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Affordable and Reliable RP-HPLC Method for Verapamil Hydrochloride Quantification in Rabbit Plasma for Pharmacokinetics

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Abstract: Background: Existing bioanalytical methods for verapamil hydrochloride (VH) are often complex, requiring advanced instrumentation and specialized expertise, which limits their use in resource-constrained laboratories. Aim: The goal of this study is to fill this gap by developing a simplified, robust RP-HPLC-UV approach for the estimation of verapamil hydrochloride in rabbit plasma. Designed to enhance accuracy and precision while minimizing sample preparation challenges, this method addresses existing limitations by providing an affordable and reliable alternative for laboratories lacking sophisticated instrumentation. Methods: The bioanalytical method was implemented on C-18 stationary phase (5 μ, 250 × 4.6 mm) using acetonitrile/0.1% tetrahydrofuran (THF) in water (80:20, in volume) as the liquid system at a 1 mL/min flow speed, employing carvedilol as an internal standard. Results: The reported retention times of verapamil hydrochloride and carvedilol were ~7.64 and 4.69 min, respectively, at sufficiently high system suitability standards. The linearity of the bioanalytical approach can be seen between 0.025 and 5.0 μg/mL (r^2 = 0.9991). The findings indicated that there was no matrix influence in terms of accuracy ($\geq$98.96 ± 2.68%), intra- and inter-day precision ($\leq$3.68%), recovery (101.98 ± 2.76%), and procedure efficiency (100.65 ± 1.82%). Benchtop, long-term, and short-term stability investigations all revealed that the verapamil hydrochloride in the bio-samples was stable. The pharmacokinetic parameters (Cmax—3.47 μg/mL; Tmax—1.59 h) were studied from time-dependent plasma concentrations of verapamil hydrochloride estimated after 40 mg oral dosing in New Zealand white rabbits. Conclusions: The developed bioanalytical method provided easier quantitative analysis of verapamil hydrochloride from rabbit plasma and was effectively used in a pharmacokinetic investigation of an oral bolus. The reliable performance of this method under practical conditions positions it as a crucial tool for advancing pharmacokinetic studies across various research environments.

Keywords: HPLC-UV; verapamil hydrochloride; bio-analytical; pharmacokinetics; rabbit plasma

1. Introduction

Verapamil hydrochloride (VH), [(9)-5-[N-(3,4-dimethoxyphenethyl)-N-methylamino]-2-(3,4-dimethoxyphenyl)-2-iso-propylvaleronitrile] ($C_{27}H_{38}N_2O_4$) has a molecular weight

of 454.59 g/mol. It is a slow calcium-ion channel antagonist [1] widely used for the cure of angina pectoris, hypertrophic cardiomyopathy, supraventricular tachyarrhythmia, ischemic heart disease [2–4], and hypertension, with low risk of developing diabetes [5].

Bioanalytical techniques are essential for biomedical and pharmaceutical research, allowing the qualitative and quantitative study of target analytes in biological matrices. Developing an efficient bioanalytical method greatly aids in the study of drug molecule and/or metabolite behavior in biological systems and is essential for drug research, pharmacokinetics, toxicology, and clinical diagnostics.

Various techniques like amperometry [6], electrophoresis [7], spectrofluorimetry [8,9], and gas chromatography [10] were used to determine VH in biological samples. However, the widespread application of the HPLC method using normal phase [11], reversed phase [12], and ion-exchange columns [13] became the method of choice for the bioanalytical study of VH because of its exceptional sensitivity, specificity, and capacity to separate complex biological matrices. The HPLC methods were also explored to study racemic mixtures and the metabolites from biological matrices.

In this context, several HPLC methods, integrated with spectrofluorometric detection [12,14–19], photodiode-array detector [20], and mass spectrometric methods [21–28], were reported to study VH in biological matrices of various species. The chromatographic technique with spectrofluorometric detectors is fluorescence-specific and restricts the application of this method in the case of the simultaneous analysis of VH with non-fluorescent drug substances. The reproducibility of quantitation with spectrofluorimetry is challenged by temperature and quenching factors. Furthermore, adopting spectrofluorometric and mass detection techniques in an HPLC system poses huge costs and sophisticated conditions to handle them.

Furthermore, the potential use of a UV detector is a topic that really interests us due to its wide applicability and availability. In this context, we found some studies that explored the bioanalytical method employing the HPLC-UV technique for studying VH from various biological matrices [3,5,29–35]. In these techniques, the method reported by Oda et al. [32] used complex methods like an automated column-switching system that has critical instrumentation and procedures. All other methods needed increased time of analysis and/or extensive procedures in the extraction of analytes from the sample matrix and/or elaborated chromatographic conditions in the study. The solid-state extraction method using molecularly imprinted polymers reported by Javanbakht et al. [36] demonstrated the extraction of VH from biological fluid and urine; however, it was not extended for chromatographic study application.

Hence, the presented study aimed to develop a simplified bioanalytical method using RP-HPLC equipped with a UV detector to quantify VH from rabbit plasma and to study the validation parameters of the developed analytical method as per the International Council on Harmonization (ICH) protocols. Furthermore, we investigated the developed bioanalytical method as a tool for in vivo pharmacokinetic study of VH in New Zealand white rabbits.

2. Materials and Analytical Methods

2.1. Chemicals and Reagents

Authentic chemical samples of carvedilol (CDL) and verapamil hydrochloride (VH) were bought from KP Labs in Hyderabad, India. Methanol and acetonitrile (ACN) of highest quality for HPLC were provided by Rankem, Hyderabad, India. Tetrahydrofuran (THF) came from SDFCL Company in Mumbai, India. For the RP-HPLC analysis, Milli-Q water was utilized. The supplier of heparinized blood-collection tubes was CML Biotech (P) Ltd. In Angamaly, Kerala, India.

