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Simultaneous estimation of anti-hypertensive drugs and a vitamin in a pharmaceutical formulation

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ABSTRACT

A simple, rapid, and precise reversed-phase high-performance liquid chromatographic method has been developed for simultaneous determination of Hydrochlorothiazide; Folic Acid; Atenolol; Aspirin; Enalapril Maleate and Atorvastatin Calcium. The six drugs were separated on an Inertsil; 150 mm; 4.6 mm; i.d. 5µm particle, C8 column. The mobile phase was buffer: methanol with a binary gradient program at a flow rate of 2.0mL min-1. The buffer was a mixture of 0.01M Sodium Di-hydrogen Ortho-Phosphate (NaH_2PO_4); 0.01M of Di-Sodium Hydrogen Ortho-Phosphate (Na_2HPO_4) and 1.0 g of Sodium-Heptane Sulphonic acid in 1000mL purified water and pH adjusted to 2.6 with ortho-phosphoric acid. UV detection was performed at 215nm. The method was validated for linearity, accuracy, and precision parameters and these parameters were acceptable in the ranges 33.043µg mL-1 to 99.129µg mL-1 for Hydrochlorothiazide, 3.659µg mL-1 to 10.977µg mL-1 for folic acid, and 126.000µg mL-1 to 378.000µg mL-1 for Atenolol, 374.000µg mL-1 to 1122.000µg mL-1 for Aspirin, 10.000µg mL-1 to 40.000µg mL-1 for Enalapril, and 49.811µg mL-1 to 149.433µg mL-1 for Atorvastatin.

KEYWORDS

High pressure liquid chromatography;
Pharmaceutical formulation;
Hydrochlorothiazide;
Folic Acid;
Atenolol;
Aspirin;
Enalapril Maleate;
Atorvastatin Calcium;
Vitamin.

INTRODUCTION

Polypill was used as single dose formulation to reduce four cardiovascular risk factors by modifying low density lipoprotein cholesterol; blood pressure; serum homocysteine and platelet function. It is a combination of Hydrochlorothiazide (6.25mg) for lowering blood pressure; Folic Acid (Vitamin) (0.80mg) to reduce serum homocysteine; Atenolol (25.0mg) for lowering blood pressure; Aspirin (75.0mg) as anti-platelet agent; Enalapril Maleate (2.50mg) for lowering blood pressure; Atorvastatin Calcium (10.0mg) for LDL cholesterol.[1]

Each drug has been used in medical practice for more than 10 years with substantial evidence on safety and efficacy. There are number of methods of analysis of each of these drugs/ analytes for qualitative and quantitative determination from their individual formulation or from formulation with combination of any two or three drugs. However for qualitative and quantitative analysis of these analytes from this combination is was not available. In this case application of available methods of analysis would be expensive and time consuming. In addition these methods would also require modification as one analyte might have interfered with the other. Large quantity of sample would have been re-

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quired for separate analysis these analytes. Therefore an attempt was made to develop single, simple, rapid and method of analysis on HPLC.[2,3]

Hydrochlorothiazide, is a thiazide diuretic that acts by inhibiting the kidneys' ability to retain water and prevent body from absorbing too much salt, which can cause fluid retention. It is also used to treat high blood pressure. Chemically as shown in Figure 1, its 6-chloro-3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7- sulfonamide 1, 1-dioxide; with a pK value of 7.9 and 9.2. It has a maximum UV detection at wavelength of λmax 226nm. It's Molecular weight is 297.74 g/mol and it is a white, or practically white, practically odorless, crystalline powder. Hydrochlorothiazide is slightly soluble in water (0.70 mg/mL in water), freely soluble in sodium hydroxide solution, in n - butylamine and in dimethylformamide, sparingly soluble in methanol, and insoluble in ether, in chloroform, and in dilute mineral acids. It is soluble in DMSO and slightly soluble in ethanol. It has a pK value of 7.9 and 9.2. It has a maximum UV detection at wavelength of λmax 226nm.

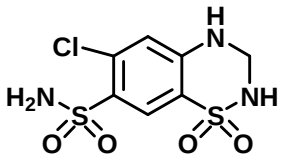

Figure 1 : Structure of hydrochlorothiazide

Folic Acid is a vitamin. It is used to reduce serum homocysteine. Chemically as shown in Figure 2 it's N-[ρ-[[(2-amino-4-hydroxy-6-pteridinyl) methyl]-amino] benzoyl]-L-glutamic acid; and it's molecular weight is 441.40 g/mol. Folic acid occurs as a yellow or yellowish-orange crystalline powder. Folic Acid is very slightly soluble in water (0.0016mg/mL in water) and insoluble in alcohol. Folic acid is readily soluble in dilute solutions of alkali hydroxides and carbonates and solutions of the drug may be prepared with the aid of sodium hydroxide or sodium carbonate, thereby forming the soluble sodium salt of folic acid (sodium folate). Aqueous solutions of folic acid are heat sensitive and rapidly decompose in the presence of light and/or riboflavin; solutions should be stored in a cool place protected from light. It has a pK value of 4.7, 6.8 and 9.0. It has a maximum UV detection at wavelength of λmax 256nm.

