Chapter 3

Analytical and Bioanalytical Method Development and Validation for Quantification of Isometamidium chloride

1. Introduction

Isometamidium chloride (ISMM) (M.W. 496 g/mol) [1] is an amphiphilic cationic moiety due to quaternary nitrogen present in phenanthridine ring and the presence of phenyl ring [2] with rapid disposition in liver and kidney and inefficient plasma concentration [3]. Therefore, there exists an urgent need for a sensitive analytical technique for the determination of ISMM from plasma. Furthermore, the cationic charge may lead to undesirable charged interaction with free silanol groups of stationary phase with increased peak asymmetry. To add on, the sensitivity of the method could be compromised due to its purple positional isomers [2] which may elute at varying retention time.

Despite its high market value in treatment and prophylaxis of Trypanosomiasis [4], miniscule efforts have been made for the development of sensitive yet rapid determination of ISMM from biological matrices. Ion-pairing solid phase extraction method using heptane sulfonate as ion-pairing reagent and triethylamine as counter-ion was developed for quantification of ISMM from serum and tissue matrices of cattle [5]. Another RP-HPLC method involved gradient elution of ISMM and diminiazene aceturate using acetonitrile and 0.05 M ammonium formate (pH 2.4) and C18 column as stationary phase (5 µm, 4.6*250 mm) with extraction of analyte using Oasis WCX solid phase extraction [6]. However, the existing RP-HPLC methods were associated with lacunae including larger volumes of biological matrices, poor resolution, tedium sample preparation technique, applicability only to biological matrices of cattle, and high cost. Therefore, the objective of the present work involved the development of a rapid, simple, cost-effective RP-HPLC method for estimation of ISMM from lipid nanoparticles, release media, and biological matrices of Wistar rat.

2. Material and methods

2.1 Chemicals, reagents and experimental animals

ISMM was procured from Marvel Drugs Ltd. (Mumbai, India). HPLC grade formic acid, acetonitrile (ACN), methanol, and acetone were purchased from Merck Limited (Mumbai, India). Sodium dodecyl sulfate (SLS), HPLC grade potassium dihydrogen orthophosphate, orthophosphoric acid, sodium hydroxide, stearic acid, docusate sodium, and dichloromethane was procured from SD Fine-Chem Limited (Mumbai, India). Tween 80 and trehalose were obtained from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Precirol® ATO 5 was a kind gift sample from Gatttefosse Co. (Mumbai, India). All other chemicals, solvents, and reagents utilized were either HPLC or analytical grade. HPLC grade water was obtained from Milli-Q system (Millipore GmbH, Germany) used throughout the analysis. The solvents and buffers prepared were suitably filtered through 0.22 Millipore™ membrane filter (Merck, Darmstadt, Germany) and suitably degassed using an ultrasonic bath for 30 min. Male Wistar rats (8–10 weeks) were procured from Central Animal Facility, BITS-Pilani, Pilani Campus, India. Institutional Animal Ethics Committee of Birla institute of technology and science, Pilani campus, Pilani approved the animal experiment protocol (IAEC), and experiments were conducted as per CPCSEA guidelines.

2.2 Analytical method development and validation for quantification of ISMM

2.2.1. Instrument

RP-HPLC method for quantification of ISMM was developed using LC-2010C HT HPLC system (Shimadzu, Kyoto, Japan) equipped with DGU-20A5R degassing unit, LC-20AT pump, SIL-20ACHT autosampler, and SPD-20A UV/VIS detector. Data analysis and processing were done using LabSolutions software (Version 5.97).

2.2.2. Liquid chromatographic conditions

For the analytical method, ISMM was separated in an isocratic mode of elution at 30°C. Phosphate buffer (pH 3.0, 20mM) and acetonitrile (55:45 %v/v) was used as mobile phase at 0.7 ml/min flow rate. ISMM was separated using C18 column (Gracesmart® 5 µm, 4.6mm* 250 mm) which was pre-equilibrated for 30 min. Sample injection volume, detection wavelength, column temperature, and total run time were 10 µl, 320 nm, 35°C, and 6 min respectively. Quantification of ISMM was done by measurement of ISMM peak area.

2.2.3 Preparation of standard solutions for analytical method of ISMM

ISMM stock solution of (1000 µg/ml) was prepared by dissolving accurately weighed quantity of ISMM (25 mg) in a volumetric flask (25 ml) and volume made up to 25 ml using methanol. Further, a working stock solution (100 µg/ml) was prepared by diluting 10 ml of stock solution up to 100 ml using phosphate buffer (pH 3.0, 20mM): acetonitrile in 55:45 v/v. The secondary ISMM standard solutions of 0.375, 0.75, 1.5, 3, 6, and 12 µg/ml were prepared by suitably diluting 0.0375, 0.0750, 0.150, 0.300, 0.600, and 1.200 ml aliquots of working standard respectively in 10 ml volumetric flask using mobile phase.

2.2.4 Preparation of sample solution for analytical method of ISMM

The supernatant obtained after separation of ISMM loaded solid lipid nanoparticles (ISMM-DS LNP) was diluted 10 times with methanol and bath sonicated for 15 min at room temperature. While, ISMM containing release media (100 µl) was diluted 10 times in methanol. Both the sample solutions were centrifuged at 15000 rpm for 10 min and the supernatant was subjected to RP-HPLC analysis.