2.2. HPLC Instrument Descriptions and Chromatographic Settings

The HPLC system (Shimadzu Company, Kyoto, Japan) comprises a dual pump (LC-20AD) with UV detection (SPD-A20), a rheodyne injecting inlet (7725i) holding a 20 µL loop

size, and LC-solutions software (1.21 SP1) was applied for acquiring chromatographic data. A 0.1 mg precise balance (Denver Instruments, New York, NY, USA) was used to weigh pure powders. Remi, India equipment, including a vortex mixer, refrigerated centrifuge, and deep freezer, was utilized to process the human plasma samples.

The C18 analytical column (Luna®, Phenomenex, Torrance, CA, USA) with dimensions 250 × 4.6 mm and 5 μ particle size was used for chromatography. Isocratic elution was performed using a mobile system involving acetonitrile: 0.1%/tetrahydrofuran in water (80:20, in volume) at a 1 mL/min flow speed and a detection wavelength of 280 nm. The eluent mixture was generated fresh, purified via a 0.45 μm membrane filtering system, and degassed using the technique of ultrasonic sonication for 30 min before every batch analysis.

2.3. Standard Solution Preparation Procedures and Quality Control (QC) Samples

Methanol was used as the solvent to generate individual parent solutions of VH and CDL (1 mg/mL), which were then kept at −20 °C until the chromatographic analysis started. The diluted working solutions (WS) of VH, including 0.25, 0.5, 1, 2.5, 5, 10, 25, and 50 μg/mL concentration levels, along with a single concentration (5 μg/mL) of CDL as internal standard (IS), were prepared using mobile phase as diluent. Prior to the chromatographic bio-analysis, the calibration standards (CSs) were generated by mixing each rabbit plasma sample (180 μL) with each WS liquid solution of VH (20 μL). This produced a plasma–drug combination (with a final volume of 200 μL) containing (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 μg/mL). Alike standard preparations, a total of 4 levels of quality control samples (QCs), from fresh stock were prepared. These levels included: Level 1 with a lower limit of quantification (LLOQ) at 0.025 μg/mL; Level 2 with a low QC (LQC) at 0.075 μg/mL (3 times LLOQ); Level 3 with a medium QC (MQC) at 2.5 μg/mL (50% of upper LOQ); and Level 4 with a high QC (HQC) at 4 μg/mL (80% of the highest concentration).

2.4. Procedures for VH Extraction from Animal Plasma Samples

With only minor adjustments, the extraction process previously published by Yin et al. [25] and Ma et al. [37] was followed when preparing the investigated samples for the chromatographic analysis. Briefly, 200 μL animal plasma (QC/CS/test/blank) sample was added to 800 μL of ice cold ACN and 20 μL of IS, then packed in an Eppendorf tube and spent a minute vortexing on a cyclomixture. The aforementioned mixture was centrifuged for 15 min at 4 °C at 5000 rpm. The 500 μL protein-free transparent supernatant was transferred to a new 1 mL Eppendorf glass tube and dried in a vacuum concentrator. After reconstituting the residue with 100 μL of the liquid phase mixture, it was vortexed for a minute and then centrifuged once more for 15 min at 4 °C at 5000 rpm. Finally, the chromatogram was produced by inserting 20 μL of the transparent supernatant into the HPLC injection inlet.

2.5. Validation Protocol for the Novel Bio-Analytical Method

The validation concept is necessary to show that the bio-analytical method is capable of serving its particular goal. In compliance with the Food and Drug Administration's (FDA) 2018 recommendations for bio-analytical method validation, the analytical method's essential criteria, such as matrix effect, LLOQ, linearity, selectivity, inter-day and intra-day precision, accuracy, recoveries, stability of the studied drug, and bio-analytical method performance, were checked.

2.5.1. Selectivity

To detect the VH in the co-existence of probable interfering substances in the plasma samples and internal standard, the chromatogram of blank plasma is compared to the chromatogram of plasma intensified with VH at LLOQ level and IS in triplicate (n = 3).

2.5.2. Assessment of Lower Limit of Quantitation and Linear Range

The measurement curve was produced using eight non-zero concentrations of VH (ranging from 0.025 to 5 μg/mL) by plotting the peak area ratio of VH:IS against the nominal concentration of VH in the rabbit plasma samples of CS (n = 3). The data were then analyzed using linear regression with a $1/x^2$ weighing factor in Microsoft Excel 2016. The calibration curve was deemed acceptable upon achieving a strong coefficient of determination ($r^2 \geq 0.99$) and meeting the required precision criteria (%CV) for each back-calculated concentration standard falling within specified limits (LLOQ: ±20% and for all other concentration standards to within ±15% of the nominal or theoretical concentration. LLOQ is the lowest substance concentration in plasma that can be identified with the lowest degree of precision (%CV, less than 20%) and the highest degree of accuracy (from 80% to 120%)) [38].

2.5.3. Accuracy and Precision

The degree to which the assessed value agrees with the theoretical or nominal concentration of the drug present in the tested matrix, expressed as %RE, is known as accuracy. It was calculated in five replicates of four quality control concentration levels (LLOQ: 0.025 μg/mL, LQC: 0.075 μg/mL, MQC: 2.5 μg/mL, and HQC: 4 μg/mL) assessed against the calibration curve (0.025 to 5 μg/mL). The aforementioned QC concentration levels were tested three times in a single day and three times on different days to find the intra- and inter-day precision (expressed as %CV), respectively. The accepted limits for precision and accuracy have been established at not more than (NMT) ± 20 for LLOQ, while it was NMT ± 15 for all other three QC concentration levels.

$$\% \text{ RE} = \left(\frac{C_p - C_n}{C_n} \right) \times 100$$

where Cp and Cn = predicted and nominal concentration of sample.

$$\% \text{ CV} = \left(\frac{\sigma}{C} \right) \times 100$$

where σ = standard deviation of mean concentration of sample, and C = mean concentration of sample.

