Chromatographic separation of (*R*) and (*S*)-enantiomers of Verapamil and Norverapamil and their simultaneous quantitation in human plasma by LC-ESI-MS/MS

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Introduction

Verapamil is a calcium ion influx inhibitor (slow-channel blocker or calcium ion antagonist) indicated for the management of hypertension, angina and arrhythmia (paroxysmal supraventricular tachycardia).

Few bioanalytical methods are reported for the separation and quantitation of (R and S Verapamil) and (R and S Norverapamil) in biological matrices. Many analytical methods of analysis of Verapamil have been reported by high-performance liquid chromatography Mikael Hedeland et al [2] have reported the simultaneous quantification of the enantiomers of Verapamil and its N-demethylated metabolite in human plasma but the processing volume is 500µL and higher spiking volume (100µL of standard solution in 500µL of blank plasma) were used. Racemic standards were used for the preparation of standards. In another method [3], simultaneous analysis of enantiomers of Verapamil and Norverapamil in rat plasma has been reported but the injection volume is 130µL which may be harmful for column life. The spiking volume is also high (25µL in 100µL plasma). The % CV for all the intra and interassay precision is about more than 6. Another method [4] describing simultaneous HPLC analysis of enantiomers of Verapamil and its metabolite in human plasma shows %CV of LLOQ more than 14. In yet another method [5] stereoselective determination of only Verapamil in serum has been quantified. The other methods [6-9] reported are describing the method for the determination of racemic Verapamil and not for the enantiomers.

The objective of the present study was to separate both the enantiomers of Verapamil and Norverapamil chromatographically in a single run and to develop a LC-ESI-MS/MS method for their quantitation in human plasma.The proposed method exhibited excellent performance in terms of sensitivity, selectivity, ruggedness and efficiency due to cleaner extracts, with simplicity of sample preparation. In the proposed method only 5 µL injection volume was used with a spiking concentration of 2%organic in plasma.

The intra assay precision observed in the proposed method was less than 3% and interassay precision was less than 6 %.

Method

Major Equipment Involved

Equipment	Make	Model
HPLC	Shimadzu	LC-VP Series
LC/MS/MS	MDS Sciex	API 2000

Liquid Chromatographic Conditions

Column	Chiracel®OD-RH, 150 × 4.6mm, 5µm
Mobile Phase	0.05%Trifluoroacetic acid : Acetonitrile (70:30, v/v)
Flow Rate	0.50 mL/minute, 70% flow splitting
Run Time	20.00 minutes

Mass Spectrometric Conditions

MRM Conditions	R & S – Verapamil: 455→165
	Verapamil-D6: 461→165
	R&S-Norverapamil:441→165
	Norverapamil-D6: 447→165
Ion Spray Voltage (KV)	4.00
Ion Spray Voltage (KV)	4.00
Nebulizer Gas (psig)	50.00
Heater Gas (psig)	60.00
Ion Source Temperature (°C)	400.00
Curtain Gas (psig)	18.00
CAD Gas (psig)	4.00
Dwell Time (ms)	600.00

Results & Discussion

Method Preparation LLE

- Aliquot 200µL of plasma in pre labeled poly propylene tubes.
- 50µL of internal standard was added to 200µL of plasma samples and mixed by vortexing.
- The samples were extracted in 2.5mL of n-hexane:diethyl ether 50;50 v/v mixture on extractor at 40rpm for 20 minutes
- The samples were centrifuged at 3200 × g (4000rpm), 10°C for 5 minutes and 2.0mL of supernatant was evaporated to dryness under nitrogen at 40±5°C.
- The dried samples were reconstituted with 100µL of 0.05%triflouro acetic acid : acetonitrile 30:70v/v mixture and 5µL was injected in chromatographic system.

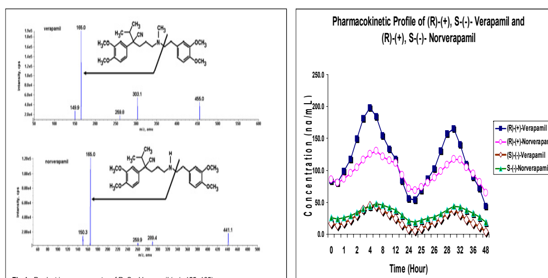


Fig.1:- Product ion mass spectra of R, S – Verapamil (m/z 455, 165) R, S – Norverapamil (m/z 441, 165) in positive ionization mode

Pharmacokinetic Profile of (R)-(+), S-(-)- Verapamil and (R)-(+), S-(-)- Norverapamil

Fig. 2:- Representative chromatograms of double blank and LLOQ in human plasma

Ion Suppression

No ion suppression or enhancement was found at the retention time of analyte and IS in presence of matrix ions through post column infusion of neat solution of analyte and IS.