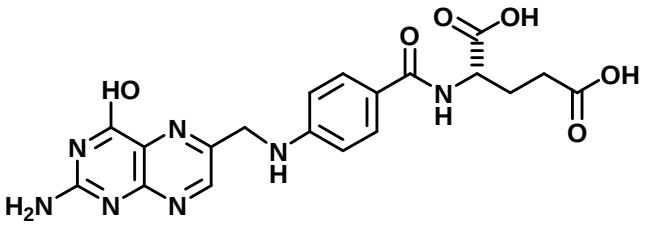

Figure 2 : Structure of folic acid

Atenolol as shown in Figure 3 is a synthetic, beta1-selective (cardioselective) adrenoreceptor blocking agent. Chemically it's 2-[4-[2-hydroxy-3-(1-methylethylamino) propoxy] phenyl] ethanamide; and it's molecular weight is 266.34g/mol. It is white to off-white solid. It is a relatively polar hydrophilic compound with a water solubility of 26.5mg/mL at 37°C and a log partition coefficient (octanol/ water) of 0.23. It is freely soluble in 1N HCl (300mg/mL at 25°C) and less soluble in chloroform (3 mg/mL at 25°C). It has a pK value of 9.5. It has a maximum UV detection at wavelength of λmax 225nm.

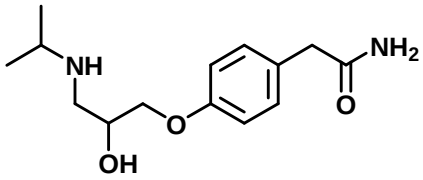

Figure 3 : Structure of atenolol

Aspirin, salicylic acid acetate, is a non-opiate analgesic, anti-inflammatory and antipyretic agent. Aspirin, sometimes known as acetylsalicylic acid or ASA, is the salicylate ester of acetic acid. As shown in Figure 4, it's 2-acetoxybenzoic acid. It's molecular weight is 180.16 g/mol. The compound occurs as a white, crystalline powder or tabular or needle-like crystals. Aspirin is slightly soluble in water and is freely soluble in alcohol. It is a weak acid with a pKa of 3.5.It has a maximum UV detection at wavelength of λmax 229nm.

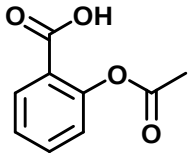

Figure 4 : Structure of aspirin

Enalapril Maleate is indicated for essential or Reno vascular hypertension and treatment of symptomatic congestive heart failure. It is usually administered in association with other drugs, particularly thiazide diuretics.

As shown in Figure 5, it's 1-[2-(1-ethoxycarbonyl-3-phenyl-propyl) aminopropanoyl] pyrrolidine-2-carboxylic acid and it's Molecular weight is 492.53 g/mol. It's white to off-white solid crystalline powder. It is sparingly soluble in water (pH 3.4), soluble in ethanol, and freely soluble in methanol and dimethylformamide. It's pK value of 3.0 and 5.5. It has a maximum UV detection at wavelength of λmax 215nm.

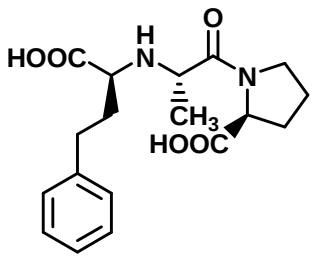

Figure 5 : Structure of enalapril

Atorvastatin calcium is a lipid-lowering agent. Atorvastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis. As shown in Figure 6, Atorvastatin is 1-[2-(1-ethoxycarbonyl-3-phenyl-propyl) aminopropanoyl] pyrrolidine-2-carboxylic acid. It's molecular weight is 1209.42g/mol. Atorvastatin calcium is a white to off-white crystalline powder. Atorvastain is insoluble in aqueous solutions of pH 4 and below. Atorvastatin calcium is very slightly soluble in distilled water, pH 7.4 phosphate buffer, and acetonitrile, slightly soluble in ethanol, and freely soluble in methanol. It has pK value of 4.5. It has a maximum UV detection at wavelength of λmax 245nm.[2-13]