2.2.5 Analytical method validation

The developed RP-HPLC method for ISMM was validated with respect to specificity, linearity, range, accuracy, precision, and robustness as per ICH Q2(R1) guideline [7].

Specificity: A standard solution of ISMM (100 µg/ml) was spiked in solution of nanoparticle (ISMM-DS LNP) excipients including precirol® ATO 5, docusate sodium, tween 80 and release media (0.2% SLS). The chromatogram was observed for any interference and % recovery of ISMM was determined.

Linearity and range: To determine the linearity and range, ISMM calibration standards including 0.375, 0.75, 1.5, 3, 6, and 12 µg/ml were injected thrice per concentration. Thereafter, the peak area corresponding to each calibration standard was recorded and the calibration curve of peak area versus concentration was contrived. Linearity was determined by ordinary least square regression analysis through the regression equation and correlation coefficient. The range of the developed analytical method was determined based on the calibration curve and % RSD of each calibration curve concentration [8].

Accuracy: The accuracy of the RP-HPLC method of ISMM was determined by estimating % recovery of known spiked ISMM concentration in three different working standards (0.75 µg/ml, 3 µg/ml and 12 µg/ml). The analysis was performed in triplicate for each concentration and %recovery and %RSD were calculated.

Precision: Precision was determined by analyzing three calibration concentrations (0.75 µg/ml, 3 µg/ml, and 12 µg/ml) each in triplicate three times in a day for intra-day precision. Whereas, intermediate precision was established by determining %RSD of nine different determinants (three concentrations of each in triplicate) for consecutive 3 days.

Limit of detection (LOD) and limit of quantitation (LOQ): LOD and LOQ were determined by measuring S/N ratio. The concentration at which S/N ratio was >3.3 and 10 confirmed LOD and LOQ respectively for developed analytical method

Robustness: Deliberate variation in chromatographic conditions including column temperature from 30°C to 33°C, flow rate (0.9 and 0.5 ml/min), pH of buffer (3.2 and 2.8)

were made, and ISMM was analyzed at 3 different concentrations (0.75 µg/ml, 3 µg/ml and 12 µg/ml). % recovery with respect to standard analytical solutions (0.75 µg/ml, 3 µg/ml and 12 µg/ml) was evaluated at each condition to confirm robustness.

2.3 Bioanalytical method development and validation for quantification of ISMM

2.3.1 Instrument

RP-HPLC method for ISMM was developed on Shimadzu LC-2010C HT HPLC system (Kyoto, Japan) equipped with DGU-20A5R degassing unit, LC-20AT pump, SIL-20ACHT autosampler, and SPD-20A UV/VIS detector. Data analysis and processing were done using LabSolutions software (Version 5.97).

2.3.2. Liquid chromatographic conditions

ISMM and ethidium bromide (EtBr) (internal standard) was separated using pre-equilibrated Waters Sunfire® C18 3.5 um ODS (4.6 x 50 mm) column. Isocratic mode of elution was utilized for quantification of ISMM from biological matrices at 30°C with a mobile phase consisting of reservoir A (ACN) and reservoir B (0.1% v/v formic acid in 5 mM sodium lauryl sulfate buffer) in 45:55 ratio (% A:% B) at 1 ml/min flow rate and detection wavelength of 365 nm. The sample injection volume was 100 µl.

2.3.3 Preparation of standard solution, working calibration standards (CS) and quality control (QC) samples

A stock solution of ISMM (10 mg/ml) and EtBr (1 mg/ml) were prepared by dissolving accurately weighed amount of ISMM and EtBR in water and methanol respectively. Working stock solutions of ISMM (1000 µg/ml) and EtBR (80 µg/ml) were prepared by suitably diluting the stock solution of ISMM and EtBR with methanol. Working stock solutions were further utilized to prepare CS of 5, 10, 20, 30, 40, 60 and 80 µg/ml for plasma and 1.25, 2.5, 5.0, 10.0, 15.0, 20.0 and 40.00 µg/ml for organ homogenate. The CS were prepared by

spiking 2.5 µl and 10 µl of working standards in plasma and tissue matrices respectively whereas; samples were spiked with 2.5 µl EtBR irrespective of any biological matrices. The CS in plasma were 125, 250, 500, 1000, 1500 and 2500 ng/ml while; for tissue matrices, the CS were 125, 250, 500, 1000, 1500, 2000 and 4000 ng/ml. QC samples were prepared by suitable dilution of stock solution at three different levels low QC (LQC, 200 ng/ml), medium QC (MQC, 750 ng/ml), and high QC (HQC, 2000 ng/ml) for plasma and skin matrices. The LQC and MQC for tissue matrices were 200 ng/ml and 750 ng/ml respectively. While, the HQC was 3000 ng/ml for liver, lungs, spleen matrices, 1750 ng/ml, and 2000 ng/ml for brain and kidney matrices respectively. All stock and working standards were stored at -20°C until further use.

