expensive and hence are not commonly available. The above said methods are high cost and the sample preparation methods were very lengthy procedure. In the present study, we developed a novel method for identification and quantification of morphine in human plasma.

Materials and Methodology

Preparation of Internal Standard solution (IS dilution)

Preparation of Internal Standard stock solution Homotropine (1mg) was weighed accurately and transferred into a 1 ml of volumetric flask. The compound was dissolved in sufficient water and the volume was made with the same to produce of 1 mg/ 1ml and stored at 20º C.

Preparation of Internal Standard stock dilution

Prior to spiking, stock dilution (IS dilution) of the internal standard stock solution, was prepared using water as diluents, as described in Table: 1

Table: 1 Preparation of Internal Standard stock dilution

Stock Conc. (ng/ml)	Stock Aliquot (mL)	Diluent Added (mL)	Total Volume (mL)	IS Dilution Conc. (ng / mL)
1000,000	0.1	0.9	1	10,0000
10,000	0.1	0.9	1	10000
5000	0.5	0.5	1	2500

Preparation of calibration curve (CC) standards

Preparation of standard stock solutions

Morphine sulphate working standard (10mg) was weighed accurately and transferred to a 10-ml volumetric flask. The compound was dissolved in sufficient Milli Q water and volume was made up with the same to produce a 1 mg /ml solution. The final concentration was corrected potency and the actual amount weighed. The solution was stored at 20º C.

Preparation of standard stock dilution for CC

Prior to spiking, stock dilutions of the compound in the concentration range of 0.93 to 120 ng/mL, using dilution solution as Diluent were prepared, as described in Table 2.

Table: 2 Preparation of standard stock dilutions for CC

Stock Conc.zng/ml)	Stock Aliquot(µl)	Diluent Added(µL)	Total Volume(mL)	Stock Dilution Conc. (ng / mL)	Stock Diluent ID
1000000	120	880	1	120	STD 006
120	500	500	1	60	STD 007
60	500	500	1	30	STD008
30	500	500	1	15	STD009
15	500	500	1	7.5	STD010
7.5	500	500	1	3.75	STD011
3.75	500	500	1	1.875	STD012
1.875	500	500	1	0.93	STD013

Preparation of spiked CC Standards

The above mentioned standard stock dilutions were spiked to the required volume of plasma in order to achieve the concentration range of 3.75 to 120 ng/ml, as described in Table: 3

Table: 3 preparations of spiked CC standards

Stock DilutionID	Stock Dilution Conc.ng / mL	Stock Dilution Aliquot(µL)	Plasma/Extraction Solution(µl)	Total Volume (ml)	Spiked CC Standard Conc.ng/ml
Sample002	1000	120	300	0.52	120
Sample003	120	250	300	0.55	60
Sample004	60	250	300	0.55	30
Sample005	30	250	300	0.55	15
Sample006	15	250	300	0.55	7.5
Sample007	7.5	250	300	0.55	3.75

Preparation of Quality Control (QC) sample

Preparation of spiked QC samples

Spiked QC samples required to achieve the concentration range of 0.93 to 120 ng/ml, as described in Table: 4 The blank plasma MOR levels were added in MQC and HQC concentration.

Table 4: Preparation of spiked QC samples

Stock DilutionID	Stock Dilution Conc.ng / mL	Stock Dilution Aliquot(µL)	Plasma/Extraction Solution(µl)	Total Volume (ml)	Spiked CC Standard Conc.ng/ml
Sample003	1000	120	300	0.52	120
Sample048	60	250	300	0.55	30
Sample066	7.5	250	300	0.55	3.75
LLOQ QC	3.75	250	300	0.55	0.93

Note: The LLQC QC and LQC sample were spiked in plasma and LLQC QC was used only for the precision and accuracy during method validation.

Sample Preparation

One set of plasma / water calibration curve standards; one or more sets of quality control samples and plasma samples to be analyzed were withdrawn from the deep freezer and allowed to thaw a room temperature or in a water bath maintained at room temperature. The thawed samples were vortexed to ensure complete mixing of contents. 100 µl of samples was pipetted into a 1ml polypropylene tube, 10 µl of 250 ng/ml internal standard dilution added (expect in blank) and vortexed. 200µl of extraction solution was added and vortexed for 1 min, followed by centrifugation for 5 min at 5000 rpm. The supernatant was separated and transferred to vials for analysis.

