Quantitative estimation of cilnidipine and valsartan in rat plasma by RP-HPLC: its pharmacokinetic application

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Abstract

Background: Cilnidipine (CLD) and valsartan (VAL) are antihypertensive agents used in the treatment of hypertension. So, pharmacokinetic study of CLD and VAL in rat plasma was carried out using chromatographic method. The chromatographic separation was performed on the Inertsil ODS column, using mobile phase methanol: water 85:15 v/v (pH 3.0) at the flow rate of 1.1 mL/min., detected at 254 nm.

Result: Cilnidipine (CLD) (1 mg/kg) and valsartan (VAL) (1 mg/kg) was administered orally in rats, and blood samples were collected at time intervals of 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 h after dosing. The retention time of plasma, CLD, and VAL was found to be 2.7, 6.6, and 4.3 min, respectively. The result was validated statistically and by recovery studies. Linearity was acceptable in the range of 1–5 and 8–40 μg/mL for CLD and VAL, respectively. Maximal concentration (C max) of CLD and VAL was observed to be 338 ± 13.85 and 1282.21 ± 39.23 (ng/mL). The half-life of CLD and VAL was found to be 1.08 ± 0.21 and 1.43 ± 0.12 h, respectively.

Conclusion: The present method was successfully applied to the pharmacokinetic study of cilnidipine (CLD) and valsartan (VAL) in rat plasma after oral administration.

Keywords: Cilnidipine, Valsartan, RP-HPLC, Rat plasma, T max, C max

Background

Hypertension is one of the risk factors for cardiovascular diseases, including ischemic and hemorrhagic stroke, dementia, heart failure, vision loss, and kidney failure. Sign of multiple underlying physiological abnormalities in elevated vital signs. Cilnidipine (CLD, Fig. 1a) is a unique calcium channel blocker used for hypertension which inhibits sympathetic voltage channels additionally to vascular dihydropyridine channel. Intracellular calcium influx is prevented by cilnidipine leading to vasodilatation. Cilnidipine possesses superior selectivity for vascular smooth muscle cells [1, 2]. Valsartan (VAL, Fig. 1b) belongs to a category of medicine called angiotensin receptor blockers (ARBs). It works by relaxing blood vessels so that blood can flow more easily. Valsartan blocks the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor in many tissues, like vascular smooth muscle and therefore the adrenal gland [3, 4] just in case with treatment of hypertension target blood pressures can't be achieved enough by use of one antihypertensive agent, and thus combination therapy is used having a different mechanism of action. Additionally, there are many patients with hypertension who take other medicines concomitantly with antihypertensive agents [3, 5–7]. The literature survey of CLD and VAL revealed various methods are used for analysis of CLD alone and together with other antihypertensive agents such as LC-MS [8, 9], UPLC [10], and RP-HPLC [11], while for VAL alone or together with other antihypertensive agents, methods like UPLC
stability indicating assay method by HPLC-DAD [15], LC-MS/MS [16–19], and LC-florescence detector [20] were reported. The tactic was developed to offer an easy, reliable, and validated bioanalytical method for the pharmacokinetic study of CLD and VAL in synthetic mixture. In the present research work, a successful attempt was made for determination of cilnidipine and valsartan in pharmaceutical tablet dosage form by reversed-phase high-performance liquid chromatography (RP-HPLC) in rat plasma. The method was developed by experimentation, based on literature survey and ascertained by statistical parameter of sampling. The simplicity, rapidity, and reproducibility of the proposed method completely fulfilled the objective of this research work. The developed method was applied successfully for pharmacokinetic studies of cilnidipine and valsartan in rats. The applicability of method suggests its further application for bioequivalence, bioavailability, and drug interaction studies. A simple and inexpensive liquid-liquid extraction procedure and an isocratic chromatography condition using a reversed phase column provided an assay well suited for real-time analyses. The developed RP-HPLC method has many advantages such as simple, accurate, sensitive, and precise for quality control and routine analysis of CLD and VAL combination. The method was compared with the previous pharmacokinetic method developed by Lee et al. [7] and located to be reliable.

Method

Chemicals and reagents

Cilnidipine and valsartan gift samples were obtained from J. B. Chemicals and Pharmaceuticals, Hyderabad, India, and Lupin Pharmaceutical Ltd., India, respectively. The purity of CLD is 99% and VAL is 98–102%. HPLC-grade acetonitrile and methanol were procured from Merck (Mumbai, India). Analytical reagent-grade (AR) triethylamine and orthophosphoric acid were also procured from SD fine chemicals (Mumbai, India).

