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# Development and Validation of HPLC Method for the Simultaneous Determination of HCTZ, AML, TELM and VAL in Human Plasma

**Jayram V. Gholave*[a], Amol V. Pansare[b], Nayan S. Gadhari[c], Suyog S. Patil[c], Sharad S. Upadhyay[c], Amol A Shedge[c], Viswanath R. Patil [c], Kiran F. Shelke[d]**

[a]*Department of Applied Chemistry, Sardar Patel College of Engineering, Andheri (W), Mumbai, India.*

[b]*Mechanical Systems Engineering, Swiss Federal Laboratories for Materials Science and Technology-Empa, 8600 Dübendorf, Switzerland.*

[c] *Department of Chemistry, University of Mumbai, Vidyanagari, Santacruz (East), Mumbai-400 098, India.*

[d]*Department of Chemistry, Late Pushpadevi Patil Arts and Science College, Risod, Dist. Washim- 444 506 (MS)*

## Abstract

A method is described for the simultaneous determination of Hydrochlorothiazide (HCTZ), Amlodipine besylate (AML), Telmisartan (TELM) and Valsartan (VAL) using gradient elution by HPLC-UV detection. The separation was carried out on a 150 x 4.6mm i.d. Zorbax $C_{18}$, 5 µm column with mobile phase A, containing 10 mM potassium dihydrogen phosphate and 10 mM butane sulphonic acid with 0.4% triethylamine (pH 3.0), ortho phosphoric acid and ammonia. Mobile phase-B composed of acetonitrile using gradient elution at a flow rate of 1 $mLmin^{-1}$ was used. The calibration curve was linear for Hydrochlorothiazide having concentration range (0.1-6.0) $\mu gmL^{-1}$, Amlodipine (0.25-10.0) $\mu gmL^{-1}$, Telmisartan (0.05-4.0) $\mu gmL^{-1}$, and Valsartan (0.05-4.0) $\mu gmL^{-1}$. Various parameters like precision, accuracy, limit of detection, limit of quantitation, robustness, ruggedness and solution stability of method were checked for validation. We have successfully developed short gradient run time; this is the main advantage of our research work. In addition to this, we have recovered four drugs simultaneously in human plasma using this short gradient method. The developed method was applied to determine these components from human plasma.

**Keywords:** Amlodipine besylate; Human plasma; Hydrochlorothiazide; Telmisartan; Valsartan.

## 1. Introduction

Valsartan (VAL), N-(1- oxopentyl)-N-[[2′-(1H-tetrazol-5-yl) [1, 1′-biphenyl]-4- yl] methyl]-L-valine (Fig. 1), is the first member of a new chemical class of a non- peptide angiotensin II receptor antagonist. Hydrochlorothiazide (HCTZ) 6-chloro-3, 4-dihydro-2H-1, {6}, 2, 4-benzothiadiazine- 7 – sulphonamide-1,1- dioxide (Fig. 1) is a diuretic of the class of benzothiadiazines [1]. It can be prescribed in a combination with other antihypertensive drugs, for example beta blockers, angiotensin receptor blockers II [2]. Combination of two antihypertensive agents is advantageous to treat some hypertension patients [3]. Amlodipine besylate (AML) is 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4-dihydro – 6 – methyl -3, 5- pyridine dicarboxylic acid 3- ethyl – 5 – methyl ester, benzene sulfonate[6] (Fig. 1) an angioselective calcium channel blocker [4] used for treatment of angina [5]. Telmisartan, (TELM) 4'-[(1, 7'-dimethyl-2'-propyl – 1H, 3'H-2, 5- bibenzimidazol-3'-yl)-methyl]biphenyl-2-

carboxylate (Fig. 1), is an angiotensin II type 1 (AT1) receptor antagonist used to treat hypertension and prevents the constriction of blood vessels [6].

Literature survey revealed different methods for estimation of HCTZ, AML, TELM and VAL either alone or in combination [7]. Simultaneous determination of HCTZ and VAL has been reported using spectrophotometery [1]. HCTZ and VAL were determined from human plasma by LC-MS-MS [2] and HCTZ analyses in urine and human plasma was derived by HPLC with Tandem Mass Spectrometer detection [3]. Some methods have been reported for the simultaneous determination of VAL and AML in combined pharmaceutical dosage forms by reversed phase HPLC [4] and TLC [5]. A method based upon UPLC was reported for simultaneous detection of HCTZ, AML and TELM in a combined dosage form [6]. Determination of Valsartan and Hydrochlorothiazide in human plasma was validated using Liquid chromatograph-MS-MS [8-9]. An isocratic HPLC method for determination of VAL and its degradants exists [10] whereas different methods exist for the determination of Valsartan. A UV, 2$^{nd}$ derivative spectrophotometric and HPLC method was developed for determination of Valsartan in a pharmaceutical formulation [11-12]. A stability assay method for Valsartan was validated with HPLC [13] and UPLC [14]. In addition, some methods have been adopted for the simultaneous determination of VAL and AML in combined pharmaceutical dosage forms by reversed phase HPLC [15-17]. Simultaneous detection of Losartan, TEL and VAL in human urine by HPLC [18]. Supercritical fluid chromatographic methods have also been used for determination of TEL and Ramipril drug [19], AML besylate and HCTZ were also electrochemically detected in pharmaceutical products [20-21], AML is determined in pharmaceutical formulation by voltammetry using nano-composite electrode [22], TEL was detected with HCTZ from human plasma by HPLC [23]. Gupta et al. simultaneously detected TEL, Ramipril and Ramiprilat in human plasma using liquid chromatography tandem mass spectrometry [24]. As a crucial part of the drug development process, a rapid, sensitive and selective assay method is necessary to measure drug concentrations in human plasma samples and formulated samples. Methods exist for simultaneous determination of VAL and HCTZ [8]; TEL, AML and HCTZ [6]. However, to the best of our knowledge, a method for simultaneous determination of HCTZ, TEL, AML, and VAL by HPLC does not yet exist. The present method was developed and validated using reverse phase HPLC-UV for marketed formulations containing either of these compounds and applied for their detection in human plasma.