Chromatographic Conditions

A summary of the chromatographic and mass spectrometric conditions are as follows:

Column HILIC HPLC column (4.6 mm × 50 mm)
Mobile Phase : 5 mM Ammonium acetate: ACN (70: 30)
Flow rate : 0.5 mL/ minute
Detection : MOR m/z - 286.1 (parent) and 152.1 (product)
Homotropine m/z- 276.1 (parent) and 142.1 (product)
Ion source : Atmospheric pressure chemical ionization (APCI)

State file information

Polarity : Positive
IS (ion spray Voltage) : 70
TEM (temperature) : 350
CUR (curtain gas) : Nitrogen
GS 1 : 3.5 kV
GS 2 : 35 V
CAD (collision gas) : Nitrogen
CE (collision energy) : -48 eV
Sample cooler temperature :- 20º C
Injection volume : 20 µl
Retention times : Morphine – 1.8 to 1.92 minutes
IS – 1.54 to 1.63 minutes

Results and Discussion

A high performance liquid chromatographic tandem mass spectrometric method for the estimation of Morphine in human plasma has been developed and validated as per principles of ICH guidelines. The plasma method was validated over a concentration range of 0.93 ng /ml to 120 ng /ml. Sample clean up was accomplished by protein precipitation method. The reconstituted samples were analysed by LC-MS/MS using HILIC column (4.6 mm × 50 mm). The results of all experiments along with all supporting documentation are given below.

Method Development
Various radioimmunoassay and GC-MS methods have been described for MOR estimation in plasma, but very few methods have reported on LC-MS/MS. The main challenges in developing and validating a method for determining MOR in human plasma are that MOR is a polar, low molecular mass (MW 286), endogenous compound which circulates at trace levels. In the current study, a reverse phase HPLC method with mass-spectrometric detection using homotropine as an IS was developed.

Mobile Phase and Flow Rate
Various approaches using combination of organic and aqueous phase were tried. The response was optimized using different proportion of 0.1 % of formic acid; acetonitrile / 5 mM ammonium acetate in water pH 3-4 (buffer). Acetonitrile lowered the baseline to a great extent than methanol. The optimum proportion of acetonitrile: water and 0.1% formic acid was 70:30. For better chromatography and to lower baseline further, buffer concentration was reduced to half (5 mM) and aqueous phase was increased in the mobile phase. The final mobile phase used was buffer: acetonitrile: 70:30.Response was evaluated from 0.2 to 0.5 ml/min flow rate. Gradient flow was used to improve peak resolution in plasma, but better results were obtained using more aqueous phase. To adjust the run time and peak shape, the flow rate was optimized to 0.5 ml/min.

Column
A variety of columns were tried in order to optimize the response.Zorbax C-18 (2.0 mm x 5mm), RP select B (4.6 mm x 10mm) and ZIC HILIC column (4.6mm × 50 mm) were tried. Better peak shape and maximum reproducibility in response was observed in Zic hilic (4.6mm × 50 mm) column.

Extraction procedure
Both liquid-liquid and solid phase extraction, protein precipitation prodedures were assessed initially for this purpose. Liquid-liquid extraction was carried out using different solvents. The equilibrated mixture of drug and IS was extracted with tertiary butyl methyl ether, ethyl acetate, dichloromethane. The extraction was carried out twice with 5 ml of solvent following a lengthy procedure (Overtaxing, centrifuging, drying reconstitution). The both liquid-liquid and solid phase extraction procedure was lengthy and low recovery.

In order to further improve recovery and sample cleanup, protein precipitation was tried. This method was alternative approach of both liquid-liquid and solid phase extraction. In this method used only small quantity of plasma samples (100µl), less time consuming procedure (2 to 3 min) and also recovery is 100 %.

System Suitability
System was evaluated for reproducibility by injecting six replicates of MQC dilution and working concentration of IS. The coefficient of variation obtained was 1.08%.The results obtained are given in Table 5. System was suitable for the determination of morphine because the co-efficient variation was less than 15 % for the analyte.

Table 5: System suitability for the determination of morphine in human plasma

	MOR		IS		Area ratio
S/NO	Area	RT (Min)	Area	RT (Min)	MOR/IS
1	1330	1.82	3650	1.59	0.3454
2	1788	1.89	3889	1.58	0.4597
3	1996	1.85	4011	1.6	0.4976
4	1894	1.87	3885	1.61	0.4875
5	1829	1.86	4342	1.54	0.4212
6	2134	1.90	4567	1.57	0.4072
Mean		1.86		1.58	0.44
SD		0.02		0.02	0.05
%CV		1.08		1.2	11.3

Chromatography
Representative chromatograms of aqueous mixture of morphine with IS, blank, blank with internal standard , blank plasma with IS LQC, MQC and HQC samples in human plasma are given in Figure 1 to 3.