2.3.4 Plasma sample preparation

Blood was withdrawn from male Wistar rats by retro-orbital plexus in heparinized tubes and plasma was separated after centrifugation of blood sample at 7500 rpm for 10 min. The plasma sample was stored at -20°C until further use. ISMM extraction from plasma matrices was done using a simple protein precipitation method. Briefly, plasma (100 µl) containing ISMM was spiked with 2.5 µl EtBr (80 µg/ml). Thereafter, the sample was vortexed using a multi spinix-vortex shaker (Tarson, USA) for 1 min. Acetone (900 µl) was then added to each plasma sample and vortexed for 30 min. The samples were centrifuged at 17000 rpm for 20 min at 4°C, supernatant separated and later evaporated overnight at 40°C. The residue was reconstituted in 100 µL of methanol: water (50:50 %v/v) and vortexed for 30 min. The samples were centrifuged (17000 rpm, 20 min, 4°C) and injected in HPLC for quantification.

2.3.5 Tissue sample preparation

For tissue sample preparation male Wistar rats were sacrificed by cervical dislocation and organs including liver, kidneys, spleen, lungs, brain, and skin were isolated and stored at -

20℃ after rinsing in phosphate buffer saline (pH 7.4). Each organ including the liver, kidneys, spleen, lungs, and brain was weighed and diluted up to 3 times the organ weight with Milli Q water and 10 times the weight of skin to mince each organ using a high shear homogenizer for 3 min. The homogenized tissue was centrifuged (7800 rpm, 20 min, 4℃) and supernatant separated to yield tissue homogenate. The protein precipitation method was used to extract ISMM from tissue homogenate. Briefly, tissue homogenate (100 µl) containing ISMM was spiked with 2.5 µl EtBr (80 µg/ml). Thereafter, the samples were vortexed using a multi spinix-vortex shaker (Tarson, USA) for 1 min. 900 µl of acetone: water (60:40 % v/v) was then added to each sample and vortexed for 30 min. The samples were centrifuged at 17000 rpm for 20 min at 4℃, supernatant separated and later evaporated overnight at 40℃. The residue was reconstituted in 100 µL of methanol: water (50:50 %v/v) and vortexed for 30 min. The samples were centrifuged (17000 rpm, 20 min, 4℃) and injected in HPLC for quantification.

2.3.6 Bioanalytical method validation

Validation of the developed bioanalytical method was done as per internationally accepted guidelines for linearity, range, selectivity, LOD, and LLOQ, respectively [9–11].

Selectivity: The selectivity of the developed bioanalytical method was determined by observing possible interference in chromatogram at retention time of ISMM when blank matrices of plasma and different tissue matrices from six different healthy rats were injected into HPLC.

Linearity and Range: Calibration curves were constructed for plasma and each tissue matrices including liver, kidneys, spleen, lungs, brain, and skin to determine linearity. The calibration curves were obtained by constructing a plot of the ratio of peak area of ISMM/EtBR versus concentration. Thereafter, the calibration curves were analyzed by ordinary least square regression analysis. Each calibration curve consists of 1 blank sample (without ISMM) and 6-

7 non-zero samples (containing ISMM and EtBr). The range of developed method was determined based on the calibration curve.

Limit of detection (LOD) and lower limit of quantification (LLOQ)**:** The LOD and LLOQ were calculated based on S/N ratio using equation 3.1

$$S/N = \frac{2H}{h} \quad \text{.....(3.1)}$$

Where, H is the peak height of the analyte from a distance of 5% of peak width up to the top. While, h is the peak height of noise from distance at 5% peak width up to the top. The desired S/N ratio for LOD and LLOQ would be 3 and 10 respectively.

% recovery and carryover effect: The % recovery for EtBR and ISMM was determined upon measuring detector response for each concentration after extraction from individual biological matrices when compared to the detector response of the analyte in the mobile phase (n=6). The carryover effect was determined by visual observation of any peak at retention time of EtBR and ISMM after injection of blank plasma preceding the upper limit of quantification calibration level (ULOQ).

2.4 Statistical analysis

The results were expressed as mean ± standard deviation (SD) obtained from three separate experiments (n=3) for the analytical method and 5 different replicates in each assay for the bioanalytical method.

3. Results and Discussion

3.1 RP-HPLC analytical method development and validation for quantification of ISMM

3.1.1 Method development

ISMM is a widely utilized cationic anti-trypanosomal drug which is highly basic due to the presence of amidino and quaternary basic nitrogen groups [8]. Furthermore, ISMM possesses a purple positional isomer and a small proportion of two analogues namely bis-compound and homidium [2,12]. Thus, the development of RP-HPLC method remains a paramount defiance due to multiple interactions of ISMM with charged silanol present in the stationary phase leading to peak tailing. To this end, phosphate buffer (20 mM, pH 3) was used in the mobile phase which reduces the interaction of charged ISMM by decreasing ionization of silanol and ISMM succoring in rapid elution with proper peak shape. Furthermore, acetonitrile: phosphate buffer (20 mM, pH 3) at 45: 55 %v/v caused avoidance of peak splitting into various positional isomers of ISMM resulting in appropriate peak area processing (Figure 3.1(a)). The elution time, and tailing factor obtained upon chromatographic separation of ISMM were 4.5 ± 0.2 min and 1.30±0.03; respectively, at 320 nm. Various calibration standard solutions (0.375, 0.75, 1.5, 3, 6 and 12 µg/ml) were injected and peak area for each was obtained; calibration curve was constructed based on peak area versus concentration (Figure 3.1(b)).

