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Simultaneous estimation of amlodipine, atenolol and hydrochlorothiazide in bulk and tablet dosage form by RP-HPLC Method

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ABSTRACT

A simple, accurate, rapid and precise isocratic reversed-phase high-performance liquid chromatographic method has been developed and validated for simultaneous determination of Amlodipine, Atenolol and Hydrochlorothiazide in capsules. The chromatographic separation was carried out on an cosmosil packed column 5C18-MS-I I analytical column (250×4.6 mm; 5 µm) with a mixture of Phosphate buffer:Acetonitrile:Methanol pH 6 adjusted with Ortho phosphoric acid (30:20:50, v/v) as mobile phase; at a flow rate of 1 ml/min. UV detection was performed at 240nm. The retention times were 2.637, 3.148 and 8.492min. for Hydrochlorothiazide, Atenolol and Amlodipine respectively. Calibration plots e linear (r^2>0.998) over the concentration range 2-12µg/ml for Amlodipine , 10-60µg/ml Atenolol and 2-12µg/ml for Hydrochlorothiazide. The method was validated for accuracy, precision, specificity, linearity, and sensitivity. The proposed method was successfully used for quantitative analysis of capsules. No interference from any component of pharmaceutical dosage form was observed. Validation studies revealed that method is specific, rapid, reliable, and reproducible. The high recovery and low relative standard deviation confirm the suitability of the method for routine determination of Hydrochlorothiazide, Amlodipine and Atenolol in bulk drug and capsule dosage form.

Introduction

Amlodipine Besylate[1], chemically is 3-ethyl 5-methyl (4*RS*)-2-[(2-aminoethoxy) methyl]-4-(2chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzene sulphonate, belongs to the class of Calcium channel blocker, used as anti-anginal.

Molecular Formula – $C_{26}H_{31}ClN_2O_8S$, Molecular Weight – 567.1 Solubility - Slightly soluble in water and in isopropyl alcohol, sparingly soluble in dehydrated alcohol, freely soluble in methanol.

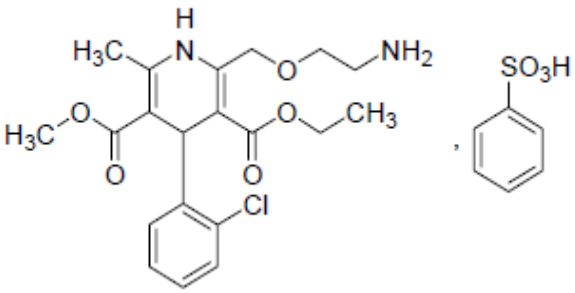

Fig 01. Chemical structure of Amlodipine Besylate

Atenolol: chemical name: (*RS*)-4-(2-hydroxy-3-Isopropylaminopropoxy) phenylacetamide, belongs to the class of β-adrenergic blocker, used as an antihypertensive drug.

Molecular Formula – $C_{14}H_{22}N_2O_3$

Molecular Weight – 266.34

Solubility – Soluble in ethanol, sparingly soluble in water**.**

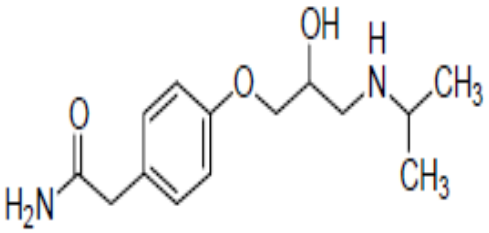

Fig 02.Chemical structure of Atenolol

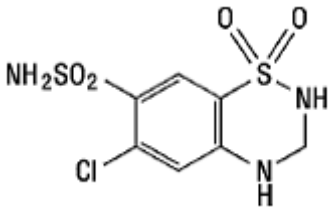

Fig.No.03.Chemical structure of Hydrochlorothiazide:

Chemical name 6-chloro-3,4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulfonamide 1,1-dioxide **Molecular formula:** $C_7H_8ClN_3O_4S_2$ **Solubile in** water, methanol, ethanol, acetone **,**Dimethyl formamide.

From the literature survey it was found that many methods are available for determination of Amlodipine Besylate, Hydrochlorothiazide and Atenolol individually and few methods in combination with other drugs. However, no stability indicating HPLC has been reported for simultaneous determination of Amlodipine Besylate, Hydrochlorothiazide and Atenolol in combination.