In reverse-phase HPLC separation, a mobile phase of low organic content is required to retain the analytes on the column. Chromatographic separation of the analytes from the major matrix materials as indicated by the retention on the column, is also important to alleviate severe matrix suppression. A mobile phase of high aqueous/low organic components should therefore be used for reverse-phase LC-MS-MS in order to retain these analytes on the column. How Eer, mobile phase with little organic modifier adversely affect sensitivity, due to the poor spray condition that results from high surface tension of the aqueous droplets. This is especially true when a conventional flow-rate and turbo ESI interface is used. The mobile phase of high Oganic content and acidic pH was required to produce good spraying condition and sensitivity for MS.However, this kind of mobile phase in reversed-phase chromatography would not provide the column retention and resolution, which are needed to avoid matrix suppression. Instead of reversed-phase chromatography, we used normal-phase LC-MS-MS for the analysis of Morphine.

Normal-phase LC (NPLC) uses a stationary phase that is relatively more polar than the mobile phase. The mobile phase consists of mainly acetonitrile. A substantial amount of water and formic acid was used to improve the analyte ionization and peak shape.

Method Validation (ICH Guidelines)

Matrix Effect

The matrix effect was evaluated by comparing aqueous response against spiked processed blanks at all QC sample concentrations. There was matrix effect observed in the analysis. The area of analyte and IS was used for matrix effect calculations. Percent matrix effect was 51.8 for anayte .The results obtained are given in Table 6.

Table 6: Matrix effect for the determination of morphine in human plasma

LQC			MQC		HQC	
Area response			Area response		Area response	
	MOR	IS	MOR	IS	MOR	IS
1	136	14400	5190	16800	12700	16200
2	143	14233	4340	16842	12652	16358
3	152	15593	4876	17523	13221	15865
Mean	143.66	14742	4802	17055	12857.6	16072
SD	7.17	663.39	384.42	362.99	282.2	328.3
%CV	4.9	4.5	8.01	2.1	2.2	2.04
% Matrix effect	40.5		55.98		59.09	
Mean % matrix effect	51.85					

Matrix effect was determined by comparing spiked processed blank plasma against same aqueous concentration for morphine. Significant matrix effect was observed. The low recovery is attributable to matrix effect. Plasma samples (n=50) were analyzed and there was no significant change in internal standard recovery. During method development three different plasma samples were spiked so as to add 10 ng/mL to endogenous morphine. The response observed in spiked plasma was proportionately increased compared to that in blank plasma concentrations and similar results were seen with QC samples. This observation implies that matrix effect is due to the presence of some constant endogenous substance other morphine or any reagent effect during sample processing. Hence, the method can be used to obtain accurate plasma morphine concentrations.

Sensitivity

The limit of quantification was 0.93ng /ml for morphine. HPLC-MS/MS in the MRM mode provide a good selectivity and sensitivity for the determination of compound in the biological samples. The representative MRM mode of the spiked plasma samples are shown in Fig 1 to 3.

No endogenous sources of interference were observed at the retention time of analyte obtained from the blank plasma.

Linearity

The linearity of the method was determined by a weighed least square regression analysis of eight point standard curve. The calibration lines were shown to be linear from 3.75 ng/ml to 120ng/ml figure (5). Best fit calibration lines of ratio of MOR to

IS peak area versus concentration of calibration standards were determined by least square regression analysis. The r^2 were consistently > 0.99 during the course of validation. The obtained results are given in Table 7.

Table 7: Concentration – response linearity data for the determination of morphine in human plasma

Concentration ng/ml	Response Area		Mean	SD	% CV	% Accuracy
	CC1	CC2				
0.93	0.00152	RS	0	0		
1.87	0.01335	RS	0	0		
3.75	0.2482	0.00345	0.125825	0.122375	0.98	98
7.5	0.4368	0.0187	0.22775	0.20905	0.90	90
15	0.5879	0.130	0.35895	0.22895	0.62	62
30	0.123	0.308	0.2155	0.0925	0.418	49
60	0.132	0.454	0.293	0.161	0.55	55
120	0.153	0.784	0.4685	0.3155	0.67	67

Note: RS rejected concentration

The calibration line was shown to be linear from 3.75 ng/ml to 120 ng/ml. Best fit calibration line of ratio of MOR to IS peak area versus concentration of calibration standard was determined by least square regression analysis . The r^2 were consistently > 0.99.

