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Determination of Verapamil in Human Plasma by Tandem Mass Spectrometry

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An analytical method based on liquid chromatography with positive ion electrospray ionization (ESI) coupled to tandem mass spectrometry detection (LC-MS/MS) was developed for the determination of verapamil in human plasma using ondansetron as the internal standard. The analyte and internal standard were extracted from the plasma samples by liquid-liquid extraction. The separation of verapamil and ondansetron was achieved on a reversed phase (C_{18}) column with a mobile phase consisting of 10 mM ammonium acetate:methanol (20:80 % v/v). The linearity range was 1 to 496 ng/mL. Inter-day and intra-day accuracy and precision was found 98.08-102.19, 7.80-10.94 and 99.74-106.55, 4.90-7.63, respectively.

Key Words: LC-MS/MS, Verapamil, Ondansetron.

INTRODUCTION

Verapamil is a calcium ion inhibitor (slow channel blocker or calcium ion anatagonist)[1,2] and it has demonstrated to be effective in the treatment of angina, arrhythmia, essential hypertension and recently modifying agent in tumours which express P-glycoprotein[3-5]. Verapamil is administered as recemic mixture of the R and S enantiomers and is not chemically related to other cardioactive drugs. The empirical formula is $C_{27}H_{38}N_2O_4$. HCl with molecular mass is 491.08 g/mol. Verapamil has been determined in plasma and other biological fluids using several types of analytical techniques such as gas chromatography, mass spectrometry[6], HPLC[7-10], liquid chromatography coupled to mass spectrometry with electron spray ionization (LC-ESI-MS)[7-12].

The purpose of this work is to develop and validate a method for determination of verapamil in human plasma. In this paper, a new method for determination of verapamil in plasma samples is proposed. A simple, sensitive and easy to operate LC-MS/MS method using liquid-liquid extraction with ethyl acetate for the determination of verapamil in human plasma. Validation study for the verapamil was performed with respect to recovery, selectivity, linearity, precision and accuracy. The procedure has been used for the analysis of plasma samples of patients taking verapamil.

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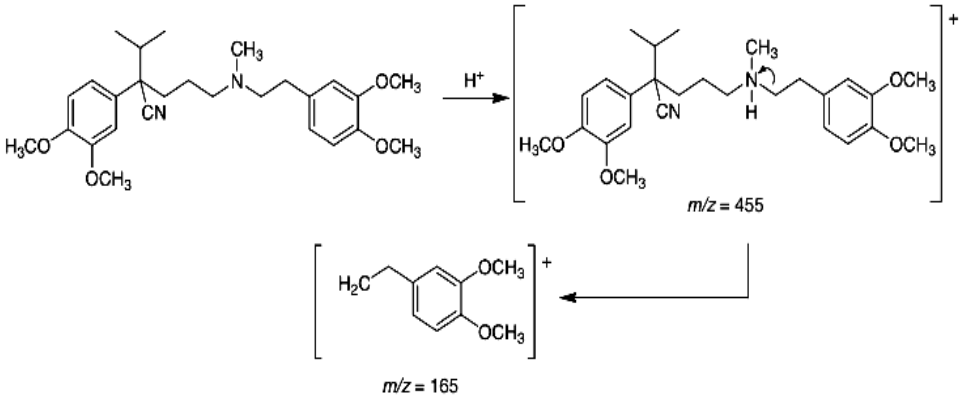

Proposed fragmentation pathway for verapamil (m/z = 455 > 165)

EXPERIMENTAL

Verapamil and ondansetron provided from Raptim Research Ltd. also supplies the COA of that drug. All reagents were of analytical grade. Ammonium acetate (Rankem), ethyl acetate (Rankem), hydrochloric acid (Merck) and methanol (HPLC, Rankem). Milli-Q water, Millipore water was used for the preparation of buffer and other aqueous solutions. Extraction method is liquid-liquid extraction. Blank human blood was collected from healthy, drug free volunteers. Human plasma was obtained by centrifugation of blood treated with the anticoagulant EDTA. Pooled human plasma was prepared and stored at *ca.* -20 °C until needed.

