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# METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF HYDROCHLOROTHIAZIDE AND IRBESARTAN IN A PHARMACEUTICAL FORMULATION BY RP-HPLC METHOD

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## ABSTRACT

An isocratic simultaneous estimation by RP-HPLC Method were developed and validated for the quantification of Hydrochlorothiazide and Irbesartan in tablet dosage form. Quantification was achieved by using a reversed-phase C18 column (Inertsil Column, 5µ, 250 mm × 4.6 mm) at ambient temperature with mobile phase consisting of Phosphate Buffer buffer(50mM): Acetonitrile (60: 40 pH: 6.0)). The flow rate was 1.0 ml/min. Measurements were made at a wavelength of 235nm. The average retention time for Hydrochlorothiazide and Irbesartan were found to be 2.85 min and 4.22. The proposed method was validated for selectivity, precision, linearity and accuracy. The assay methods were found to be linear from 90-210µg/ml for Hydrochlorothiazide and 7.5 to 17.5µg/ml for Irbesartan. All validation parameters were within the acceptable range. The developed method was successfully applied to estimate the amount of Hydrochlorothiazide and Irbesartan in tablet dosage form.

## KEYWORDS

Hydrochlorothiazide, Irbesartan, RP-HPLC method, Inertsil Column, Methanol, Acetonitrile, KH2PO4, Ortho phosphoric acid and Validation.

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## INTRODUCTION

Hydrochlorothiazide (Figure No.1) is thiazide diuretic often considered the prototypical member of this class. It reduces the re absorption of electrolytes from the renal tubules. This results in increased excretion of water and electrolytes, including sodium, potassium, chloride, and magnesium. It has been used in the treatment of several disorders

including edema, hypertension, diabetes insipidus, and hypoparathyroidism.

Hydrochlorothiazide, a thiazide diuretic, inhibits water reabsorption in the nephron by inhibiting the sodium-chloride symporter (SLC12A3) in the distal convoluted tubule, which is responsible for 5% of total sodium reabsorption. Normally, the sodium-chloride symporter transports sodium and chloride from the lumen into the epithelial cell lining the distal convoluted tubule. The energy for this is provided by a sodium gradient established by sodium-potassium ATPases on the basolateral membrane. Once sodium has entered the cell, it is transported out into the basolateral interstitium via the sodium-potassium ATPase, causing an increase in the osmolarity of the interstitium, thereby establishing an osmotic gradient for water reabsorption. By blocking the sodium-chloride symporter, hydrochlorothiazide effectively reduces the osmotic gradient and water reabsorption throughout the nephron.

**Pharmacodynamics**

Thiazides such as hydrochlorothiazide promote water loss from the body (diuretics). They inhibit $Na^+/Cl^-$ reabsorption from the distal convoluted tubules in the kidneys. Thiazides also cause loss of potassium and an increase in serum uric acid. Thiazides are often used to treat hypertension, but their hypotensive effects are not necessarily due to their diuretic activity. Thiazides have been shown to prevent hypertension-related morbidity and mortality although the mechanism is not fully understood. Thiazides cause vasodilatation by activating calcium-activated potassium channels (large conductance) in vascular smooth muscles and inhibiting various carbonic anhydrases in vascular tissue.

Irbesartan (Figure No.2) is an angiotensin receptor blocker (ARB) used mainly for the treatment of hypertension. It competes with angiotensin II for binding at the AT1 receptor subtype. Unlike ACE inhibitors, ARBs do not have the adverse effect of dry cough. The use of ARBs is pending revision due to findings from several clinical trials suggesting that this class of drugs may be associated with a small increased risk of cancer.

Irbesartan is a nonpeptide tetrazole derivative and an angiotensin II antagonist that selectively blocks the binding of angiotensin II to the $AT_1$ receptor. In the renin-angiotensin system, angiotensin I is converted by angiotensin-converting enzyme (ACE) to form angiotensin II. Angiotensin II stimulates the adrenal cortex to synthesize and secrete aldosterone, which decreases the excretion of sodium and increases the excretion of potassium. Angiotensin II also acts as a vasoconstrictor in vascular smooth muscle. Irbesartan, by blocking the binding of angiotensin II to the $AT_1$ receptor, promotes vasodilation and decreases the effects of aldosterone. The negative feedback regulation of angiotensin II on renin secretion is also inhibited, but the resulting rise in plasma renin concentrations and consequent rise in angiotensin II plasma concentrations do not counteract the blood pressure lowering effect that occurs. The action of ARBs is different from ACE inhibitors, which block the conversion of angiotensin I to angiotensin II, meaning that the production of angiotensin II is not completely inhibited, as the hormone can be formed via other enzymes. Also, unlike ACE inhibitors, irbesartan and other ARBs do not interfere with response to bradykinins and substance P, which allows for the absence of adverse effects that are present in ACE inhibitors (eg. dry cough).