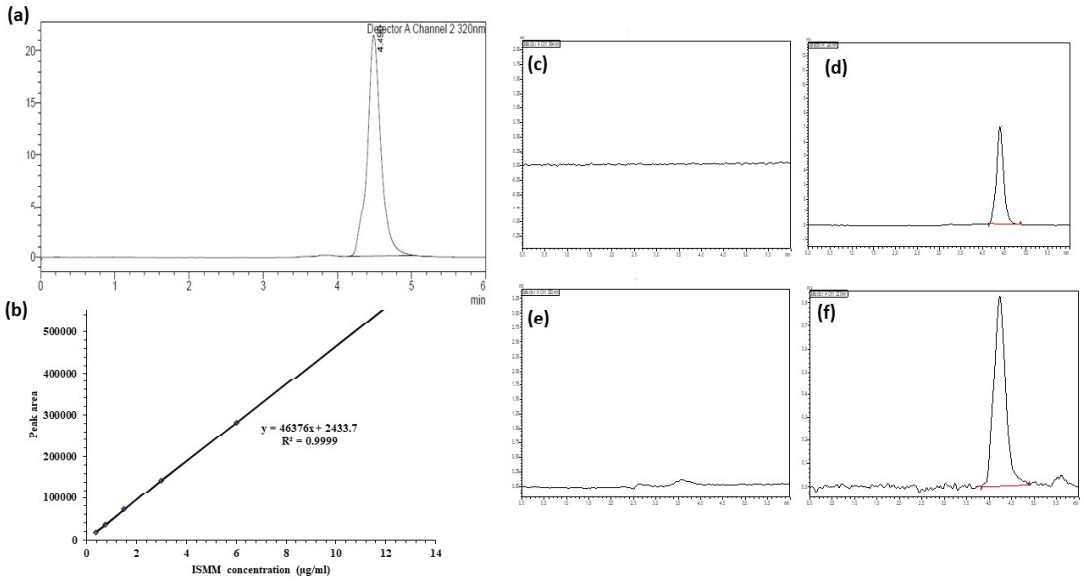

Figure 3.1 (a) Chromatogram of Isometamidium chloride (b) Calibration curve for Isometamdium chloride. Specificity of RP-HPLC method for Isometamidium chloride depicted through chromatogram of (c) Blank lipid nanoparticles (LNP) (d) Isometamidium chloride loaded LNP (e) release media and (f) Isometamidium chloride in release media.

3.1. 2 Analytical method validation

3.1.2.1 Linearity and range

Linearity was determined by ordinary least square; wherein, 6 calibration standards were found to be linear in the concentration range of 0.375 µg/ml-12 µg/ml. The regression equation was obtained from the constructed calibration curve (equation 3.2). The correlation coefficient was found to be 0.999.

y=46376x+2433.7…….(3.2)

where, y is the peak area for corresponding x concentration (µg/ml) [13]. The range of developed methods was determined from the calibration curve (Table 3.1). % bias determined for each concentration of calibration standard aided in determining the range of the analytical method (Table 3.1).

Table 3.1 %bias of calibrated concentration range of Isometamidium chloride

Concentration (ng/ml)	% bias
0.375	-4.36 ± 6.21
0.750	-2.75 ± 2.11
1.500	-1.46 ± 6.11
3.000	-0.50 ± 5.15
6.000	-0.76 ± 2.55
12.00	-0.52 ± 1.45

3.1.2.2 Specificity

The developed RP-HPLC method was found to be specific for ISMM with no interfering peak at 4.5 ± 0.2 min in the chromatogram obtained after extraction of ISMM in presence of formulation excipient and release media (Figure 3.1(c)-(f)).

3.1.2.3 Accuracy and precision

The accuracy of the analytical method determines the degree of closeness between derived and true value. The overall % recovery of ISMM at 0.75, 3, and 12 µg/ml was found to be 89.6±7.05%. Thus the developed method was found to be accurate [11]. The random error during repeated measurements is determined by precision. The overall % RSD was found to be <2% for 0.75, 3 and 12 µg/ml, making the method precise for ISMM analysis (Table 3.2)

3.1.2.4 LOD and LOQ

The LOD and LOQ of the developed RP-HPLC method for ISMM were determined by measuring S/N ratio. The LOD and LOQ of the developed RP-HPLC method were found to be 0.148 µg/ml and 0.45 µg/ml, respectively.

Table 3.2 Accuracy and precision at different levels of Isometamidium chloride in standard solution

Accuracy(n=3)			
% Target	%Recovery	SD	% RSD
80	81.93	2.23	2.72
100	91.15	0.41	0.45
120	95.79	0.11	0.11
Intra-day precision(n=3)			
Concentration (µg/ml)	Measured conc. (µg/ml)	SD	% RSD
0.75	0.72	0.002	0.28
3	3.16	0.029	0.92
12	12.13	0.075	0.62
Inter-day precision(n=3)			
0.75	0.70	0.02	2.22
3	2.89	0.03	0.12
12	11.78	0.25	2.16

3.1.2.5 Robustness

Deliberate change in chromatographic parameters did not cause a significant change in the retention time of ISMM. The % recovery of ISMM was found to be between 82.36%±0.46% to 134.70%+1.01% upon change in several parameters including column temperature, flow rate, and pH of buffer (Table 3.3).