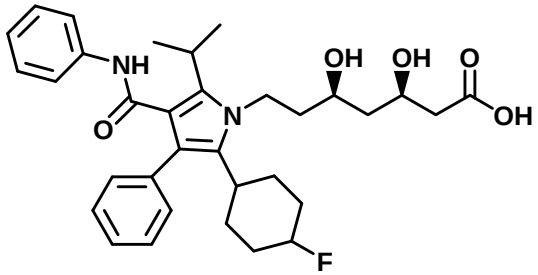

Figure 6 : Structure of atorvastatin

EXPERIMENTAL

Chemicals and materials

Working standards of Hydrochlorothiazide; Folic Acid; Atenolol; Aspirin; Enalapril; Atorvastatin were obtained from Accutest Research Laboratories, Navi-Mumbai, India. Polypill tablets, manufactured by Macleods, Mumbai, India, were obtained. Acetonitrile, methanol, Di-methyl Sulphoxide and orthophosphoric acid were from Qualigens. Sodium Di-hydrogen Ortho-Phosphate (NaH_2PO_4) and Di-Sodium Hydrogen Ortho-Phosphate (Na_2HPO_4) and Heptane Sulphonic acid was from S.D. Fine Chem. Purified water was obtained from Milli Q A10 gradient water purification system (Millipore, Banglore, India) and was used throughout the work. All dilutions were performed in standard volumetric flasks.

High performance liquid chromatographic condition

An HPLC system consisting of a Binary Solvent Manager (pump) and Sample Manager (autosampler) were used for the entire analysis. The chromatographic system consisted of Inertsil C8 (150 x 4.6 mm i.d., 5 μ particle size) analytical column. The flow rate of the mobile phase under binary gradient conditions was kept at 2.0 mL/min. The auto sampler temperature was set at room temperature and the injection volume employed was 30 μL. The mobile phase consisted of buffer (mixture of 0.01M Sodium Di-hydrogen Ortho-Phosphate (NaH_2PO_4); 0.01M of Di-Sodium Hydrogen Ortho-Phosphate (Na_2HPO_4); 1.0 g of Sodium-Heptane Sulphonic acid in 1000mL purified water) pH adjusted to 2.6 with Orthophosphoric acid: methanol with a binary gradient program as shown in TABLE 1. Detection of all analytes was performed on ultraviolet HPLC detector. Class VP software was used to control all parameters of HPLC. Quantitation was performed using appropriate integration parameters. Figure 7 shows the representative chromatogram. The total run time was 20.0 min.

TABLE 1 : Gradient program

Time (min.)	Mobile phase A Buffer	Mobile phase B Methanol
0.01	85	15
3	65	35
8	58	42
9	45	55
15	30	70
16	30	70
18	85	15
20	85	15

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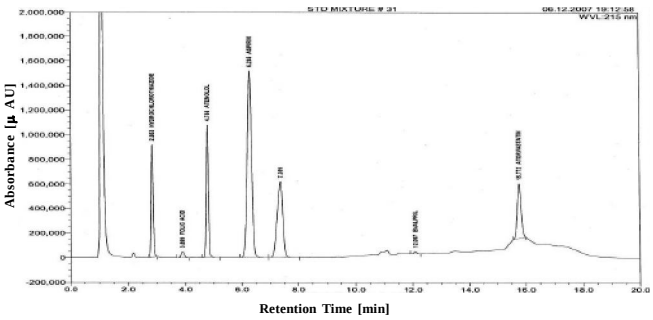

Figure 7 : Representative chromatogram

Analytical data processing

Chromatographic data were collected and integrated using Class VP software. Concentrations of individual analyte in unknown samples were calculated using external standard method. Peak area response of individual analyte in standard and sample chromatogram was compared for calculation. The best-fit equation (y = mx + c), where y is the peak area response was also used to calculate linearity of the individual analyte.

Standard and quality control preparation

Preparation of diluent: Weigh and dissolve about 1.4gms of Di-sodium hydrogen phosphate (Na_2HPO_4) in about 1000mL water.

Preparation of standard: Standard stock solutions were prepared by dissolving 25mg of Enalapril; 10 mg of Folic Acid; 65mg of HCTZ to 50mL volumetric flask in 10mL of Acetonitrile in then diluting it with diluent to give a final concentration of 1000µg/mL. Weigh 10mg of Atorvastatin; 75mg of Aspirin; 25mg of Atenolol in same 100mL volumetric flask. Add 2mL of Dimethyl sulfoxide (DMS); sonicate to dissolve. Add 5mL of each of Enalapril and HCTZ (1000µg mL^{-1}) and 4mL of Folic Acid (1000µg mL^{-1}) and dilute it with diluent.

Sample preparation: Weigh and crush the content of 20 Tablets and calculate the average weight. Weigh powder-containing equivalent to average weight of a tablet in a 100mL volumetric flask. Add 2.0mL of Dimethyl sulfoxide; sonicate for 5 min. Add 10mL of Acetonitrile and dilute up to the mark with diluent. Shake well. Filter the solution through GF/C 70 mm filter paper.