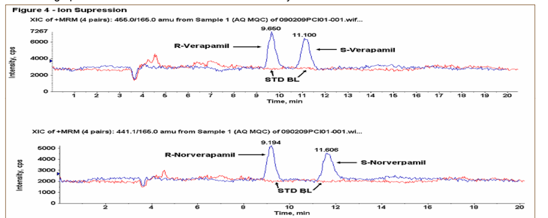


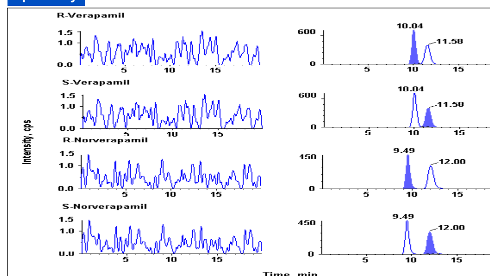
Fig.3:- The post column infusion spectra of analyte and ISTD.

Precision & Linearity

QC ID	Nominal Concentration (ng/mL)	Mean Concentration observed (% Mean Accuracy, %CV)			
		Verapamil		Nor-Verapamil	
		R	S	R	S
		Intra-batch (n=5)			
HQC	192	192 (100, 2.3)	193 (101, 1.5)	191 (99.5, 1.7)	195 (102, 2.1)
MQC	99.1	101 (102, 1.7)	102 (103, 1.3)	101 (102, 0.8)	102 (103, 1.6)
LQC	29.7	29.9 (101, 2.0)	29.9 (102, 2.1)	30.0 (102, 1.7)	29.9 (101, 2.5)
LLOQ	10	10.4 (103, 1.4)	10.5 (104, 0.7)	10.3 (102, 1.8)	10.4 (103, 2.1)
		Inter-batch (n=25)			
HQC	192	194 (101, 5.3)	197 (103, 5.2)	195 (102, 5.0)	198 (103, 5.4)
MQC	99.1	99.8 (101, 2.9)	101 (102, 2.7)	101 (102, 3.1)	101 (102, 3.2)
LQC	29.7	30.8 (104, 5.8)	30.9 (104, 5.4)	30.6 (103, 5.4)	30.7 (103, 5.9)
LLOQ	10	10.3 (102, 2.8)	10.4 (103, 4.1)	10.3 (102, 4.4)	10.4 (103, 4.1)

Table 1 - Intra-batch / inter-batch precision & accuracy for R, S – Verapamil and R, S - Norverapamil

Specificity



Representative chromatograms of double blank and LLOQ in human plasma

Stability, Selectivity and Recovery Parameters*

*(For All Analytes)

Bench Top	:	Up to 7 hours
Autosampler	:	Up to 69 hours
Dry Extract (-20°C)	:	Up to 47 hours
Freeze Thaw	:	Three Cycles at -20°C & -70°C
Ruggedness	:	Analyst, Column and Instument
Specificity	:	No significant interference
% Recovery	:	More than 92%
Matrix Effect	:	ISTD normalization Matrix factor 1.0
Dilution	:	Up to 10 time

Conclusion

- The proposed method successfully demonstrates chromatographic separation of R and S verapamil and R and S Norverapamil from human plasma with high resolution
- The bioanalytical methodology for their simultaneous determination is highly specific, rugged and rapid for therapeutic drug monitoring.
- The method involved a simple and specific sample preparation by liquid-liquid extraction followed by isocratic chromatographic separation in 20 min. The overall analysis time is promising compared to other reported procedures for both enantiomers.
- The established LLOQ and a wide linear dynamic range is adequate to conduct a pharmacokinetic study with 240mg or higher dose formulations of R and S verapamil,and R and S Norverapamil in healthy human volunteers.
- The method showed a good reproducibility and good separation for all the four analytes.
- The method was validated over the range of 10ng/mL -250ng/mL with excellent linearity r2 > 0.99.

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