Precision and Accuracy

The precision of the assay was measured by the percent coefficient of variation over the concentration range of LLOQ, low, middle and high quality control samples, respectively during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ, low. Middle and high to their respective nominal values, expressed in percent. The precision and accuracy were determined by analyzing drug free plasma samples with known concentration of morphine.

With in –batch precision and accuracy

Within-batch precision ranged from 1.2% to 3.16% and the within batch -accuracy ranged from 88.5% to99.7 %. The results are summarized in Table 8.

Table - 8. With in –batch precision and accuracy for the determination of morphine in human plasma

BATCH	QC-CONC	Number			Mean	S.D	% CV	% Accuracy
		QC1	QC2	QC3				
I	LLOQ	0.916	0.887	0.825	0.876	0.042	4.7	94.2
	LQC	3.56	3.38	2.88	3.27	0.31	9.4	87.2
	MQC	30.02	29.97	31.03	30.34	0.530	1.7	101
	HQC	114.1	117.5	118.2	116.3	1.96	1.68	96.9
II	LLOQ	0.912	0.897	0.815	0.874	0.046	5.2	93.9
	LQC	3.46	3.18	1.98	3.20	0.215	6.7	85.3
	MQC	30.12	29.87	33.13	31.04	1.62	5.2	103.4
	HQC	113.9	119.2	115.8	116.3	2.40	2.06	96.9
II	LLOQ	0.916	0.852	0.890	0.886	0.028	3.16	95.2
	LQC	3.56	3.44	2.98	3.32	0.27	8.1	88.5
	MQC	30.02	29.47	30.3	29.93	0.37	1.2	99.7
	HQC	115.1	117.5	119.2	117.26	1.84	1.5	97.71

Intra – day precision and accuracy

Intra-day precision ranged from 1.2% to8.1% and intra-day accuracy ranged from 88.5% to 99.7%. The results are summarized in Table 9.

Table 9: Intra – day precision and accuracy for the determination of morphine in human plasma

BATCH	QC-CONC	Number			Mean	S.D	% CV	% Accuracy
		QC1	QC2	QC3				
I	LLOQ	0.916	0.887	0.825	0.876	0.042	4.7	94.2
	LQC	3.56	3.38	2.88	3.27	0.31	9.4	87.2
	MQC	30.02	29.97	31.03	30.34	0.530	1.7	101
	HQC	114.1	117.5	118.2	116.3	1.96	1.68	96.9
II	LLOQ	0.914	0.892	0.815	0.875	0.001	0.11	94.1
	LQC	3.51	3.28	2.93	3.23	0.05	1.5	86.13
	MQC	30.07	29.92	32.08	30.69	0.49	1.5	103
	HQC	114.05	118.35	117.0	116.3	0.070	0.1	96.9

Between-batch / inter-day precision and accuracy

Between-batch precision ranged from 0.4% to1.5% and the between-batch accuracy ranged from 87.2% to101 %. The results are summarized in Table 10.

Table 10: Between-batch / inter-day precision and accuracy for the determination of morphine in human plasma

QC-ID	Concentration added	Mean	S.D	%CV	% Accuracy
LLOQ	0.93	0.88	0.01	1.1	94.6
LQC	3.75	3.27	0.05	1.5	87.2
MQC	30	30.31	0.43	1.4	101
HQC	120	116.78	0.55	0.4	97.3

Recovery

The absolute recovery of MOR and internal standard was calculated for replicate spiked quality control samples (LQC, MQC and HQC). Three sets of recovery comparison samples were prepared by adding 100µl of plasma sample having concentration (LQC, MQC and HQC) and 10µl of internal standard (250 ng/ml). All dilutions for recovery of MOR and IS were prepared in reconstituting solution. Results indicate the overall recovery of 99.8% for MOR.The obtained results are given in Table 11.

Table 11: Recovery of morphine from human plasma

Sample concentration	QC-ID			Mean	SD	%CV	% Recovery	% Overall recovery
	QC1	QC2	QC3					
LQC	3.46	3.32	3.56	3.44	0.1	2.9	91.7	
MQC	30.3	33.3	30.15	31.25	1.4	4.5	104	97.4 %
HQC	113.4	116.8	117.52	115.9	1.7	1.5	96.58	

In previously reported methods, recovery was obtained by comparing processed plasma concentrations against spiked processed blank plasma samples. This approach to determine the recovery is not appropriate; because any constant endogenous substance can reduce the response (in presence of matrix effect) and calculated recovery will not be the actual recovery. During validation recovery was calculated by comparing processed plasma concentration of morphine and against same aqueous concentrations of morphine.

