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RP-HPLC method for simultaneous determination of atenolol and indapamide in pharmaceutical dosage forms, human blood and milk

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ABSTRACT

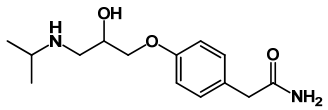
A simple, sensitive and precise high performance liquid chromatographic method for the analysis of atenolol and indapamide with UV detection at 231 nm, has been developed, validated and used for the determination of these compounds in pharmaceutical dosage forms, in human blood and in human milk. The compounds were well separated on a Hypersil BDS C_{18} reversed-phase column with mobile phase consisting of, pH = 3.5, 0.01 M potassium dihydrogen orthophosphate buffer-acetonitrile (60:40; v:v) at a flow rate of 1.0 mL/min. The method showed good linearity in the range of 5-30 µg/mL for atenolol and 0.25-1.50 µg/mL for indapamide. Both the drugs were eluted within 5 minutes and give sharp peak with high theoretical plate count and low tailing factor. The reaction time for atenolol and indapamide was found to be 2.29 and 3.83 min, respectively. The validation was carried according to International Conference on Harmonisation (ICH) guidelines. In linearity curve correlation coefficients for atenolol and indapamide were found to be 0.9995 and 0.9991, respectively. The percent recovery was 99.81-100.02 for atenolol and indapamide indicating accuracy and reliability of method. So the method can be used for estimation of these drugs in tablet dosage form, human blood and milk.

1. Introduction

Development of the rapid and reproducible analytical methods for estimation of multicomponent drugs is very important part of quality control and for social awareness which is established in present work. Nowaday's new multicomponent formulations in market increasing with alarm rate which have better synergetic effect it is very essential that two or more number of drugs should be estimated simultaneously. Atenolol in combination with indapamide used as antihypertention and β blocker in cardiac and diuretic conditions as sustained release tablets.

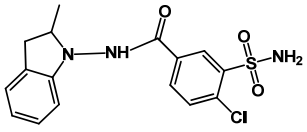
Atenolol (Figure 1) [(R.S)-4-(2-hydroxy-3-isopropyl-amino-propoxy) phenyl actetamide], is a cardio selective β-blocker. It may be used alone or concomitantly with other antihypertensive agents including thiazide-type diuretics, hydralazine, prazonin and α-methyl dopa [1]. It is reported to lack intrinsic sympathomimetic activity and membrane-stabilizing properties. Various adverse effects (Skin eruptions, Skin necrosis and connective tissue disease) are reported for atenolol. It is also contraindicated in pregnant women and lactating mothers [2].

Several analytical methods have been reported for the determination of atenolol in Pharmaceutical formulations. The United States Pharmacopeia (2003) describes a method that uses high performance liquid chromatography (HPLC) with UV detection for assay of atenolol tablets [3]. The method recommended by British Pharmacopoeia (2001) involves UV spectrophotometry [4]. In Brazilian Pharmacopoeia, however a method for assay of atenolol was not found. Other methods reported in the literature for the determination of atenolol in pharmaceutical formulations include visible spectrophotometry [5-10], UV derivative spectrophotometry [11,12], HPLC [13], high performance thin layer chromatography [14,15], potentiometry [16-18], capillary electrophoresis [19-21]and voltammetry [22,23].



2-(4-(2-hydroxy-3-(isopropylamino)propoxy)phenyl)acetamide

"Atenolol"



4-chloro-*N*-(2-methylindolin-1-yl)-3-sulfamoylbenzamide

"Indapamide"

Figure 1. Chemical structures of atenolol and indapamide.

Indapamide is a diuretic with actions and uses similar to those of thiazide diuretics, even though it does not contain a thiazide ring system. It is used for hypertension and also for oedama, including that associated with heart failure [24]. Indapamide is chemically 3-(amino sulfamoyl)-4-chloro-*N*-(2,3-dihydro-2-methyl-1*H*-indol-1-yl) benzamide [25].