In the proposed study an attempt will be made to develop a stability indicating HPLC method for simultaneous estimation of

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Amlodipine Besylate, Hydrochlorothiazde and Atenolol in pharmaceutical formulation (tablets).

Pharmaceutical grade of Amlodipine, Hydrochlorothiazide and Atenolol were kindly supplied as gift samples by Morpen laborastories, New Delhi, India, certified to contain > 99% (w/w) on dried basis. Commercially available B-Amlol (Themis Formulation Pvt. Ltd., India) tablets claimed to contain 25mg hydrochlorothiazide; 5 mg Amlodipine and 50 mg Atenolol have been utilized in the present work. All chemicals and reagents used were of HPLC grade and were purchased from Agenta Chemicals, Hyderabad, India.

Chromatographic system and conditions:

The HPLC system (Analytical Technologies Gujarat, India) consisted of pump. The Analytical column a cosmosil packed column 5C18-MS- I I(250mm x 4.6mmi.d., 5µ particle size)was operated at ambient temperature (20 $\pm$ 1^{o}c) . Isocratic elution with Phospate buffer: Acetonitrile: Methanol (30:20:50 v/v pH 6)was used at flow rate at 1ml/min . column (150×4.6 mm; 5 µm). The mobile phase. Before analysis the mobile phase was filtered through a 0.2 µm membrane and degassed by ultrasonification. Detection was monitored at 240nm and injection volume was 20µl. All the experiments were performed at ambient temperature.

Pharmaceutical grade of Hydrochlorothiazide, Amlodipine and Atenolol were kindly supplied as gift samples by morpen Pharmaceuticals, new Delhi , India, certified to contain > 99% (w/w) on dried basis. Commercially available B-Amlol(Themis Formulation Pvt. Ltd., India),tablets claimed to contain 25mg Hydrochlorothiazide; 5 mg Amlodipine and 50 mg Atenolol have been utilized in the present work. All chemicals and reagents used were of HPLC grade and were purchased from Agenta Chemicals, India.

Standard solutions and calibration graphs for chromatographic measurement:

Stock standard solutions were prepared by dissolving separately 5mg Hydrochlorothiazide,5mg Atenolol and 5mg Amlodipine in 50 ml acetonitrile (1000 µg/ml). The standard calibration solutions were prepared by appropriate dilution of the stock solution with acetonitrile to reach a concentration range of 2-12 µg/ml for Hydrochlorothiazide, 2-12µg/ml for Amlodipine and 10-60 µg/ml for Atenolol. Triplicate 20 µl injections were made for each concentration and chromatographed under the optimized conditions described above. The peak area were plotted against the corresponding concentrations to obtain the calibration graphs.

Sample preparation:

Twenty capsule contents were accurately weighed, their mean weight was determined and they were mixed and finely powdered. A portion equivalent to about one capsule was accurately weighed and transferred into a 100 ml volumetric flask containing 50 ml acetonitrile, sonicated for 15 min and diluted to 100 ml with acetonitrilel. The resulting solution was centrifuged at 100 rpm for 15 min. Supernatant was taken and after suitable dilution the sample solution was then filtered using 0.45 µ filter (Millipore, Milford, MA). The original stock solution was further diluted to get sample solution of drug concentration of 25 µg/ml Hydrochlorothiazide, 50µg/ml Atenolol and 5 µg/ml Amlodipine. A 20 µl volume of sample solution was injected into HPLC, six times. The peak areas for the drugs were measured at 233 nm and amounts of Hydrochlorothiazide, Atenolol and Amlodipine were determined using the related linear regression equations.

Method validation:

The developed method was validated according to the ICH guidelines [24]. The system suitability was evaluated by six replicate analyses of Hydrochlorothiazide, Atenolol and Amlodipine mixture at a concentration of 25 µg/ml Hydrochlorothiazide, 50 µg/ml Atenolol and 5 µg/ml Amlodipine. The acceptance criteria were a R.S.D. of peak areas and retention times less than 2%, Theoretical plate numbers (N) at least 2500 for each peak and tailing factors (T) less than 1% for Hydrochlorothiazide, Atenolol and Amlodipine.