![Chemical Structure of CLD and VAL](image)

**Fig. 1 a, b** The chemical structure of CLD and VAL

![Optimized Chromatogram](image)

**Fig. 2** Optimized chromatogram of cilnidipine and valsartan in spiked rat plasma in methanol to water (85:15, v/v, pH 3.0 adjusted with orthophosphoric acid)
Instrumentation
HPLC (Waters 600 controllers) instrument equipped with a model code 6CE In-Line Degasser Af, reciprocating pump, Rheodyne 7725i Manual Injector with a 20-μl fixed loop and HPLC syringe of 100 μl, using a UV-visible detector, was used for separation and quantitation.

Chromatographic condition
Chromatographic separation was achieved on RP-18, Inertsil ODS column, (250 mm × 4.6 mm × 5 μ), particle size 5 μm. The mobile phase used for separation of analytes was methanol to water, adjusted at pH 3.0 with orthophosphoric acid in the ratio 85:15 (v/v) used throughout the analysis. The flow rate of the mobile phase was 1.1 mL/min, using injection volume 20 μl of analyte and detected at 254 nm, as shown in Fig. 2.

Preparation standard solutions
The standard calibration curve was carried out by preparing primary stock solutions using QC samples which were weighed separately and prepared for a concentration of cilnidipine (1 mg/mL) and valsartan (1 mg/mL) using methanol as solvent. Working standard solutions were prepared by transferring different volumes of 0.1–0.5 mL stock of CLD and 0.8–4 mL stock of VAL using a mixture of methanol and water pH 3.0 (85:15 v/v) as a diluent.

Preparation of CC standards and QC samples
Working standard solutions of 1–5 ppm of CLD and 8–40 ppm of VAL were prepared from the stock solution. Volumes of 20 μl of working standard solution were added to 500 μl of plasma to obtain drug concentration levels of 1–5 μg/mL and 8–40 μg/mL for cilnidipine and valsartan, respectively. Quality control samples were prepared separately and pooled at three different concentration levels of 1, 3, and 5 μg/mL and 8, 24, and 40 μg/mL for cilnidipine and valsartan as low, medium, and high, respectively.

Preparation of plasma sample
Before processing the stored plasma samples, they were allowed to soften at room temperature. A liquid-liquid extraction procedure was used for the extraction of drugs from plasma. ACN is used as an extracting solvent. Plasma was centrifuged at 4000–4500 rpm. For 10 min, an aliquot (0.5 mL) was pipetted into a 10-mL polypropylene tube and acetonitrile (2.0 mL) was added. The mixture was vortex mixed briefly and after standing for 5 min at room temperature the mixture was centrifuged at 7000–7500 rpm for 10 min. The supernatant was carefully transferred into vials filtered through a syringe filter 0.2 μm and then injected into the HPLC system.

Method validation
The proposed analytical method was validated according to US FDA guidelines [21]. The assay was validated for specificity, linearity, accuracy, and precision. Six different lots of rat plasma samples were taken and the specificity of the method was determined. The interfering substances or background noises responses at the retention time of cilnidipine and valsartan were acceptable less than 20% of the response of the lower limit of quantification (LLOQ). Linearity was performed in the concentration range for cilnidipine and valsartan from 1000 to 5000 ng/mL and 8000 to 40,000 ng/mL, respectively. For

### Table 1 The calibration curves for cilnidipine and valsartan

| Parameters     | CLD       | VAL       |
|----------------|-----------|-----------|
| Slope          | 110.1     | 36.92     |
| Y-intercept    | 0.74      | – 8.916   |
| Correlation coefficient | 0.999 | 0.999 |
| Regression equation | y = 110.1 x + 0.74 | y = 36.92 x – 8.916 |
| Linearity range| 1–5 μg/mL | 8–40 μg/mL|
| LOD            | 0.023 μg/mL | 0.069 μg/mL |
| LOQ            | 0.078 μg/mL | 0.235 μg/mL |

### Table 2 Mean value of system suitability study

| Parameters     | CLD       | VAL       |
|----------------|-----------|-----------|
| HETP           | 0.1024    | 0.2372    |
| Retention time | 6.6 ± 0.01 | 4.3 ± 0.02 |
| Tailing factor | 1.37 ± 0.02| 1.45 ± 0.02|
| Resolution     | 13.66 ± 0.15| 9.36 ± 0.14|

### Table 3 Recovery study

| Drug | Amount taken (μg/mL) | Amount added (μg/mL) | Amount found (μg/mL) | % Recovery |
|------|----------------------|----------------------|----------------------|------------|
| CLD  | 20                   | 1.6                  | 1.582 ± 0.03         | 98.87 ± 2.08 |
|      | 2.0                  | 2.008 ± 0.05         | 100.4 ± 1.58         |            |
|      | 2.4                  | 2.396 ± 0.03         | 99.83 ± 1.54         |            |
| VAL  | 16                   | 12.8                 | 12.62 ± 0.26         | 98.59 ± 2.07 |
|      | 16                   | 16.02 ± 0.04         | 100.12 ± 0.29        |            |
|      | 19.2                 | 19.16 ± 0.07         | 99.79 ± 0.40         |            |