Note: Prepare fresh solution and inject immediately.

Final concentrations of all analytes in standard and sample solution are as follows:

Enalapril	25	µg mL^{-1}
Folic Acid	8	µg mL^{-1}
HCTZ	65	µg mL^{-1}
Atorvastatin	100	µg mL^{-1}
Aspirin	750	µg mL^{-1}
Atenolol	250	µg mL^{-1}

Method validation

A thorough and complete method validation of method for simultaneous determination of Hydrochlorothiazide; Folic Acid; Atenolol; Aspirin; Enalapril Maleate and Atorvastatin Calcium in formulation was done following the Validation of Analytical Procedures: Methodology, ICH Harmonised Tripartite guidelines, Adoption on 6 Nov, 1996[20]. The method was validated for selectivity, linearity, accuracy and precision, recovery, stability.

The selectivity towards endogenous and exogenous sample matrix components was assessed by analyzing diluent and sample solution at working concentration level.

The linearity of the method was determined by analysis of standard solutions having a concentration range of 50% to 150% of the working concentration level. Five linearity levels 50%; 80%, 100%, 120% and 150 % of the working concentration were analyzed. Best-fit calibration curves of peak area response versus concentration were drawn. The simple linear equation for calibration levels with respect to the drug concentration without weighting factor was determined. Correlation Coefficient was greater than 0.999 with equation (y=mx + c). The peak area response values of calibration standards were found to be proportional to the concentration of the drug over the range tested.

Precision was evaluated at working concentration level in six different sample preparations. Mean and standard deviation (SD) were obtained for calculated drug concentration at replicate sample preparation. Accuracy and precision were evaluated in terms of relative error (RE).

Recovery presents the extraction efficiency of a method. It was performed at working concentration level. The absolute recoveries were evaluated by comparing peak area of spiked samples to that of standards.

Stability experiments were performed to evaluate

the analyte stability in stocks solutions at room temperature. Stock solution stability was performed by comparing area response of stability sample of analyte with the area response of sample prepared from fresh stock solutions and the stocks were found to be stable for a minimum of 6Hrs.

RESULTS AND DISCUSSION

Method development[14-19]

For optimum detection at working concentration level of all analytes in sample, it was necessary to adjust not only the chromatographic conditions but also to develop an efficient sample preparation that gives consistent and reproducible recovery of analytes. The formulation under research has six different drugs with varying label claim. After detailed literature and few laboratory tests, it was revealed that all six drugs have different physical and chemical properties. However for simultaneous estimation of drugs it was necessary to prepare a single solution of all drugs without compromising their stability. Due to difference in solubility and stability of these six drugs, sample preparation to have all analytes in a single sample solution was a challenge and for that, different solvents and mixture of solvents were tried to check solubility; stability and compatibility of analytes in resultant solution. Commonly used solvents such as water, methanol, Acetonitrile were tried.

Folic acid was insoluble in water therefore water was not found to be suitable for sample preparation.

In methanol, all drugs except Folic acid were soluble. However, Aspirin was found to be unstable in methanol as it gets hydrolysed to salicylic acid.

Acetonitrile was preferable solvent in HPLC as it has very low UV absorbance. It in turns results in low interference and more sensitivity. In addition, Aspirin has shown some improved stability. However, Atorvastatin was practically insoluble and Folic acid was sparingly soluble. Therefore there was need to select other solvent, buffer preferable with pH or combination of solvents.

Therefore, different solvents were tried to dissolve Folic Acid and Atorvastatin with optimum stability of Aspirin. After literature search and practical trials, Dimethyl Sulphoxide was selected to dissolve Atorvastatin as it is freely soluble in it. Similarly, Folic Acid was found to be freely soluble in 0.01M of Di-Sodium phosphate buffer.

However it was required to prepare standard and sample solution having same concentration of individual drug. While preparing such a solution it was also required to have simple and same treatments such as dilutions or extractions given to standard and sample solution.

Therefore several permutations and combination of solvents were tried. Several trails were performed with different sequence of dilutions and treatments to sample and standards.

Finally following procedure was developed for standard and sample preparation.

Dimethyl Sulphoxide for Atorvastatin, 0.01M of Di-Sodium phosphate buffer for Folic Acid and Acetonitrile for other drugs were selected. 0.01M of Di-Sodium phosphate buffer was used as diluent to prepare different dilutions and volume make up. Standard and sample solutions were prepared as described above.

The column was selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilitates a wide range of columns like octadecylsilane (C18), cyano, propyl, nitro, amino etc. Different columns containing octyl, octadecyl and phenyl stationary phase were tried for the separation and resolution. It was found that octyl silane C8 column offered more advantages over the other columns tried. Most of drugs in this formulation under research were found to be polar. As C8 column packings are more polar compared to C18 columns due to availability of short carbon chain on silanols in C8. Therefore, C8 column was selected for method. In addition C8 column was chosen for the study since it is the most retentive, rugged and widely available.