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The structure of indapamide is shown in Figure 1. It produces anti-hypertensive effect by diuresis which causes decrease in plasma extracellular fluid volume, cardiac output and sodium concentration intracellularly in vascular smooth muscle wall and also dampens responsiveness to constrictor stimuli like those of angiontensin II or nor adrenaline. Numerous methods have been reported for determination of indapamide including spectrophotometric [26,27] and liquid chromatographic [28,29] methods. Indapamide active pharmaceutical ingredient (API) of official in British Pharmacopoeia [30] and United States Pharmacopoeia [31], while indapamide tablets are official in British Pharmacopoeia [32] and United States Pharmacopoeia [33].

Simultaneous determination of atenolol and indapamide is not official in any Pharmacopoeia. Combination of atenolol and indapamide result in synergistic effect and thus superior blood pressure lowering. Many methods have been described in the literature for the determination of atenolol and indapamide individually and in combination with other drugs.

The validation of method carried out as per ICH guidelines. This proposed method is suitable for the quality control of the raw materials, formulations, dissolution studies and can employed for bioequivalence studies [34,35].

To the best of our knowledge, no RP-HPLC (Reverse phase high performance liquid chromatography) method has been described for simultaneous estimation of both drugs in dosage form, human blood and milk. Therefore, it was thought-worth while to develop simple, precise, accurate RP-HPLC methods for simultaneous determination of atenolol and indapamide in tablet and biological fluids.

2. Experimental

2.1. Instrumentation

HPLC was performed with a Shimadzu (Japan) SPD-10 A VP system comprising an LC-10AT VP pump, an autosampler, and an SPD-10 A VP detector. Data processing was by Shimadzu Class-VP software on a Hewlett-Packard computer. Compounds were separated on a 250 mm x 4.6 mm, 5 µm particle, Hypersil BDS C_{18} column.

2.2. Materials

Pharmaceutical grade atenolol and indapamide were pursued as gift sample by Torrent Research Center (Gandhinagar, India) Acetonitrile and ethanol of HPLC grade were purchased from Qualigens fine chemicals (Mumbai, India). Water HPLC grade was obtained from a Milli-Q water purification system. Whatman filter paper (No. 1) was obtained from Merck and Potassium dihydrogen orthophosphate of analytical reagent grade was obtained from S.D fine chemicals (Mumbai, India).

2.3. Chromatographic conditions

The optimum composition of mobile phase was determined to be 0.01 M potassium dihydrogen ortho phosphates: acetonitrile (60:40, v:v). The pH of this mobile phase was adjusted to 3.5 with ortho phosphoric acid (85%) prior to delivering into the system. It is filtered through 0.45 µm filter and degassed using a sonicator. The analysis was carried out under isocratic conditions using a flow rate 1.0 mL/min at room temperature. Chromatograms were recorded at 231 nm.

2.4. Preparation of standard solution

Stock solutions of 1000 µg/mL were prepared by dissolving 25 mg of atenolol and 1.25 mg of indapamide in 50 mL volumetric flasks dilute to volume with mobile phase. From these stock solutions, working standard solutions having concentration 100 µg/mL each were prepared by appropriate dilution.

2.5. Sample preparation

2.5.1. Analysis of pharmaceutical dosage forms

10 mg atenolol and 10 mg of indapamide were dissolved in ethanol in 100 mL volumetric flask and made up the volume with the same solvent (stock solution of 100 µg/mL). Aliquots were appropriately diluted. Twenty tablets were weighed; their average weights determined and were finally powdered. The correct amount of powder was dissolved in ethanol by stirring for 30 min, the excipients were separated by filtration. Appropriate aliquots were subjected to above methods and the amount of atenolol and indapamide.

2.5.2. Serum

Blood was obtained from healthy volunteers and serum was separated by centrifugation at 5,000 g for 10 min, standard drug solution containing 8-32 and 2-10 µg/mL of atenolol and indapamide were added to 1 mL serum to give five concentrations, and the contents of the tubes were vigorously shaken. Mobile phase was added to deproteinate the serum, at ratio of one part serum to three parts mobile phase, and the mixture was vigorously mixed by means of shaker. The precipitated proteins were separated by centrifugation at 5,000 g for 10 min. Clear supernatant was diluted with water and injected directly for HPLC analysis under the conditions described above.

