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Original Article

AN IMPROVED LC-MS/MS METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF TRANDOLAPRIL AND VERAPAMIL IN HUMAN PLASMA

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ABSTRACT

Objective: A simple, sensitive and rapid LC-MS/MS technique was developed for the quantitation of trandolapril (TDL) and verapamil (VPL) in a biological matrix and validated.

Methods: Sample preparation processed by SPE (Solid Phase extraction) on phenomenex cartridge using Ledipasvir as an internal standard. Two drugs were eluted on waters symmetry-RP_{18} (5µ, 150 mm×4.0 mm) column with the mobile composition of 10 mmol ammonium formate and ACN(acetonitrile) in the ratio of 70:30 %V/V. Detection and quantitation were processed by electrospray ionization in positive ionization mode.

Results: The quantification approach was validated in 5-1500 ng/ml linear concentration range for TDL and 1-2000 ng/ml for VPL. The intraday and inter-day precision and accuracy were found to be 0.58% to 5.69% and 93% to 104% for two drugs. The average recoveries for TDL and VPL were found to be 92.9% and 93.5% respectively.

Conclusion: The developed work was validated and can be applicable to the routine analysis of TDL and VPL simultaneously in a biological matrix.

Keywords: Trandolapril, Verapamil, Cancer, ACE inhibitor, Accuracy, LC-MS/MS, Validation

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INTRODUCTION

TDL is a nonsulfhydryl prodrug that is de-esterified to trandolaprilat in the liver, belongs to the class of an angiotensin-converting enzyme (ACE) inhibitor medications [1]. It obstructs ACE, which shows a significant role in the conversion of angiotensin-I (AT-I) to angiotensin-II (AT-II). AT-II controls blood pressure and is a main constituent of the RASS (renin-angiotensin aldosterone system) [2]. It helpful in hypertension treatment and increase survival subsequent myocardial infarction patients suffering from left ventricular dysfunction. It acts as an adjunct in heart failure and reduces the rate of progress of the renal disease in patients with hypertension, diabetes and overt nephropathy. It chemically designated as (2S, 3aR, 7aS)-1-[(2S)-2-{[(2S)-1–ethoxy-1-oxo-4–phenylbutan-2-yl] amino} propanol]–octahydro-1H–indole-2-carboxylic acid [3, 4].

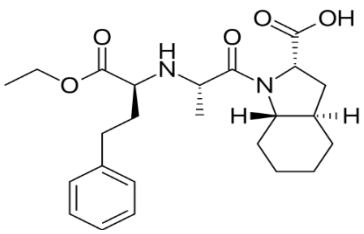

Fig. 1: Structure of trandolapril

VPL is useful in the treatment of high blood pressure by dilating the blood vessels, angina by increasing the oxygen and blood supply to the heart and supraventricular tachycardia. It also useful in the prevention of a migraine and cluster headaches. The drug was administered either by oral route or through injection into a vein. It acts by blocking the voltage-dependent calcium channel and this drug considered as a class-IV antiarrhythmic agent in cardiac pharmacology [4, 5]. IUPAC name of the compound was (*RS*)-2-(3, 4-Dimethoxyphenyl)-5-{[2-(3, 4–dimethoxyphenyl) ethyl]-(methyl) amino}-2–prop-2-yl pentanenitrile [4, 6]. Chest pain is controlled by using verapamil regularly.

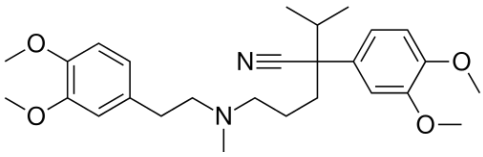

Fig. 2: Structure of verapamil

Literature survey of TDL and VPL revealed that several analytical techniques were employed such as high-performance thin layer chromatography (HPTLC) [7], high-performance liquid chromatography (HPLC) [8-12] and LC-MS/MS [13, 14]. Aim of the present work was to develop an improved, accurate and rapid LC-MS/MS technique with low retention time.