Generally, a longer column provides a better separation due to higher theoretical plate numbers, but it is reported that only a few centimeters of the columns exerts the dominant effect of separation and hence short columns can provide separation that are achieved on longer columns, if the mobile phase composition is suitably adjusted. Shorter columns packed with the smaller particles are often used for shorter analysis times. Therefore, a 15 cm column was employed for the analysis. For analytical purposes a 0.4–0.6 cm internal diameter column having a particle size between 3 – 10 μm are

used. As a particle size decreases the surface area available for coating increases. Columns with 5µm particle size give the best compromise of efficiency, reproducibility and reliability. In this case, the column selected had a particle size of 5µm and an internal diameter of 4.6mm.

Finally based on results obtained for peak symmetry, tailing factor and resolution of individual analyte, Inertsil C8, (4.6 mm x 150 mm, 5µ), column was employed as the stationary phase.

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute – stationary phase, the solute – mobile phase and the mobile phase - stationary phase. For a given stationary phase, the relation of the given solute depends directly upon the mobile phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. Solvent polarity is the key word in chromatographic separations since the polar mobile phase, which give rise to low solute retention in normal phase and high solute retention in reversed phase liquid chromatography.

The choice of mobile phase for a given separation constitutes a very important stage in producing a good separation in HPLC. Acetonitrile and Methanol are the most popularly used organic solvents in HPLC. Acetonitrile is the best initial choice of organic solvent for the mobile phase. Acetonitrile – Water mixtures are used to initiate the development because it has lower UV cut-off (185 to 210 nm) and low viscosity leading to higher plate numbers and lower column backpressures. However methanol which has a relatively lower UV cut-off (205nm) is also a reasonable alternative. In addition, during gradient analysis, ghost peaks are larger than when Acetonitrile is used. Elution strength differs with Acetonitrile and methanol and, if mixed with water-based solvents, Acetonitrile exhibits the greater elution strength. When conditions using Acetonitrile are substituted for use with methanol, increased ratio of organic solvent is suggested to use. On the other hand, when the ratio of organic solvent is 100% or very close to this, the nature (polarity) of the solvent itself comes to the fore and the elution strength of methanol is greater. Separation selectivity differs with Acetonitrile and methanol, and the degree of separation and elution order sometimes changes. A cause of this is considered to lie in the difference of the chemical nature (methanol: protic, Acetonitrile: aprotic) of organic solvent molecules. However, in some cases, components that cannot be separated by water/Acetonitrile solvents can be separated by water/methanol solvents.

Because the viscosity of methanol is higher than that of Acetonitrile, the pressure applied on the column by water/methanol solvents is higher than that of water/Acetonitrile solvents. However despite this entire methanol was used as organic solvent in mobile phase for better and faster elution of drugs such as Atorvastatin.

Whenever acidic or basic samples are to be separated it is advisable to control mobile phase pH by adding a buffer solution. For reverse phase chromatography, a buffer concentration of 10-50 mM is usually adequate. Reversed phase HPLC separations are generally carried out with pH ranges 2.0 and 8.0. Thus buffers able to control pH between 2.0 to 8.0 are desirable. In that respect for simultaneous elution of six drugs different phosphate buffers at concentration ranging from 10mM to 30mM were tried with varying mobile phase pH ranging from 2.5 to 6.0. Finally simultaneous elution of drugs except Atorvastatin, in combination of 0.01M Sodium Di-hydrogen Ortho-Phosphate (NaH_2PO_4) and 0.01M of Di-Sodium Hydrogen Ortho-Phosphate (Na_2HPO_4) with pH 2.6 adjusted with Ortho-phosphoric acid and methanol in the ratio of 60:40v/v was found to be satisfactory. However the resolution and peak symmetry was not satisfactory. In addition the sixth drug Atorvastatin was not eluted. Therefore 1.0 g of Sodium-Heptane Sulphonic acid was used in addition to other buffers to get better resolution among hydrochlorothiazide, Atenolol, Enalapril and folic acid. However still Atorvastatin was retained on column. Therefore finally gradient program was employed to mobile phase composition with buffer 0.01M Sodium Di-hydrogen Ortho-Phosphate (NaH2PO4) and 0.01M of Di-Sodium Hydrogen Ortho-Phosphate (Na2HPO4) and 1.0 g of Sodium-Heptane Sulphonic acid in 1000mL purified water pH adjusted to 2.6 with Ortho-phosphoric acid and methanol as organic solvent. It gave better resolution among drugs especially between Aspirin and Salicylic Acid. The simultaneous determination of drug in short analysis time was achieved using following mobile phase and gradient program.