2.5.3. Milk

Drug-free human milk was obtained from a healthy volunteer and spiked with atenolol and indapamide in the concentration range 2-64 and 1-32 µg/mL. Deproteination and chromatographic analysis were described for analysis of human serum.

3. Results and discussion

3.1. Optimization of chromatographic conditions

Because mobile phase pH, concentration of the organic modifier, and instrumental conditions have a substantial effect on the selectivity and sensitivity of HPLC, the effects on the chromatographic separation of atenolol and indapamide were investigated. The effect of mobile phase in the pH range 2.5-4.0 on the separation behaviour of was investigated first. The retention time and peak area of atenolol and indapamide increased with increasing mobile phase pH. Atenolol and indapamide were not stable and strange peaks were observed on use of mobile phase of pH = 2. These were plateau between pH = 3 and 4 and the central point of the plateau, pH = 3.5 was chosen as the optimum mobile phase pH.

The effects of mobile phase buffer-acetonitrile concentration on peak shape and retention time were examined for mobile phase concentrations 60:40 (v:v) at pH = 3.5. As expected increasing the concentration of organic solvent (32-40) reduced analysis time. Peak shape was almost unchanged for all mobile phase concentrations. On the basis of efficiency, retention time and peak symmetry, buffer - acetonitrile (60:40; v:v) was regarded as suitable.

Another important condition affecting the retention behavior of atenolol and indapamide is mobile phase flow rate. When different flowrates from 0.6 to 1.2 mL/min were tested, parabolic decreases in atenolol and indapamide retention time and peak areas were observed with increasing flowrate. The flow rate corresponding to the smooth part of the parabola, 1.0

mL/min was chosen as optimum on the basis of retention time, column pressure and peak symmetry.

Results from system suitability testing are presented in Table 1. Good agreement values were observed. As a consequence the optimum conditions were determined as mobile phase containing buffer-acetonitrile (60:40; v:v) at pH = 3.5 and flow rate 1.0 mL/min, 20 µL loop volume, and detection wavelength at 231 nm. The chromatogram obtained from atenolol and indapamide under these conditions was shown in Figure 2.

Table 1. Validation parameters of atenolol and indapamide by HPLC.

Parameters	Atenolol	Indapamide
Linearity & range µg/mL	5-30	0.25-1.50
Correlation coefficient	0.9995	0.9991
Slope	13.738	157.640
Intercept	10.670	2.295
Standard deviation	0.630	0.280
Resolution Factor	5.859	8.859
Tailing Factor	1.292	1.337
Theoretical plates	3550	7474
Limit of Detection (µg/mL)	0.520	0.011
Limit of Quantification (µg/mL)	1.636	0.034
Human serum LOD	0.325	0.070
LOQ	0.55	0.58
Human milk LOD	0.057	0.034
LOQ	1.720	0.175

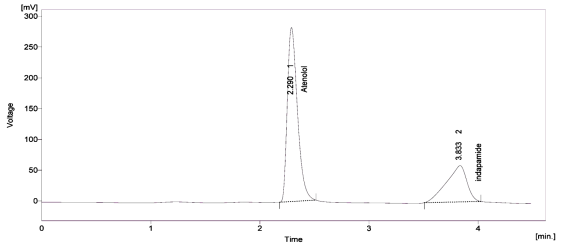

Figure 2. Chromatogram obtained from atenolol and indapamide raw material.

3.2. Method validation

3.2.1. Precision

Under the optimized conditions the precision of the method was determined by measurement of repeatability (intra-day) and intermediate precision (inter-day) both expressed as RSD% (RSD: Relative standard deviation) of a series of measurements. Statistical evaluation of the results from determination of intra-day precision showed RSD was 0.174 and 0.172 statistical evaluation of the result from determination of inter-day variability, calculated from assays on 3 days, showed RSD was 0.041 and 0.144, respectively. RSD values below 2% are indicative of sufficient method precision, so both values are acceptable analytically.