2.5.4. Investigation of Process Efficiency (PE), Extraction Efficiency (EE), and Matrix Effect (ME)

Three separate sets were used for each of the three distinct QC concentration levels (LQC: 0.075, MQC: 2.5, and HQC: 5 μg/mL), as well as the internal standard (IS) in triplicate, to study the aforementioned items. Set No. 1: QC concentrations made in methyl alcohol; Set No. 2: QC concentrations inserted into blank plasma liquid extracts; and Set No. 3: sample obtained from plasma that has been enriched with QC concentrations. The ME was identified by matching the results of set No. 1 with Set No. 2; recoveries were assessed through the comparison of Set No. 2 with Set No. 3. Furthermore, the PE was computed by comparison of Set No. 1 with Set No. 3. The goal of assessing EE should be to ensure consistent results, not necessarily to maximize the percentage recovery [39].

2.5.5. Investigation of Stability Studies

Stability assessments were carried out to assess the integrity of VH in working plasma samples under varying circumstances (bench top—at ambient condition; thermal stress—freeze thawing; long term—storage at −20 °C). QC samples were measured in triplicate at low (0.075 μg/mL) and high (4 μg/mL) concentrations to evaluate the stability of VH in the matrix. The integrity of VH and IS was assessed in blood, reconstituted samples, and extracted residue samples for a 6 h period under bench-top conditions. QC plasma samples underwent 3 freeze–thaw cycles (from −25 °C to 4 °C) to assess their stability under stress conditions. Each cycle involved freezing for 18–20 h and thawing for 4–6 h.

The QC samples were thawed gently to room temperature following each freeze–thaw cycle and analyzed to determine precision. To evaluate long-term stability, QC samples were stored at $-20\ °C$ and analyzed after thawing to room temperature at 0, 1, 2, and 3 months to assess precision. For all the aforementioned stability studies, the initial samples (time 0/cycle 0) served as controls, and their differences from other samples were analyzed using one-way ANOVA to assess significance [40].

2.6. Pharmacokinetic Application of Bio-Analytical Method

2.6.1. Research Protocol for In Vivo Biological Study

The in vivo biological assay of VH buccal film was conducted on New Zealand white rabbits (3 rabbits) following the ethical code (IAEC/52/SRU/578/2017) issued by the official animal ethics committee of the Center for Toxicology and Developmental Research at Sri Ramachandra University, Chennai. Furthermore, healthy male rabbits, weighing around 2.5 kg, were selected and kept without food overnight prior to the study. A single 40 mg VH tablet was given orally. Heparin-coated tubes were used to collect 2 mL blood samples from the marginal ear vein at 0, 0.5, 1, 2, 3, 4, and 6 h after dosing. The blood samples were centrifuged at $5000\times g$ for 15 min at $4\ °C$, and the clear liquid (plasma) was separated. Then, 200 μL of rabbit plasma was mixed with 800 μL of acetonitrile (ACN) for extraction, and 20 μL of a CDL (IS) solution (5 μg/mL) was added. The mixture was spun in a cold centrifuge at $5000\times g$ for 15 min, and the clear liquid without protein (500 μL) was transferred to a new tube and dried in a vacuum concentrator. The dried residue was reconstituted with 100 μL of mobile phase mixture, shaken, and centrifuged again at $5000\times g$ for 15 min at $4\ °C$. The clear supernatant without particles (20 μL) was inserted into the HPLC sample inlet to start the chromatographic assay.

2.6.2. Drug Behavior in the Pharmacokinetic Evaluation

The pharmacokinetic behavior of VH was analyzed based on the time-dependent plasma concentration profile. Different pharmacokinetic parameters were calculated using the mathematical formulas of each item. The elimination rate constant (K_E), representing the rate at which the drug is eliminated from the body, was determined from the slope of the terminal log-linear phase of the plasma concentration–time curve plotted in Microsoft Excel$^{®}$. The absorption rate constant (K_a) was calculated from the slope of the residual line resulting from plotting the log-residual drug concentration against time. The mean absorption half-life ($t_{½-ab}$) and the mean biological half-life ($t_{½-E}$) were computed as $0.693/K_a$ and $0.693/K_E$, respectively. The maximum plasma concentration (C_{max}) was estimated by the formula $C_{max} = (FX_0/Vd)e^{-KE*Tmax}$ and the incident time (T_{max}) was computed via the equation $T_{max} = [2.303/(K_a - K_E)]Log(K_a/K_E)$. The total area under the plasma drug concentration curve from time 0 to time t (AUC_{0-t}) was calculated using the linear trapezoidal rule. The AUC between time 't' and 'infinity' ($AUC_{t-\infty}$) were computed using C_t*/K_E, where C_t* denotes the assessed VH concentration level in rabbit plasma at time t*. The total area under the curve from time 0 to infinity ($AUC_{0-\infty}$) is obtained by adding AUC_{0-t} and $AUC_{t-\infty}$. The area under the first moment curve ($AUMC_{0-\infty}$) is calculated by integrating the product of VH plasma concentration and time plotted against time. The determination of other items such as total body clearance (CL_T) by $Vd*K_E$, volume of distribution (V_d) by $FX_0/(K_E*AUC_{0-\infty})$, and Mean Residence Time (MRT) by the percentage of $AUMC_{0-\infty}$ and $AUC_{0-\infty}$ were considered [41–43].

2.7. Analysis and Interpretation of Data

All results were reported as the average value with standard deviation and/or percentage relative error. The regression statistics were applied to calibration study data. The test of significance (paired *t*-test) was applied to compare the means of the study data. All the statistical studies were performed with Microsoft Excel$^{®}$, 2016.