## *2. Materials and methods*

### *2.1 Reagents, standards and samples*

All solvents were HPLC grade and all chemicals were analytical reagent grade. Acetonitrile (ACN), methanol and potassium dihydrogen phosphate ($KH_2PO_4$), 1-butane sulphonic acid sodium salt, triethylamine, ortho phosphoric acid and ammonia were purchased from Merck Specialties Private Limited, Mumbai, India. Double distilled water used was filtered through 0.45µm membrane filter prior to analysis. All solvents were degased before use and solutions were filtered through a membrane filter of filtration units (Millipore filter units, polyethylene, 0.45 µm pore size). Reference standards of HCTZ, AML, TELM and VAL were obtained from local pharmaceutical industries and were used without further purification. All pharmaceutical formulations [Eslo-D, Eslo-5, Eslo-Tel and Valzaar] were purchased from local market.

### *2.2 Instrumentation*

The HPLC system (Waters Corporation, Milford Massachusetts, USA) used included a Waters US 600 controller pump, In-Line degasser and a 2489 dual wavelength UV-VIS detector (Milford, MA). A Waters US 717 auto sampler injector was used for injection of sample and an Agilent Eclipse XDB C18 (4.6 x150) mm 5µm particle size column (Agilent Technologies) was used for separation of all drug components. Double distilled water was prepared from LAB-SIL quartz double distiller (LAB-SIL Instruments Pvt. Ltd., India)

***2.3 Chromatographic conditions***

Mobile phase-A was 10 mM $KH_2PO_4$ buffer containing 10 mM butane sulphonic acid and 0.4% triethylamine adjusted to pH 3.0 with ortho-phosphoric acid, Mobile phase-B was acetonitrile. All analysis were done at ambient temperature under gradient condition using mobile phase A and mobile phase-B (Table 1). A flow rate of 1 $mLmin^{-1}$ was used for sample injection of 10 µL. Detection was done at 254 nm. Mobile phase were freshly prepared and degased prior to analysis.

***2.4 Method development and optimization of chromatographic conditions***

The chromatographic conditions were optimized by using different columns (C8, C18), mobile phase buffer, pH (7.0, 5.5, and 3.0), wavelength (241nm, 248nm, 254nm, 264nm, 269nm) and column temperature (ambient to 45°C) and injection volume (5, 10, 20, 50µL).

***2.5 Standard solution preparation***

Accurately weighed standards of 10 mg each of Hydrochlorothiazide, Amlodipine, Telmisartan and Valsartan were individually transferred to separate 100 ml standard volumetric flasks containing 20 ml methanol, sonicated for 15 min and diluted with methanol to 100ml. All solutions were freshly prepared prior to analysis.

***2.6 Sample preparation***

Twenty tablets of each sample Eslo-D (containing 12.5 mg of HCTZ and 2.5 mg of AML), Eslo-5 (containing 5 mg of AML), Eslo-Tel (containing 40 mg of TELM & 2.5 mg of AML), Valzaar tablets (containing 40 mg of VAL) were individually weighed and finely powdered to obtain a homogenous mixture. The amount of powder equivalent to the sample (12.5 mg for HCTZ, 5 mg for AML, 40 mg for TELM and 40 mg for VAL) was transferred to 100 ml standard volumetric flasks and diluted with methanol. The solutions were sonicated for 15 min and used as sample solutions.

***2.7 Plasma preparation***

Extraction of the drug from plasma was carried out by precipitation method [22]. A 4 ml aliquot of free plasma was spiked with 5 mg of each standards viz. HCTZ, AML, TELM and VAL. A 8 mL of methanol was added as a precipitating agent and vortexed for 5 min, and then centrifuged for 15 min at 7500 rpm. The supernatant solution was separated and filtered through 0.45 µm and run under the conditions derived for standards of the four drugs.

***2.8 Optimization of method***

In order to validate the method for analysis of HCTZ, AML, TELM and VAL in pharmaceutical formulations, preliminary tests were performed to obtain optimum conditions.

***2.9. Validation of the method***

The present method was validated as per International Conference on Harmonisation Q2 (R1) guidelines for various parameters such as linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), robustness, ruggedness and solution stability.

***3.0. Results and discussion***

The selection of the HPLC method depends on the nature of the sample (ionic or neutral molecule), its pKa, solubility, molecular weight, functional groups and polarity. The RP-HPLC method for the simultaneous determination of these four drugs in the current study was selected because of the polar nature of these compounds.

***3.1. Optimization of the chromatographic condition***

Optimization of the method for the simultaneous estimation of the HCTZ, AML, TELM and VAL as single component and combined dosage forms was carried out.

***3.1.1 Selection of column***

Trials were taken on different columns C6, C8, C18 endcapped with phenyl, cyano, amino to achieve separation of four analyte peaks from blank and other extraneous plasma peaks to obtain better resolution. On C8 column, AML peak asymmetry factor was observed more than 2.0 and peak shape was not well resolved. The separation between TELM and VAL was not proper on C8 stationary phase, while

on C18 stationary phase peak asymmetry factor, resolution, theoretical plates and peak shapes were good for all drugs.