MATERIALS AND METHODS

Chemicals and reagents

The TDL, VPL reference standards and internal standard (Ledipasvir) were obtained from Hetero drugs, Hyderabad. HPLC-grade ACN and methanol were procured form SD-Fine Chemicals, Mumbai, India. Phenomenex SPE-cartridges were bought from Phenomenex, Bengaluru, India. Analytical grade ammonium formate and formic acid were bought from Qualigens chemicals, Mumbai, India.

Mass spectrometric and liquid chromatographic conditions

Liquid chromatographic system (Shimadzu, Japan) consisted of a binary LC-20A CE pump, solvent degasifying system (DGU-20A), autosampler (SIL-HTC) and temperature controller (CTO-10 AS) for maintaining column temperature was used for the chromatographic elution of the two drugs and internal standard (IS). Separation of

analyte peaks was carried out with Waters symmetry-RP_{18} (150 mm×4.0 mm, 5µ) column at 40 °C oven temperature with the mobile composition of ACN and 10 mmol ammonium formate in the ratio of 70:30 v/v. The mobile phase flow of 0.9 ml/min was processed through an analytical column with 5 °C autosampler temperature.

Mass spectrometric detection was processed using API-4000 mass spectrometer (MDS-SCIEX, Toronto, Canada) combined with ESI (electrospray ionization). The MRM transitions for the quantitation of TDL, VPL and Ledipasvir were m/z 431.2/234.1, m/z 455.0/165.0 and m/z 889.4/130.0 respectively. The optimized mass parameters like ion-spray voltage, curtain gas, Gas-1, Gas-2, collisional activation dissociation, and turbo heater temperature were processed at 2500 V, 30 psi, 40 psi, 65 psi, 8 psi and 450 °C respectively. The parameters depends on the compound, namely declustering potential(DP), entrance potential, collision cell exit potential (CEP) and collision energy were adjusted to 90 V, 9 V, 12 V and 25 V for TDL and 80 V, 9 V, 10 V and 25 V for VPL respectively.

Quality and calibration control samples

TDL and VPL stock solutions (200 µg/ml) were freshly processed by dissolving accurate quantities of drugs in methanol as solvent separately. The quality control (QC) samples and calibration standards (CS) were processed by spiking with suitable volumes of working standard solutions made from intermediate stocks for the two drugs to blank plasma. The QC concentrations for TDL/VPL were LLOQ (lower limit of quantification): 5/1 ng/ml, Low QC (low-quality control): 10/2 ng/ml, Medium QC (medium-quality control): 600/600 ng/ml and High QC (high-quality control): 1100/1500 ng/ml. The CSs were 5, 10, 150, 300, 400, 600, 850, 1100 and 1500 ng/ml for TDL and 1, 2, 40, 200, 350, 600, 900, 1500 and 2000 ng/ml for VPL. Ledipasvir (LPR) 100 µg/ml stock solution was prepared by dissloving an accurate amount of IS in methanol. LPR 8 µg/ml working standard was made from 50% methanol. Standard and stock sample solutions were kept at 2 to 8 °C and QC standards and CSs were kept at-70 °C till actual sample analysis.

Preparation of sample solution

Prior to the separation, all samples subjected for freezing, QCs and CSs sample concentrations were equilibrated to room temperature. To 250 µl of spiked sample, 50 µl of LPR was mixed and subjected to vortex for 30 sec. Further 150 µl of buffer (10 mmol ammonium formate pH 3.2) was added and placed in a centrifuge for 15 min at 4500 rpm. Further samples were processed through pre-treated phenomenex cartridges with methanol and 10 mmol ammonium formate buffer. The sample matrix was exhausted out from the cartridges by the application of positive pressure (nitrogen). The cartridges were cleaned with 1 ml of 10 mmol ammonium formate buffer and 1 ml of 50% methanol. The IS and drugs were separated with 500 µl of the mobile phase into pre-labeled vials.

System suitability and sensitivity

System suitability experimentation was executed by introducing six repeated injections, using an extracted sample of TDL/VPL and internal standard at the starting of each lot. Analytical technique selectivity was analysed for potential interfering matrix components in 10 dfferent sources (6 Na-heparinized, 2-haemolysed and 2-lipemic) of blank plasma sample by extraction and examination of the resultant chromatograms for interfering matrix components peaks [15-18]. Analytes stock solutions were processed by dissolving required quantity in methanol. Working solutions were processed in 50 % methanol and 5 µl was introduced in to the system to check for possible interfering constituents at the elution time of the drugs and IS.