3. Results and Discussions

3.1. Improvement of the Chromatographic Settings

To develop an accurate and affordable HPLC bio-analytical approach for assay of VH from rabbit plasma several investigations were performed. Carvedilol (CDL) was selected as an internal standard (IS) due to its structural relevancy to VH, solubility, and stability profile in the solvents used. Moreover, CDL showed good response at the common detection wavelength (280 nm) used in this study. The mobile phase compositions employed in the chromatogram development are shown in Table 1 and Figure 1. The higher proportion of methanol in the mobile phase achieved significant resolution between VH and IS with poor peak symmetry. Replacing the methanol with an aprotic solvent like acetonitrile maintained good resolution between VH and IS in the chromatogram with acceptable peak symmetry, but peak tailing was observed. Addition of 0.1% THF in the water (mobile) phase resolved the peak tailing and enhanced the peak symmetry.

Table 1. Trials in mobile phase optimization.

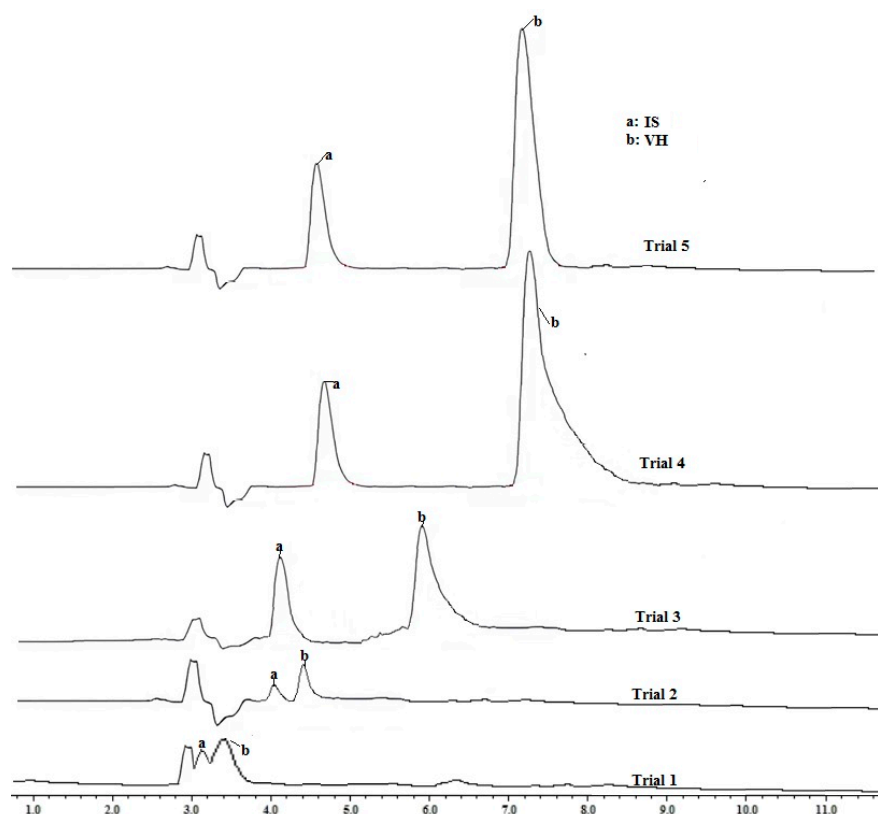| Trial	Mobile Phase Composition	Ratio *v/v*	Observation
1	Methanol:Water	50:50	Solvent front resulted
2	Methanol:Water	65:35	No resolution
3	Methanol:Water	80:20	Tailing with Peak asymmetry
4	ACN:Water	80:20	Tailing with resolution
5	ACN:0.1% THF in water	80:20	Resolution with Symmetry

Figure 1. Trial chromatograms generated during optimizing chromatographic condition. a: IS and b: VH. Trial 1—methanol/water (50:50, *v/v*), Trial 2—methanol/water (65:35, *v/v*), Trial 3—methanol/water (80:2, *v/v*), Trial 4—ACN/water (80:20, *v/v*), Trial 5—ACN/01%THF (80:20, *v/v*).

The best separation of VH and IS was achieved on reversed-phase HPLC using a mobile phase composed of acetonitrile/0.1% tetrahydrofuran in water (80:20, in volume) and a reversed-phase stationary material (250 × 4.6 mm; 5 μ particle size). Extraction of the target analytes (VH and IS) from the rabbit plasma is an essential process in the development of the chromatogram. A straightforward liquid–liquid extraction protocol was used to extract VH and IS from plasma samples using different solvents, including acetonitrile, methanol, dichloromethane, and ethyl acetate. The liquid extraction procedure involving ACN was found to be simple to follow and more efficient than all the other solvents tried.

The chromatogram developed by using the optimized procedure is shown in Figure 1-Trial 5. The recorded retention times (Rt$_s$) of IS and VH were ~4.69 and ~7.64 min, respectively. The chromatographic condition achieved relatively faster elution of VH than the reported Rt value of ~11 min [12], ~7.98 min [35], and little higher value than the Rt value of ~7.3 min [30]. The system suitability factors for the novel bio-analytical approach were assessed in the following manner: numbers of theoretical plates for IS and VH were 2107 and 2619, respectively. Asymmetry factors were 1.49 and 1.41 for IS and VH, respectively, and the resolution between VH and the internal standard was 3.802. All these measured system suitability items, theoretical plates (>2000), asymmetry factors (<2), and resolution value (>1.5), under the described chromatographic conditions indicate the VH and IS were well separated with adequate peak symmetry and resolution.

3.2. Validation of Bio-Analytical Method

3.2.1. Selectivity

The selectivity of the chromatographic method is studied to predict the ability of the particular procedure to separate and detect more than one analyte without any interference. A blank rabbit plasma sample was compared to a rabbit plasma sample Fortified with IS and VH by analyzing their chromatograms (Figures 2A and 2B, respectively). In addition, Figure 2C illustrates the chromatogram of a rabbit plasma sample collected 0.5 h after a 40 mg oral dose of VH with added IS. The selectivity of the bio-analytical approach was confirmed by the absence of significant endogenous interference peaks around the aforementioned retention times of VH and IS in the chromatogram of the blank plasma sample.