Table 3.3 Robustness results of Isometamidium chloride RP-HPLC method at different analytical conditions

Condition	**% Recovery ± SD***		
	0.75 µg/ml	3 µg/ml	12µg/ml
Column temperature (37°C)	93.23 ± 0.11	94.85 ± 3.73	99.32 ± 0.40
Column temperature (33°C)	94.32 ± 0.84	99.26 ± 0.16	100.48± 0.23
Flow rate (0.9 ml/min)	82.36 ± 0.46	85.99 ± 0.33	87.49 ± 0.52
Flow rate (0.5 ml/min)	121.26 ± 1.05	131.37 ± 2.04	134.70 ± 1.01
Buffer pH (pH=3.2)	88.08 ± 0.86	93.23 ± 0.95	97.87 ± 1.91
Buffer pH (pH=2.8)	90.13 ± 1.37	93.47 ± 1.70	98.31 ± 1.17

*Data represented as mean±SD, n=3

3.2 Bioanalytical method development and validation for quantification of ISMM

3.2.1 Method development

3.2.1.1 Optimization of stationary and mobile phase

The bioanalytical method development of ISMM was initiated with a goal of rapid elution of ISMM, easy and least sample preparation technique, and time with consistent recovery, peak shape, and resolution between ISMM and EtBr. ISMM is highly water-soluble (6% w/v) [2] which may impede its retention on the hydrophobic C18 column. However, the presence of quaternary nitrogen interacting with free silanol groups and hydrophobic π- π interaction of aromatic phenanthridine ring present in ISMM with hydrophobic alkyl groups of C18 column may contribute to its retention [14]. Initially, acetonitrile (ACN) and potassium dihydrogen orthophosphate (KH_2PO_4)(20 mM, pH 3) was utilized as the mobile phase for elution of ISMM and EtBR. Acetonitrile was utilized due to its lower viscosity compared with methanol when used as an organic modifier. Furthermore, ISMM did not elute when methanol was utilized as an organic modifier even up to 30 min. KH_2PO_4 decrease the undesirable interaction with the free silanol group which causes prominent interaction with the cationic basic molecules leading to an increase in tailing factor [15]. Similar reports have been established in the past for estimation of ISMM using ACN and KH_2PO_4 (20 mM, pH 3) [12]. EtBR did not elute in an isocratic mode of elution using ACN and KH_2PO_4. Therefore, a continuous gradient RP-HPLC method composed of A-ACN and B- KH_2PO_4 (20 mM, pH 3) was developed consisting of Tmin/% B (v/v) 0.01/70, 1.31/65, 2/50. 10.01/70 as gradient at 0.7 ml/min flow rate. The gradient method led to elution of both EtBR and ISMM with poor resolution (<2.0) using gracesmart® C18 column (5µm pore size, 4.6*250 mm) (Table 3.4). Ion pairing reagents have been well established for elution of charged moieties by hydrophobic complexation [5]. Therefore, to establish better resolution between biological

matrices, EtBr, and ISMM; ion-pairing agent (SLS) has been incorporated into the mobile phase. Similar reports have been found in the past wherein basic compounds of pharmaceutical interest consisting of various isomers were well resolved using ACN and SLS (10 mM) in phosphate buffer (0.01-0.0066 M, pH 2) as mobile phase [16]. It appeared that SLS led to charged interaction with cationic quaternary nitrogen of ISMM thereby causing enhanced retention on hydrophobic C18 stationary phase and decrease the undesirable interaction of ISMM with free silanol groups. ISMM did not elute when Gracesmart® C18 column (5µm pore size, 4.6*250 mm) due to increased hydrophobicity of ISMM upon ion-pairing. Enhanced resolution between EtBR and ISMM with quick elution was obtained when Waters Sunfire® C18 3.5 um ODS (4.6 x 50 mm) was used as a stationary phase (Table 3.4). Moreover, an increase in the ratio of ACN (A): SLS buffer (5 mM, pH 3) from 45:55 to 47:53 led to increased peak splitting of EtBR. While, further decrease in ACN (<45% v/v) caused increased retention of ISMM on stationary phase leading to increased elution time. Thus, ISMM and EtBR were extracted from biological matrices using ACN and 0.1% formic acid in SLS buffer (5 mM, pH 3) in 45:55% v/v ratio at 1 ml/min flow rate.

Table 3.4 Optimization of stationary and mobile phase for Isometamidium chloride bioanalytical method

Parameter	Limits	Observation	
Mode of elution	-	Gradient	Isocratic
Mobile phase (A:B)	-	ACN:K_2HPO_4(20 mM, pH3)	ACN: 0.1% v/v formic acid in SLS buffer (5 mM, pH 3)
Mobile phase ratio (Tmin/B % v/v)	-	0.01/70, 1.31/65, 2/50. 10.01/70, 20/70	0-22/55
Stationary phase	-	Gracesmart® C18 column (5µm pore size, 4.6*250 mm)	Waters Sunfire® C18 3.5 um ODS (4.6 x 50 mm)
Flow rate (ml/min)	-	0.7	1.0
Retention time (min)*	-	3.8±0.01	10.9±0.09
Resolution*	>2	1.9±0.13	5.4±0.87
Tailing factor (EtBR, ISMM)*	0.8-1.5	0.94±0.119, 1.23±0.017	1.00±0.092 1.34±0.190

