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DETERMINATION OF LOPERAMIDE IN HUMAN PLASMA BY UPLC-MS/MS

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ABSTRACT

Bioanalysis is the term utilized to depict the quantitative assessment of drugs / metabolite in biological fluids. LC-MS combines the chromatographic separation and mass analysis capabilities. Loperamide in human plasma were validated using UPLC-MS/MS technique. Loperamide is the drug which is mainly used for the treatment of sudden diarrhea. Internal standard used in this method is Fluconazole and 5 mM Ammonium acetate is used as buffer. Acetone-M, Formic acid, and 5mM Ammonium acetate (80:20:01%v/v/v) is used as solvent. ZORBAX Eclipse XDB-C8(100mm × 4.6mm, 3.5µm) column used. The bio-analytical method SOP No.MTR-BA-M-452-00, Loperamide in K_2EDTA human plasma in the concentration range 24.8560-5063.9260pg/ml was successfully developed and validated.

Keywords: Bio-analytical method, Loperamide, Fluconazole, UPLC, Human plasma, Calibration curve

INTRODUCTION

Bioanalysis is the term utilized to depict the quantitative assessment of drugs / metabolite in biological fluids **[1]**. LCMS combines the chromatographic separation and mass analysis capabilities **[2, 3]**. UPLC is the progressed strategy which gives high resolution, sensitivity, and speed and also save the time and solvent utilized **[4,5]**. Chemically Loperamide : 4-(4-chlorophenyl)-4-hydroxy-N,N-dimethyl-α,α-

diphenyl-1-piperidinebutanamide Hydrochloride **[6]**. Loperamide an opioid agonist having anti-diarrheal activity **[7, 8]**. Loperamide acts on the μ receptors in intestinal mucosa, reducing the GI motility through reducing circular and longitudinal smooth muscle activities **[9, 10]**. Analysis of Loperamide in various pharmaceutical products and bulk drugs are explained in the literature **[11 – 15]**. For drug profile refer **Figure 1 and Table 1**.

Table 1: Drug profile

Profile	Loperamide	Fluconazole
Drug Name	Loperamide Hydrochloride	Fluconazole
IUPAC	4-(4-chlorophenyl)-4-hydroxy-*N*-*N*-dimethyl-α,α-diphenyl-1-piperidinebutanamide Hydrochloride.	2-(2,4-difluorophenyl)-1,3-bias(1,2,4-triazole-1-yl)propan-2-ol.
Chemical Formula	C29H34ClN2O2	C13H12F2N6O
Mol. Wt.	513.50 and 477.03(free salt)	306.27
Solubility	Methanol	Slightly soluble in water
Physical Properties	White solid	White crystalline powder
Half Life	10.8hours – 14.4hours	30hours

MATERIALS AND METHODS

Preparation of Buffer-1 (5mM Ammonium Acetate): Weighed about 385.40 mg of Ammonium acetate and add 1000 milliliter of water to dissolve. Mix well, Sonicate and filter.

Preparation of Mobile phase [Acetone-M: Buffer-1: Formic Acid (80:20:0.1%, v/v/V)]:

Measure 800 milliliter of Acetone –M and add 200 milliliter of buffer-1 and 1ml of formic acid mix well. Filter through a 0.2 μm membrane filter and degas the solution by placing it in an ultrasonic bath for 2 minutes.

Preparation of Loperamide Stock Solution (1mg /mL) for CC: Weigh accurately about 2 mg of Loperamide and transfer into 2 mL of volumetric flask add 1mL of Acetone-M to dissolve and make up to the volume with Acetone-M. Calculate final concentration of Loperamide in μg/mL by:

$$\frac{\text{Wt. of Loperamide taken in milligram}}{2\ \text{mL}} \times \frac{\text{Potency(as is basis)}}{100} \times \frac{M_1}{M_2} \times 1000$$

Where M_1 is the Mol. Wt. of Loperamide (Free), M_2 is Mol. Wt. of Loperamide (Salt).

