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# RP-HPLC method for simultaneous estimation of atorvastatin calcium and ramipril from plasma.

Potdar VH,[1] Mule VS,[1] Pishawikar SA,[2] Jadhav SD,[2] Thamake SL.[2]

1. Department of Pharmacology. Tatyasaheb Kore College of Pharmacy, Warananagar. 2. Department of Pharmaceutical Chemistry, Bharati Vidyapeeth College of Pharmacy, Morewadi.

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Correspondence: Jadhav SD.
Department of Pharmaceutical Chemistry,
Bharati Vidyapeeth College of Pharmacy, Morewadi,
Kolhapur-416013(M.S.) India.
e-mail: swapnil.mpharm@gmail.com

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**ABSTRACT**

Solid lipid microparticles (SLMs) loaded with ketoprofen were prepared by single emulsion-solvent evaporation method, in which glyceryl monostearate and Tween 80 were employed. The particle size was found to be 99.80±2.1µm. Microparticles observed by scanning electron microscope (SEM) showed spherical shape. The entrapment efficiency (EE %) and drug loading capacity (DL %) were found to be 72.60±1.6 % and 17.98±0.7% respectively. Results of stability evaluation showed relatively long term stability after storage at 4˚C for 3 months. The in-vivo study revealed slightly better per cent inhibition of pain i.e. 74% in comparison with 68% produced by plain drug.

**KEYWORDS:** Solid lipid microparticles, ketoprofen, solvent evaporation method.

**RESUMEN**

Las micropartículas lipídicas sólidas (MLS) cargadas con ketoprofeno se prepararon a través del método de evaporación del disolvente en emulsión simple, en el que se ha utilizado monoestearato de glicerilo y Tween 80. El tamaño de la partícula resultó ser de 99,80±2,1 µm. Las micropartículas observadas a través del microscopio electrónico de barrido (MEB) mostraron una forma esférica. La eficacia de captura (EC %) y la capacidad de carga (CC %) del fármaco resultaron ser del 72,60±1,6% y 17,98±0,7%, respectivamente. Los resultados de la evaluación de estabilidad mostraron una estabilidad relativa a largo plazo, después de una conservación a 4˚C durante 3 meses. El estudio in vivo reveló un ligero mejor porcentaje en la inhibición del dolor, concretamente, un 74% en comparación con un 68% producido por un fármaco corriente.

**PALABRAS CLAVE:** Micropartículas lipídicas sólidas, ketoprofeno, método de evaporación del disolvente.

## INTRODUCTION

Atorvastatin [R-(R*, R*)]-2-(4-fluorophenyl)-β,∂ dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenyl amino) carbonyl]-1H-pyrrole-1-heptanoic acid calcium salt for trihydrate] is Anti-lipidemic agent act by inhibiting HMG CoA reductase enzyme.[1] Ramipril [2S-[1[R*(R*)], 2α, 3αβ, 6αβ]]-1-[2[[1(Ethoxycarbonyl)-3-phenylpropyl] amino]-1-oxon propyl] octahydro cyclopenta [b] pyrrole-2-carboxylic acid.] is ACE inhibitor.[2,3] The combination of both drugs is used in treatment of Hypertension. Several HPLC,[4-6] HPTLC,[7] Liquid Chromatography–electro Spray Ionization Tandem Mass Spectrometry[8] methods have been reported for atorvastatin alone or in combination with other drugs.[9-11] Only one UV spectroscopy i.e. derivative spectroscopy method has been reported for simultaneous estimation of Atorvastatin [ATR] and Ramipril [RAM].[12] The RP-HPLC method for simultaneous estimation of atorvastatin and ramipril have been reported from capsule dosage form.[13] The present method (Internal Standard RP-HPLC method) quantifies both drugs simultaneously extracted from plasma by eliminating interference of endogenous plasma components. The method was validated using USFDA guidelines for bio-analytical methods.

## MATERIALS AND METHODS:

### Instrumentation:

The separation of ATR and RAM was carried out on an isocratic JASCO RP-HPLC system using C18 column [150 x 4.6 mm internal diameter, particle size 5µm].The pump used in this HPLC system was PU 2080 pump [Dual piston with gear driven pump]. The 20 µl sample solutions were injected to chromatographic system using Rheodyne Injector. The UV detector used in this HPLC system was Czerny turners mount monochromater with deuterium lamp as light source. The chromatographic and the integrated data were recorded using Hercule 2000 [Interface] computer system. Data processing was carried out using Borwin® Version 1.5software.