Chromatographic separation of the sample was performed on a Merck, Purosphere C_{18} column (150 mm × 4.6 mm i.d.). The LC-MS/MS equipment comprised of a solvent delivery system (Shimadzu) and Mass detector. The auto ampler (Shimadzu) was used to sample injection. Analyst software was used for data analysis. A lab India pH meter was used to measure the pH of the aqueous mobile phase. The mobile phase was delivered at an isocratic rate of 1.0 mL/min with a pump pressure of approximately 850 psi. Total run time for plasma samples was 1.5 min.

Chromatographic conditions: A liquid chromatography Shimadzu Corporation and Auto injector was used throughout. An aliquot (10 µL) of each plasma extract was injected into purosphere column C_{18} (150 mm × 4.6 mm) 5 µ, operating at room temperature. The compounds were eluted by pumping the mobile phase 10 mM ammonium acetate:methanol (20:80 % v/v) at flow rate 1.0 mL/min. Under these conditions, standard retention times were 1.2 min for verapamil and 0.5 min for ondansetron and back pressure values of *ca.* 780 psi was observed. The temperature of the autosampler was kept at 8 °C, the injection volume was 10 µL and run time was 1.5 min. The mass spectrometer (Lab India) equipped with electronspray ionization source running in positive mode (ES^+), was set up in multiple reaction monitoring (MRM), monitoring the transitions 455.2 > 165.2, 294.061 > 170.2 for verapamil

and ondansetron, respectively. In order to optimized all the MS parameters, a standard solution of analyte and IS was infused into the mass spectrometer, for both verapamil and ondansetron. In order to optimize the MS parameters, a standard solution of the analyte and IS was infused into the mass spectrometer. The following optimized parameters were obtained. The dwell time and the collision gas 20 pressure were 0.2 s and DP, FP, EP were 29.2, 400.0 and 10.0, respectively. Data acquisition and analysis were carried out using software MassLynx.

Preparation of stock solution: Stock solution of verapamil was prepared in methanol (1000 ppm), weigh accurately 25 mg of verapamil transfer to volumetric flask, add 10 mL of methanol sonicate it, adjust the volume with methanol. Solutions were stored at 2-8 ºC was used to spike plasma samples.

Preparation of calibration curve standards and quality control samples: Assay performance was determined in accordance with FDA guidance for bioanalytical method validation for human studies. Linearity was determined to assess the performance of the method. A linear least-squares regression with a weighting index of 1/x was performed on the peak area ratio of verapamil and ondansetron *versus* verapamil nominal concentrations of the eight plasma standards (1.00, 2.00, 15.50, 31.00, 62.00, 124.00, 248.00 and 496.00) to generate the calibration curve.

The quality control samples were prepared in blank plasma at concentration 3.12, 223.20 and 446.40 ng/mL. (LQC, MQC and HQC, respectively). Calibration curves were generated using weighted least squares regression analysis and obtained over the respective standard concentrations in plasma. All standards and quality control samples were stored at -20 ºC until analysis.

Preparation of plasma samples: Take 0.5 mL of plasma in ria vial, add 100 µL of 0.1 N HCl, vortex the vials on vortexer, then add 3 mL of ethyl acetate, vortex vials for 5 min on vortexer, then centrifuge the vial in cold centrifuge for 10 min at 4500 rpm on 4 ºC. Separate 2 mL of upper layer of organic solvent in vial, evaporate to dryness at 50 ºC under a steady stream of nitrogen. Following reconstitution of the residue in 200 µL of mobile phase, the mixture was transferred to a micro insert and 10 µL was then injected on to LC-MS/MS.

Precision and accuracy: Standards and replicate quality control samples of plasma at each concentration were analyzed on three consecutive days, after which inter- and intra-day means, % nominal and coefficients of variation (% CV) were calculated by standard method.

Method validation: Method validation were carried out according to the currently accepted USFDA (Food and Drug Administration) bioanalytical method validation guidelines.

Selectivity: Blank plasma samples of the healthy human used for testing specificity of the method were obtained from six different sources. Each blank sample was tested for the visible interference. Six blank samples and six lower limits of concentration samples were analyzed for specificity.

Linearity: Calibration curves were generated by using the analyte peak area. The sample concentrations were calculated using weighted (1/x) least squares regression. Acceptance criteria were not more than 20 % deviation at LLOQ and not more than 15 % deviation at above LLOQ levels. The LOD values were calculated by using a signal-to-noise ratio of 3.2, while LOQ value was calculated by using a signal-to-noise ratio of 10.