Preparation Internal Standard Stock Solution (1mg/mL): Weigh accurately about 2 mg of Fluconazole and add 1 milliliter of Acetone-M and make up using Acetone-M. Calculate final concentration of Loperamide D6 in μg/mL by:

$$\text{Wt. of Fluconazole taken in milligram} \times \text{Potency(as is basis)} \times M_1 \times 1000$$

$$\frac{\text{2mL}}{\text{100}} \quad M_2$$

Where M_1 is the Mol. Wt. of Fluconazole (Free), M_2 is Mol. Wt. of Flucanazole (Salt)

Preparation of Internal Standard Stock Dilution: Prepare a solution of internal standard dilution in the concentration of 250 ng /ml of Fluconazole in diluent as described in **Table 2**.

Table 2: Preparation Internal standard solution for Fluconazole

Stock Conc.(microgram/milliliter)	Stock aliquot(milliliter)	Diluent used (milliliter)	Final volume (milliliter)	Final Conc. (microgram/milliliter)
1000.0000	0.020	1.980	2.000	10.0000
10.0000	0.500	19.500	20.000	250.0000ng/mL

Spiked Calibration Curves Standards for Loperamide: Measure 0.040 mL of stock aliquot of comparing concentrations of mentioned Loperamide stock dilutions and make to 2 milliliter with screened and pooled K_2EDTA plasma. CC concentrations mentioned (**Table 3)**.

Table 3: Preparation of Loperamide Spiked CC Curves STD

Stock CCID	Stock Concentration (µg/milliliter)	Stock Aliquot (milliliter)	Plasma Used (milliliter)	Final Volume (milliliter)	Final Concentration (nano gram/ml	Spiked CCID
STD SS H	253.1963	0.040	1.960	2.000	5063.9260	STDH
STD SS G	189.8972	0.040	1.960	2.000	3797.9440	STDG
STD SS F	94.9486	0.040	1.960	2.000	1898.9720	STDF
STD SS E	47.4743	0.040	1.960	2.000	979.4860	STDE
STD SS D	21.3634	0.040	1.960	2.000	427.2680	STDD
STD SS C	9.0794	0.040	1.960	2.000	181.5880	STDC
STD SS B	3.3140	0.040	1.960	2.000	66.2800	STDB
STD SS A	1.2428	0.040	1.960	2.000	24.8560	STDA

600µL of aliquot spiked standards stored in ultra low temperature freezer at -70◦C±15◦C

Preparation of Loperamide stock solution (1mg/mL) for QC: Weigh accurately about 2 milligram of Loperamide STD and add 1 milliliter of Acetone-M and makeup using Acetone-M. Calculate final concentration of Loperamide in µg/mL by **Table 4**.

$$\frac{\text{Weight of Loperamide taken in milligram}}{\text{2mL}} \times \frac{\text{Potency(as is basis)}}{\text{100}} \times \frac{M_1}{M_2} \times 1000$$

Where, M_1 is the Mol. Wt. of Loperamide (free), M_2 is the Mol. Wt. of Loperamide (salt)

Table 4: Preparation of Loperamide Stock Dilutions for Quality Control

Stock ID	Stock Conc. (microgram/milliliter)	Stock Aliquot (milliliter)	Diluents used (milliliter)	Final Volume (milliliter)	Final Conc. (microgram/milliliter)	Stock QCID
LOPE	945624.9653	0.020	1.980	2.000	9456.2497	INT-1
INT-1	9456.2497	0.800	9.200	10.000	756.5000	SS DQC
SS DQC	756.5000	5.000	5.000	10.000	378.2500	SS HQC
SS HQC	378.2500	2.500	2.500	5.000	189.1250	SS MQC
SS MQC	189.1250	1.130	3.870	5.000	42.7423	SS INTQC

SS INTQC	**42.7423**	**1.560**	**8.440**	**10.000**	**6.6678**	**SS LQC**
SS LQC	**6.6678**	**1.890**	**3.110**	**5.000**	**2.5204**	**SS LOQQC**

Method validation: Sample preparation

Vortexes the thawed sample. Add fifty micro liter of IS (20 nano gram/milliliter of Loperamide D6) to all RIA vials excluding blank. Add 500 micro liters of plasma samples and vortex. 100 micro liters of Buffer-1 added and vortex. 2.5 micro liters of Extraction solvent added and vortex. Samples centrifuged at 3500rpm for 5mins (2-8◦C). Collect 2.0 milliliter of supernatant into respective RIA vials and evaporate in LV. Reconstitute the sample using 300 micro liter of solvent and placed in LC-MS/MS.