### Reagents and Chemicals:

The solvents used were of HPLC/AR grade. Pure drug samples of ATR, RAM and Valsartan [VAL] are obtained as gift samples from Cipla India Pvt. Ltd. Kurkumbh, Mumbai. Plasma is prepared by centrifugation of blood collected from blood bank.

### Chromatographic Conditions:

The analysis was carried out by HPLC using Acetonitrile: 0.02 M Potassium dihydrogen phosphate (pH 3.2) [80:20 %V/V] as a mobile phase and HIQ SII C18 column -10 [4.5 mm x 250 mm] as a stationary phase at a flow rate of 1ml / minute in an isocratic elution mode. Before delivering the mobile phase in to the system, it was degassed and filtered through 0.20µm syringe filter. The injection volume was 20 µl and the detection was performed at 217 nm.[14]

### Preparation of Standard Drug Solutions:

To the 5 ml of blood sample, 10 mg ATR, 20 mg RAM and 10 % of 0.2 ml trichloroacetic acid were added. Sample was centrifuged at 3000 r/m for 35 min. to settle RBCs at bottom [15]. The supernatant was separated and volume was made up to 10 ml to get the solution 1000 µg/ml of each drug. This solution was filtered through syringe filter of size 0.45 µm. These solutions were diluted with mobile phase to get 100 µg/ml [ATR-10] and 200 µg/ml [RAM-100] stock solutions of ATR and RAM respectively. Standard stock solution of VAL was prepared by dissolving 10 mg of drug in 80 ml of mobile phase. It was then sonicated for 10 min and then final volume was made up to 100 ml with mobile phase to get 100 µg/ml of solution in 100 ml volumetric flask.

### Calibration Curves:

For each drug, appropriate aliquots were pipetted out from each standard stock solution into a series of 10 ml volumetric flasks. The volume was made up to mark with mobile phase to get solutions having concentrations 0.4-25.6 µg/ml , 0.8-51.2 µg/ml [ increasing geometrically] and 25 µg/ml in each flask for RAM, ATR and VAL respectively. Triplicate dilutions of each concentration of each drug were prepared separately. From these triplicate solutions, 20 µl injections of each concentration of each drug were injected into the RP-HPLC system separately and chromatographed under the conditions as described above. Evaluation of both drugs was performed with UV detector at 217 nm. Peak areas were recorded for all the peaks. Retention factors were calculated and plotted against the concentrations to obtain the standard calibration curves [Fig. 1]. Using these calibration curve data like slope and intercept, it will be possible to quantitate concentrations of both drugs.

### Analysis of Quality Control Sample:

For each drug, appropriate aliquots were pipetted out from each standard stock solution into three 10 ml volumetric flasks. The volume was made up to mark with mobile phase to get solutions having concentrations for ATR 0.5, 2.5 and 25 µg/ml ,for RAM 1, 7 and 50 µg/ml and for VAL 25 µg/ml in each flask. Nearly 20 µl injections of each concentration of each drug were injected into the RP-HPLC system separately and chromatographed under the conditions as described above [Fig. 2]. Evaluation of both drugs was performed with UV detector at 217 nm. Peak areas were recorded for all the peaks. Retention factors were calculated. The concentrations of both drugs were calculated using calibration curve data.