Precision and accuracy: The intra- and inter-day precision and accuracy was determined by analyzing six replicates of spiked plasma samples of verapamil at each quality control (3.12, 223.20 and 446.40 ng/mL) levels. Inter-day and intra-day precision and accuracy was observed in three consecutive batches. The accuracy was expressed by (mean concentration)/(spiked concentration) × 100 % and the precision by relative standard deviation (% RSD). The concentration of each sample was calculated using standard curve prepared and analyzed on the same day.

Recovery: Recovery of verapamil was performed at three concentration levels. Recovery was evaluated by comparing detector response for analyte obtained from extracted plasma samples with the true concentration of analyte (unextracted)

Stability: The stability of verapamil in the reconstituted solution was assessed by placing quality control samples in auto sampler conditions for 42 h. The freeze-thaw stability of verapamil was also assessed by analyzing quality control samples undergoing three freeze (-20 ºC)-thaw (room temperature) cycles. Short term and long term stock solution stability (for 8.0 h and 8 d), short term and long term (for 8 d and 28 d) stability of drug in matrix was evaluated.

Anticoagulant effect: Anticoagulant effect was performed by comparing high and low quality control samples prepared in EDTA (ethylene diamine tetraacetic acid) anticoagulant based human plasma and compared against calibration curve prepared in CPDA anticoagulant based plasma.

Matrix effect: This exercise was done to assess the matrix effect throughout the application of this method. Matrix has a lot of inherent variability and can affect the response of analyte during the method validation and subsequently in subject analysis. The quantification of verapamil from plasma can be grossly affected by a significant matrix effect.

Ion supression: Suppression of the MS signal can be caused by contaminants in the LC eluant entering the MS. Thus, a non specific extraction procedure may produce ion suppression that could interfere with the analysis of the samples. The effect of the sample preparation method on the variability of the electronspray ionization response should be determined. To assess the effect of ion suppression on the MS/MS signal of the analyte, verapamil and internal standard, ondansetron was evaluated.

Biological application: To demonstrate the applicability of the procedure for measurement of verapamil levels in pharmacokinetics studies on healthy male volunteers. The study on new coating formulation is also performing.

RESULTS AND DISCUSSION

Method development: To develop a sensitive, selective and simple method for the extraction and quantification of verapamil during method development different options were evaluated to optimize detection and chromatography parameters. First we have to find out the mass in tuning mode and also set the following parameters, declustering potential (DP), entrance potential (EP), GS1, GS2 and CXP. Then select the mobile phase varying the composition and pH, optimize it. It is well known that verapamil is not stable at low pH, further optimization in chromatography conditions increased the response of analyte. A mobile phase containing 10 mM ammonium acetate:methanol (20:80 % v/v) resulted in good response. Use of purosphere (C_{18} 150 × 4.6 mm × 5 µm) column resulted in run time of 1.5 min.

Selectivity: Selectivity of the method was demonstrated by the absence and presence of endogenous interfering peaks at the retention times of drug in 6 different lots of extracted blank plasma.

Linearity and LLOQ: The peak area of calibration standards was proportional to the concentration of analyte in each assay over the nominal concentration range of 1.00 to 496.00 ng/mL for verapamil. The calibration curves appeared linear and were well described by least squares lines. A weighting factor of 1/X concentration was chosen to achieve homogeneity of variance. The correlation coefficients were ≥ 0.99 (n = 4) for verapamil. The mean (± SD) slopes and intercept of the calibration curves (n = 4) for verapamil were 0.0179 (± 0.0011) and 0.0136 (± 0.0028), respectively. Results of four representative standard curve for HPLC determination of Verapamil are given in Table-1. The lower imit of quantification for verapamil proved to be 1.00 ng/mL.

TABLE-1
RESULTS OF FOUR CALIBRATION CURVES FOR DETERMINATION OF VERAPAMIL IN HUMAN PLASMA

Assay/Conc. (ng/mL)	1.00	2.00	15.50	31.00	62.00	124.00	248.00
1	0.980	1.750	13.950	31.940	58.770	118.910	255.700
2	1.000	1.820	14.490	33.910	64.920	124.250	258.900
3	0.960	1.820	17.090	30.280	63.640	135.910	225.740
4	0.960	1.820	17.090	30.280	63.640	135.910	225.740
Average	0.975	1.804	15.654	31.603	62.743	128.746	241.520
SD	0.020	0.040	1.670	1.720	2.720	8.560	18.270
%RSD	1.870	1.990	10.670	5.460	4.330	6.650	7.560
%Nominal	97.53	90.19	100.99	101.94	101.20	103.830	97.390

Precision and accuracy: Intra-batch and inter-batch precision and accuracy of the method for verapamil are presented in Tables 2 and 3. The precision deviation values for intra-batch and inter-batch are all within 15 % of the relative standard deviation (RSD) at each quality control level. The accuracy deviation values for intra-batch and inter- batch are all within the (100 ± 15) % of the actual values at each quality control level.