Sample Analysis

The concentrations of MOR were determined using the validated method in human plasma samples. A histogram depicting the spread of MOR concentration in human plasma is given Figure 6 and the obtained results are given in Table 12.

Table 12: Concentration of morphine determined by developed method in patient plasma

S. NO	SAMPLE ID	MOR ng/mL
1	Sample 001	58.66
2	Sample 002	37.16
3	Sample 003	38.66
4	Sample 004	11.5
5	Sample 005	42.5
6	Sample 006	10.66
7	Sample 007	99.33
8	Sample 008	82.66
9	Sample 009	23.83
10	Sample 010	17.55

Morphine plasma concentrations were read against IS calibration curve in water. The use of surrogate analyte limits the of bioanalytical software's. Microsoft Excel was used for calculations with IS calibration curve. Therefore the concentration was not reported. Analyst software was used for calculating plasma MOR concentrations in all samples.

The validated analytical method developed in this study was used to establish the baseline value of MOR in human plasma, and the spread in its estimate. Several different batches of blood were used to obtained plasma, which was subsequently frozen. These batches of frozen plasma were analyzed for MOR levels.

Figure 1: Representative chromatogram of aqueous mixture of morphine with internal standard

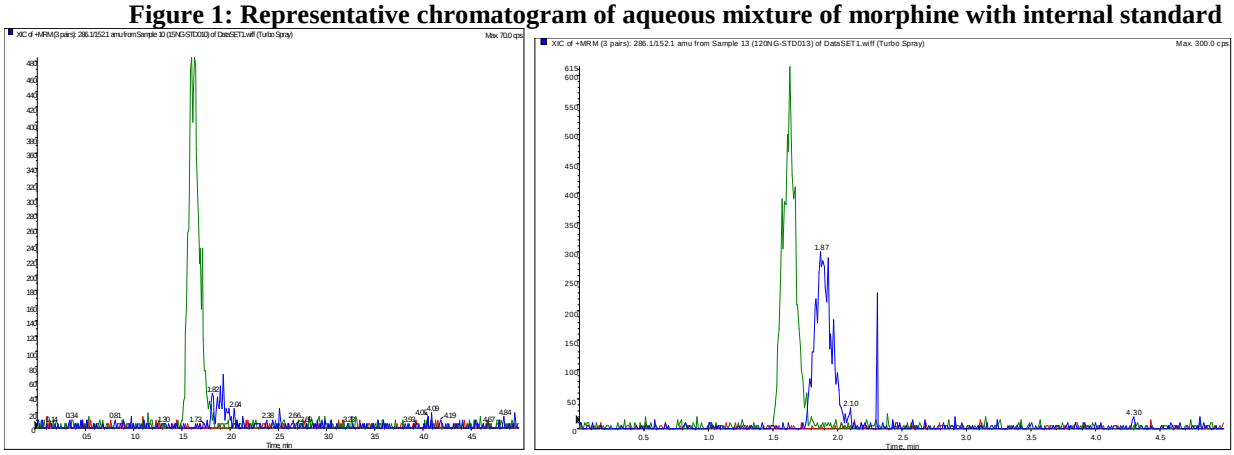


Figure 2: Representative chromatogram of LLOQ and LQC in human plasma

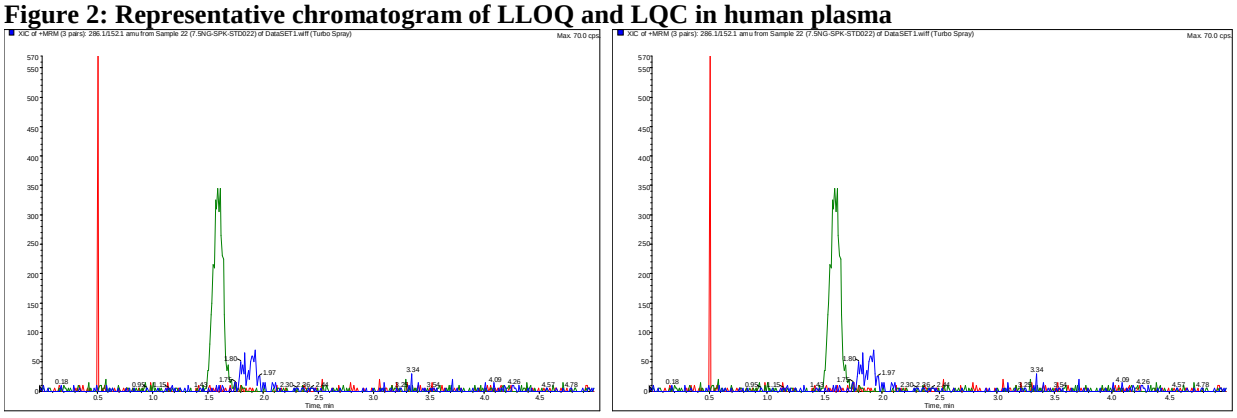


Figure 3: Representative chromatogram of MQC and HQC in human plasma

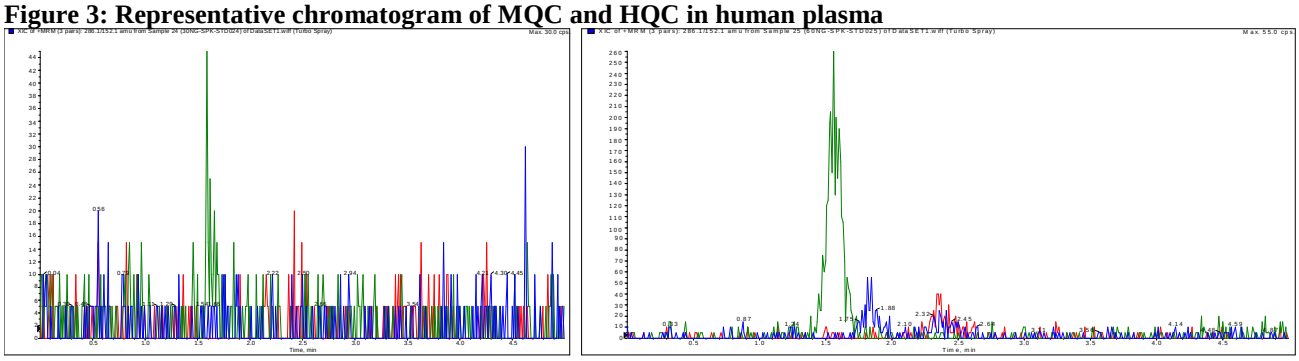


Figure 4: Representative chromatogram of patient plasma samples

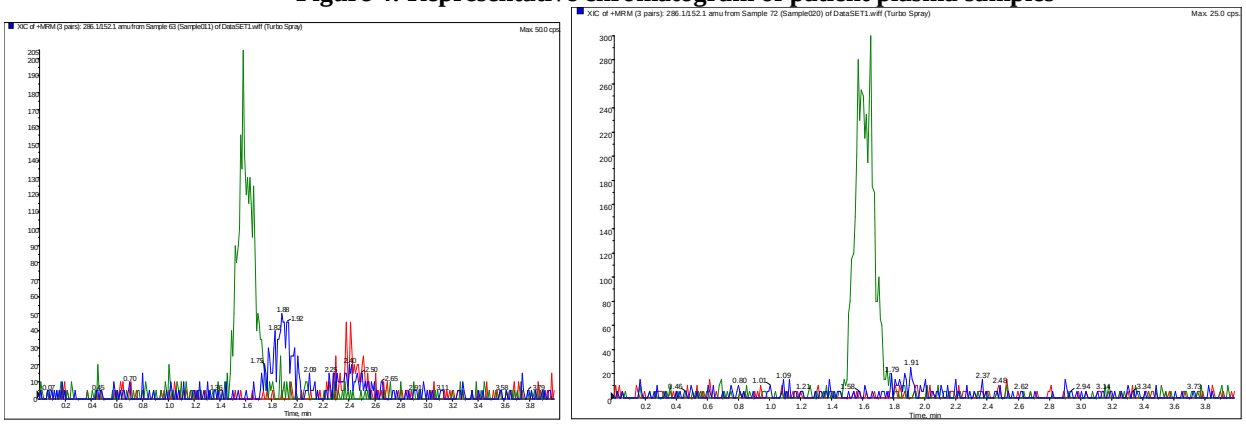


Figure 6: Spiked plasma standard curve for morphine

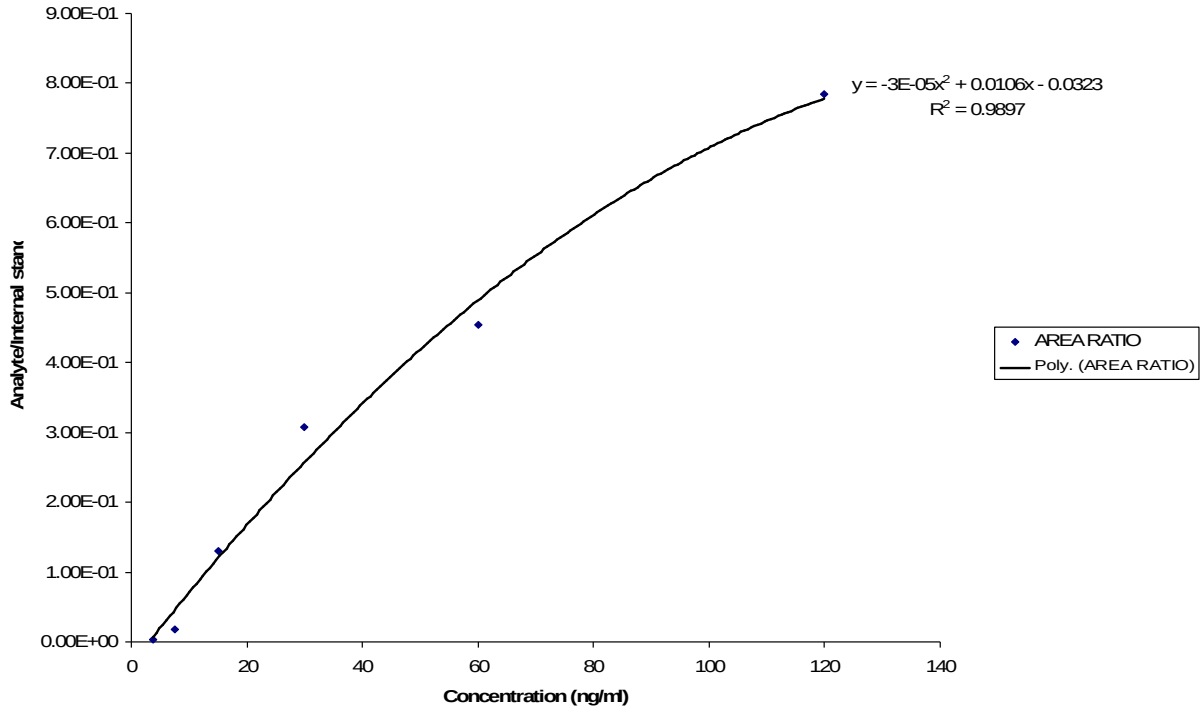
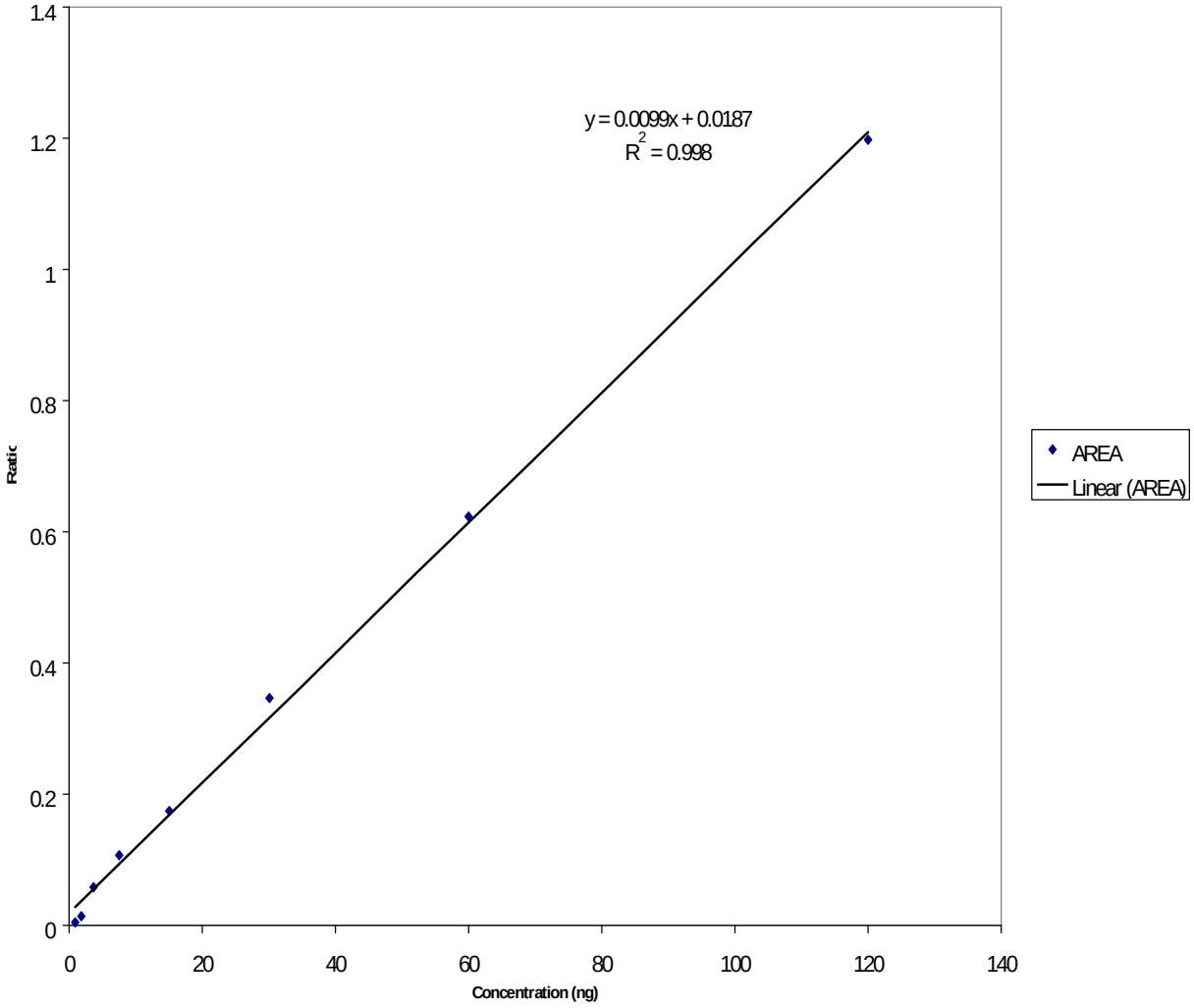