**Figura 1. Overalain chromatogram of Physical Mixtures**

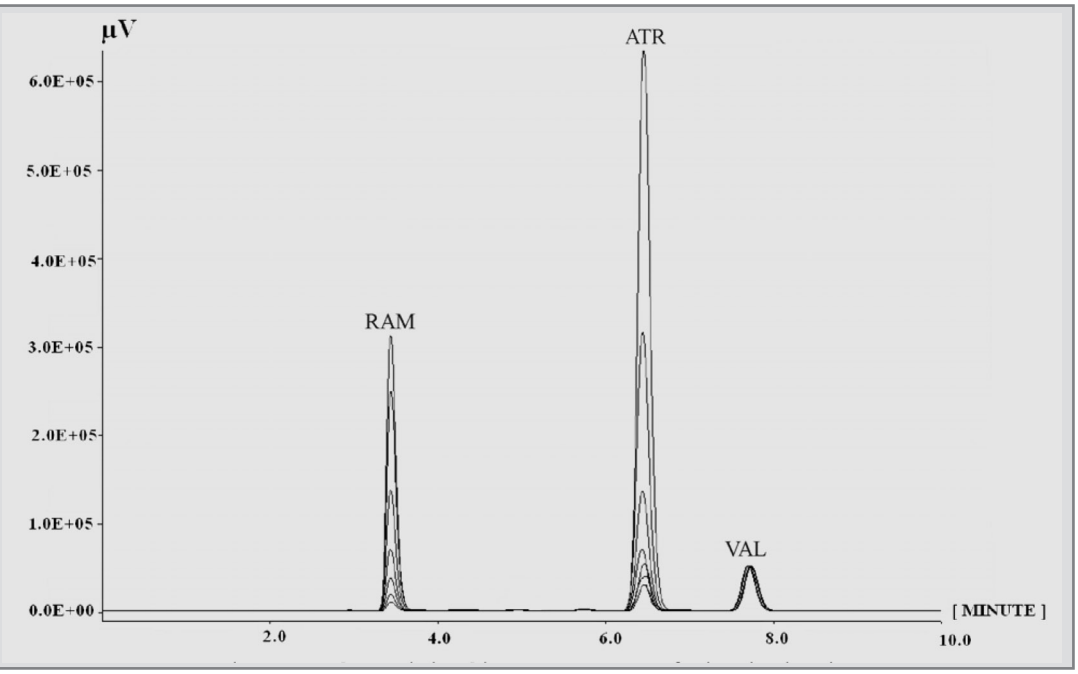


**Figure 2. Chromatogram of quality control sample**

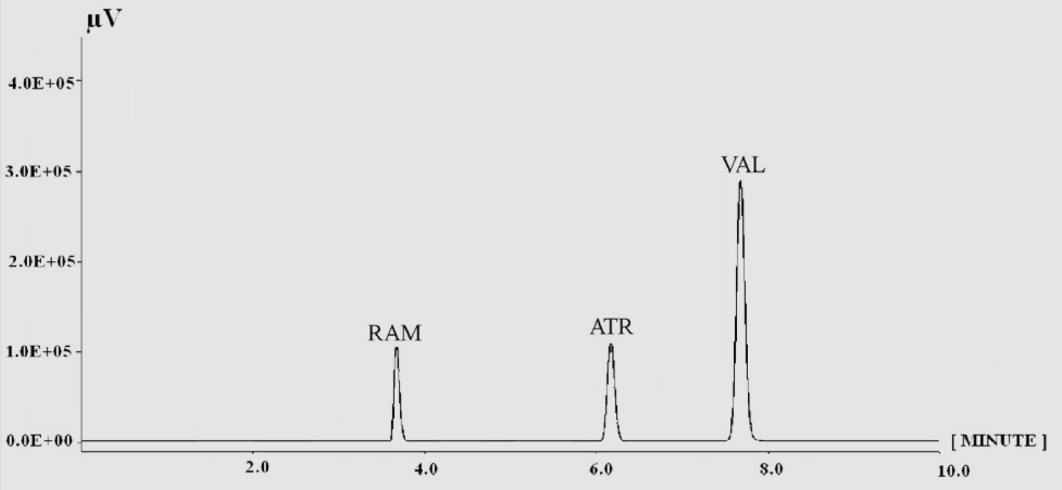


**Figure 3. Chromatogram of blank plasma sample**

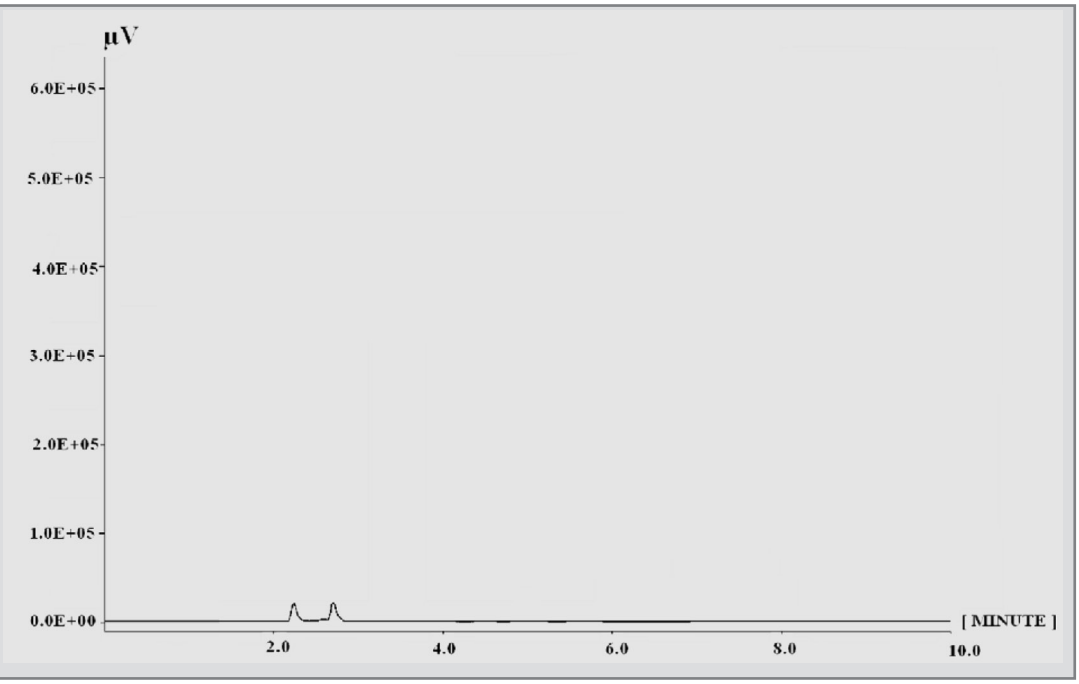

**Table 1: Results of Accuracy and Precision study**

| Day | Time | % concentration found* [Mean ± SD] | | RSD | |
|---|---|---|---|---|---|
| | | ATR | RAM | ATR | RAM |
| I | T1 | 92.41± 3.7755 | 91.23 ± 2.9673 | 4.0322 | 2.8542 |
| | T2 | 94.14± 3.1389 | 93.72 ± 4.1569 | 3.0246 | 4.2789 |
| | T3 | 90.45± 2.3314 | 97.41 ± 2.2195 | 2.5673 | 2.2785 |
| II | T1 | 89.20± 1.2104 | 93.72 ± 4.0325 | 1.3756 | 4.1678 |
| | T2 | 92.62± 3.2799 | 95.03 ± 4.4897 | 3.3054 | 4.6213 |
| | T3 | 96.25± 3.8973 | 90.18 ± 1.5486 | 3.8249 | 1.4987 |
| III | T1 | 97.04± 2.9310 | 95.13 ± 2.0032 | 3.0482 | 2.1852 |
| | T2 | 92.36± 3.1942 | 94. 87± 4.1025 | 3.3954 | 4.2149 |
| | T3 | 97.93± 2.1083 | 91.20 ± 3.4326 | 2.2547 | 3.4219 |

SD: Standard Deviation; RSD: Relative Standard Deviation. *Average of fifteen determinations.

**VALIDATION:**

The proposed bio-analytical method was validated as per USFDA guidelines.[16]

**Selectivity:**

The selectivity of the RP-HPLC method was determined by comparison of the chromatogram of blank plasma sample [Fig. 3] and quality control samples. The retention times peaks present in plasma sample are different than same of both drugs thereby proving selectivity of method to analyze both drugs with best resolution.

**Accuracy and Precision Study:**

The accuracy and precision study was carried out by analyzing quality control sample at three concentration levels for five times. Precision study was performed to find out intra-day and inter-day variations. The results of these studies are reported in Table 1.

**Extraction Recovery Study:**

The recovery of an analyte is the retention factor obtained from an amount of the analyte added to and extracted from plasma compared to same obtained for the true concentration of the quality control sample. Recovery experiments has been performed by comparing the analytical results for extracted samples at three concentrations as specified in quality control sample analysis with unextracted standards that represent 100% recovery. The results of extraction recovery are reported in Table 2.