3.2.2. Investigation of Lower Limit of Quantitation (LLOQ) and Linear Range

The standard concentration curve established for the VH in plasma samples is shown in Figure 3. The bio-analytical HPLC method demonstrated a good linearity over a concentration range of 0.025 to 5 μg/mL and all the back-calculated concentrations of CS fell within the 95% confidence level of the predicted concentration with acceptable variability (≤10.26%). The LLOQ is the lowest detectable amount of the analyte in the standard calibration graph with adequate precision, and accuracy was established as 0.025 μg/mL. The percentage recovery of the LLOQ was determined as 99.35 %w/w with bias value (%RE) of −0.65 %w/w and precision (%CV) of 1.86 %w/w, which agrees with the limits for LLOQ (%RE and %CV NMT ±20 %w/w) as per bio-analytical analytical method guidelines, USFDA, 2018. The regression statistics for the calibration curve points resulted in coefficients for x-variable and y-intercept of 0.1813 and 0.008, respectively. The r^2 value of 0.9991 with the standard error of 0.0383 supports the linearity claim of the calibration curve.
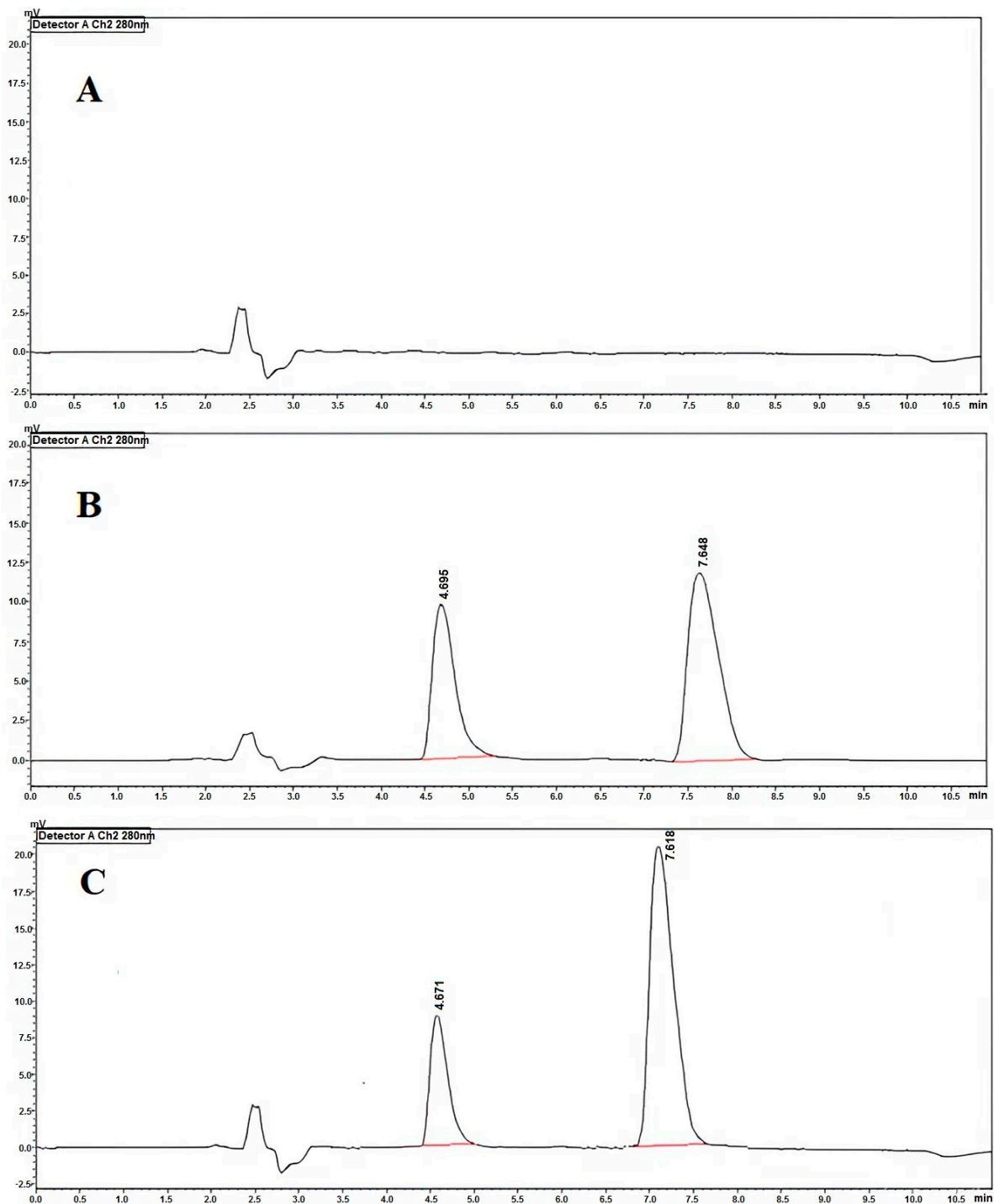
Figure 2. Chromatograms of blank (**A**). Rabbit plasma sample spiked with IS and VH sample (1 μg/mL) (**B**). Rabbit plasma sample taken 0.5 h after receiving a 40 mg oral dose of VH and fortified with IS (**C**).

