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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF TIZANIDINE IN K2EDTA HUMAN PLASMA BY USING LC-MS/MS

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ABSTRACT:

Bioanalytical method Validation employed for quantitative determination of drug and their metabolites in biological fluids. Comprises all criteria determining data quality, such as selectivity, accuracy, precision, recovery and senstivity. The main purpose of method validation is to demonstrate that a specific Bioanalytical method can reliably determine the concentration of drug in study sample with high degre of confidence. Validation does not means that method is perfect, but validation means method has met a set of criteria to ensure that it is reliable and consistent. Tizanidine is a central alpha 2 adrenergic agonist –inhibits release of excitatory amino acid in the spinal interneurones. It may facilitate the inhibitory transmitter glycine as well. It inhibits polysyneptic reflexes reduce muscle tone and frequency of muscle spasms without reducing muscle strength.

Following oral administration, tizanidine is essentially completely absorbed .The absolute oral bioavailability of tizanidine is approximately 40%, due to extensive first-pass hepatic metabolism. Tizanidine is extensively distributed throughout the body with a mean steady state volume of distribution of 2.4 L/kg following intravenous administration in healthy adult volunteers .tizanidine is approximately 30% bound to plasma proteins.

Keywords: Bioanalytical method Validation, LC-MS/MS, Human Plasma, Tizanidine, HPLC.

INTRODUCTION

The main purpose of method validation is to demonstrate that a specific Bioanalytical method can reliably determine the concentration of drug in study sample with high degre of confidence. Bioanalytical method Validation employed for quantitative determination of drug and their metabolites in biological fluids.

Instrumentation of LC-MS/MS

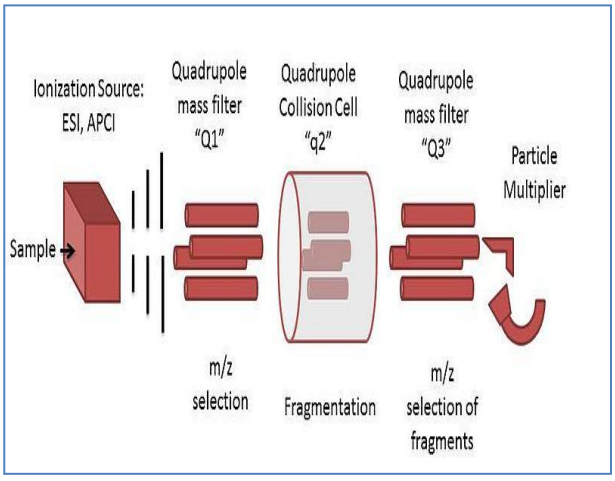

Figure 1:

Table 1: Drug Profile

Physicochemical Profile	
Name	Tizanidine
Emperical Formula	$C_9H_8ClN_5S$
IUPAC Name	5-chloro-4-(2-imidazolin-2-ylamino)-2,1,3-benzothiodiazole
Molecular Weight	253.712g/mol
Appearance	White to off white
Merck Index no.	10171
Density	1.37g/cm3
CAS Number	64461-82-1
Solubility	Soluble in water (20mg/ml), methanol and DMSO(100mM)
Melting point	280°C
Flash Point	190
Log P	1.4
pka	7.49
Boiling point	391.2°C
Category	Centrally Acting Muscle Relaxants
Storage condition	Store at room temperature
Pharmacological profile	
Therapeutic	Centrally Acting Muscle Relaxant
Mechanism of action	Tizanidine is a central alpha 2 adrenergic agonist –inhibits release of excitatory amino acid in the spinal interneurones .it may facilitate the inhibitory transmitter glycine as well .it inhibits polysyneptic reflexes reduce muscle tone and frequency of muscle spasms without reducing muscle strength.
Absorption	Following oral administration , tizanidine is essentially completely absorbed .The absolute oral bioavailability of tizanidine is approximately 40% ,due to extensive first-pass hepatic metabolism.
Distribution	Tizanidine is extensively distributed throughout the body with a mean steady state volume of distribution of 2.4 L/kg following intravenous administration in healthy adult volunteers .tizanidine is approximately 30% bound to plasma proteins
Metabolism	Tizanidine has linear pharmacokinetics over a dose of 1 to 20 mg. tizanidine has a half life of approximately 2.5 hours .approximately 95% of an administered dose is metabolized . The primary cytochrome P450 Isoenzyme involved in tizanidine metabolism is CYP1A2
Excretion	Following single and multiple oral dosing the excretion of tizanidine occur through urine and feces.
Half life	2-3 hour
Dose	Adult: 2 mg 3 times daily, max.24 mg/day with food

The objectives of current research are

- To optimize sample preparation and Extraction technique.
- Optimization of Column, mobile phase, and operating parameters.
- To Develop rapid, economic,and selective method that is useful in Bioequivalence study.