While selecting the wavelength, the interest of minor component in the formulation or component with low extraction co-efficient needs special consideration. Variable wavelength detector or a photo diode array detector is used.

Wavelengths of maximum absorbance(s) of six drugs are as follows:

Hydrochlorothiazide : 226 nm
Folic Acid : 256 nm
Atenolol : 225 nm
Aspirin : 229 nm
Enalapril : 225 nm
Atorvastatin : 245 nm

In this particular method, 215nm wavelength was selected as a detection wavelength because Enalapril among six drugs have low absorbance at low UV range and other five drugs exhibited good absorbance at this wavelength.

Method validation

A thorough and complete method validation of method for simultaneous determination of Hydrochlorothiazide, Atenolol, Folic Acid, Aspirin, Enalapril and Atorvastain from polypill tablet using HPLC was done following the ICH guidelines. The method was validated for specificity, linearity, accuracy and precision, recovery, solution stability.

System suitability

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. It should be performed prior to initiation of analysis.

Standard solution having assay concentration of individual analyte was prepared as described in method details and injected to HPLC. System suitability parameters (e.g., Tailing factor, Capacity Factor, theoretical plates) were established to ensure the validity of the analytical procedure even in controlled or non-controlled variations in the method parameters. Please refer TABLE 2.

TABLE 2 : System suitability parameters

Name	Capacity Factor	Tailing Factor	Theoretical Plates
Acceptance criteria	NLT 1.5	NMT 2.0	NLT 5000
Hydrochlorothiazide	1.66	1.04	9581
Folic Acid	2.63	1.08	9625
Atenolol	3.84	0.86	21823
Aspirin	5.71	1.00	11808
Enalapril	10.68	1.02	71064
Atorvastatin	14.25	0.96	77196

TABLE 3 : Results of the linearity

Drug Name	Slope	Intercept	Correlation coefficient (r)
Hydrochlorothiazide	65726	955340	0.9992
Folic Acid	48933	803	0.9997
Atenolol	22543	17002	0.9998
Aspirin	19689	-1335070	0.9998
Enalapril	5404	-7161	0.9991
Atorvastatin	36150	-265694	0.9997

Specificity

Specificity is a measure of the degree of interference (or absence thereof) in the analysis of complex sample mixtures such as an analyte present in matrix containing endogenous substances, and related chemical compounds, etc.

To validate specificity of method following samples were prepared as described above in method details:
Blank solution i.e. diluent
Blank + individual drug having assay concentration of individual drug
Blank + all drugs having assay concentration of individual drug
Sample solution having assay concentration of individual drug
To demonstrate the ability of the method to specifically determine individual drugs from other sample components above samples were individually injected to HPLC and chromatograms were recorded. There were no interferences at the retention time of individual dugs due to other components of sample solution and also from gradient changes. Resolution between subsequently eluting analytes was determined.

Hydrochlorothiazide - Folic Acid : 7.56
Folic Acid- Atenolol : 8.60

Atenolol-Aspirin : 9.92
Aspirin – Enalapril : 23.53
Enalapril – Atorvastatin : 18.09

Specificity was also examined during solution stability at 12 hrs. and found that there was no interference of degraded products of any of drugs. Salicylic acid degraded from Aspirin was well resolved with a resolution between aspirin and salicylic acid was 4.70. Based on results it was concluded that the method is specific.

Linearity, accuracy and precision, recovery

The peak area ratios of calibration standards were proportional to the concentration of analyte in each assay over the nominal concentration range of 33.043µg mL-1 to 99.129µg mL-1 for Hydrochlorothiazide, 3.659µg mL-1 to 10.977µg mL-1 for folic acid, and 126.000µg mL-1 to 378.000µg mL-1 for Atenolol, 374.000µg mL-1 to 1122.000µg mL-1 for Aspirin, 10.000µg mL-1 to 40.000µg mL-1 for Enalapril, and 49.811µg mL-1 to 149.433µg mL-1 for Atorvastatin. The calibration curves appeared linear and were well described by lines with correlation coefficient ≥ 0.99 for individual analyte. The results obtained were as shown in TABLE 4.

The precision and accuracy of the developed method was determined by analysis of six samples in each of method precision and intermediate precision. As a part of Method Validation, Intermediate precision was also examined by carrying out the same assay procedure on a different instrument on a different day. The experimental conditions were kept the same but the HPLC system was changed. In this exercise, six samples were prepared separately just as in Method Precision and analysed. The values thus obtained were compared with those obtained from Method Precision. The results obtained were as shown in TABLE 9. The percent relative standard deviation of individual drugs on comparing both Method Precision and Intermediate Precision are less than 2.0 hence it can be concluded that the method is precise.