## EXPERIMENTAL

**Equipments**

The chromatographic technique performed on a Shimadzu LC20-AT Liquid chromatography with SPD-20A prominence UV-visible detector and Spinchrom software, reversed phase C18 column (Inertsil 5µ, 250 mm × 4.6 mm) as stationary phase. Thermo Electron Corporation double beam UV-visible spectrophotometer (vision pro-software), Ultrasonic cleaner, Shimadzu analytical balance AY-220, Vaccum micro filtration unit with 0.45µ membrane filter was used in the study.

**Materials**

Pharmaceutically pure sample of Hydrochloro thiazide and Irbesartan were obtained as gift samples from Chandra Labs, Prashanthi nagar, Kukatpally, Hyderabad, India. The purity of the drug was evaluated by obtaining its melting point and ultraviolet (UV) and infrared (IR) spectra. No impurities were found. The drug was used without further purification.

HPLC-grade Acetonitrile was from standard reagents pvt ltd. $KH_2PO_4$ (AR grade) was from Merck.

A tablet formulation of Hydrochlorothiazide and Irbesartan (150 mg and 12.5 mg label claims) were procured from local market (Irovel-H, Sun Pharmaceuticals, India).

**Chromatographic conditions**

The sample separation was achieved on a C18 (5 µm, 25 cm X 4.6 mm i.d.) Inertsil column, aided by mobile phase mixture of Phosphate Buffer buffer: Acetonitrile (60:40 pH:6.0)), that was filtered and degassed prior to use, at a flow rate of 1ml/min. Injection volume is 20 µl and detected at 235 nm at ambient temperatures.

**Preparation of mobile phase**

**Buffer Preparation**

Weigh accurately about 6.8 gms of $KH_2PO_4$ and dissolve with 500ml of HPLC Grade water than make up to 1000 ml with HPLC grade water then adjust the pH: 6.0 with ortho phosphoric acid or sodium hydroxide.

**Mobile phase**

Then add 60 volumes of buffer, 40volumes of Acetonitrile and sonicated for 15 min and filtered through a 0.45 µ membrane filter.

**Analysis of formulation**

**Preparation of standard solution**

A 150mg of standard Hydrochlorothiazide and 12.5 mg Irbesartan ware weighed and transferred to 100 ml of volumetric flask and dissolved in mobile phase. The flask was shaken and volume was made up to mark with mobile phase to give a primary stock solution containing 1500µg/ml Hydrochlorothiazide and 125µg/ml of Irbesartan. From the above solution 5ml of solution is pipette out into a 50 ml volumetric flask and volume was made up to mark with mobile phase to give a solution containing 150µg/ml Hydrochlorothiazide and 12.5µg/ml of Irbesartan.

**Preparation of sample solution (Tablet Formulation)**

For the estimation of the drug in tablet formulation twenty tablets were weighed and their average weight was determined. The tablets were then finely powdered. Appropriate quantity equivalent to 150mg Hydrochlorothiazide and 12.5 mg Irbesartan ware accurately weighed and The powder was transferred to 100 ml volumetric flask and shaken vigorously with mobile phase and sonicated for 15 min and volume made up to the mark with mobile phase. The solution was shaken vigorously and filtered by using whatmann filter no.41. From the above filtered clear solution 5ml of sample pipetted out into a 50 ml volumetric flask volume made up to the mark with mobile phase to give a solution containing 150µg/ml Hydrochlorothiazide and 12.5µg/ml of Irbesartan.

## RESULTS AND DISCUSSION

**Determination of working Wavelength (λmax)**

10 mg of the Hydrochlorothiazide standard drug is taken in a 10 ml volumetric flask and dissolved in methanol and volume made up to the mark, from this solution 0.1ml is pipette out into 10 ml volumetric flask and made upto the mark with the methanol to give a concentration of 10 µg/ml. The above prepared solution is scanned in UV between 200-400 nm using methanol as blank. The λmax was found to be 289nm.