***3.1.2 Selection of buffer, pH and mobile phase***

Buffers are aqueous systems that resist changes in pH when small amounts of acid or base are added. For achieving chromatographic separation, the pH of the buffer plays an important role because of its dependence on the pKa value. The pKa values for HCTZ, AML, TELM and VAL are 7.9, 8.6, 6.1, and 3.6 respectively. The mobile phase was selected in terms of its components and then proportionality. Initially ammonium acetate containing acetic acid, acetonitrile and $KH_2PO_4$ containing phosphoric acid were studied for isocratic and gradient elution modes. However, the separation of all four drug components was not proper using these mobile phase. Then 0.4% triethylamine (TEA) with ACN at pH 3.0 in different proportions was tested as the mobile phase. Another mobile phase involving gradient elution using 0.4% TEA with ion pairing agent 10 mM butane sulphonic acid with ACN was studied. The separation and elution of all four drug components was favorable but the resolution was poor and the peak shape was not good. Finally, mobile phase A and mobile phase B were selected as mentioned in the section under chromatographic condition.

***3.1.3 Selection of wavelength***

The proper selection of wavelength in UV detection determines the sensitivity of the HPLC method. The wavelength observed for HCTZ was 269 nm, AML showed 241 nm, TELM showed 254 nm, VAL showed 241 nm (Fig. 2). By comparing all spectra of drugs, suitable sensitivity was obtained between 250 to 260 nm. Intense absorption for detection of all compounds on 254 nm was observed. A region 248 to 264 nm was selected for detection in which all four components showed response at 254 nm for quantitative detection.

***3.1.4 Optimization of elution profile***

Initially isocratic mode of elution using 70:30 and 60:40 proportion of mobile phase A and mobile phase B was used. As the peaks were not well separated, a gradient proportion using 67.0 -33.0 to 33.0 - 67.0 (v/v) was tried and the elution was better with improved retention time and good resolution for all drug components was obtained. The retention times for HCTZ, AML, TELM and VAL were 2.35 min, 6.16 min, 10.60 min and 12.12 min respectively (Fig. 3). The chromatograms for the separations of the components in tablet - Eslo-D, Eslo-5, Eslo-Tel, and Valzaar are presented in Fig. 4a-b and Fig. 5a-b.

***3.2 Validation procedure***

***3.2.1 Specificity***

The specificity of the HPLC method is shown in Fig. 6; well separated peaks of standard drugs HCTZ, AML, TELM, and VAL were seen without any interference at the main peaks retention time. The sample stock solution of marketed sample (Eslo-D, Eslo-5, Eslo-Tel, and Valzaar) spiked in human plasma with different concentrations was evaluated for interference due to any other extraneous peaks of human plasma at the main peak retention time (Fig. 6).

***3.2.2 System suitability***

Freshly prepared standard solutions were injected to perform the system suitability test and mean retention time, resolution, tailing factor, number of theoretical plates and percent relative standard deviation (% RSD) were evaluated in replicates using the working standard solution (Table 2).

***3.2.3 Calibration curve and linearity***

To check the linear range of the drugs, eight different concentrations (8.0, 6.0, 4.0, 2.0, 1.0, 0.5, 0.3, 0.1 µg/ml for HCTZ, 20.0, 15.0, 10.0, 5.0, 2.5, 1.25, 0.75, 0.25 µg/ml for AML, 4.0, 3.0, 2.0, 1.0, 0.5, 0.25, 0.15, 0.05 µg/ml for TELM and 4.0, 3.0, 2.0, 1.0, 0.5, 0.25, 0.15, 0.05 µg/ml for VAL) were prepared in methanol. Each solution was injected into the column and a plot of peak areas against concentrations (n=3) was recorded. A linear regression analysis fitted with the least squares method and the correlation coefficients for HCTZ, AML, TELM, and VAL were 0.9999, 0.9999, 0.9993, and 0.9998 respectively (Fig. 7), suggesting that this HPLC method was found to be linear in nature.

### *3.2.4 Accuracy*

The accuracy of an analytical procedure is termed as trueness towards to its reference value [23]. The sample containing drug component was spiked with different quantity of standard HCTZ, AML, TELM, and VAL by standard addition method at three different levels of 50%, 100%, 150%, with three times repetition. The percent recoveries for each drugs and relative error are presented in Table 3 and indicates good accuracy.

### *3.2.5 Precision*

Precision was evaluated by performing six different preparations of same sample for all individual and combination products. The samples were analyzed for repeatability; the relative standard deviation was less than 2%. Intra-day analysis and inter-day analysis proves method was precise. The responses of all standard and sample solutions were determined in replicate (n=6) injections (Table-4).

### *3.2.6 Limit of detection and Limit of quantitation*

In order to estimate the LOD and LOQ, the mobile phase was injected six times, and the noise level was determined. The LOD and LOQ were calculated using the following equations;

$$LOD = 3.3\sigma/S \text{ and } LOQ = 10\sigma/S$$

Where, σ is the lowest standard concentration and S is the slope of calibration curve for the standard solution of respective drug [23]. LOD and LOQ for Hydrochlorothiazide were 0.063µgmL$^{-1}$ and 6.38 µgmL$^{-1}$; for Amlodipine 0.145 and 1.45 µgmL$^{-1}$; for Telmisartan 0.214 and 2.151 µgmL$^{-1}$; for Valsartan 0.044 µgmL$^{-1}$ and 0.440 µgmL$^{-1}$ respectively.

### *3.2.6 Robustness*

To determine the robustness of the method; the experimental conditions were slightly changed. Organic mobile phase was changed by ±1% and pH ±0.1 was used for aqueous mobile phase. No significant change in retention time of the peaks was observed.

### *3.2.7 Ruggedness*

Ruggedness of HPLC method was evaluated by comparing the results obtained by two different analysts using different columns of same type. Same result and retention times were obtained. It proved that time to time and analyst to analyst method was rugged as shown in Table 4.

### *3.2.8 Solution stability*

The solution stability of mobile phase, diluents, standard solution and sample solution was evaluated by storing at ambient (25°C) temperature for 48 hours. The stability of these standard solutions and sample solutions was studied by comparing the results obtained for stored solutions (48hours), such as changes in system suitability parameters i.e. resolution, retention, asymmetry and theoretical plates of the peaks with the freshly prepared solutions as given in Tables 5 and 6.