### Table 4 Intraday precision for cilnidipine

| Statistical data | CLD       | VAL       |
|------------------|-----------|-----------|
| Mean             | 100.03    | 99.60     |
| S.D.             | 0.56      | 0.076     |
| %R.S.D.          | 0.569     | 0.0764    |
the determination of linearity, standard calibration curves containing five points were plotted and checked. The volume of biological matrix precision was carried out using five independent sample preparations of a single lot of bulk drug and formulation. Minimum three concentrations in the range of expected study were recommended. The precision is determined at each concentration level which does not exceed 15% of the coefficient of variance (CV) except for the LLOQ, where it should not exceed 20% of the CV.

**Result**

**Selectivity**

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other component in the sample. Analysis of blank samples of the appropriate biological matrix of six samples. Each blank sample should be tested for interference and selectivity should be ensured at the LLOQ. If the method is intended to quantify, more than one analyte should be tested to ensure that there is no interference. Six individual rat blank plasma samples were analyzed for investigation of selectivity of the method. Each blank sample was tested for interference using the present analytical method and it was compared with spiked samples whose concentration of the analyte was at the LLOQ.

**Linearity**

The linearity was performed by making appropriate working stock solutions with the mobile phase to obtain concentration ranging from 1 to 5 μg/mL of cilnidipine and 8 to 40 μg/mL of valsartan. The calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficients. Limit of detection and quantification of CLD and VAL were found to be 0.023 μg/mL and 0.069 μg/mL, and 0.078 μg/mL and 0.235 μg/mL, respectively. The result is shown in Table 1.

**System suitability study**

System suitability parameters such as retention time, HETP, resolution, and peak tailing are determined. The results obtained are shown in Table 2.

**Recovery study**

The recovery study was performed by comparing the analytical result for extracted samples at three different concentrations (low, medium, and high) with an unextracted standard that represents 100% recovery, as shown in Table 3.

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**Table 5 Intermediate precision**

| Statistical data | CLD            | VAL            |
|------------------|----------------|----------------|
|                  | Day to day | Analyst to analyst | Day to day | Analyst to analyst |
| Mean             | 99.82      | 99.25          | 99.75      | 99.56               |
| S.D.             | 0.06       | 0.268          | 0.12       | 0.20                |
| %R.S.D.          | 0.0656     | 0.271          | 0.126      | 0.208               |

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**Fig. 3** Mean plasma concentration-time profile CLD and VAL

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Precision
Repeatability
The repeatability was performed for three replicate at five concentrations in linearity range 1–5 μg/mL for cilnidipine and 8–40 μg/mL for valsartan. Results of repeatability for cilnidipine and valsartan are reported in Table 4.

Intermediate precision
It was performed within laboratory variation on a different day in three replicates at three different concentrations. A result of day to day variation and analyst to analyst variation was performed. Cilnidipine and valsartan are reported in Table 5.

Robustness
The capacity of a method to remain unaffected by small deliberate variations in method parameters, the RSD of peak areas of Cilnidipine and Valsartan were found to be well within the acceptable limit of 2%. The tailing factor for both the peaks was found to be (Symmetrical) < 1.5.

LOD and LOQ
The developed method has calculated the limit of detection and quantification based on the standard deviation response and slope of the linearity curve.

Plasma estimation of cilnidipine and valsartan in rat plasma
The plasma concentrations of cilnidipine and valsartan vs time are shown in Fig. 3. The C_{max} and T_{max} after oral administration of cilnidipine and valsartan were 338 ± 13.85 and 1282.21 ± 39.23 ng/mL and 3 and 6 h, respectively. The biological half-life (t_{1/2}) of cilnidipine and valsartan was 1.08 ± 0.21 and 1.43 ± 0.12 h, respectively. The result is shown in Table 6.

Discussion
Pharmacokinetic applications
The developed method has been successfully applied to quantify cilnidipine and valsartan concentrations in rat plasma [23–33]. Six healthy rats (200–400 g) were used for conducting in vivo studies. After an initial period of acclimatization for 1 week to laboratory conditions, the rats were randomly selected and administered the dose. In this study, a single dose was designed as, one-way cross over study with a washout period of 14 days. The protocol followed in experimental was under Animal Ethical Guidelines (reg. No.-1153/PO/Re/S/08/CPCSEA) for investigations in laboratory animals and approved by the Animal Ethics Committee. Animals have fasted for 2 h after dose administration. A synthetic mixture of CLD (1 mg/kg) and VAL (1 mg/kg) dose was administered orally. A total of 13 blood collection time points including the pre-dose sample (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 h). Vacutainers containing K3EDTA as an anticoagulant was used for collecting blood samples separately. The obtained plasma sample was stored until analysis. The sample was separated from plasma by using a cooling centrifuge at 4000–7500 rpm for CLD and VAL. After analysis, the pharmacokinetic parameters were computed using Kinetica software version 5.0.