10 mg of the Irbesartan standard drug is taken in a 10 ml volumetric flask and dissolved in methanol and volume made up to the mark, from this solution 0.1ml is pipette out into 10 ml volumetric flask and made upto the mark with the methanol to give a concentration of 10 µg/ml. The above prepared solution is scanned in UV between 200-400 nm using methanol as blank. The λmax was found to be 228nm.

The Isosbestic Point of Hydrochlorothiazide and Irbesartan were found to be 235nm. The U.V Graph shown in Figure No.5.

After several initial trails with mixtures of methanol, water, ACN and buffer in various combinations and proportions, a trail with a mobile phase mixture of Phosphate Buffer: Acetonitrile (60:40 pH:6.0) brought sharp and well resolved peaks. The chromatogram was shown in Figure No.6.

## METHOD OF VALIDATION

### Linearity

Linearity was studied by analyzing five standard solutions covering the range of 90-210µg/ml for Hydrochlorothiazide and 7.5 to 17.5µg/ml for Irbesartan of the drug. From the primary stock solution 0.6ml, 0.8ml, 1.0ml, 1.2ml, 1.4 ml of aliquots are pipette into 10 ml volumetric flasks and made up to the mark with the mobile phase to give a concentrations of 90µg/mL, 120µg/mL, 150 µg/mL, 180µg/mL and 210 µg/mL of Hydrochlorothiazide and 7.5µg/mL, 10µg/mL, 12.5 µg/mL, 15µg/mL, 17.5µg/mL of Irbesartan.

Calibration curve with concentration verses peak areas was plotted by injecting the above prepared solutions and the obtained data were subjected to regression analysis using the least squares method.

### Method precision (repeatability)

The precision of the instrument was checked by repeated injections and measurement of peak areas and retention times of solutions (n = 6) for, 150 µg/ml of Hydrochlorothiazide and 12.5 µg/ml of Irbesartan without changing the parameter of the proposed chromatographic method.

### Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve by using the equations (2) and (3), respectively.

$$LOD = 3.3\ \delta/S \quad \text{……. (3)}$$

$$LOQ = 10\ \delta/S \quad \text{…….. (4)}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

### Accuracy (recovery study)

The accuracy of the method was determined by calculating the recoveries of Hydrochlorothiazide and Irbesartan by the standard addition method. Known amounts of standard solutions of Hydrochlorothiazide and Irbesartan were added at 10% concentration to pre quantified sample solutions of Hydrochlorothiazide (120, 150, 180/ml) and Irbesartan (10, 12.5, 15µg/ml) (Figure No.7.1 and 7.2). The amount of Hydrochlorothiazide and Irbesartan recovered was estimated by using the following formulas.

$$\%\ Recovery = \frac{amount\ found}{Amount\ added} \times 100$$

$$Amount\ Found(mcg/ml) = \frac{Mean\ test\ area}{Mean\ standard\ area} \times Standard\ concentration$$

### Specificity

In an assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay results are unaffected by the presence of these extraneous materials. There should be no interference of the diluents, placebo at retention time of drug substances.

### Robustness

Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied ±2nm and flow rate was varied ±0.2 ml/min. The results were shown in (Table No.4).

### Ruggedness

The ruggedness of the method was studied by analyzing the sample and standard preparations by two analysts. The % RSD assay values between two analysts was calculated i.e.,(limit <2%). This indicates the method was rugged. The results were shown in Table No.5.

## DISCUSSION

In RP HPLC method, the primary requirement for developing a method for analysis is that the using different solvents and buffers and columns to get better retention time and theoretical plates, and better cost effective and time saving method than the previously developed methods. The Isobestic Point of Hydrochlorothiazide and Irbesartan were found to be 235nm by scanning in UV region. The chromatographic method was optimized with mobile phase consisting of $KH_2PO_4$ Buffer (50mM): Acetonitrile (60:40) and C18 Inertsil column. All the validation parameters were studied at a the wavelength 235nm. Accuracy was determined by calculating the recovery (Table No.3) and the results were in acceptable range (limit 98-102%). The method was successfully used to determine the amount of Hydrochlorothiazide and Irbesartan present in the Tablet. The results obtained were in good agreement with the corresponding labeled amount (Table No.3). The method was linear in the concentration range of 90 to 210µg/ml for Hydrochlorothiazide and 7.5 to 17.5µg/ml for Irbesartan (Figure No.1, Table No.1). Precision was calculated (% RSD) for the drug (Table No.7 Robustness and ruggedness results were in acceptable range (Table No.4 and Table No.5). Summary of all validation parameters for method is given in Table No.8. By observing the validation parameters, the method was found to be simple, sensitive, accurate and precise. Hence the method can be employed for the routine analysis Hydrochlorothiazide and Irbesartan in tablet dosage form.