### *Conclusion*

The method developed in the present investigation for simultaneous determination of HCTZ, AML, TELM and VAL were very simple, reliable, precise and fast using gradient elution for their separation. Method was validated for LOD, LOQ, linearity, precision, accuracy, robustness, ruggedness and solution stability. The applicability of the procedure was checked for the simultaneous determination of HCTZ, AML, TELM, and VAL in human blood plasma. The method is advantageons in terms of short elution time and low LOD and LOQ. Method can be useful for pharmaceutical formulation and clinical industries for simultaneous determination of assay of four drugs in future.

### *Conflicts of interest*

The authors declare no conflicts of interest.

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## Tables

**Table 1:** Programme for Gradient elution of HCTZ, AML, TELM, and VAL

| Run Time (min) | %Mobile phase A | % Mobile phase B |
|---|---|---|
| 0.0 | 67.0 | 33.0 |
| 2.0 | 67.0 | 33.0 |
| 6.0 | 40.0 | 60.0 |
| 10.0 | 50.0 | 50.0 |
| 13.0 | 67.0 | 33.0 |

% A-10 mM Potassium dihydrogen phosphate containing 10 mM butane sulphonic acid and 0.4% triethylamine having pH 3.0 with ortho phosphoric acid and ammonia; % B – acetonitrile

**Table 2:** System suitability parameters

| Parameter | HCTZ | AML | TELM | VAL |
|---|---|---|---|---|
| Resolution (Rs) | - | 14.31 ± 0.20 | 16.20 ±0.50 | 8.20 ± 0.30 |
| Retention time | 2.35 ±0.03 | 6.16 ± 0.09 | 10.60 ± 0.04 | 12.12 ± 0.10 |
| Tailing factor | 1.23 ± 0.16 | 1.15 ± 0.08 | 1.18 ± 0.15 | 1.13 ± 0.11 |
| Number of theoretical plates | 2527 | 4801 | 43665 | 71036 |
| (% RSD) Peak area | 0.537 | 0.885 | 0.730 | 0.647 |

**Table 3:** Accuracy of the method

| Drug | Drugs Samples (%) added | Added Conc. ( mg/100 ml ) | Found Conc. ( mg/100 ml ) | % Recovery | % RSD | % R.E |
|---|---|---|---|---|---|---|
| HCTZ | 50 | 6.12 | 6.071 ± 0.024 | 99.26 | 0.394 | -0.756 |
| | 100 | 12.18 | 11.966 ± 0.019 | 98.22 | 0.157 | -1.815 |
| | 150 | 18.10 | 18.199 ± 0.013 | 100.59 | 0.071 | -0.583 |
| AML | 50 | 2.6 | 2.518 ± 0.021 | 98.38 | 0.851 | -1.659 |
| | 100 | 5.0 | 5.027 ± 0.029 | 100.53 | 0.580 | 0.527 |
| | 150 | 7.5 | 7.496 ± 0.033 | 99.95 | 0.445 | -0.047 |
| TELM | 50 | 20.3 | 20.36 ± 0.004 | 100.56 | 0.020 | -0.548 |
| | 100 | 40.2 | 39.84 ± 0.06 | 98.85 | 0.142 | -1.167 |
| | 150 | 60.1 | 59.18 ± 0.08 | 98.42 | 0.139 | -1.604 |
| VAL | 50 | 20.3 | 20.191 ± 0.084 | 99.63 | 0.417 | -0.376 |
| | 100 | 40.3 | 40.070 ± 0.104 | 99.43 | 0.259 | -0.573 |
| | 150 | 59.7 | 59.364 ± 0.284 | 99.38 | 0.478 | -0.622 |

R.E = Relative Error

**Table 4:** Intraday and interday assay precision analysis data of the proposed method

| Drug | Actual Conc. ( mg/100 ml ) | Intraday. ( mg/100 ml ) | Inter day ( mg/100 ml ) | % RSD |
|---|---|---|---|---|
| HCTZ | 6.0 | 6.1 | 6.0 | 0.41 |
| | 12.0 | 12.1 | 12.1 | 0.18 |
| | 18.0 | 17.9 | 18.0 | 0.06 |
| AML | 2.5 | 2.6 | 2.5 | 0.91 |
| | 5.0 | 5.0 | 5.1 | 0.60 |
| | 7.5 | 7.4 | 7.6 | 0.51 |
| TELM | 20.0 | 20.1 | 20.1 | 0.05 |
| | 40.0 | 39.9 | 40.1 | 0.13 |
| | 60.0 | 60.1 | 60.2 | 0.15 |
| VAL | 20.0 | 20.1 | 20.0 | 0.40 |
| | 40.0 | 40.1 | 40.1 | 0.27 |
| | 59.0 | 59.9 | 60.1 | 0.46 |

**Table 5:** Solution stability of the Standard drug solutions of the proposed method

| Parameter | HCTZ 0Hrs, 24Hrs, 48Hrs | AML 0Hrs, 24Hrs, 48Hrs | TELM 0Hrs, 24Hrs, 48Hrs | VAL 0Hrs, 24Hrs, 48Hrs |
|---|---|---|---|---|
| Resolution (Rs) | - | 14.32, 14.33, 14.30 | 16.19, 16.20 | 8.21,8.22,8.20 |
| Retention time | 2.33, 2.36, 2.35 | 6.15,6.16,6.15 | 10.60,10.61,10.63 | 12.12,12.11,12.13 |
| Tailing factor | 1.22, 123, 1.22 | 1.16,1.15,1.14 | 1.18,1.15,1.16 | 1.13,1.12,1.11 |
| Number of theoretical plates | 2527, 2628, 2611 | 4801,4845, 4823 | 43665,43788, 43697 | 71036,71049, 71011 |
| (% RSD) Peak area | 0.537, 0.531, 0.535 | 0.885, 0.881, 0.883 | 0.730, 0.732, 0.731 | 0.647, 0.645, 0.665 |