CHROMATOGRAPHIC CONDITIONS: UPLC Conditions

Column : ZORBAX Eclipse XDB-C8 (100mm × 4.6mm, 3.5µm)

Mobile Phase : Acetone - M: Buffer-1:Formic acid (80:20:0.1, v/v/v)

Column Oven Temp. : 40◦C **Flow Rate :** 0.5 mL/minutes

Auto sampler Temp. : 10◦C **Total run time :** 3.0 minutes

Injection Volume : 10 microliter

Expected Retention Time: Analyte-2.10minutes ± 0.30 minute, **IS-** 2.09minutes ± 0.30minutes

MS/MS Conditions: Instrument ID: MTR-BA-LCMS/MS-20 (Waters TQS)

Table 5: MS/MS Conditions

Molecule	Parent (m/z)	Daughter (m/z)	Dwell (Volts)	Conc.(volts)	Collision Energy(eV)
Loperamide	**477.1700**	**266.1100**	**0.200**	**50**	**25**
Fluconazole	**307.1900**	**220.1600**	**0.200**	**50**	**20**

Source conditions:

Capillary (kV) : 3.00, Desolvation gas (L/Hr) : 1000

Desolvation temperature (◦c) : 350, Cone gas flow : 150

RESULT AND DISCUSSION

SYSTEM SUITABILITY

Table 6: System Suitability

S. No.	RETENTION TIME		AREA RATIO
	ANALYTE	INTERNAL STANDARD	
1	**2.13**	**2.116**	**1.1836**
2	**2.130**	**2.109**	**1.1993**
3	**2.130**	**2.116**	**1.1908**
4	**2.130**	**2.109**	**1.2009**
5	**2.123**	**2.102**	**1.1854**
6	**2.109**	**2.102**	**1.1445**
MEAN	**2.12533**	**2.10900**	**1.18408**
STD DEV.	**0.008477**	**0.006261**	**0.020631**

% CV	0.40	0.30	1.74
Result	PASS	PASS	PASS

Acceptance Criteria:

- % Coefficient of Variation of area ratio should be ≤ 5%
- % Coefficient of Variation of analyte and IS retention time should be less than or equal to 15%

SELECTIVITY AND SPECIFICITY

Table 7: Selectivity and Selectivity

TOTAL No. OF LOTS	9	No. OF PASSING LOTS	9

Plasma Lot ID	Specificity (Blank)		Selectivity (Spiked LLOQ)		% Interference in Blank		Area Ratio
	Analyte	IS peak	Analyte	IS peak	Analyte (<20%)	Internal standard (<5%)	Analyte/Internal standard
MAT-17-0406-I	28	0	4167	169492	0.6719	0.0000	0.0246
MAT-17-0408-I	35	0	4122	168249	0.8491	0.0000	0.0245
MAT-17-0409-I	0	0	4359	177736	0.0000	0.0000	0.0245
MAT-17-0410-I	0	0	3958	155276	0.0000	0.0000	0.0255
MAT-17-0402-II	0	0	4232	165285	0.0000	0.0000	0.0256
MAT-17-0405-II	0	0	4035	164036	0.0000	0.0000	0.0246
MAT-17-0374-I(H)	70	0	3811	154522	1.8368	0.0000	0.0247
MAT-17-0322-IV(L)	0	0	3457	131399	0.0000	0.0000	0.0263
MAT-CMP-0398-VIII(Hep)	0	0	4038	152196	0.0000	0.0000	0.0265
				Mean	0.37309	0.00000	0.02520
						SD	0.000798
% of Lots passing =	100.00	%				%CV	3.17
						Result	Pass

Acceptance Criteria:

- Responses of the interfering peak in blank at retention time of the analyte and or metabolite should be less than 20 percent of the responses of the corresponding LLOQ sample.
- Response of interfering peak in blank at the retention time of IS should be less than 5 percent of the average responses of the internal standard observed in the sample of LLOQ run along with the batch.
- % Coefficient of Variation of the area ratio (analyte/IS) in extracted LLOQ should be ≥ 5.

- S/N ratio of LLOQ samples should be greater than or equal to 5.