3.2.3. Accuracy and Precision

Accuracy and precision are the most expected qualifications for the bioanalytical method to quantify an analyte from the biological matrix. Accuracy is the estimate that indicates the similarity of the experimentally found values to the nominal values. Precision measures the degree of closeness between the individual measurements performed for a single sample. The percentage relative error (%RE) and percentage coefficient of variation (%CV) of the percentage recovery were used to assess accuracy and precision (Table 2).
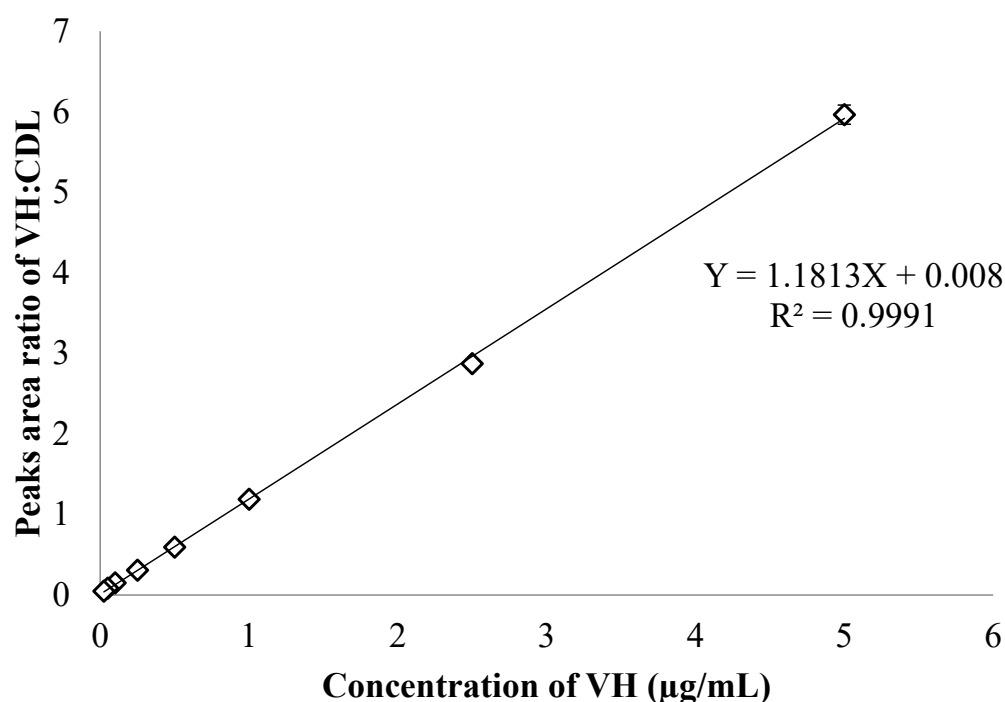
Figure 3. Calibration curve for bio-analytical study of VH in rabbit plasma samples.

Table 2. The outcomes of precision and accuracy for the novel bioanalytical approach.

Spiked Conc. (µg/mL)	Assay *	SD	RE	Found Conc. * (µg/mL)	SD (µg/mL)	CV
During a Day						
0.025	102.16	3.25	2.16	0.02554	0.000813	3.18
0.075	99.57	2.47	−0.43	0.07467	0.00247	3.31
2.5	101.28	2.19	1.28	2.532	0.05475	2.16
4	100.13	1.96	0.13	4.0052	0.0784	1.95
Inter-Day						
0.025	100.96	3.72	0.96	0.02524	0.00093	3.68
0.075	98.96	2.68	−1.04	0.07422	0.00268	3.61
2.5	102.06	2.36	2.06	2.5515	0.059	2.31
4	101.14	2.05	1.14	4.0456	0.082	2.03

* Values represents mean (n = 5).

The accuracy of the assay results varied from 98.96 ± 2.68 to 102.16 ± 3.25, with a percentage relative error (%RE) ranging from −1.04 to 2.16. The intra-day precision (%CV) ranged from 1.95 to 3.18, while the inter-day precision (%CV) ranged from 2.03 to 3.68. All outcomes for accuracy (%RE) and precision (%CV) were within the standard limits of ±2.16 and ±3.68, respectively, which comply with the official recommendations (%RE and %CV not more than ±20 for the lower limit of quantification [LLOQ] and not more than ±15 for the other three QC concentrations). This confirms the accuracy and precision of the bio-analytical approach for measuring the target drug. The recovery of VH in this study was shown to have higher accuracy than the results reported by Lau-cam and Piemontese [3], which ranged from 85.6% to 93%. Furthermore, these results are relevant to the results of accuracy value ≥ 95.7% with the intra- and inter-day precision (%CV) as 1.96 to 9.06% and 0.6 to 3.1%, respectively, as reported by Jhee et al. [12]. These results indicate that the developed method achieved a good level of accuracy and precision in the recovery of VH from the sample matrix.

3.2.4. Investigation of Process Efficiency (PE), Extraction Efficiency (EE), and Matrix Effect (ME)

PE, EE, and ME were assessed by comparing paired sets, as outlined in the aforementioned methods, and the results are displayed in Table 3. These three items were calculated at three QC concentrations, 0.075, 2.5, and 4 µg/mL, in three parallel measurements, and the mean values for ME, EE, and PE were 98.74 ± 3.17, 101.98 ± 2.76, and 100.65 ± 1.82, respectively, deemed satisfactory. The extraction method reported by the current study involved simple steps and has achieved relatively higher recovery results than the reported study with 72.56 ± 7.2 to 84.36 ± 8.8 recovery of verapamil from rabbit plasma through a method based on n-hexane–isobutyl alcohol (98:2, *v/v*) [30] and another study that recovered 89.58% to 92.12% of verapamil from human plasma through an acetonitrile-based method [34].

Table 3. Outcomes of process efficiency (PE), extraction efficiency (EE), and matrix effect (ME).

Drug Conc. (µg/mL)	Assay of Sample *			ME *	EE *	PE *
	Methanol	Un-Extracted	Extracted			
0.075	102.15 (±2.45)	97.25 (±3.17)	101.24 (±2.19)	95.21 (±2.37)	101.1 (±1.82)	99.11 (±3.42)
2.5	98.64 (±1.29)	99.94 (±2.84)	98.81 (±3.07)	101.32 (±0.98)	98.87 (±2.56)	100.17 (±2.83)
4	99.62 (±3.52)	99.31 (±2.21)	102.27 (±1.87)	99.69 (±1.84)	102.98 (±3.27)	100.65 (±3.18)
Mean **	100.14	98.83	100.77	98.74	101.98	100.65
%RSD **	1.81	1.41	1.78	3.17	2.76	1.82

* Value represents mean (±SD); n = 3, ** n = 9.