**Table No.1: Concentration verses peak areas of Hydrochlorothiazide**

| S.No | Concentration (µg/ml ) | Peak Area |
|---|---|---|
| 1 | 90 | 207.72 |
| 2 | 120 | 277.79 |
| 3 | 150 | 359.53 |
| 4 | 180 | 425.87 |
| 5 | 210 | 493.89 |

**Table No.1.1: Concentration verses peak areas of Irbesartan**

| S.No | Concentration (µg/ml) | Peak Area |
|---|---|---|
| 1 | 7.5 | 2127.51 |
| 2 | 10.0 | 2757.08 |
| 3 | 12.5 | 3575.88 |
| 4 | 15.0 | 4201.17 |
| 5 | 17.5 | 4752.49 |

**Table No.2: LOD and LOQ values calculated from calibration curve**

| S.No | Hydrochlorothiazide | | | Irbesartan | |
|---|---|---|---|---|---|
| | | Mcg | Area | mcg | Area |
| 1 | LOD | 65.19 | 156.66 | 0.05 | 13.08 |
| 2 | LOQ | 197.56 | 474.72 | 0.15 | 39.62 |

**Table No.3: Recovery data**

| S.No | Level | Amount of Sample taken (%) | Amount of Standard Spiked (%) | % Recovery of Hydrochlorothiazide | % Recovery of Irbesartan |
|---|---|---|---|---|---|
| 1 | I | 80 | 10% | 100.42% | 100.85% |
| | | 80 | 10% | | |
| | | 80 | 10% | | |
| 2 | II | 100 | 10% | 99.82% | 101.19% |
| | | 100 | 10% | | |
| | | 100 | 10% | | |
| 3 | III | 120 | 10% | 100.94% | 98.42% |
| | | 120 | 10% | | |
| | | 120 | 10% | | |

**Table No.4: Results of Robustness study**

**Irbesartan**

| S.No | Parameter | Rt | Tailing factor | Theoretical plates |
|---|---|---|---|---|
| 1 | Flow Rate(0.8ml) | 4.263 | 1.250 | 5301 |
| 2 | 1.2ml | 2.150 | 1.200 | 5412 |
| 3 | 1.0ml | 2.891 | 1.260 | 5612 |
| 4 | Wave Length235nm±2 | 2.885 | 1.483 | 5001 |
| 5 | 237nm | 2.887 | 1.483 | 5021 |
| 6 | 233nm | 2.893 | 1.467 | 5423 |

**Hydrochlorothiazide**

| S.No | Parameter | Rt | Tailing factor | Theoretical plates |
|---|---|---|---|---|
| 1 | Flow Rate(0.8ml) | 6.067 | 1.470 | 4012 |
| 2 | 1.2ml | 3.301 | 1.478 | 4011 |
| 3 | 1.0ml | 5.103 | 1.471 | 4254 |
| 4 | Wave Length235nm±2 | 5.040 | 1.470 | 4154 |
| 5 | 237nm | 5.050 | 1.356 | 4087 |
| 6 | 233nm | 5.087 | 1.328 | 4114 |

**Table No.5: Results of Ruggedness**

| S.No | Analyst | Drug | % Assay | % RSD |
|---|---|---|---|---|
| 1 | Analyst-1 | Irbesartan | 100.21 | **0.38%** |
| 2 | Analyst-2 | | 100.75 | |
| 3 | Analyst-1 | Hydrochlorothiazide | 100.63 | **0.15%** |
| 4 | Analyst-2 | | 100.41 | |