**Table 6:** Solution stability of the Sample solutions of the proposed method

| Drug | % Assay Initial | % Assay 24hours | % Assay 48hours | Mean | SD | % RSD |
|---|---|---|---|---|---|---|
| **HCTZ** | 98.22 | 98.15 | 98.31 | 98.23 | 0.080 | 0.082 |
| **AML** | 100.53 | 100.40 | 100.24 | 100.39 | 0.145 | 0.145 |
| **TELM** | 98.85 | 98.92 | 98.71 | 98.83 | 0.107 | 0.108 |
| **VAL** | 99.43 | 99.32 | 99.51 | 99.42 | 0.095 | 0.096 |

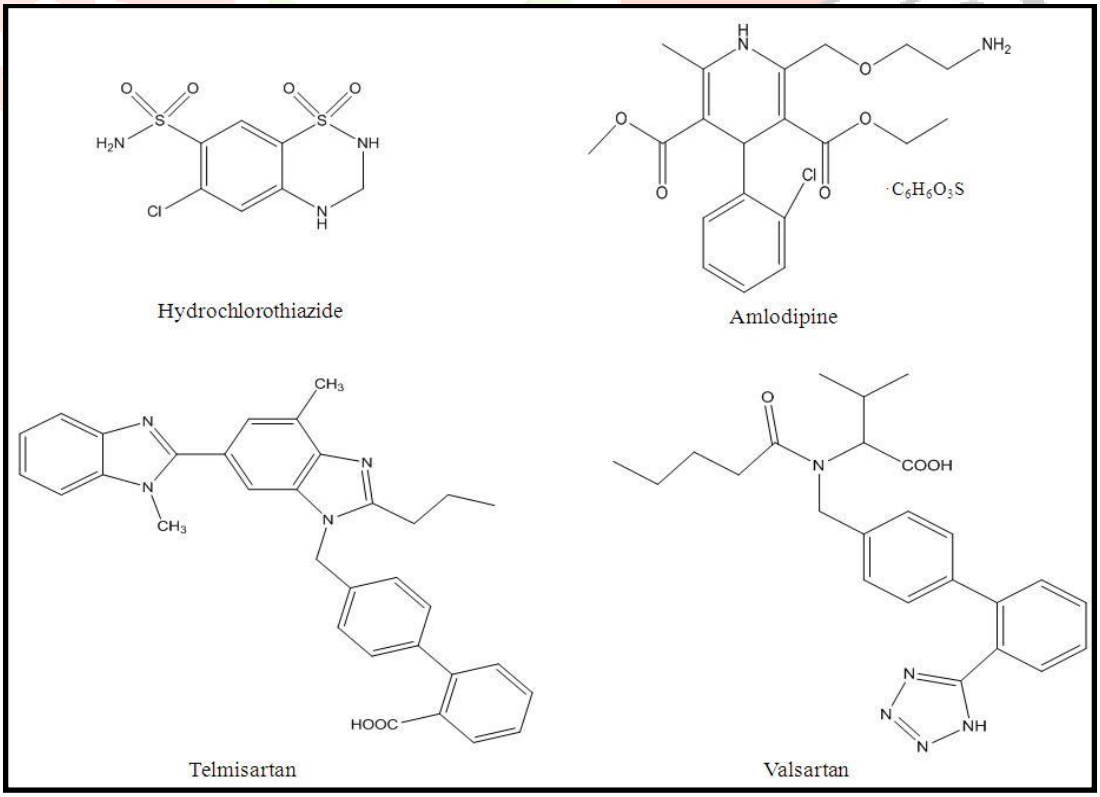


**Fig. 1** Chemical structures of HCTZ, AML, TELM, and VAL

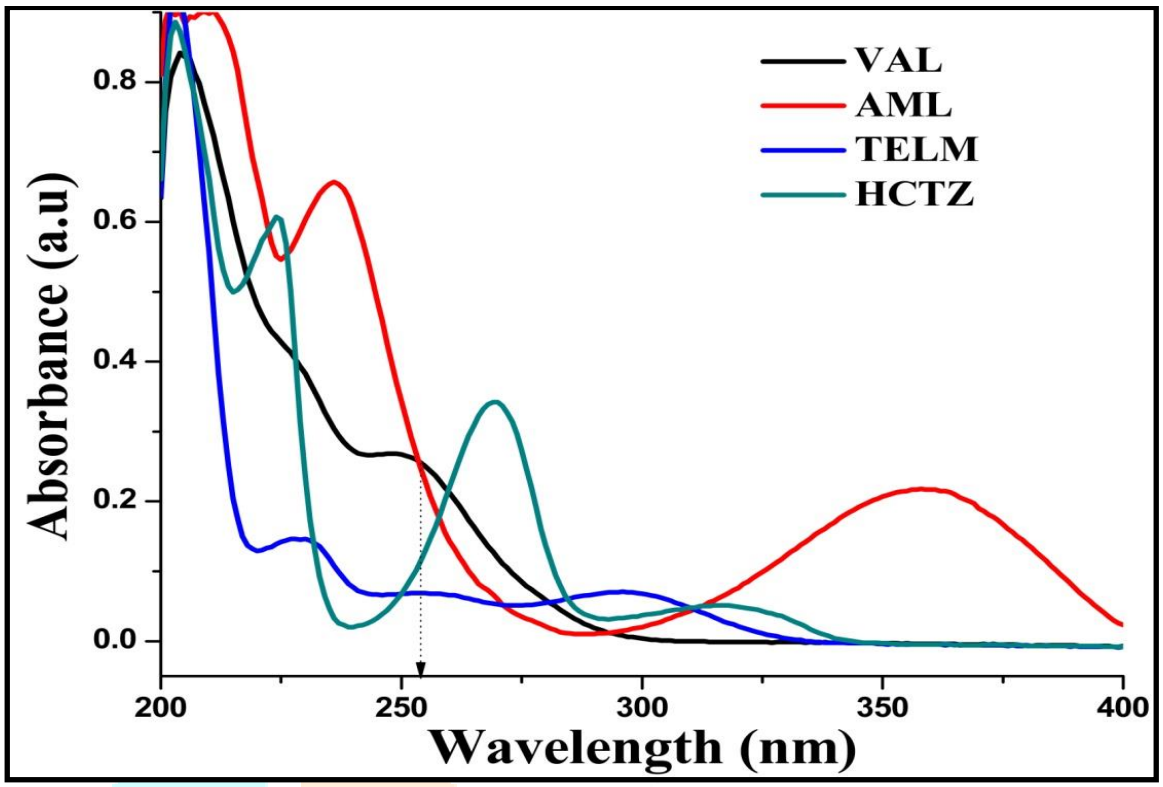


**Fig. 2** UV spectra of HCTZ, AML, TELM, and VAL

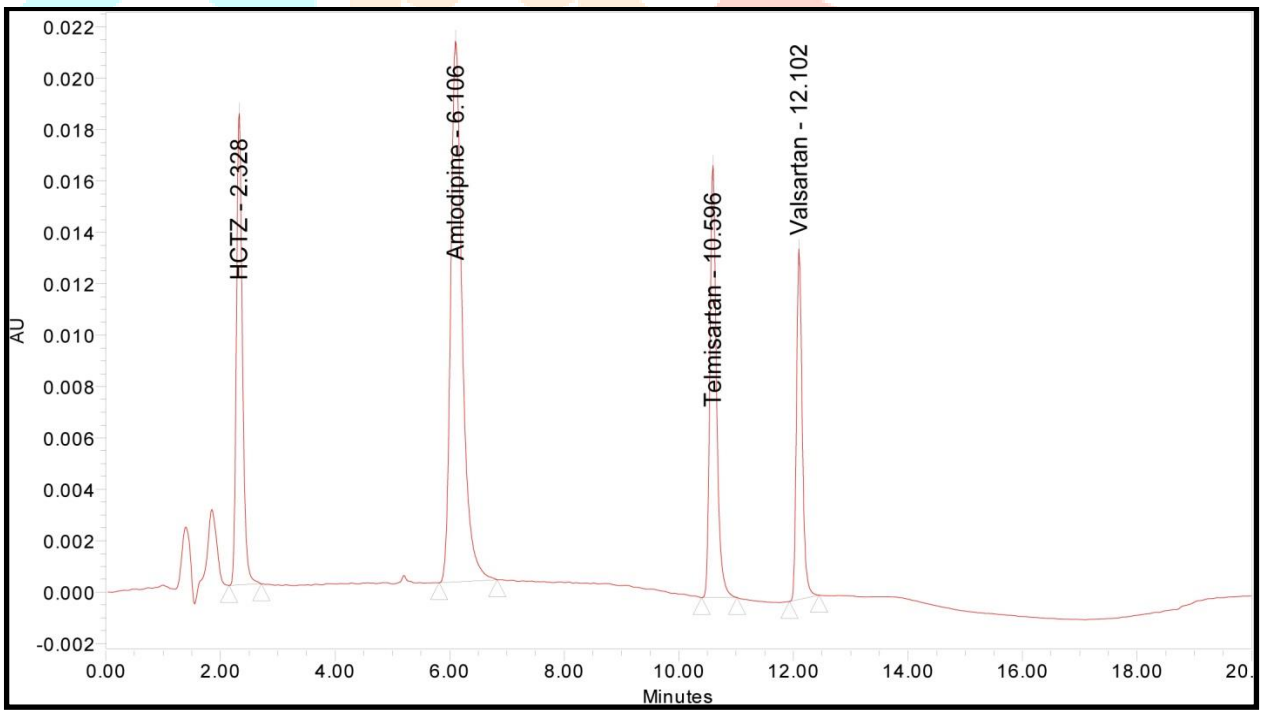