**Table 2: Result of Extraction Recovery**

| Analyte | Mean Recovery* ± SD | RSD |
|---|---|---|
| ATR | 92.25± 3.8973 | 3.9345 |
| RAM | 91.72 ± 4.0325 | 4.1925 |

SD: Standard Deviation; RSD: Relative Standard Deviation. *Average of fifteen determinations.

**Limit of Detection [LOD] and Lower Limit of Quantitation [LLOQ]:**

The LOD and LLOQ were separately determined based on the calibration curve data. The standard deviation of analyte response and slope of the regression line were used. Results of LOD and LLOQ are given in Table 3.

**Stability Study:**

The stability tests were performed in low and high QC samples in terms of short-term storage, long-term storage, freeze/thaw stability, post preparative stability and stock solution stability. The short-term (room temperature) stability was assessed at room temperature for 24 hours and long-term stability was assessed at 200C for 45 days. Freeze–thaw stability was performed by freeze-thawing for 4 times specifically. Freezing was performed at 200C for 24 hours and thawed at room temperature. Post-preparative stability was tested by comparing after-day analysis with the first intra-day analysis results. It has estimated by storing the extracted samples at 100C in an auto-sampler for 30 hours. The stock solution stability samples were prepared in methanol with water [1:1 ratio] and stored at 2–80C for 35 days. The samples were further diluted before injection. The obtained results were compared with the results which were obtained from the freshly prepared solutions of the same concentration.

**System Suitability Parameters:**

System suitability parameters were analyzed on freshly prepared standard stock solutions of ATR and RAM. Both the drugs were injected into the chromatographic system under the optimized chromatographic conditions. Parameters that were studied to evaluate the suitability of the system were number of theoretical plates, tailing factor, resolution, separation factor etc. These parameters are reported in Table 3.[17]

**Table 3: System Suitability Parameters**

| Sr. No. | Parameters | RAM | ATR | VAL |
|---|---|---|---|---|
| 1. | Theoretical Plates | 3483.15 | 2878.32 | 3145.37 |
| 2. | Asymmetry | 1.11 | 1.29 | 1.24 |
| 3. | Capacity Factor | 319 | 255 | 276 |
| 4. | Resolution | 12.32 | | |
| | | | 4.93 | |
| 5. | Retention Time in minutes | 3.43 | 6.42 | 7.69 |
| 6. | Calibration Range [µg/ml] | 0.8-51.2 | 0.4-25.6 | 25 |
| 7. | LOD [µg/ml] | 0.29 | 0.13 | - |
| 8. | LLOQ [µg/ml] | 0.79 | 0.40 | - |

**RESULT AND DISCUSSION:**

The proposed method describes a RP-HPLC procedure employing a C18 column and a mobile phase containing Acetonitrile: 0.02 M Potassium dihydrogen phosphate (pH 3.2) [80:20 %V/V]. The detection wavelength was 217 nm which was selected by analyzing overlain UV spectra of ATR and RAM of concentration 10 µg/ml each. Valsartan has been selected as an internal standard after observing elution behavior and retention times of most of drugs. The flow rate of 1ml/min. gives good resolution of both drugs.

The ATR and RAM were found be linear in concentration range of 0.4 –25.6 µg/ml and 0.8 – 51.2 µg/ml respectively. The accuracy and precision study has been carried out by analyzing five determinations per concentration [three concentrations representing the entire range of the standard curve]. The results of accuracy were found be in range of 89.76 % to 99.89 %. The precision study has been done for intra and inters day variations for three consecutive days. Extraction recovery was determined by spiking the pure drug sample in previously analyzed sample which is more than 50 percentages. The stability studies have also proved precision of method over long period. The LOD and LLOQ values for Atorvastatin were 0.13µg/ml and 0.40 µg/ml, for Ramipril 0.29 µg/ml and 0.79 µg/ml respectively thus proving sensitivity of method.

The developed method has found to have least interference of endogenous substances form plasma for simultaneous estimation of ATR and RAM. The Extraction recovery studies revealed excellent efficiency of extraction and precision studies showed that method is accurate and precise. Low LOD and LLOQ have proven sensitivity of method to detect minute quantities of ATR and RAM with better accuracy and precision. Thus developed HPLC method was found to be more accurate, precise, sensitive, selective and reproducible. The developed method has its application in pharmacokinetic and bioequivalence study of marketed formulations of ATR and RAM.

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