* Data represents mean±SD, n=3

3.2.1.2 Optimization of sample extraction procedure

Method of ISMM and EtBr extraction including liquid-liquid extraction (LLE) and protein precipitation was attempted after suitable optimization of the mobile phase. Both ISMM and EtBR being highly hydrophilic, did not partition in either dichloromethane or ethyl acetate by LLE from plasma matrices. Therefore, protein precipitation using trifluoroacetic acid, ACN, and acetone was attempted. ISMM is highly labile at extreme pH [2] causing no elution of ISMM upon extraction with trifluoroacetic acid. Unacceptable tailing factor (>1.2) for ISMM and peak splitting of EtBR was observed when ACN was used for extraction (Table 3.5). Therefore, protein precipitation of plasma was done using acetone which led to acceptable tailing factor and resolution. However, water was required for the extraction of ISMM from tissue matrices due to its ability to elicit higher protein binding. Therefore, extraction of ISMM from tissue matrices was done using 900 µl acetone: water (60:40% v/v) respectively (table 3.5).

Table 3.5 Optimization of Isometamidium chloride sample extraction procedure

Parameter	**Results***				
Biological matrices	Plasma			Tissue matrices	
Solvent	ACN	acetone		acetone:water	
			90:10	80:20	60:40
Volume (µl)	100	900	900	900	900
Retention factor	1.73±0.041	2.71±0.030	2.27±0.045	2.26±0.043	2.17±0.020
Tailing factor (EtBR, ISMM)	1.0±0.12, 1.7±0.19	1.3±0.61, 1.4±0.41	1.2±0.19, 1.3±0.31	1.0±0.20, 1.4±0.04	0.9±0.02, 1.5±0.10
Resolution	5.4±0.87	5.9±0.32	8.1±2.37	6.9±0.89	6.4±0.53
Extraction efficiency	55.7±3.9	62.2±16.7	74.5±4.3	69.1±10.7	78.8±5.8

*Data represented as mean±SD, n=3

The retention time, tailing factor and HETP for ISMM upon extraction from different biological matrices are mentioned in table 3.6.

Table 3.6 Retention time, asymmetric factor and HETP for Isometamidium chloride eluted from biological matrices

	Results				
Biological matrix	**retention time**	**Tailing factor**	**HETP**	**LOD (ng/ml)**	**LLOQ (ng/ml)**
Plasma	10.8±0.10	1.72±0.17	240.6±38.7	48.8	136.7
Liver	17.0±1.65	0.76±0.03	71.3±6.2	66.6	222.0
Kidneys	14.22±0.26	1.43±0.05	118.8±27.2	29.3	97.8
Spleen	15.1±1.5	1.44±0.17	112.3±28.0	23.2	77.4
Lungs	15.3±0.32	1.5±0.23	128.6±1.84	55.6	185.5
Brain	16.6±0.17	1.22±0.05	144.2±15.9	52.9	176.3
Skin	12.18±0.27	2.14±0.34	744.2±337.9	40.1	133.9

Data represented as mean±SD, n=6

3.2.2 Method validation

3.2.2.1 Selectivity, LOD and LLOQ

The selectivity of ISMM bioanalytical method was determined by the ability of optimized chromatographic conditions to selectively detect ISMM from biological matrices as depicted in Figure 3.2. Blank chromatograms of plasma and individual tissue matrices from six different Wistar rats showed no interference at a retention time of ISMM confirming selectivity of ISMM bioanalytical method.