Figure 5: Aqueous mixture of standard curve for morphine



SUMMARY AND CONCULSION

Morphine is an opioid analgesic used for the treatment of moderate to severe pain. Some clinical report indicated that large doses of morphine may produce more side effects such as allodynia, decreased gastric motility and vomiting. In case of low doses of morphine not giving adequate pain relieve in cancer patient. A non-traditional normal-phase LC-MS/MS method was developed for the determination of morphine in human plasma with in a 3 min run time. The separation and quantification were achieved on a HILIC (4.6mm x 50 mm) column using a mobile phase Acetonitrile, 5mM ammonium acetate buffer with 0.1 % formic acid at the flow rate of 0.5 ml /minute. The biological sample was prepared by protein precipitation method. Sensitivities of Multiple reaction modes (MRM) were optimized by with an infusion of 0.93 ng/ml morphine with water. The drying gas (nitrogen) flow rate of the Turbo Ion Spray was 8 ml/ min. The electro spray source was operated with a capillary voltage of 4.5 KV, an orifice voltage of 35 V, and a source temperature of 350º C. The preselected protonated precursor ion masses, which passed the first MS, went into the collision cell. The fragmentation occurred at collision energies of -48 eV for morphine. Nitrogen was used as collision gas. The product ions with preselected masses produced from the fragmentation in the collision cell passed the second MS and was detected. The following ions were monitored: MOR, m/z = 286 → 152.1 and Homotropine m/z- 276.1 → 142.1, several product ions were observed but the most abundant one was chosen. The LLOQ is 0.93 ng /ml for morphine. The method showed good linearity in the concentration range of 3.75 to 120 ng/ml with the correlation coefficient of 0.98 for morphine. The recovery of the sample

was 97 %. The precision of the quality control samples were less than 15% of standard deviation and accuracy of the quality control samples were 85 to 103 %.A sensitive, reliable and highly automated LC-MS-MS method for the measurement of morphine in human EDTA plasma has been successfully developed and validated. Homotropine is analyte minimized the potential bias caused by inconsistent matrix effects and improved the method ruggedness. The sample preparation method is simple and less time consuming, the recovery was good. A silica column and an aqueous-organic mobile phase were used to achieve chromatographic resolution of the analyte which is important for avoiding artificial over-estimation of morphine due to in-source deconjugation of morphine-3-glucuronide. The LLOQ is 0.93 ng ml^{-1} for morphine using only 0.2 ml plasma. The developed automated method may be used for the determination of morphine in human plasma.

Reference

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