Accuracy and precision

Intra-day accuracy and precision were evaluated by identical analysis of samples on the same day. The sample runs consist of calibration standards and 6 replicates of LLOQ, Lower-QC, Medium-QC, and High-QC samples. The inter-day accuracy and precision were evaluated by determination of 3 accuracy and precision lots on three di fferent days [19-21]. The % CV at each sample concentration should be<15%. Similarly, an average accuracy should be between 85%–115% all and for LLOQ it should be 80%–120% of the nominal concentration.

Linearity and re-injection reproducibility

The five calibration curve lines were plotted by preparing and analysing 9 different concentration solutions for the estimation of linearity. Linear ($1/x^2$), least squares regression algorithm was used to construct linearity plot from the data of peak response ratio (analyte/IS) versus concentration. The linearity formula was used to estimate the projected concentrations in samples within the analytical runs. The correlation coefficient of calibration curves should to be more than 0.99 for the two analytes [16]. Re-injection reproducibility estimated for extraction samples by re-injecting the sample after storing at 5 °C.

Method ruggedness

To find out method ruggedness of analytes, two lots were studied for accuracy and precision. The first lot was assessed on two Symmetry-ShieldRP$_{18}$-(150 mm×3.9 mm, 5 µm) analytical columns with different batch numbers and the second lot was studied by two different analysts.

Carryover effect

Carryover effect was assessed to confirm that the washing solution used to clean the infusing needle and the port is able to evade any carry forward of an introduced sample in preceding runs. Autosampler carryover was estimated by consecutively introducing extracted blank plasma, duplicate LLOQ, duplicate ULOQ sample followed by introducing duplicate blank plasma. Cross selectivity test was executed to check the conversion of TDL to VPL and vice versa during successive steps of analysis [14]. This test was performed at LLOQ and HQC levels for both the analytes in duplicate and processed along with two blank plasma samples.

Matrix effect and Recovery

Extraction retrieval of the drugs and IS from biological matrix was assessed in six duplicates by comparing the maverage peak responses of pre-extraction fortfied samples to those of post - extraction fortified samples. Absolute matrix effect was evaluated by paralleling the average peak responses of post-extraction fortified samples to those of neat samples prepared in elution solution. To evaluate the relative matrixffect in different plasma lots, post - extraction fortified samples were prepared in duplicate at Low-QC and High-QC concentrations and % CV was assessed. Effect of matrix ions on drugs sensitivity were estimated by analyte infusion [13]. Briefly, TDL, VPL and Ledipasvir (at ULOQ level) standard solution was injected post-column through the mobile phase having infusion pump. 5 µl aliquots of extracted control samples were then infused into the column and anlyte chromatograms were estimated.

Stability assessment

TDL and VPL stock solutions and IS were tested at room conditions (short-term stability at 25 °C) and at 5 °C (long-term stability). Stability findings were estimated by determining the peak area (analyte/IS) of samples against freshly processed samples with same concentration. The % difference should be±10% for both stability solutions. Freeze-thaw (at −70 °C) stability, bench-top stability, autosampler (at 5 °C) stability, processed sample (at 25 °C) stability and long term (at −70 °C) stability were processed at Low-QC and High-QC levels using 6 replicates. The stability solutions were estimated against freshly processed calibration standards [22-24].

Dilution integrity

It was processed to validate the test for dilution, performed on higher concentrations of analyte (above ULOQ). It was processed at 1.6 times the ULOQ concentration and 6 duplicate samples of ¼ and ½ concentrations were processed. The concentrations were estimated by the application of dilution factor 4 and 2 respectively by comparing with the freshly processed calibration standards [15, 16].