**Fig. 3** Chromatographic separation of HCTZ, AML, TELM, and VAL

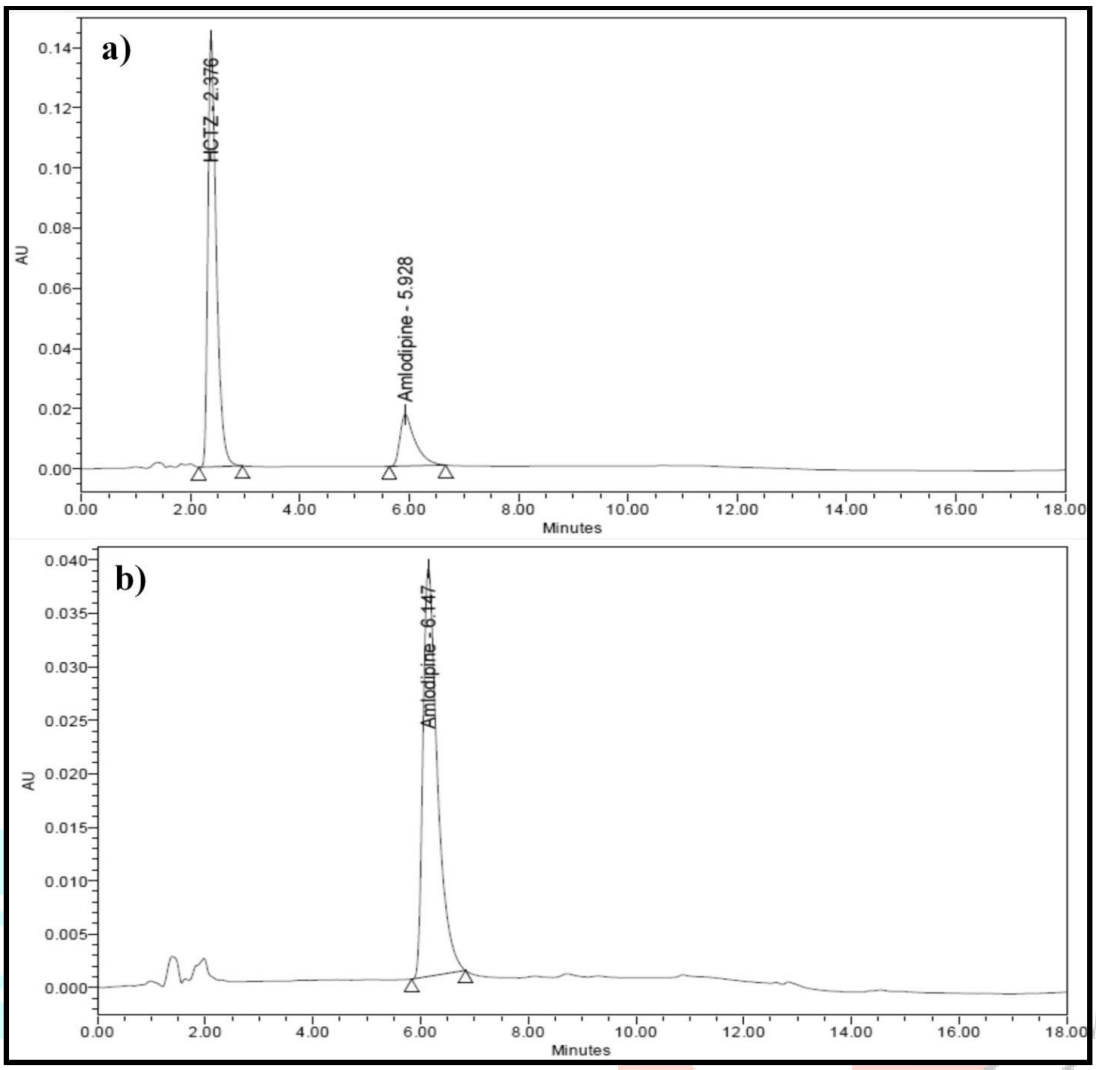


**Fig. 4 a)** Chromatographic separation for Tablet Eslo-D, **b)** Chromatographic separation for Tablet Eslo-5

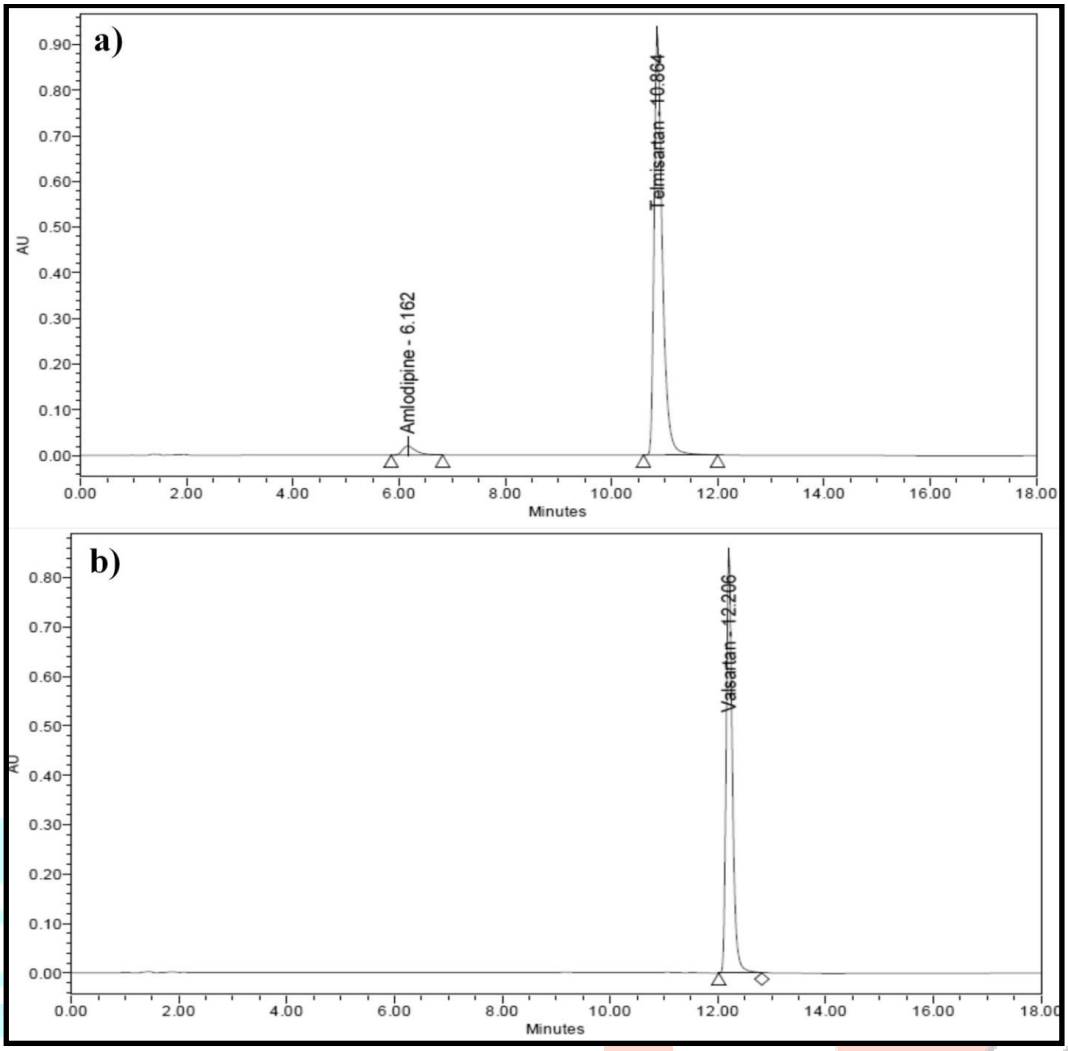


