Sensitive, Reproducible and Simultaneous Bioanalytical method of Isosorbide Dinitrate and its metabolites Isosorbide 2-mononitrate and Isosorbide 5-mononitrate in human matrix samples for Pharmacokinetics analysis

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Purpose:

To Develop and validate the reliable method for simultaneous determination of Isosorbide dinitrate (ISDN) and its metabolites Isosorbide 2-mononitrate (2-ISMN) and Isosorbide 5-mononitrate (5-ISMN) in Human plasma Samples by using liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The method was developed and validated using 0.7mL of plasma sample over the calibration range of 0.4ng/mL to 100ng/mL for ISDN and 2.000 ng/mL to 500.000 ng/mL for both 2-ISMN and 5-ISMN to generate pharmacokinetic data as a component of bioequivalence studies for generic Isosorbide dinitrate formulations.

Background:

Isosorbide dinitrate is a nitrate that dilates (widens) blood vessels, making it easier for blood to flow through them and easier for the heart to pump. Isosorbide dinitrate is used to treat or prevent attacks of chest pain (angina) in coronary artery disease. After administration of ISDN tablets orally, ISDN is metabolized by enzymatic denitration followed by formation of glucuronide. The primary initial metabolites, Isosorbide 2-mononitrate generally referred as 2-ISMN and Isosorbide 5-mononitrate referred as 5-ISMN are presumed to be responsible, at least in part, for the therapeutic efficacy of ISDN.

A liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method was developed for simultaneous quantitation of Isosorbide dinitrate (ISDN) and its metabolites Isosorbide 2-mononitrate (2-ISMN) and Isosorbide 5-mononitrate (5-ISMN) in human plasma samples. Liquid-liquid extraction method allowed the selective extraction of ISDN, 2-ISMN and 5-ISMN from plasma. The measurement of 2-ISMN and 5-ISMN requires chromatographic separation of both isomers which were separated by using C18 stationary phase followed by gradient elution. ISDN, 2-ISMN and 5-ISMN were monitored simultaneously using negative ion acetate-adduct multiple reaction monitoring (MRM) mode transitions by mass spectrometric detection. ISDN and 13C6ISDN eluted at about 13.0 min while 2-ISMN and 13C6 2-ISMN eluted at about 6.0 min and 5-ISMN and 13C6 5-ISMN eluted at about 7.50 min with the total run time of 16.0 min. For the quantitation, individual calibration curves were used for all the three analytes. The calibration curves were found linear over the range of 0.4 ng/mL to 100 ng/mL for ISDN and 2.000 ng/mL to 500.000 ng/mL for both 2-ISMN and 5-ISMN. The method was found sensitive with LOQs of 0.400 ng/mL for ISDN and 2.000 ng/mL for both 2-ISMN and 5-ISMN.

Key Features of the Research Work:

The analytical method developed in past for the determination of Isosorbide dinitrate and its two isomeric metabolites has used capillary gas chromatography with electron capture detection technique to give LOQ 2.5ng/mL for ISDN, 2.6ng/mL for 2-ISMN and 2.3ng/mLfor 5-ISMN.However, no LC-MSMS method has been developed for the simultaneous determination of Isosorbide dinitrate and its two isomeric metabolites. The method described here is more sensitive that produce LOQ concentration 0.4ng/mL for ISDN and 2.0ng/mL for 2-ISMN & 5-ISMN. A few publications have reported simultaneous estimation of ISDN and 5-ISMN while some of the publications have reported estimation of 5-ISMN alone in human plasma by LC-MS/MS, however no one has reported simultaneous LC-MSMS method for the estimation of Isosorbide dinitrate and its mononitrate metabolites. Patel et al have reported simultaneous estimation of 2-ISMN and 5-ISMN in rat and human plasma using LC-MS/MS employing chiral chromatography. The usage of SPE cartridges and chiral column attributes the method cost. According to USFDA guidance estimation of Isosorbide dinitrate and both metabolites (2-ISMN and 5-ISMN) are required in plasma samples, Hence, we have developed the simple, selective, sensitive and cost effective bioanalytical method for the estimation of ISDN and both the metabolites in human plasma samples.

The Various method attributes aligning with the major regulatory guidelines were evaluated during method validation activity. This method was successfully employed for the analysis of ISDN, 2-ISMN and 5-ISMN in plasma samples collected during a human pharmacokinetic study from healthy subjects who received single oral dose of Isosorbide dinitrate 30 mg tablet as either Test or Reference treatment.