Similarly during the above exercise comparison of method precision and Intermediate Precision shows mean percent assay were 100.21 for hydrochlorothiazide, 99.75 for Folic Acid, 99.69 for Atenolol, 99.51for Aspirin, 100.04 for Enalapril and 99.68 for Atorvastatin. The percentage value for the assay lies close to the theoretical value (100 %), which indicates that the method is accurate. The results obtained were as shown in TABLE 4.

TABLE 4 : Result of comparison between method precision and intermediate precision analysis

	% ASSAY					
	HCTZ	Folic Acid	Atenolol	Aspirin	Enalapril	Atorvastatin
M.P 1	98.89	100.23	99.11	98.25	98.82	100.64
M.P 2	100.73	99.55	99.63	100.54	100.40	100.21
M.P 3	100.16	100.15	99.04	100.26	99.27	98.55
M.P 4	98.50	99.48	99.67	99.13	98.82	98.12
M.P 5	100.68	99.03	100.77	98.21	100.57	100.77
M.P 6	99.45	98.49	100.41	100.13	100.18	100.36
I.P 1	100.07	100.43	99.85	99.85	100.71	99.54
I.P 2	100.72	100.19	100.96	100.96	101.03	99.61
I.P 3	100.65	101.17	100.29	100.29	100.26	99.64
I.P 4	100.71	99.70	99.30	99.30	99.10	99.72
I.P 5	100.88	98.24	98.52	98.52	100.92	100.48
I.P 6	101.09	100.32	98.67	98.67	100.44	98.48
Min.	98.50	98.24	98.52	98.21	98.82	98.12
Max.	101.09	101.17	100.96	100.96	101.03	100.77
MEAN	100.21	99.75	99.69	99.51	100.04	99.68
S.D	0.84	0.85	0.80	0.95	0.81	0.89
R.S.D.	0.83	0.85	0.80	0.96	0.81	0.89

Sample powder equivalent to one tablet weight was weighed and transferred to 100mL standard volumetric flask. To this flask, 1mL, 2.5mL and 5mL of standard solution i.e. mixture of drugs having assay concentration of individual drugs was added to achieve different levels namely 10%, 25% and 50% of the estimated amount of the drug. Solutions were further prepared as described in the method details. Three replicates of each level were prepared.

From each of the three samples prepared at each level, two replicate injections were made and the mean of the two values was considered for statistical analysis. The % recovery was calculated using the formula,

$$\text{Percentage Recovery} = \frac{\text{Amount of drug found}}{\text{(Label Claim + Amount of drug added)}} \times 100$$

The results obtained were as shown in TABLE 5.

TABLE 5 : Result of percent recovery

Name	Level-1 (10%)	Level-2 (25%)	Level-3 (50%)
Hydrochlorothiazide (6.25mg)	100.08	99.45	99.95
Folic Acid (0.8mg)	100.05	100.46	101.02
Atenolol (25mg)	101.61	100.47	99.70
Aspirin (75 mg)	101.44	100.85	99.37
Enalapril (2.5mg)	100.03	100.00	100.56
Atorvastatin(10.0mg)	99.65	100.04	100.35

Stability

For checking the solution stability, standard and sample solutions having assay concentration of individual analyte was prepared as per the method given above. Injections from these solutions were analysed initially and at different time intervals of 6 hrs and 12 hrs at laboratory temperature.

The areas of individual drugs in sample solution after each time interval were compared against the areas obtained at the initial time point. The results obtained for the sample solution were well within the acceptance criteria indicating the high stability of drugs until about 6 hrs. The results obtained were as listed in the TABLE 11 and 12 for 6hrs and 12 hrs respectively. Percent assay of individual analyte was also consistent until about 6 hrs. The results obtained were as shown in TABLE 6 and 7 for 6hrs and 12 hrs. respectively.

TABLE 6 : Result of solution stability experiment 6Hrs.

Drug		HCTZ	Folic Acid	Atenolol	Aspirin	Enalapril	Atorvastatin
Area	Initial	4489206	358266	5667028	14534451	134512	3382375
	6 hrs	4464259	358753	5674738	14251904	131997	3346543
Mean		4476733	358510	5670883	14393178	133255	3364459
S.D.		17640	344	5452	199791	1778	25337
R.S.D.		0.39	0.10	0.10	1.39	1.33	0.75
%Diff.		-0.56	0.14	0.14	-1.94	-1.87	-1.06
% Assay		98.13	98.82	98.2	98.41	99.38	99.14

TABLE 7 : Result of solution stability experiment 12Hrs.