**Fig. 5 a)** Chromatographic separation for Tablet Eslo-Tel, **b)** Chromatographic separation for Tablet Valzaar

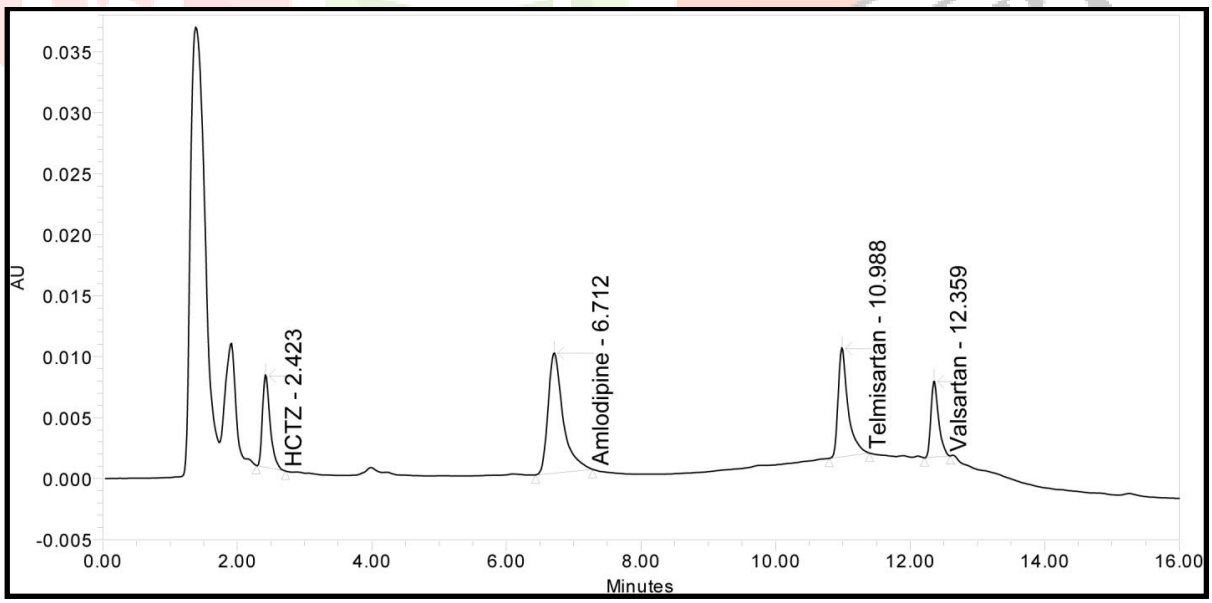


**Fig. 6** Chromatogram of human plasma spiked by standard samples

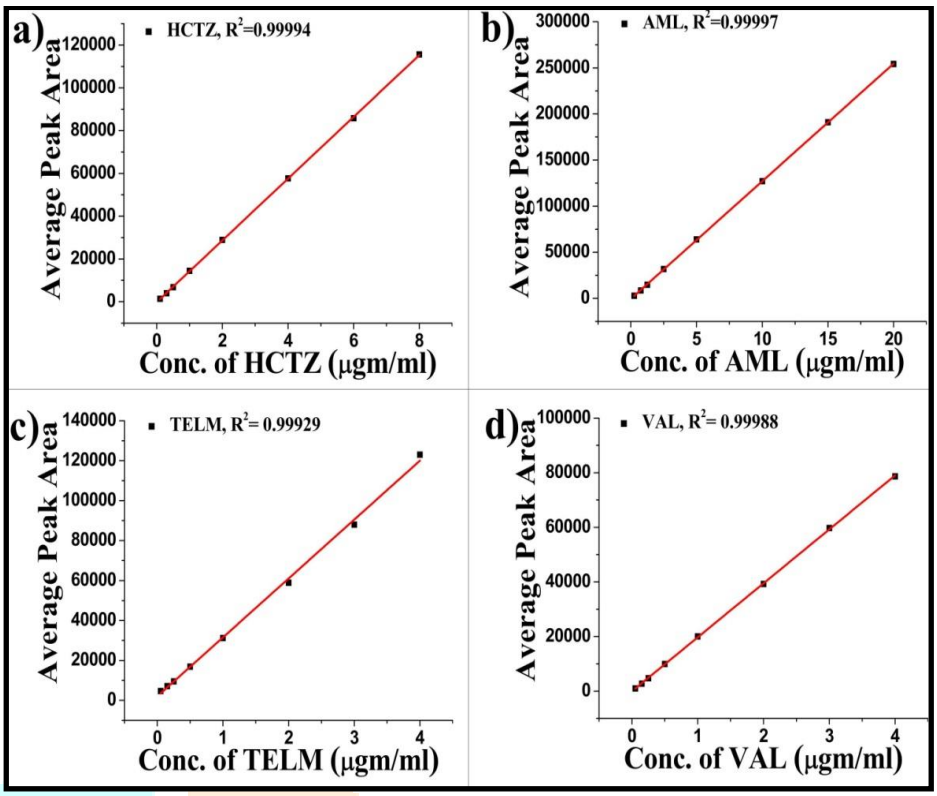


**Fig. 7** Calibration and linearity of curves for standards of HCTZ, AML, TELM, and VAL