TABLE-2
INTRA BATCH PRECISION AND ACCURACY OF THE METHOD FOR DETERMINATION OF VERAPAMIL IN HUMAN PLASMA

Intra-batch precision and accuracy (n = 18)			
Concentration (ng/mL)	Concentration Mean ± SD, (ng/mL)	Accuracy (%)	Precision (%)
3.12	3.20 ± 0.18	102.69	5.75
223.20	222.61 ± 16.98	99.74	7.63
446.40	475.62 ± 23.33	106.55	4.90

TABLE-3
INTER BATCH PRECISION AND ACCURACY OF THE METHOD FOR DETERMINATION OF VERAPAMIL IN HUMAN PLASMA

Intra-batch precision and accuracy (n = 18)			
Concentration (ng/mL)	Concentration Mean ± SD, (ng/mL)	Accuracy (%)	Precision (%)
3.12	3.08 ± 0.24	98.82	7.89
223.20	218.92 ± 17.07	98.08	7.80
446.40	456.19 ± 49.93	102.19	10.94

Recovery: Results of extraction efficiency measured for verapamil was consistent, precise and reproducible. The mean related extraction recovery of verapamil at each quality control level (3.12, 223.20 and 446.40 ng/mL) was 81.34, 78.55 and 85.32 %, respectively.

Stability: Results of autosampler stability, freeze thaw stability, long term stability are given in Table-4. Stability data gives the idea about reliable stability of verapamil in tested conditions. Verapamil was found stable during short term and long term stock solution stability. During short term stock solution stability % mean stability of verapamil was found 98.37 %. Long term stock solution stability was performed for 8 d and % mean stability of verapamil was found 101.68 %.

TABLE-4
STABILITY OF VERAPAMIL IN HUMAN PLASMA UNDER TESTED CONDITIONS

Concentration (ng/mL)	% Accuracy (Mean ± SD)	
	3.12 ng/mL	446.40 ng/mL
Autosampler Stability (26 h, 10 ºC)	95.02 ± 5.43	102.10 ± 47.76
Freeze thaw stability (3 cycles, -20 ºC-room temperature)	99.67 ± 5.87	100.98 ± 55.07
Bench top stability (8 h, room temperature)	93.97 ± 8.77	101.75 ± 51.96
Short term stability in matrix (6days, -20 ºC)	95.76 ± 3.67	98.99 ± 20.15
Long term stability (19 days -20 ºC)	100.32 ± 6.75	102.46 ± 34.87

Anticoagulant effect: As method validation was performed in CPDA base human plasma anticoagulant effect was performed for EDTA base human plasma. Results of anticoagulant effect are summarized in Table-5.

TABLE-5
RESULTS OF ANTICOAGULANT EFFECT

Concentration (ng/mL)	% Accuracy (Mean ± SD)	
	3.12 ng/mL	446.40 ng/mL
Anticoagulant effect	104.12 ± 10.56	102.76 ± 39.56

TABLE-6
MEAN PHARMACOKINETIC PARAMETERS OBTAINED FROM 6 VOLUNTEERS AFTER ADMINISTRATION OF EACH 80 mg VERAPAMIL TABLET FORMULATION

	Reference	Test
t_{max}	1.30 ± 0.03	2.27 ± 0.81
c_{max}	141.87 ± 2.60	144.85 ± 2.56
$\log_{max}$	4.3533 ± 0.018	4.9741 ± 0.0797
$t_{½}$	1.29 ± 0.47	0.96 ± 0.28
$k_{elimination}$	1.598 ± 0.315	1.498 ± 0.304
AUC_{0-t} ng/mL h	624.01 ± 44.83	669.03 ± 80.25
$INAUC_{0-t}$	6.4073 ± 0.06781	6.4434 ± 0.1083

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