**Table No.6: Assay Results**

| S.No | Hydrochlorothiazide | | | Irbesartan | | |
|---|---|---|---|---|---|---|
| 1 | Standard Area | 1 | 3467.35 | | 349.71 | |
| | | 2 | 3391.87 | | 368.64 | |
| | | 3 | 3403.27 | | 357.98 | |
| | | 4 | 3410.80 | | 348.50 | |
| | | 5 | 3415.22 | | 353.98 | |
| | | Average | 3417.70 | Average | 355.76 | |
| 2 | Sample area | 1 | 3414.15 | | 350.18 | --- |
| | | 2 | 3421.66 | | 349.04 | |
| | | 3 | 3469.74 | | 349.70 | |
| | | 4 | 3476.75 | | 356.85 | |
| | | 5 | 3408.76 | | 370.14 | |
| | | Average | 3438.21 | Average | 355.76 | |
| 3 | Tablet average weight | | 302.20 | mg | 302.20 | Mg |
| 4 | Standard weight | | 150.25 | mg | 12.54 | Mg |
| 5 | Sample weight | | 303.30 | mg | 303.30 | Mg |
| 6 | Label amount | | 150.00 | mg | 12.50 | Mg |
| 7 | std.purity | | 99.80 | % | 99.70 | % |
| 8 | Cal.: | | 150.30 | mg | 12.46 | Mg |
| | | % Assay | 100.20 | % | 99.66 | % |

**Table No.7: Method Precision (Repeatability)**

| S.No | Irbesartan | | Hydrochlorothiazide | |
|---|---|---|---|---|
| | Rt | Area | Rt | Area |
| 1 | 2.98 | 356.04 | 4.46 | 3536.12 |
| 2 | 2.97 | 342.15 | 4.44 | 3520.52 |
| 3 | 2.94 | 349.99 | 4.42 | 3497.85 |
| 4 | 2.95 | 349.70 | 4.41 | 3502.25 |
| 5 | 2.95 | 347.48 | 4.39 | 3497.11 |
| 6 | 2.95 | 342.86 | 4.41 | 3473.10 |
| Avg | 2.96 | 348.04 | 4.42 | 3504.49 |
| st.dev | 0.02 | 5.15 | 0.03 | 21.66 |
| % RSD | 0.52 | 1.48 | 0.57 | 0.62 |

**Table No.8: Validation parameters of evaluated method**

| S.No | Parameter | Limit | Value Obtained |
|---|---|---|---|
| 1 | Accuracy (% Recovery) | 98-102% | 99.82 to 100.94% (Hydrochlorothiazide) 98.42 to 101.19% (Irbesartan) |
| 2 | Linearity concentrations Range (µg/mL) Regression coefficient (R2 value) | NLT 0.99% | 90 to 210µg/ml (Hydrochlorothiazide) $R^2$=0.998 and 7.5 to 17.5µg/ml (Irbesartan) $R^2$=0.995 |
| 3 | Method precision (Repeatability) (% RSD, n = 6) | NMT 1% (For Rt) NMT 2% (For Area) | % RSD of Rt=0.57% and % RSD of Area 0.62% (Hydrochlorothiazide) % RSD of Rt=0.52% and % RSD of Area 1.48% (Irbesartan) |
| 4 | Robustness | Should meets with system suitability | Complies |
| 5 | Ruggedness (% RSD analyst to analyst variation) | NMT 2% | Complies |