3.2.2. Linearity

Calibration plots (Figure 3) for atenolol and indapamide standard in the mobile phase were constructed by plotting the concentration of atenolol and indapamide against the atenolol and indapamide peak area. Linearity was good in the concentration range to 5-30 and 0.25-1.50 µg/mL. Results from linear regression analysis of the plots are listed in Table 1. Resolution was always good in the linear range studied.

3.2.3. Detection limit

The limits of detection (LOD) and quantification (LOQ) were calculated from integrated peak areas from the HPLC chromatogram. The values, estimated by dividing the standard deviation of the regression equation by the slope of the equation and multiplying by 3.3 and 10.0, respectively. The minimum limits at which the analyte can be readily detected (LOD) and quantified (LOQ) for atenolol and indapamide were 0.520, 0.011 µg/mL and 1.636, 0.034 µg/mL, respectively, in bulk material.

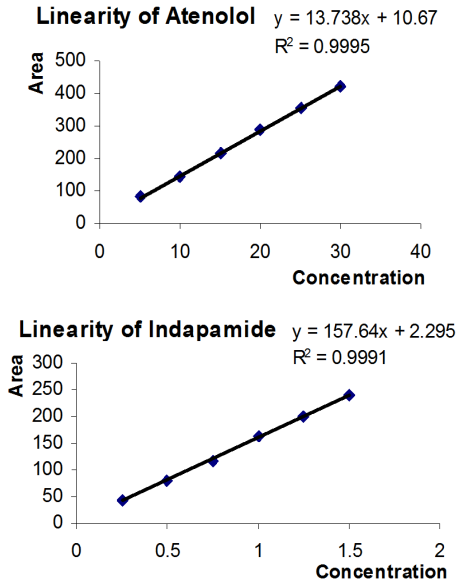

Figure 3. Calibration plots atenolol and indapamide

3.2.4. Accuracy

Accuracy was calculated by use of the Equation 1.

Relative error (%) = (Conc. found – spiked conc. / Spiked conc.) x 100 (1)

and precision was evaluated by determination of the coefficient of variation CV%, RSD% [(SD/mean)x100] at low, central and high concentrations in the linear range. The RSD% values were also much lower than the acceptance criteria, showing the precision of the proposed method is good, as is apparent from Table 2. The accuracy values for between and within day studies at low, medium and concentrations of atenolol and indapamide in serum and milk were with in acceptable limits.

Table 2. Results from determination of method accuracy for atenolol and indapamide.

Amount added µg/mL	Amount found µg/mL, mean, *n*=6	Recovery %	Accuracy %	RSD %
Atenolol				
0.5	0.499	99.84	-0.16	0.329
1.0	1.002	100.02	0.02	0.258
1.5	1.499	99.986	-0.013	0.109
2.0	1.997	99.89	-0.11	0.292
Indapamide				
0.5	0.498	99.72	-0.28	0.390
1.0	0.999	99.94	-0.06	0.328
1.5	1.499	99.93	-0.06	0.170
2.0	1.997	99.85	-0.15	0.278

3.2.5. Specificity

Specificity was checked by using the same column for analysis of the inactive ingredients of the tablet, to ensure these ingredients did not interfere with the peaks, so specificity was regarded as sufficient for application of the method to tablet analysis, human blood and milk. Representative chromatograms was generated to show other components that could be present in the sample matrix are resolved from the parent analytes. No change was observed in the chromatogram of

atenolol and indapamide in the presence of common excipients. The specificity was also determined by injecting human serum and milk samples. Therefore, the proposed method is selective and specific for the drugs