RESULTS AND DISCUSSION

Method development

Present method was projected to improve upon the existing methods in order to achieve higher sensitivity, lower sample volume requirement for extraction and use of Ledipasvir internal standard

for better precision and accuracy. Moreover, none of the reported method afforded baseline chromatographic separation of TDL and VPL under the established chromatographic conditions. Both TDL and VPL were processed in the positive mode and the ions of precursor and product were optimized for two drugs and ledipasvir 150 ng/ml solutions by injecting in mass range of 50–500 Da. The Q-1 full scan for two drugs and ledipasvir primarily contains protonated precursor $[M+H]^+$ ions at *m/*m/z431.2, 455.0 and 889.4 for TDL, VPL and IS respectively. The corresponding stable and abundant productions in Q-3 MS were detected at m/z 234.1, 165.0 and 130.0 respectively. The MRM transitions for the quantification of TDL, VPL and IS were m/z431.2/234.1, m/z 455.0/165.0 and m/z 889.4/130.0 respectively. The chromatographic parameters were optimized to increase peak response and reduce the interference of impurity peaks. In order to achieve this, several reversed-phase columns like Hypersil-Gold (5 µ, 150 mm × 4.6 mm), Hypurity-Advance (5 µ, 150 mm × 4.6 mm), BDS Hypersil-C_{18} (5 µ, 150 mm × 4.6 mm) and symmetry-RP_{18} (5µ, 150 mm×4.0 mm) column were verified using similar mobile phase. All four columnsffarded chromatographic elution of the analytes but the response was not adequate especially at the LLOQ levels of TDL and VPL. However, the signal intensity of the drugs were fairly high on Symmetry Shield-RP_{18} column compared to other columns, with comparatively less peak tailing in VPL. Further optimization was made by the change in the aqueous/organic ratios of the mobile phase using the same column. Increase in the organic phase (>70%) result in improper peak shapes, while ratio at 50:50 %V/V the elution time was more than 4.0 min. Mobile phase with 10 mmol ammonium formate and ACN in a proportion of 30:70 %V/V produce symmetric peaks, better retention and good peak response with less matrix effect.