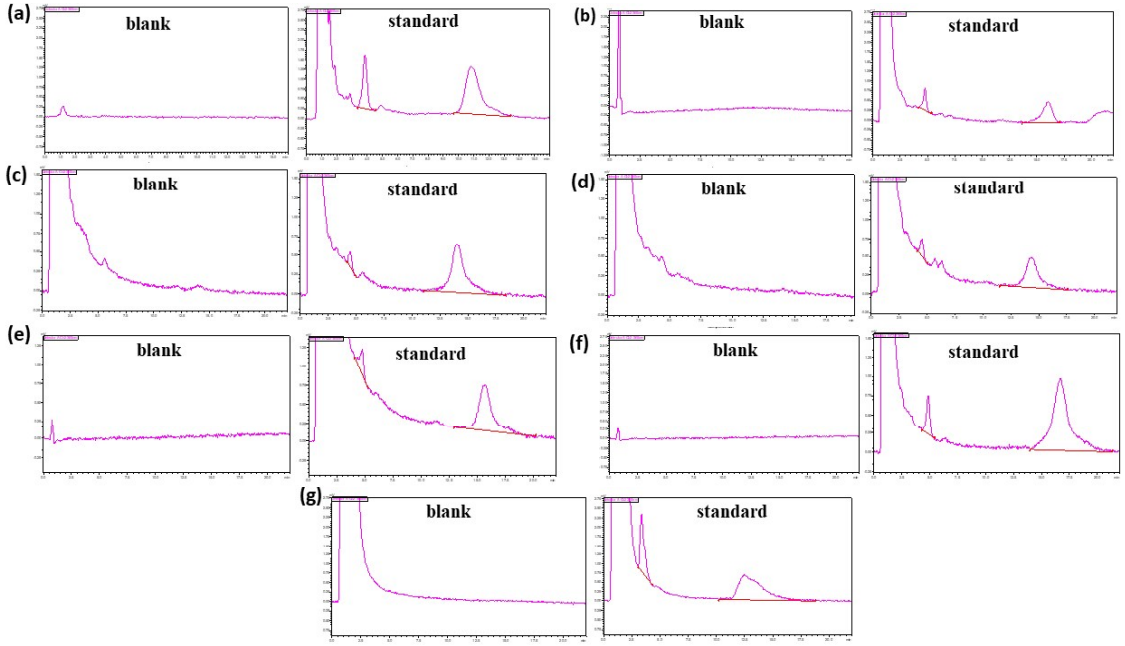

Figure 3.2 Representative chromatogram of Isometamidium chloride standard (1000 ng/ml) in biological matrices (a) Plasma (b) Liver (c) Kidneys (d) Spleen (e) Lungs (f) Brain (g) skin

Whereas, LOD and LLOQ were determined as per equation 3.1 for plasma and tissue matrices (Table 3.6). The LLOQ was further confirmed by visual observation as determined by % RSD (<±20%) determined upon 6 replicate injections.

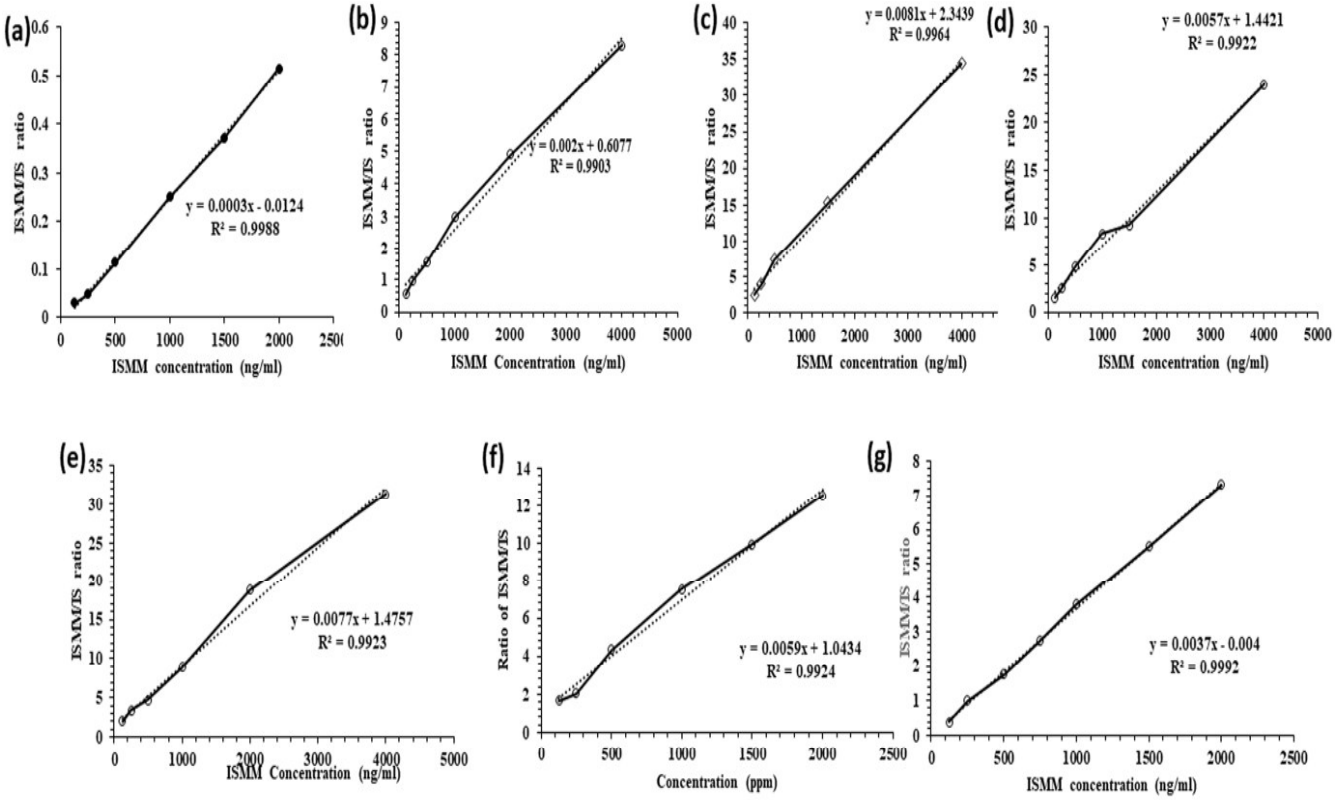

Figure 3.3 Calibration curves for Isometamidium chloride in different biological matrices (a) plasma (b) liver (c) kidneys (d) spleen (e) lungs (f) brain (g) skin

3.2.2.2 Linearity and range

The linearity of ISMM bioanalytical method was determined from constructed calibration curves for individual tissue and plasma matrices (Figure 3.3) (Table 3.7). Calibration curves were analyzed by the ordinary least square method and the regression coefficient was determined for each (R^2>0.99); further confirming the linearity of the method. The range for individual plasma and tissue matrices has been presented in Table 3.7.