Standard calibration curves were prepared in the mobile phase with six concentrations ranging from 2-12 µg/ml for Hydrochlorothiazide and 2-12µg/ml for Amlodipine and 10-60 µg/ml for Atenolol into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs. To study the reliability and suitability of the developed method, recovery experiments were carried out at three levels 50, 100 and 150%. Known concentrations of commercial capsules were spiked with known amounts of Hydrochlorothiaide, Atenolol and Amlodipine. At each level of the amount six determinations were performed and the results obtained were compared with expected results. Recovery for pharmaceutical formulations should be within the range 100±5%. The percent R.S.D. of individual measurements was also determined. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) for 2 consecutive days. Three different concentrations of Hydrochlorothiazide, Atenolol and Amlodipine were analyzed in six independent series in the same day (intra-day precision) and 3 consecutive days (inter-day precision).. The repeatability of sample application and measurement of peak area for active compounds were expressed in terms of percent RSD.

All chromatgrams were examined to determine if compounds of interest co-eluted with each other or with any additional excipients peaks. Marketed formulations were analyzed to determine the specificity of the optimized method in the presence of common capsule excipients. Limit of detection (LOD) and limit of quantitation (LOQ) were estimated from the signal-to-noise ratio. LOD and LOQ were calculated using 3.3σ/s and 10σ/s formulae, respectively, where, σ is the standard deviation of the peak areas and *s* is the slope of the corresponding calibration curve. To evaluate robustness of HPLC method a few parameters were deliberately varied. The parameters included variation of flow rate, percentage of buffer in the mobile phase, and pH of mobile phase.

Results And Discussion

During the optimization of HPLC method, columns C18 5µm; cosmosil packed column 5C18-MS- I I(250mm × 4.6 mm), Three organic solvents (phosphate buffer,acetonitrile, and methanol), buffer (phosphate) at two different pH values (3 and 4) were tested. Initially phosphate buffer: acetonitrile: methanol, acetonitrile: water, acetonitrile: phosphate buffer, methanol: phosphate buffer were tried in different ratios at pH 3 and 6. Amlodipine , Atenolol and Hydrochlorothiazide eluted with the tried mobile phases.. Then, with acetonitrile: phosphate buffer only the two drugs eluted. The mobile phase conditions were optimized so the peak from the first-eluting compound did not interfere with those from the solvent, excipients. Other criteria, *viz*. time required for analysis, appropriate k range (1<k<10) for eluted peaks, assay sensitivity, solvent noise were also considered. Finally a mobile phase consisting of a mixture of Phosphate buffer: Acetonitrile: Methanol pH 6 adjusted with 30% orthophosphoric acid in ratio 30:20:50 (v/v), was selected

as mobile phase to achieve maximum separation and sensitivity. Flow rates between 0.5 to 1.2 ml/min were studied. A flow rate of 1 ml/min gave an optimal signal to noise ratio with a reasonable separation time. Using a reversed phase C18 column, the retention times for Hydrochlorothiazide, Amlodipine and Atenolol were observed to be 2.637, 8.492 and 3.148 min. min, respectively. Total time of analysis was less than 10 min. The chromatogram at 240 nm showed a complete resolution of all peaks (fig. 2)