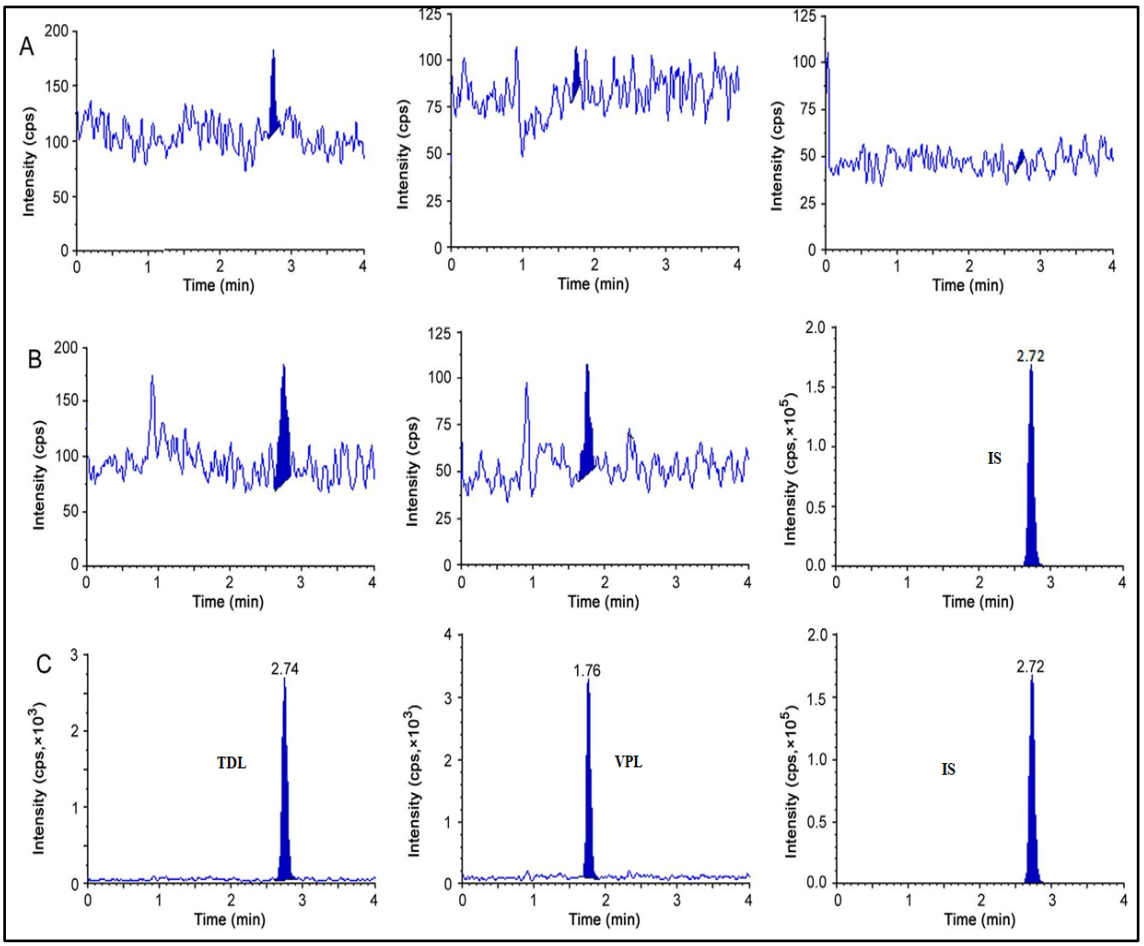

Fig. 3: Typical chromatograms of A) Double blank plasma B) Blank plasma with IS C) TDL, VPL at LLOQ level with IS

It was necessary to have the chromatographic analysis time of 4.0 min for higher sensitivity and better ionizatffiency. The chromatograms in fig. 3A-C showed no interference with an elution time of TDL and VPL against endogenous components. However, as they eluted much ahead of VPL there was no interference in the quantitation and no further attempt was made towards their identification. The resolution factor between the analytes under the established conditions was 3.8, while the capacity factors for TDL and VPL were 2.01 and 0.93, respectively. Further, there was no nosiness of medications at the elution time of the two drugs. Post-column injection chromatograms showed the lake of matrix effects with no signal improvement or suppression at TDL and VPL retention times (fig. 3).

Protein precipitation and liquid-liquid extraction methods were not providing precise and accurate results. As a result, Solid-phase extraction was processed on different separation cartridges, namely Bond Elut C_{18}, Oasis-HLB, Phenomenex Strata-X, and Lichrosep-DVB. It was found that the addition of 2 mmol ammonium formate buffer was essential during all stages of workup to obtain adequate response and consistency in the recovery with minimal matrix interference. The extracts found with Phenomenex cartridges were cleaner than remaining cartridges [13, 14].

Method validation results

The % CV values for system suitability were obtained between 0.09% to 0.32 % for the elution times and 0.52% to 1.57% for the peak response of two analytes and internal reference standard. For the determination of selectivity, the response for 10 different blank plasma lots at the elution times of analytes was equated with the peak response (at LLOQ). None of the blank plasma sources

exhibited any obvious interference (≤0.52% of LLOQ sample for TDL and VPL) at elution times of analytes.

The method was evaluated for carryover for column and auto-sampler with blank plasma after consequent infusion of ULOQ of analytes and the findings were ≤0.64%. Peak response ratios (analyte/IS) against the sample concentrations were plotted to calculate linear regression. The linear curves were obtained over 5-1500 ng/ml range for TDL and 1-2000 ng/ml range for VPL. The corresponding regression equations for TDL and VPL were y = (0.00089±0.00002) x+(0.00052±0.00021) and y=(0.00025 ±0.00010) x+(0.00038±0.00008) respectively, with a correlation coefficient (r^2) of ≥ 0.9994. For both the analytes, the bias of back-calculated values in calibration range was within 7.6% of the nominal concentration values. The signal-to-noise ratio at the LLOQ concentration was ≥ 15 and ≥ 29 for TDL and VPL respectively. The inter and intraday precision and accuracy findings were tabulated in table 1. The relative standard deviations were ≤ 5.69% and the overall mean accuracy ranged from 93.00% to 104.00% for the two drugs.