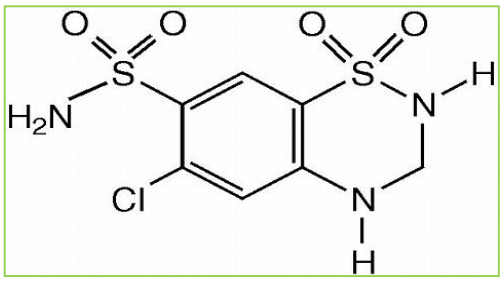


**Figure No.1: Structure of Hydrochlorothiazide**

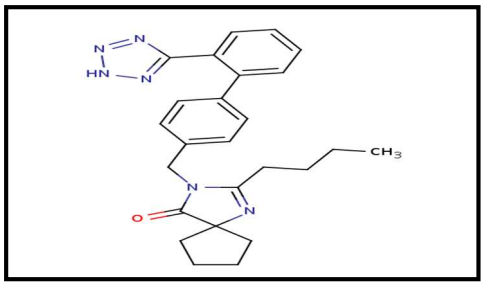


**Figure No.2: Structure of Irbesartan**

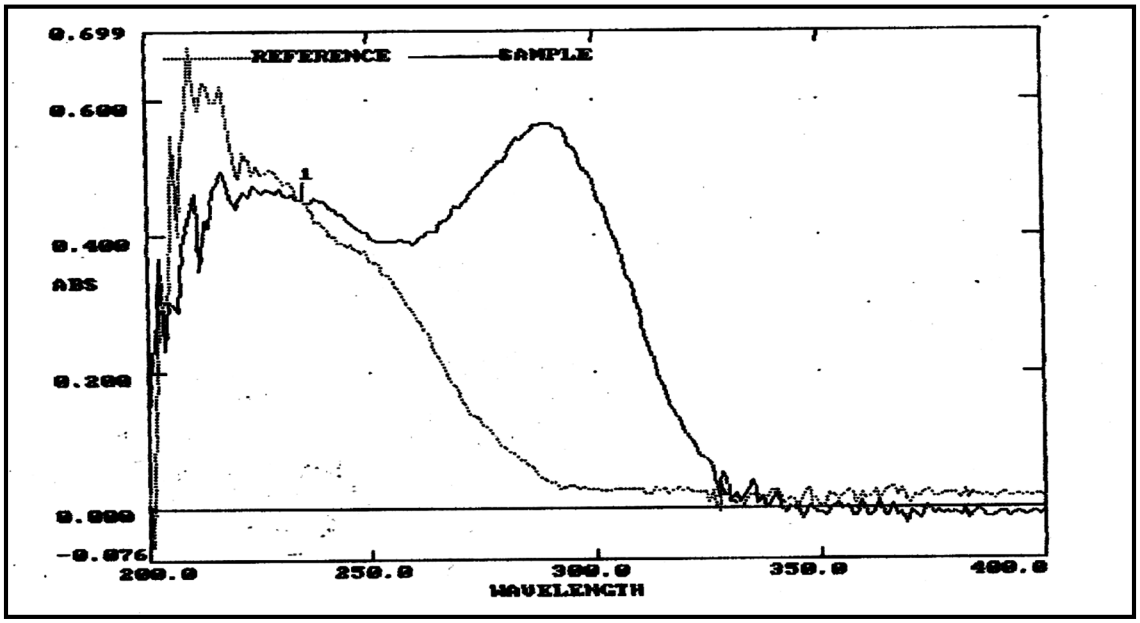


**Figure No.3: U.V Graph of Hydrochlorothiazide and Irbesartan**

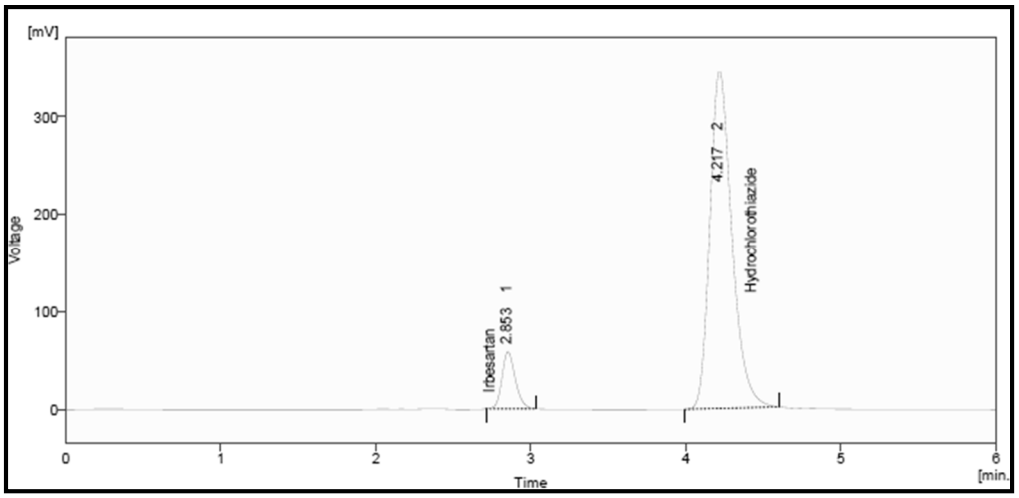


**Figure No.4: Chromatogram of Hydrochlorothiazide and Irbesartan**

## CONCLUSION

From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation of Hydrochlorothiazide and Irbesartan was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in meant in industries, approved testing laboratories.

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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