To validate the developed method as per USFDA Guideline

Table 2: MATERIAL AND METHODS

MAJOR EQUIPMENT USED		
EQUIPMENTS	**MAKE**	**MODEL**
HPLC ESI MS/MS	Shimadzu MDS Sciex MDS Sciex	LC-20-AD API 4000 API 4000
MINOR EQUIPMENT USED		
EQUIPMENTS	**MAKE**	
Centrifuge Evaporator Extractor (Rotospin) Vortex Shaker Eazypress Ultra Sonicator Weight balance Deepfreezer Refrigerator Multipipette Micropipette	Thermo Scientific Eppendorf Eppendorf Spinix Orochem Technology Sanyo Metller Toledo Sanyo Sanyo Eppendorf Eppendorf	

Table 3: MATERIAL USED

S.N.	**NAME OF MATERIAL**	**PURPOSE**	**GRADE**
1	Tizanidine	Drug	Working standard
2	Tizanidine d4	Internal standard	Working standard
3	Methanol	Solvent	HPLC
4	Acetonitrile	Solvent	HPLC
5	Milli-Q-water	Solvent	In-house
6	Acetic acid	Buffer	Emparta
7	Ortho phosphoric acid	Extraction buffer	Emparta
8	Formic acid	Buffer	Emparta
9	Ammonium formate	Buffer	GR
10	Ammonium acetate	Buffer	GR
11	Tert butyl methyl ether	Solvent	HPLC
12	Ethyl acetate	Solvent	Emparta
13	Ammonia	Extraction buffer	AR
14	n-hexane	Solvent	HPLC
15	NaOH	Buffer	AR
16	HCl	Buffer	AR
17	Extraction cartiridges	Method development	Orochemhlb, strata, grace C18
18	Column	Method development	Kinetix,inertsustain,ace, Gemini,luna,hypurity
19	Human Plasma	Blank plasma	In-house

EXPERIMENTAL WORK

Table 4: Mass Tuning Parameters

COMMON MASS PARAMETERS		
Parameters	**Tizanidine**	**Tizanidine D4**
Ion mode	Electro spray ionization(ESI)	Electro spray ionization(ESI)
Scan type	Multiple reaction monitoring (MRM)	Multiple reaction monitoring(MRM)
Polarity	Positive	Positive
Declustering Potential(DP)	80	80
Enterance Potential	7	7
Collision Energy	50	50
Collision Cell exit potential(CXP)	3	3
Dwell time(millisecond)	200	200
Parent	254.1	258.4
Daughter	44.3	48.3

Table 5:

Source Dependent Parameters	
Collision gas(L/H)	9.00
Curtain gas(L/H)	20.00
Nebulizer gas(L/H)	20.00
Heater gas(L/H)	80.00
Ion spray voltage	5500.00
Temperature	450
Interface Heater	ON

Table 6: HPLC PARAMETERS

Optimization of Chromatographic Condition	
Column	Kinetex C18 (100×4.6mm) 5µ
Mobile Phase	0.1% formic acid in water : Acetonitrile(20:80%v/v)
Flow rate	1ml/minute
Autosampler temperature	5±3°C
Column oven temperature	40±3°C
Volume of injection	10µL
Detector	Mass
Retention time	3.90 minute
Run time	5.5 minute
Rinsing volume	1000µl
Needle stroke	52mm
Rinsing speed	35µl/second
Sampling speed	5.0µl/second
Purge time	1.0 minute
Rinse dip time	2 second
Rinse mode	Before and after aspiration