Table 1: Trandolapril and verapamil data for intra and inter-day precision and accuracy

Nominal concentration (ng/ml)	Intra-day		Inter-day	
	Accuracy (%)	%RSD	Accuracy (%)	%RSD
Trandolapril				
5	103.6	1.23	103.2	5.67
10	93.2	4.9	97.1	5.2
600	103.2	3.5	103.2	4.2
1100	103.6	7.9	102.9	2.9
Verapamil				
1	104.5	5.2	107.5	5.1
2	94.2	3.2	93.6	4.21
600	98.2	4.8	94.6	5.1
1500	95.9	0.59	95.1	0.92

Table 2: Trandolapril and verapamil data for extraction recovery

Analyte and QC levels	peak response		Extraction recovery A/B (%)
	Pre-extraction spiking (A)	Post-extraction spiking (B)	
Trandolapril			
LQC	36525	37526	97.33
MQC	378455	365214	103.63
HQC	10005236	10506345	95.23
Verapamil			
LQC	35568	37523	94.79
MQC	543985	532852	102.09
HQC	5263521	5405892	97.37
Ledipasvir			
LQC	925345	945852	97.83
MQC	935621	975623	95.89
HQC	945654	986521	95.86

Table 3: Matrix effect data for trandolapril and verapamil

Analytes/IS	Average area response (n = 6) Post-extraction spiking (A)		Neat samples in elution solution (B)		Matrix factor (A/B)	
	LQC	HQC	LQC	HQC	LQC	HQC
Trandolapril	45615	13025468	46523	14086955	0.9804828	0.924648
Verapamil	46750	6486531	48523	6542586	0.9634606	0.991432
Ledipasvir	1045263	1203564	1123758	1305462	0.9301496	0.921945

Table 4: Stability data for trandolapril and verapamil

Storage condition	QC level	Accuracy (%)		Precision (% CV)	
		TDL	VPL	TDL	VPL
Bench-top stability	LQC	35.14	98.23	1.25	2.01
(8 h, 25 °C)	HQC	95.65	95.23	0.52	1.3
Freeze-thaw stability	LQC	94.57	94.95	1.52	1.52
(at −70 °C, 5cycles)	HQC	96.12	96.24	0.98	0.35
Auto-sampler stability	LQC	94.21	97.84	0.58	0.84
(74 h, 5 °C)	HQC	95.32	95.69	0.89	0.98
Processed sample	LQC	94.68	98.23	0.68	0.74
stability (16 h, 25 °C)	HQC	98.41	97.46	1.35	0.25
Long-term stability (at −70 °C for 116 d,)	LQC	97.25	96.58	1.25	0.26
	HQC	97.66	97.32	0.36	1.25

The average recoveries for TDL and VPL were found to be 92.9% and 93.5% respectively (table 2). The average recovery of the internal standard was 90.87%. These findings specifies the developed technique was improbable to be modified by intra or inter-individual differences in the biomatrix and that the technique has good accuracy and reproducibility. The effects of matrix components suppression or improvement, represented as IS normalized matrix effects, ranged from 0.965 to 1.245 for both the analytes (table 3). The % CV findings for relative matrix effect in lipemic, sodium-heparin plasma and haemolysed plasma lots also represents that no ion improvement or suppression of the analytes (intensities) (table 2).

The TDL and VPL stock solutions and ledipasvir were stable in methanol at refrigerated and room conditions for 2 w and 24 h respectively. The bench top stability of analytes in plasma were proven up to 8 h. Both the analytes were stable during 5 freeze-thaw cycles and for at least 74 h in the autosampler. Processed sample stability of the analytes was established up to 16 h at 25 °C with no obvious difference in the concentration of TDL and VPL. The long-term stability (at-70 °C) samples were stable for 116 d and findings were tabulated in table 4.

Precision and accuracy results obtained in order to establish method ruggedness with different RP-columns and different analysts were ranged from 0.83% to 4.25% and 97.60% to 98.37%, respectively for TDL and VPL. Likewise, the % CV and accuracy for dilution reliability of ½ and ¼th dilution varied from 1.67% to 3.27% and 95.6% to 99.25%, respectively for two drugs.

CONCLUSION

An LC–MS/MS technique was developed for the sensitive and specific determination of trandolapril and verapamil in human plasma and validated using the ledipasvir internal reference standard. There was no interference or matrix effect from endogenous substances in the quantitative analysis. The calibration range established for TDL and VPL was adequate using a small sample volume for the analysis. The optimized SPE protocol gave highly precise and consistent recovery for the two analytes with no further stages of dehydration and reconstitution.

AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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