3.2.5. Outcomes of Stability Studies

The impact of maximum handling time on analyte integrity in the matrix was assessed in a bench-top stability study using dried, reconstituted samples after extraction and whole blood samples, two various QC concentrations in three parallel measurements. The outcomes of the bench-top stability investigation (Table 4) for LQC and HQC were determined from percentage recovery of VH from the samples, found to be ≥98.47 ± 3.21 with $p \geq 0.46$ for LQC and ≥100.14 ± 3.03 with $p \geq 0.52$ for HQC, which indicates that VH is stable in all kinds of samples (plasma, reconstituted and dry states) at both QC levels. VH remained stable in both wet and dry samples throughout a standard bench-top working period of 6 h.

Table 4. Outcomes of bench-top stability investigation (up to 6 h) for sample matrices in bioanalytical study of VH from rabbit plasma.

QC Conc.	Conc. (µg/mL)	Accuracy *	%RSD	*p*-Value
Plasma				
LQC	0.075	98.47	3.21	0.65
HQC	4	100.14	3.03	0.75
Reconstituted				
LQC	0.075	101.33	4.39	0.64
HQC	4	100.26	1.98	0.83
Dry state				
LQC	0.075	99.14	1.77	0.46
HQC	4	101.13	2.4	0.52

* Value represents mean (n = 3).

The results of the freeze–thaw stress study are summarized in Table 5. The percentage recovery of the LQC and HQC were found as ≥85.91 %*w/w* and ≥82.15 %*w/w*; precision (%CV) as ≤10.83 and ≤14.55; *p*-value ≥ 0.059 and ≥0.061, respectively. The short-term (stress induced) stability test results are shown, and both the QC levels managed to maintain chemical integrity in the plasma sample up to the studied three-stress cycle.

Table 5. Findings from the short-term stability assessment of biological samples in bioanalytical study to estimate VH from rabbit plasma.

QC Conc.	Conc. (µg/mL)	Accuracy *	% CV	*p*-Value
		Cycle 0		
LQC	0.075	100.41	2.95	-
HQC	4	101.23	1.96	-
		Cycle 1		
LQC	0.075	98.96	5.14	0.69
HQC	4	96.12	6.95	0.23
		Cycle 2		
LQC	0.075	95.13	10.55	0.88
HQC	4	94.27	8.96	0.63
		Cycle 3		
LQC	0.075	85.91	10.83	0.059
HQC	4	82.15	14.55	0.061

* Value represents mean (n = 3).

Further, the long-term stability of VH in plasma was studied by storing the plasma samples at -20 °C and the results are displayed in Table 6. The VH in plasma samples was found to be unchanged up to the end of 1 month ($p \geq 0.29$). At the second month, the stability of LQC was found to be insignificant because the %CV (15.16 %w/w) exceeds the maximum limit (± 15 %w/w), and the stability of HQC was found to be significant (6.87 %w/w). At end of the third month, both QC levels failed for stability as a result of the percentage recovery of LQC falling out of the 95% confidence interval of the nominal value ($p = 0.029$), and the precision (%CV) of HQC exceeded the accepted limit (± 15 %w/w) even though the percentage recovery fell within the 95% confidence interval of the nominal value ($p = 0.075$).

Table 6. Results of long-term stability study of biological samples in bioanalytical study to estimate VH from rabbit plasma.

Time	QC Conc.	Conc. (µg/mL)	Accuracy *	% CV	*p*-Value
0 Month	LQC	0.075	102.45	3.96	-
	HQC	4	101.12	1.85	-
1 Month	LQC	0.075	96.13	5.48	0.29
	HQC	4	95.46	6.81	0.302
2 Month	LQC	0.075	85.02	15.16	0.114
	HQC	4	90.6	6.87	0.059
3 Month	LQC	0.075	80.07	12.99	0.029
	HQC	4	82.32	15.58	0.075

* Results represents mean (n = 3).