Sl. No.	EXPERIMENTAL PARAMETERS.	ACCEPTANCE RANGE OR CRITERIA	RESULTS
1.	IS normalized matrix factor LQC	• Matrix factor should be within 0.85-1.15 • %CV of matrix factor should be ≤15%	PASS 0.95-1.09 % CV-4.09
	IS normalized matrix factor	• Matrix factor should be within 0.85-1.15 • %CV of matrix factor should be ≤15%	PASS 0.97-1.03 % CV-1.87
2	Precision and Accuracy	• % Nominal(LOQQC) 80-120, %Nominal(LQC, INTQC,MQC,HQC)-85-115	PASS LOQQC-95.33 LQC-113.88 INTQC-102.59 MQC-100.63 HQC-100.95
		• %CV(LOQQC)≤20, %CV(LQC,INTQC,MQC,HQC)	PASS LOQQC-5.56 LQC-2.67 INTQC-0.66 MQC-1.84 HQC1.97
3	Auto sampler carryover test	• For analyte % carryover < 20 of LLOQ area	PASS Blank 1=-2.86 Blank 2=-3.00
		• For IS % carryover < 5 for IS area	PASS Blank 1=0.00 Blank 2=0.00
4	Recovery	• % Recovery for analyte or IS should not be more than 110%	PASS
		• % CV of the mean recovery of LQC, MQC and HQC sample together should be ≤ 15%	5.90
		• Difference of % recovery between any two individual QC should be ≤ 15%	LQC, MQC- 6.17 LQC , HQC- 2.11 MQC, HQC- 4.07

CALIBERATION CURVE

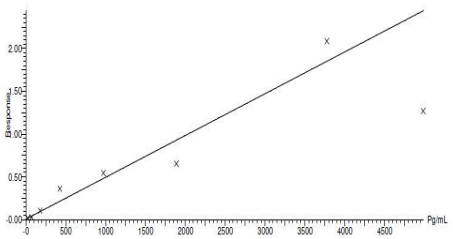

Figure 2: CC of Loperamide

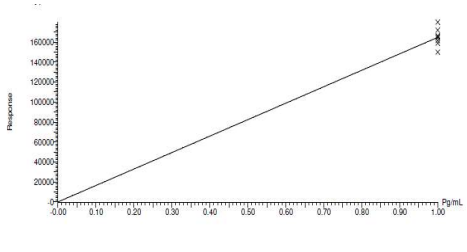

Figure 3: Calibration Curve of Fluconazole

r = 0.921875, r^2 = 0.849853 **RRF SD: 8850.87, % Relative SD: 5.37171**

AQSCCQC

Table 14: AQSCCQC

ID	TYPE	STD.CONC	RT	AREA	IS AREA	RESPONSE	pg/ml	%Dev
MP	Blank							
STD-A	Standard	24.874	2.24	3175	166352	0.0191	26.8033	7.76
STD-B	Standard	71.888	2.247	4468	161560	0.0277	44.3515	-38.3
STD-C	Standard	179.722	2.247	17133	165308	0.1036	199.9682	11.27
STD-D	Standard	425.376	2.24	59259	164276	0.3607	726.4507	70.78
STD-E	Standard	972.29	2.24	81751	149929	0.5453	1104.369	13.58
STD-F	Standard	1887.942	2.24	117558	179938	0.6533	1325.665	-29.78
STD-G	Standard	3775.882	2.24	331417	158913	2.0855	4258.684	12.79
STD-H	Standard	4984.662	2.24	218215	171870	1.2697	2587.848	-48.08
LQC	QC	71.894	2.24	14681	154377	0.0951	182.4714	153.81
INTQC	QC	424.906	2.24	91481	151029	0.6057	1228.167	189.04
MQC	QC	1880.116	2.247	274890	163941	1.6768	3421.562	81.99
HQC	QC	3760.232	2.24	543192	151964	3.5745	7307.92	94.35