In vivo data analysis
To determine the various pharmacokinetic parameters, the plasma concentration of cilnidipine and valsartan at different time intervals was subjected to quantitative analysis shown in Fig. 3. Pharmacokinetic parameters like maximum plasma concentration (C_{max}), time to reach maximum concentration (T_{max}), and area under the plasma concentration-time curve (AUC_{0-∞}) was calculated using the Kinetica software version 5.0 [22].

Comparative study of the developed method
The developed method compared with the previously reported method by Lee et al., [7] the C_{max} observed by the previous method were 8.3 ± 4.5 and 4612.6 ± 2302.4

Table 6 Pharmacokinetic parameters
| Parameters | CLD | VAL |
|------------|-----|-----|
| C_{max} (ng/mL) | 338 ± 13.85 | 1282.21 ± 39.23 |
| T_{max} (h) | 3.0 ± 0.81 | 4.0 ± 0.89 |
| AUC_{max} (ng h/mL) | 1069.04 ± 36.93 | 51328.2 ± 76.76 |
| AUC_{extra} (ng h/mL) | 1117.84 ± 54.90 | 558489 ± 125.7 |
| Half-life (h) | 1.08 ± 0.21 | 1.43 ± 0.12 |

Table 7 Comparative study of pharmacokinetic parameters
| Parameters | Developed pharmacokinetic method | Previous pharmacokinetic method [7] |
|------------|---------------------------------|------------------------------------|
| C_{max} (ng/mL) | CLD | VAL | CLD | VAL |
| 338 ± 13.85 | | 1282.21 ± 39.23 | 8.3 ± 4.5 | 46126 ± 23024 |
| T_{max} (h) | 3.0 ± 0.81 | 4.0 ± 0.89 | 2.5 | 4 |
| AUC_{max} (ng h/mL) | 1069.04 ± 36.93 | 51328.2 ± 76.76 | 38.7 ± 19.7 | 25,864.5 ± 12,611 |
| AUC_{extra} (ng h/mL) | 1117.84 ± 54.90 | 558489 ± 125.7 | 43.1 ± 22.5 | 27,498.1 ± 13,676 |
ng/mL. $T_{\text{max}}$ were found to be 2.5 and 4 h, $\text{AUC}_{\text{last}}$ were reported as 38.7 ± 19.7 and 25,864.5 ± 12,611 ng/mL, and $\text{AUC}_\infty$ were reported as 43.1 ± 22.5 and 27,498.1 ± 13,676 ng × h/mL for CLD and VAL, respectively [7].

In the developed method, $C_{\text{max}}$ were found to be 338 ± 13.85 and 1282.21 ± 39.23 ng/mL, $T_{\text{max}}$ were found to be 3.0 ± 0.81 and 4.0 ± 0.89 h, $\text{AUC}_{\text{last}}$ were 1069.04 ± 36.93 and 5132.8 ± 76.76 ng × h/mL, and $\text{AUC}_\infty$ were 1117.84 ± 54.90 and 5584.89 ± 125.7 ng × h/mL for CLD and VAL, respectively. The result is shown in Table 7.

**Conclusion**

A simple, accurate, precise, and rapid UV–liquid chromatographic method for simultaneous estimation of cilnidipine and valsartan in rat plasma is validated as per the USFDA guidelines. The method is found to be suitable for the study of pharmacokinetic application in rats. The cost-effectiveness, simplicity of the assay, and usage of liquid–liquid extraction make it an attractive procedure in high-throughput bioanalysis of cilnidipine and valsartan. From the results of all the validation parameters, we can conclude that the developed method can be useful routinely for bioavailability and bioequivalence studies and therapeutic drug monitoring with the desired precision and accuracy.

**Abbreviations**

RP-HPLC: Reversed-phase high-performance liquid chromatography; ODS: Octa-decyl silane; v/v: Volume by volume; $C_{\text{max}}$: Concentration maxima; $T_{\text{max}}$: Therapeutic maxima; CLD: Cilnidipine; VAL: Valsartan; CV: Coefficient of variance; LOD: Limit of detection; LOQ: Limit of quantitation; DAD: Diode array detector

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**Authors’ contributions**

RNK was design and optimized method. The manuscript was drafted by RNK. The method was performed and validated by SSY. AGM have contributing in grammatically molding and writing of manuscript and gives their scientific suggestion. All authors have read and approved the manuscript.

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**Availability of data and materials**

All data and materials are available upon request.

**Ethics approval and consent to participate**

Not applicable

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Not applicable

**Competing interests**

The authors declare that they have no competing interests.

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