3.3. Pharmacokinetic Application of Bio-Analytical Method

The practical application of the novel bio-analytical approach for in vivo assay of VH was demonstrated in healthy male New Zealand white rabbits. Time dependent VH concentrations in rabbit plasma were estimated with the developed RP-HPLC method and are depicted in Figure 4. The possible pharmacokinetic parameters for VH as per compartmental and non-compartmental pharmacokinetic modeling were calculated followed by oral bolus administration (Table 7). The absorption rate was found to be 1.54 ± 0.37 h^{-1} with an absorption half-life of 0.47 ± 0.1 h. The elimination rate of VH was determined as 0.17 h^{-1} and the corresponding biological half-life was calculated as 4.18 h. The C_{max} value of VH was found to be 3.47 ± 0.31 µg/mL at a T_{max} of 1.59 h after oral dosing. The apparent volume of distribution (V_d) is the hypothetical volume of biological fluids of a rabbit in which the VH distribution was estimated to be 8.63 ± 1.04 L and the total clearance (CL_T) value of VH was found to be 1.43 ± 0.18 L/h.
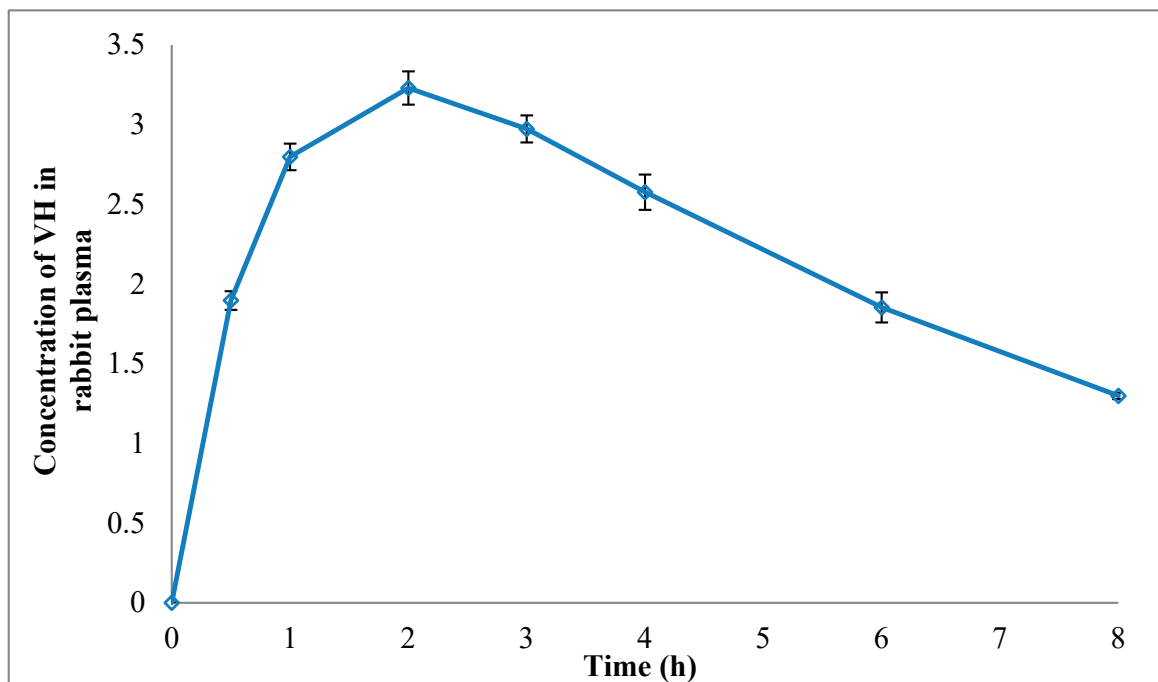
Figure 4. In the bioanalytical method estimate of VH plasma concentration in rabbits, represented as mean ± SD (n = 3).

Table 7. Results of pharmacokinetic parameters of in vivo study on rabbits.

PK Items	Unit	Oral		
		Mean *	**SD**	**SE**
C_{max}	μg/mL	3.47	0.31	0.16
T_p	h	1.59	0.22	0.11
K_a	h^{-1}	1.54	0.37	0.20
$T_{1/2\text{-ab}}$	h	0.47	0.10	0.05
K_E	h^{-1}	0.17	0.00	0.00
$T_{1/2\text{-E}}$	h	4.18	0.05	0.02
AUC_{0-6}	μg/mL/h	18.12	0.60	8.29
$AUC_{6-\infty}$	μg/mL/h	7.10	1.20	2.93
$AUC_{0-\infty}$	μg/mL/h	25.21	1.56	11.28
$AUMC_{0-6}$	μg.h/mL	66.04	2.35	30.14
$AUMC_{6-\infty}$	μg.h/mL	96.27	22.02	37.96
$AUMC_{0-\infty}$	μg.h/mL	162.31	22.89	68.45
MRT	h	6.75	0.06	3.19
V_d	L	8.63	1.04	0.54
CL_T	L/h	1.43	0.18	0.10

* Results represents mean (n = 3).

Generally, the whole biological performance of VH will be evaluated using the total area under the plasma drug concentration curve from time 0 to infinity ($AUC_{0-\infty}$). The total $AUC_{o-\alpha}$ of VH for the oral bolus route was found to be 25.21 ± 1.56 μg/mL/h per 40 mg of dose administered (0.63 μg/mL/h per unit dose 1 mg). The mean residence time (MRT) was calculated as the ratio of the area under the first moment curve (AUMC) to the area under the curve (AUC) and it was found to be 6.75 ± 0.06 h for oral bolus. This shows VH administered as a buccal patch formulation resides for a longer period of time than oral bolus. All these estimated pharmacokinetic results have precision comparable with earlier reported pharmacokinetic results. Assessment of these pharmacokinetic parameters in small animal models may be useful to assess the in vivo performance of VH from various pharmaceutical formulations.

4. Conclusions

The developed RP-HPLC-UV bio-analytical approach for the in vivo analysis of verapamil hydrochloride in rabbit plasma demonstrated outstanding performance in terms of accuracy, selectivity, sensitivity, and precision. The method utilized a C18 analytical column with a mobile liquid phase of acetonitrile and 0.1% THF in water, achieving clear separation and adequate retention times for both verapamil hydrochloride and the internal standard, carvedilol. The bio-analytical method demonstrated a linear relationship within a concentration range of 0.025 to 5 µg/mL with a high coefficient of determination ($r^2 = 0.9991$), indicating reliable quantification across this range. The novel approach exhibited adequate accuracy and intra- and inter-day precision, with recovery and process efficiency metrics aligning with acceptable bioanalytical standards, showing no significant matrix effect. Stability studies confirmed that the drug (verapamil hydrochloride) remained stable under various environments, ensuring the method's reliability for pharmacokinetic applications. Consequently, the pharmacokinetic study of verapamil hydrochloride was productively conducted using the validated RP-HPLC method following a 40 mg oral dose in New Zealand white rabbits, yielding valuable pharmacokinetic parameters. This method is thus suitable for further pharmacokinetic investigations and potential clinical applications.

Future Aspects

Future research could adapt this RP-HPLC-UV method for quantifying verapamil hydrochloride in human plasma, enhancing its clinical relevance. Expanding to include metabolite quantification and correlating pharmacokinetic data with pharmacodynamic effects would deepen understanding of the drug's efficacy and safety. Additionally, applying this method in various disease models and developing multianalyte approaches could optimize therapy. Investigating new drug delivery systems and aligning the method with regulatory standards would broaden its application.

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