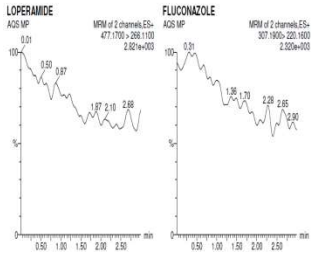

Figure 4: AQS Blank

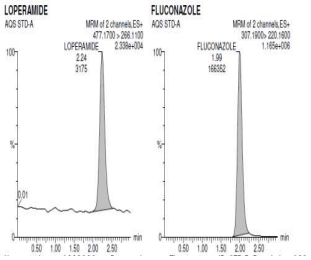

Figure 5: AQS STD-A

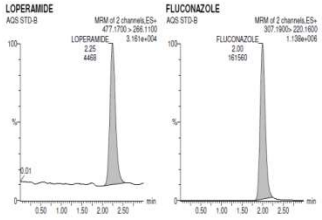

Figure 6: AQS STD-B

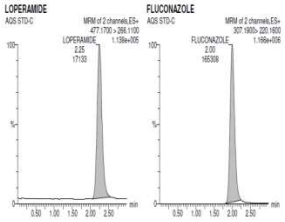

Figure 7: AQS STD-C

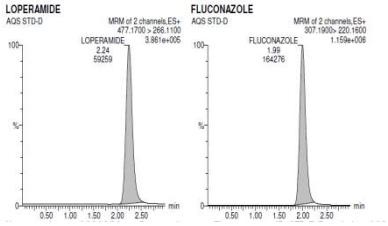

Figure 8: AQS STD-D

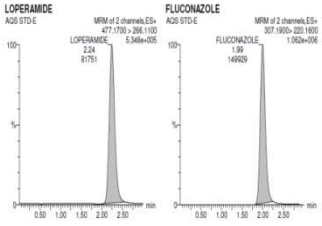

Figure 9: AQS STD-E

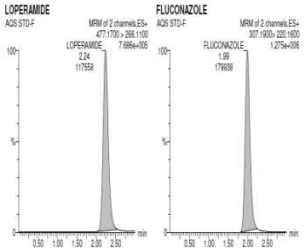

Figure 10: AQS STD-F

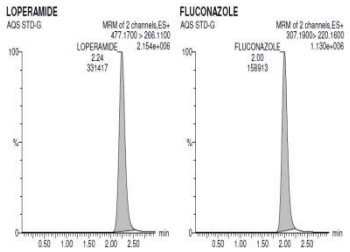

Figure 11: AQS STD-G

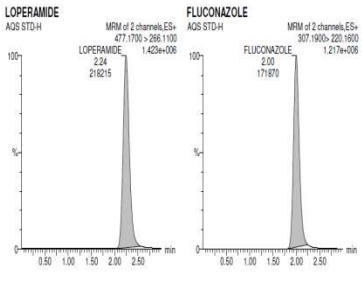

Figure 12: AQS STD-H

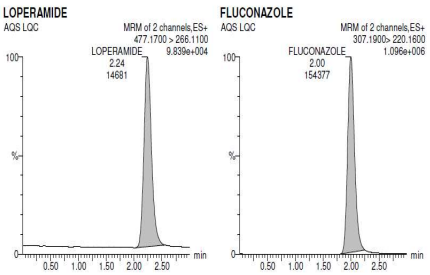

Figure 13: AQS LQC

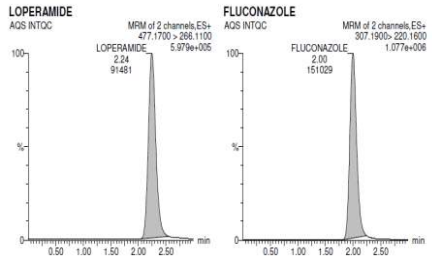

Figure 14: AQS INTQC

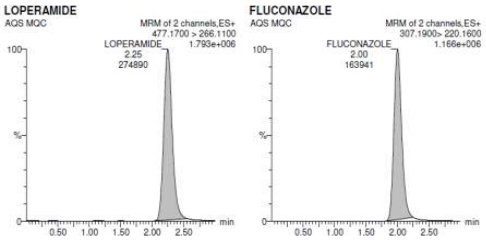

Figure 15: AQS MQC

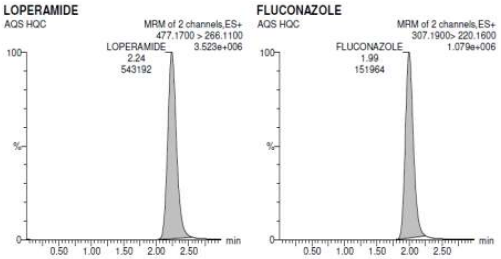

Figure 16: AQS HQC

CONCLUSION

The bio-analytical method SOP No.MTR-BA-M-452-00, Loperamide in K_2EDTA human plasma in the concentration range 24.8560-5063.9260pg/ml was successfully validated.

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