Table 3.7 Linearity and range of bioanalytical method for Isometamidium chloride in biological matrices

	Results			
Biological matrix	**Range (ng/ml)**	**Regression equation**	**Correlation coefficient**	**%Recovery**
Plasma	125-2000	y=0.0003x-0.0124	0.998	87.7±17.6
Liver	125-4000	y=0.002x+0.6077	0.9903	118.8±15.7
Kidneys	125-4000	y=0.0081x+2.3439	0.9964	90.02±31.1
Spleen	125-4000	y=0.0057x+1.4421	0.9922	120.5±7.4
Lungs	25-4000	y=0.0077x+1.4757	0.9923	86.1±7.0
Brain	125-2000	y=0.0059x+1.0434	0.9924	95.1±13.6
Skin	125-2000	y=0.0037x-0.004	0.9992	95.8±9.7

3.2.2. 3 Accuracy and precision

The intra- and inter-day precision at LLOQ, LQC, MQC, and HQC has been represented in Table 3.8. The % bias of ISMM at all 4 levels was found to be within the limit. Thus, the method was acceptable for estimation of ISMM from biological matrices (% bias and RSD <±20% and LLOQ and <±15% at LQC, MQC, and HQC).

3.2.2.4 Recovery and carryover effect

Consistency in the recovery of analyte is desirable for reproducibility. The mean % recovery of ISMM was found to be 99.14% (Table 3.7). Further, the absence of carryover effect was confirmed by the absence of any peak at retention time of ISMM and EtBr upon injection of blank plasma and tissue matrices preceding the ULOQ sample.

Table 3.8 Accuracy and Precision of Isometamidium chloride from different biological matrices of Wistar rat (n=5)

Biological matrix	**Level**		**Intra-day precision**			**Inter-day precision**		
		Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Precision (%CV)	Accuracy (%bias)	Measured concentration (ng/ml)	Precision (%CV)	Accuracy (%bias)
Plasma	LLOQ	125	139.9±10.6	10.85	-11.94	141.4±31.6	12.77	-13.15
	LQC	200	174.8±25.6	14.96	-0.56	182.2±21.0	11.28	-13.48
	MQC	750	703.5±55.6	7.90	6.19	823.4±114.8	13.75	13.92
	HQC	2000	2011.2±253.9	12.62	12.59	1844.7±205.7	11.02	-7.76
Liver	LQC	200	180.5±8.21	4.54	9.71	177.3±9.5	5.54	11.30
	MQC	750	741.1±9.13	1.23	1.17	747.2±11.7	1.56	0.602
	HQC	3000	3250.3±91.22	2.80	11.21	2887.8±309.8	11.37	10.25
Kidneys	LQC	250	211.6±14.4	6.80	2.50	219.1±20.7	9.44	12.34
	MQC	1000	1281.9±72.5	11.45	-5.29	1027.3±360.0	12.52	-2.73
	HQC	4000	3963.4±370.4	13.3	0.91	3775.8±251.4	6.65	5.60
Spleen	LQC	250	283.8±14.8	5.24	-13.53	251.1±28.8	11.47	-0.445
	MQC	750	824.6±68.4	8.30	-9.95	841.0±46.1	5.47	-12.14
	HQC	1500	1352.2±7.4	0.55	9.84	1373.3±32.7	2.35	8.44
Lungs	LQC	200	162.6±20.4	12.58	18.6	192.7±32.4	3.60	15.09
	MQC	750	647.9±23.9	3.69	13.61	650.4±22.9	3.74	13.28
	HQC	2000	1839.3±176.5	9.59	8.03	1975.5±342.6	14.90	1.22
Brain	LQC	250	238.1±3.2	1.36	4.75	223.3±17.6	8.35	10.65
	MQC	750	682.1±61.7	9.04	9.05	693.5±37.8	6.29	7.52
	HQC	150	1691.1±204.8	12.11	3.36	1672.9±141.2	8.4	4.4
Skin	LQC	200	225.4±6.9	3.09	-12.73	214.1±5.8	2.81	-7.08
	MQC	750	614.8±7.22	1.17	18.02	665.7±74.4	9.85	11.23
	HQC	2000	1884.9±68.49	3.63	5.75	1762.0±140.1	8.08	5.75

4. Conclusion

Present work revealed the development of analytical and bioanalytical methods for efficient elution and quantification of ISMM from formulation excipient, release media, and biological matrices with acceptable recovery (80-120%). The acidic pH of the mobile phase caused complete ionization of the analyte thereby resulting in enhanced separation. Furthermore, the undesirable charged interaction of the analyte with free silanol groups was reduced using phosphate buffer and SLS buffer in analytical and bioanalytical methods, respectively. Additionally, SLS buffer functioned as an ion-pairing agent for the separation of cationic hydrophilic ISMM on the C18 column. The analytical and bioanalytical methods for estimation of ISMM were validated as per ICH Q2(R1) and USFDA guidance for industry respectively. Thus, the developed methods were suitable for routine analysis of ISMM from various formulations and biological matrices in a simple and cost-effective manner without the need for sophisticated instruments.

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