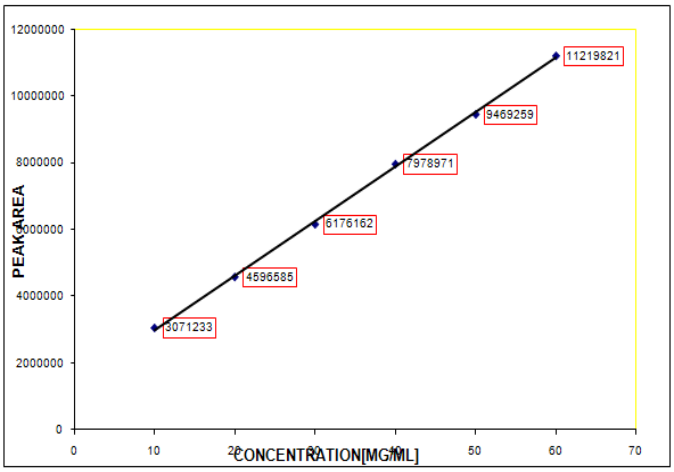

Fig 04.Calibration Curve of Amlodipine

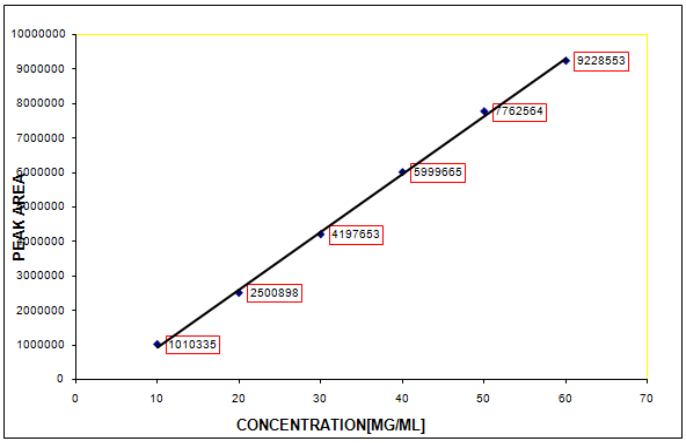

Fig 5. Calibration Curve of Atenolol

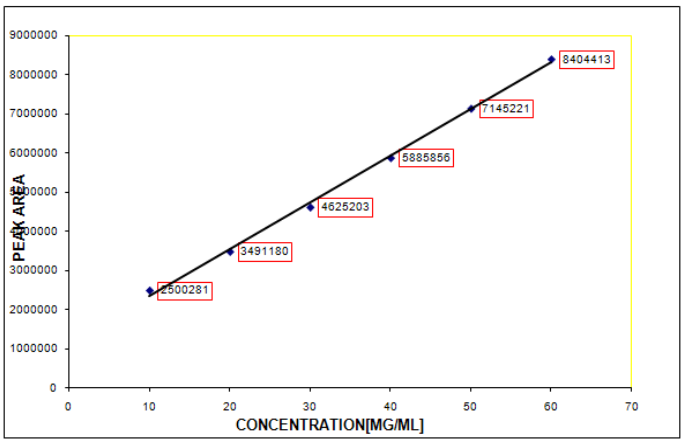

Fig 6.Calibration Curve of Hydrochlorothiazide

Representative chromatograms of standard solutions (a) Standard solution of Hydrochlorothiazide (25 µg/ml); (b) standard solution of Atenolol (50 µg/ml); (c) standard solution of Amlodipine (5 µg/ml) and (d) a standard solution containing 25 µg/ml Hydrochlorothiazide, 50 µg/ml Atenolol, 5 µg/ml Amlodipine.

Validity of the analytical procedure as well as the resolution between different peaks of interest is ensured by the system suitability test. All critical parameters tested met the acceptance criteria on all days. As shown in the chromatogram, all three analytes are eluted by forming symmetrical single peaks well separated from the solvent front

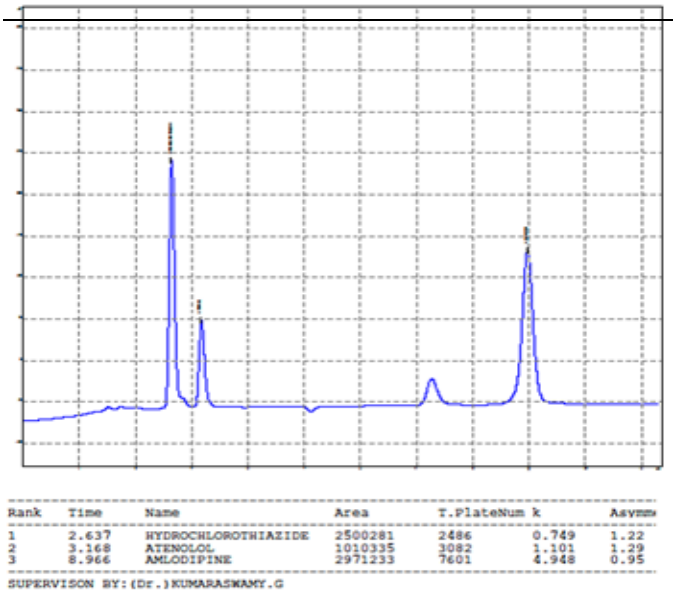

Fig 7. Typical Chromatogram for Mixture of AMDP,ATN and HCT

Excellent linearity was obtained for all the three drugs in the range of 2-12 µg/ml for Hydrochlorothiazide, 10-60 Atenolol and 2-12 µg/ml Amlodipine. The correlation coefficients (r^2) were found to be greater than 0.999 (n=6) in all instances. The results of calibration studies are summarized in Table 1. The proposed method afforded high recoveries for Hydrochlorothiazide, Atenolol and Amlodipine tablets. Results obtained from recovery studies presented in Table 2, indicate that this assay procedure can be used for routine quality control analysis of this ternary mixture in capsules. Precision of the analytical method was found to be reliable based on % RSD (< 2%) corresponding to the peak areas and retention times. The % RSD values were less than 2, for intra-day and inter-day precision. Hence, the method was found to be precise for all the three drugs.