Drug		HCTZ	Folic Acid	Atenolol	Aspirin	Enalapril	Atorvastatin
Area	Initial	4489206	358266	5667028	14534451	134512	3382375
	12 hrs	4435950	357451	5668706	13340030	131984	3450433
Mean		4462578	357859	5667867	13937241	133248	3416404
S.D.		37658	576	1187	844583	1788	48124
R.S.D.		0.84	0.16	0.02	6.06	1.34	1.41
%Diff.		-1.19	-0.23	0.03	-8.22	-1.88	2.01
% Assay		98.06	98.32	97.85	97.04	99.09	99.13

Application

The validated method has been successfully used to quantitate Hydrochlorothiazide; Folic Acid; Atenolol; Aspirin; Enalapril Maleate; Atorvastatin Calcium in their combined dosage forms. In addition to this it can be applied for as estimation tool for the dissolution of polypill tablet.

CONCLUSION

The developed simultaneous HPLC assay for Hydrochlorothiazide; Folic Acid; Atenolol; Aspirin; Enalapril Maleate; Atorvastatin Calcium is selective, rugged and suitable for routine analysis of polypill formulations. This method has significant advantages in terms of single and reproducible sample preparation and a single chromatographic run time of 20 min. The sample preparation gave consistent and reproducible recoveries for analytes. The gradient chromatography leads to the better resolution among peaks of all analytes in addition to the impurities and other co-eluting peaks. This method can be extended for analysis of the related substances of formulations of combination of these six drugs.

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REFERENCES

[1] N.J.Wald, M.R.Law; BMJ, bmj.com, **326**, 1419, 28 June **(2003)**.

[2] 'The United State Pharmacopoeia XXIII', United states pharmacopoeial convention, Inc., USA, **(1995)**.

[3] 'British pharmacopoeia **I** and **II'**, Her majesty's stationary office, London, **(1993)**.

[4] 'The Merck Index', 14th Ed., Merck and Co., Inc., USA, **(2006)**.

[5] V.Das Gupta, Arvind B.Dhruv; 'Quantitation of Hydrochlorothiazide in Combination with Methyldopa and Propranolol Hydrochloride by High-Performance Liquid Chromatography', Invamed, Inc., Fairfield, NJ, 07006.

[6] Center for drug evaluation and research application number: 020884 printed labeling for aspirin.

[7] PRODUCT MONOGRAPH VASOTEC. enalapril maleate tablets 2.5, 5, 10 and 20 mg Merck Frosst Std.

[8] Atenolol Qualification in human plasma by HPLC→ Applical; to be AAPs pharm Sci., **5(2)**, article 21 **(2003)**.

[9] S.A.Shah, I.S.Rathod, C.J.Shishoo, S.S.Savale, M.C.Saba, K.M.Bhat; Indian Journal of Pharmaceutical Sciences, **62(3)**, **(2000)**.

[10] Indian journal of pharmaceutical Sciences, **68(2)**, 275-277 for determination of Atorvastatin & Amlodipine Simultaneous **(2006)**.

Full Paper

[11] D.A.Shah, K.K.Bhatt, M.B.Shankar, R.S.Mehta, T.R.Gandhi, S.L.Baldania; Indian Journal of Pharmaceutical Sciences, **68(6)**, 796-799 **(2006)**.

[12] S.S.Yadav, D.V.Mhaske, A.B.Kakad, B.D.Patil, S.S.Kadam, S.R.Dhaneshwar; Indian Journal of Pharmaceutical Sciences, **67(2)**, **(2005)**.

[13] A stability-indicating high performance liquid chromatographic (HPLC) assay for the simultaneous determination of atorvastatin and amlodipine in commercial tablets Technologies in the Biomedical and Life Sciences, **846(1/2)**, 215-221, ISSN 1570-0232.

[14] B.A.Bidlingmeyer, Ed.; 'Preparative Liquid Chromatography', **38**, (Elsevier Scientific, New York), **(1987)**.

[15] L.R.Snyder, J.J.Kirkland, J.L.Glajch; 'Practical HPLC Method Development', 2nd ed. (Wiley & Sons, New York), pp. 50-56 **(1997)**.

[16] W.Long, R.Majors; 'Preparative HPLC Scale-Up of Antibiotics,' Application Note, Agilent Technologies, Inc., Wilmington, Delaware, February **(2004)**.

[17] B.K.Sharma; 'Instrumental methods of Chemical Analysis', Goel publishing House, Meerat, **(1998)**.

[18] Kevin Robards, Paul R.Haddad, Peter E.Jackson; 'Principles and Practice of Modern Chromatographic Methods', Academic Press Limited London, 203 **(1994)**.

[19] J.Sherma, B.Fried; 'Handbook of Thin Layer Chromatography', CRC Press Inc. USA, 2nd Edition, 71, **(1996)**.

[20] Validation of Analytical Procedures: Methodology, ICH Harmonised Tripartite guidelines, Adoption on 6 Nov, **(1996)**.