Table 7: Mobile Phase optimization Trials

S.N.	COLUMN	MOBILE PHASE	RESULTS
1	INERTSUSTAIN(10*4.6 mm)	0.1%formic acid:Acetonitrile(70:30)	Response was poor, Peak shape was not good.
2	ACE (10*4.6mm)	10mm Am. formate: ACN(40:60), flow 1.0ml 0.1%FA:ACN,flow 0.7ml	Peak shape was not good,tailing was observed at LLOQ level of analyte
3	GEMINI(10*4.6)	10MM A FORMATE:MEOH(10:90) 0.5ML	Peak shape was not found good
4	LUNA	2mm a.a.:ACN(80:20) pH 5.0 flow 0.3	Peak shape was not found good
5	Hypurity(50*4.6mm)	0.1%F.A.:ACN(90:10) FLOW O.3	Peak shape was good for both drug and ISTD but tailing was observed.
6	KINETEX C18 (100*4.6mm) 5µm	0.1%formic acid in water: Acetonitrile(20:80%v/v),flow rate 1ml/minute,injection volume 10µl.	Peak shape was sharp, no tailing factor observed.

Table 8: Preparation of Calibration curve Spiking solution

Solution ID	Parent Solution Conc. (ng/mL)	Vol. Taken (mL)	Vol. of Diluent (mL)	Total Vol. (mL)	Spiking Solution Conc. (ng/mL)	Spiking Solution ID
Drug Intermediate Solution	3000.000	2.000	10.000	12.000	500.000	SS STD1
SS STD1	500.000	4.000	1.000	5.000	400.000	SS STD2
SS STD2	400.000	3.125	1.875	5.000	250.000	SS STD3
SS STD3	250.000	2.000	3.000	5.000	100.000	SS STD4
SS STD4	100.000	2.500	2.500	5.000	50.000	SS STD5
SS STD5	50.000	2.000	3.000	5.000	20.000	SS STD6
SS STD6	20.000	2.500	2.500	5.000	10.000	SS STD7
SS STD7	10.000	2.500	2.500	5.000	5.000	SS STD8

Table 9: Spiked calibration curve standards

SS ID	Spiking Solution Concentration (ng/mL)	Spiking Volume (mL)	Plasma Volume (mL)	Final Volume (mL)	Spiked Concentration (ng/mL)	STD ID
Methanol	0.000	0.200	9.800	10.000	0.000	STDBL
SS STD1	500.000	0.200	9.800	10.000	10.000	STD1
SS STD2	400.000	0.200	9.800	10.000	8.000	STD2
SS STD3	250.000	0.200	9.800	10.000	5.000	STD3
SS STD4	100.000	0.200	9.800	10.000	2.000	STD4
SS STD5	50.000	0.200	9.800	10.000	1.000	STD5
SS STD6	20.000	0.200	9.800	10.000	0.400	STD6
SS STD7	10.000	0.200	9.800	10.000	0.200	STD7
SS STD8	5.000	0.200	9.800	10.000	0.100	STD8

Optimized Extraction Method Optimization-LLE

- Retrieve the required number of samples from the deep freezer, thaw them at room temperature or in water bath maintained at room temperature and vortex the tubes to mix. Transfer 0.400mL of sample into pre-labeled tube.
- Add 50µL of ISTD dilution, 50ng/mL to all the samples except STD Blank and vortex to mix.
- Add 50µL Methanol in STD BL sample and vortex to mix.
- Add 50µL of 0.1N Sodium Hydroxide in Water to all the samples and Vortex to mix
- Add 2.500 ml of Tert Butyl Methyl Ether and extract samples on Rotospin for 20 minutes at 50 rpm.
- Centrifuge the samples at 4500 rpm at 10±2°C for 5 minutes.
- Transfer 2.000 ml of organic supernatant in to pre-labeled tubes and evaporate the samples to dryness under stream of nitrogen gas at 40±05 ºC
- Reconstitute the residue with 200 µL of Mobile Phase, Vortex to mix.
- Centrifuge the samples at 4500 rpm at 10±2°C for 5 minutes and transfer appropriate volume of samples into pre-labeled Auto sampler vials and inject by using LC-ESI-MS/MS

RESULT & DISCUSSION

S.N.	PARAMETER OPTIMIZED	RESULTS
1	Mobile phase: 0.1% formic acid in Water: acetonitrile (20:80v/v). column: kinetex c18 (100*4.6mm)	Better resonse and peak shape was sharp,good area of response
2	Extractor solvent: tert butyl methyl ether	All calibration curve and qc standard passed within criteria.
3	Extraction buffer: 0.1%v/v,formic acid in water.	Best in terms of response and matrix effect
4	Internal standard: tizanidine d4	Results was found to be best as is due to stable response and consistency and proper quantification

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