The chromatograms were checked for the appearance of any extra peaks. It was observed that single peak for Hydrochlorothiazide (R_t±SD, 4.415±0.01), Atenolol (R_t±SD, 3.001±0.01) and Amlodipine (R_t±SD, 3.688±0.01) were obtained under optimized conditions, showing no interference from common capsule excipients and impurities. Also the peak areas were compared with the standard and % purity calculated was found to be within the limits. These results demonstrate the specificity of the method.

LOD and LOQ were found to be 0.0478µg/ml and 0.0145 µg/ml for hydrochlorothiazide, 0.1379 µg/ml and 0.4180 µg/ml for Atenolol and 0.0677 µg/ml and 0.2051 µg/ml for Amlodipine. In all deliberately varied conditions, the SD of retention times of Hydrochlorothiazide, Atenolol and Amlodipine were found to be well within the acceptable limit. The tailing factor for all the three peaks was found to be < 1.5 (Table 3). The validated method was used in the analysis of marketed conventional capsules B-AMLOL with a label claim: 25mg Hydrochlorothiazide, 50 mg Atenolol and 5 mg Amlodipine per capsule. Representative chromatogram is shown in (fig. 4). The results for the drugs assay show a good agreement with the label claims.

Table 1. Linearity parameters for the simultaneous estimation of hydrochlorothiazide, atenolol and amlodipine (*N*=6)

PARAMETERS	ATENOLOL	AMLODIPINE	HYDRO CHLOROTHIAZIDE
λ_{max} (nm)	240	240	240
Beers law limit (µg/ml)	2.5-15	1 - 6	5-30
Correlation coefficient (r)	0.999032	0.999072	0.999618
Regression equation (y=mx+c)	y= 0.772151x + 2424786	y=0.326938 x + 542963.5	y= 0.488661x + 465108.4
Slope (m)	0.772151	0.326938	0.488661
Intercept (c)	2424786	542963.5	465108.4
LOD (µg/ml)	0.1379	0.0677	0.0478
LOQ (µg/ml)	0.4180	0.2051	0.0145
Standard Error	0.001295	85052.77	0.002046

Table 2. Recovery analysis of formulation (B-AMLOL) by RP – HPLC

NAME OF DRUGS	PERCENTAGE	%RECOVERY	S.D	%RSD	S.E
HYDROCHLOROTHIAZIDE	50% 100% 150%	97.94 98.71 99.22	0.6444	0.6580	0.3720
AMLODIPINE	50% 100% 150**%**	100.10 99.75 99.16	0.4751	0.4746	0.2743
ATENOLOL	50% 100% 150%	100.66 99.44 101.06	1.1069	1.0996	0.6391

Table 3. System suitability parameters for the optimized chromatogram by RP - HPLC

PARAMETERS	ATENOLOL	AMLODIPINE	HYDROCHLOROTHIAZIDE
Tailing factor	1.21	1.22	1.16
Asymmetrical factor	1.29	0.95	1.22
Theoretical plates	3082	7601	2486
Capacity factor	1.101	4.948	0.749
Theoretical plate per unit length	202.19	232.05	314.70
Resolution	Between ATEN and AMLO 2.04	Between AMLO and HCTZ 1.82	

The developed HPLC method is simple, specific, accurate and precise for the simultaneous determination of Hydrchlorothiazide, Atenolol and Amlodipine from capsules. The developed method provides good resolution between Hydrochlorothiazide, Atenolol and Amlodipine. It was successfully validated in terms of system suitability, linearity, range, precision, accuracy, specificity, LOD, LOQ and robustness in accordance with ICH guidelines. Thus, the described method is suitable for routine analysis and quality control of pharmaceutical preparations containing these drugs either as such or in combination.

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