Sample Preparation ISDN, 2-ISMN & 5-ISMN:

Calibration curve standards, Quality control samples and unknown samples (0.700mL Plasma Samples) were transferred in a vial for sample preparation. 0.050 mL of mixed ISTD dilution was added to all samples except Blank samples. 0.300 mL of Formic acid in water (0.5% v/v) was added in all samples and mixed properly. Ethyl acetate solvent was added to all samples for extraction. Samples were extracted on rotospin for 25 minutes at 50 rpm. Samples were centrifuged at 5°C for 25 minutes at 4000 rpm. Organic layer phase was transferred from samples and dried under nitrogen gas. 0.100 mL of Reconstitution solution (1mM Ammonium Acetate in water: Methanol, 50:50 V/V) was added to all samples and mixed. The final samples were arranged in an auto-sampler and acquired by applying pre-defined equipment parameters for LC/MS/MS.

Method Summary ISDN, 2-ISMN & 5-ISMN:

Analytical Technique	Liquid chromatography coupled with mass spectroscopy
MS/MS	Triple QUAD 5500 - Sciex
Auto-sampler	UFLC XR Prominence - Shimadzu
Software used	Analyst software version No 1.6.3 (for analysis) and WATSON LIMS 7.3 for final regression
Ion source	Turbo Ion Spray
Scan Type	Multiple Reaction Monitoring
Column type	Gemini C18 5µm 110, 150*4.6mm
Mobile Phase	Milli-Q water (Pump A): Methanol (Pump B); 1mM ammonium acetate in Methanol (Pump C)
Flow Rate	1.0 mL/min
Biological Matrix	Human Plasma
Internal Standard	Isosorbide 13 C6, Isosorbide 13C6 2-mononitrate and Isosorbide -13C6 5-Mononitrate
Quantification	Area Ratio
Regression & Equation	Linear, y = ax + b
Weighting Factor	1/X²

	ISDN	2-ISMN	5-ISMN
Sample Processing Volume		0.700ml	
Linearity Range (ng/mL)	0.400 – 100.000	2.000 – 500.000	2.000 – 500.000
Validated LLOQ (ng/mL)	0.400	2.000	2.000
Validated LLOQ QC (ng/mL)	0.400	2.000	2.000
Validated LQC (ng/mL)	1.200	6.000	6.000
Validated MQC (ng/mL)	30.000	150.000	150.000
Validated HQC (ng/mL)	75.000	375.000	375.000
Validated AUL QC (ng/mL)	500.000	2500.000	2500.000
Validated ULOQ (ng/mL)	100.000	500.000	500.000

Chromatographic System and MS/MS Condition

Experiments were conducted on Shimadzu LC-20 AD XR system (Shimadzu Corp, Japan) coupled with TQ 5500 triple quadrupole mass spectrometric detector (AB Sciex, USA) equipped with an electrospray ionization source. Chromatographic separations of ISDN, 2-ISMN and 5-ISMN were achieved on Gemini C18 (150 × 4.6 mm, 5 µm) column using water, methanol as mobile phase with gradient condition (LC binary gradient programme) and ammonium acetate was continuously infused for adduct formation.

Details of the gradient programme are as follows: Milli-Q water (Pump A):
Methanol (Pump B)

Time (min)	Module	Events	Parameter (%B)
8.50	Pumps	Pump B conc.	10
9.00	Pumps	Pump B conc.	50
13.50	Pumps	Pump B conc.	50
13.60	Pumps	Pump B conc.	95
15.50	Pumps	Pump B conc.	95
15.60	Pumps	Pump B conc.	10
16.00	System Controller	Stop	

Stability Experiment Details ISDN, 2-ISMN & 5-ISMN:

Parameters	ISDN , 2-ISMN and 5-ISMN
Stability of Extract (SE) at Ambient Temperature	43 Hours at Ambient Temperature in Reconstitution solution (1mM Ammonium Acetate in water: Methanol, 50:50 V/V)
Stability of Extract (SE) in Refrigerator	210 Hours at 5±3°C in Reconstitution solution (1mM Ammonium Acetate in water : Methanol, 50:50 V/V)
Dry Extract stability	161 Hours at -20±5°C
Freeze Thaw (FT)	V Cycles at -20±5°C and -78±8°C
Bench Top (BT)	13 Hours at wet ice bath at below 10°C, Protected from normal light
Auto-sampler Re-Injection Reproducibility	214 Hours at 5±3°C in Reconstitution solution (1mM Ammonium Acetate in water: Methanol, 50:50 V/V)
Long Term Stability of Drug in Matrix (LTM)	93 Days at -20±5°C and -78±8°C
Batch Size Experiment	Total samples 155 including Calibration Curve
Dilution Integrity (DI)	10 fold DQC, 500.000 ng/mL for ISDN and 2500.000 ng/mL for 2- ISMN and 5-ISMN (Dilution medium used - human Plasma)

Validation Experiments:

Parameters	ISDN , 2-ISMN and 5-ISMN
Selectivity	< 3.0% interference in blank samples processed from different human plasma lots at the retention time of each analyte
Overall % Recovery	69.62%, 55.17% and 69.02%
Matrix effect by evaluation matrix factor	% CV of IS Normalized factor at HQC & LQC (1.89 & 2.82),(1.89 & 1.69), (1.92 & 1.53)
Cross selectivity(Impact analysis)	Significant interference was not observed in presence of another simultaneously analysed analyte(s)

Intra – Inter Precision & Accuracy ISDN, 2-ISMN & 5-ISMN:

ISDN	Precision			% Bias		
	RUN 1	RUN 2	RUN 3	RUN 1	RUN 2	RUN 3
Intra batch precision and % Bias (Accuracy) (HQC, MQC and LQC)	0.47 to 1.07	0.73 to 3.15	1.57 to 3.18	-0.27 to 1.31	-2.08 to 3.33	2.25 to 5.21
Intra batch precision and % Bias (Accuracy) (LLOQ QC)	5.25	3.89	9.13	0.00	9.25	15.00
Inter batch precision and % Bias (Accuracy) (HQC, MQC and LQC)		< 3.07			0.42 to 3.28	
Inter batch precision and % Bias (Accuracy) (LLOQ QC)		8.56			8.00	

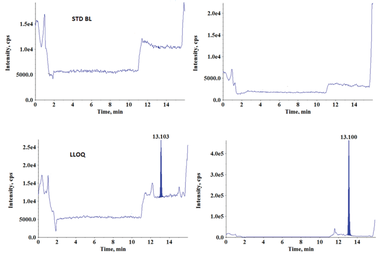
2-ISMN	Precision			% Bias		
	RUN 1	RUN 2	RUN 3	RUN 1	RUN 2	RUN 3
Intra batch precision and % Bias (Accuracy) (HQC, MQC and LQC)	0.44 to 1.38	0.47 to 5.49	0.47 to 5.49	-2.20 to 2.33	-8.85 to 4.85	2.07 to 5.58
Intra batch precision and % Bias (Accuracy) (LLOQ QC)	4.84	13.8	13.8	-2.85	3.60	9.00
Inter batch precision and % Bias (Accuracy) (HQC, MQC and LQC)		2.04 to 7.01			-3.00 to 3.92	
Inter batch precision and % Bias (Accuracy) (LLOQ QC)		9.64			3.25	

5-ISMN	Precision			% Bias		
	RUN 1	RUN 2	RUN 3	RUN 1	RUN 2	RUN 3
Intra batch precision and % Bias (Accuracy) (HQC, MQC and LQC)	0.95 to 1.50	0.77 to 1.33	0.91 to 3.07	-1.38 to 3.63	-2.40 to 5.39	-1.07 to 6.65
Intra batch precision and % Bias (Accuracy) (LLOQ QC)	1.77	1.94	3.19	-1.00	5.50	0.20
Inter batch precision and % Bias (Accuracy) (HQC, MQC and LQC)		1.28 to 2.09			-1.25 to 5.22	
Inter batch precision and % Bias (Accuracy) (LLOQ QC)		3.64			1.55	

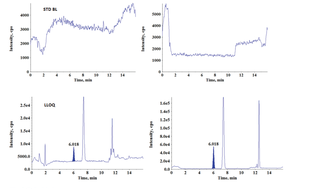
Calibration Curve Parameters:

Analyte	CC parameters		
	Slope	intercept	R-Squared
ISDN	0.0950	-0.0031	0.9992
2-ISMN	0.0550	-0.0071	0.9991
5-ISMN	0.0799	-0.0123	0.9994

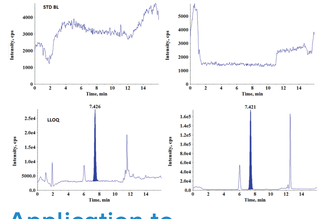
Representative Chromatograms:



2-ISMN



5-ISMN



Application to Pharmacokinetics study:

The study objective was to compare and evaluate a single-dose oral bioavailability of Isosorbide dinitrate 30mg Tablets. This method was successfully employed for the analysis of ISDN, 2-ISMN and 5-ISMN in plasma samples collected during a human pharmacokinetic study from healthy subjects who received single oral dose of Isosorbide Dinitrate 30 mg tablet as either Test or Reference treatment.

Conclusion:

The bioanalytical methodology described in this research work provides a simple, sensitive, cost-effective analytical method for simultaneous estimation of ISDN, 2-ISMN and 5-ISMN in human plasma samples. This method offers advantages over those previously reported methods in terms of Quantification of ISDN and its two isomeric metabolites in single injection method. The method was successfully employed for the analysis of ISDN, 2-ISMN and 5-ISMN in human plasma samples collected during a pharmacokinetic study.

Acknowledgment:

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Reference:

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