3.2.6. Recovery

Recovery was almost 100% for the drug substance from the drug product and accuracy was much better than the acceptance criteria. The same concentration was used to evaluate precision as repeatability. The RSD% values were also much lower than the acceptance criteria, showing the precision of the proposed method is good, as is apparent from Table 3. Blank serum and milk samples from healthy volunteers were collected tested for the matrix interferences with determine drugs. An example of blank serum and milk spiked with 0.5 to 6.0 µg/mL of drugs. As can be seen, the drugs are well resolved with no apparent interferences from serum and milk component. The recoveries of atenolol were 100.02±0.258, 100.01±0.022, 100.0±0.027, respectively, and those of indapamide were 99.94±0.328, 99.94±0.117, 99.98±0.051. These results suggested that there were no relevant differences in serum and milk treatment recovery at different concentration levels for atenolol and indapamide.

3.3. Application of the method to human serum samples

HPLC analysis of drugs in biological samples, for example human serum, usually requires time-consuming and expensive sample-preparation procedures. The serum deproteination technique used in this method was quite simple. The serum proteins are precipitated by addition of buffer:acetonitrile (60:40) and, after centrifugation at 5,000 g, the supernatant is injected directly for analysis. Chromatograms obtained from serum spiked with 8-32 and 2-10 µg/mL atenolol and indapamide are presented in Figure 4. Peak shape and other characteristics are not different from those for standards. When the ratio of peak areas of atenolol and indapamide in serum were evaluated statistically RSD% was 0.15 indicative of good intra-day precision. Inter-day variability was calculated from assays on three days and RSD% was 0.098. Intra and Inter day precision can be regarded as acceptable for biological samples [36]. The results obtained from three determinations of each concentration are given in Table 3.

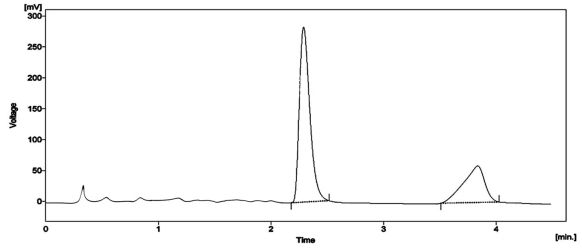

Figure 4. Chromatogram obtained from analysis of serum spiked with atenolol and indapamide

Table 3. Results from determination of method accuracy for serum spiked with atenolol and indapamide.

Amount added µg/mL	Amount found µg/mL, mean, *n*=6	Recovery %	Accuracy %	RSD %
Atenolol				
2.0	1.998	99.90	-0.10	0.262
4.0	3.9984	99.96	-0.04	0.128
6.0	6.0004	100.0	0.006	0.022
Indapamide				
2.0	1.998	99.90	-0.10	0.262
3.0	2.997	99.91	-0.08	0.171
4.0	3.997	99.94	-0.05	0.117

3.4. Application of the method to human milk

The sample-preparation procedure for milk was the same as for serum. It is apparent from Figure 5 that peak shape and other characteristics after extraction of spiked human milk were not different from those for standards (Table 4). The atenolol and indapamide were evaluated statistically and the results showed that RSD was 0.27 % indicative of good intra-day precision. Inter day variability was calculated from assays on three days and RSD was 0.381 not indicative of excellent precision for human milk. LOD and LOQ calculated for human milk were 0.057, 0.034 and 1.720, 0.175 µg/mL for atenolol and indapamide, respectively.

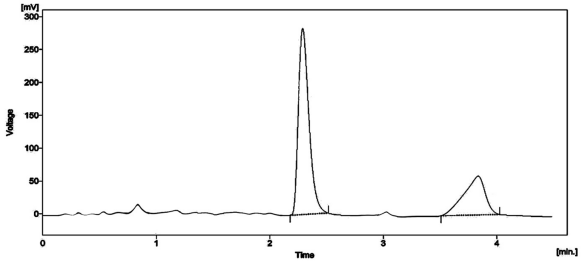

Figure 5. Chromatogram obtained from analysis of human milk spiked with atenolol and indapamide.

Table 4. Results from determination of method accuracy for human milk spiked with atenolol and indapamide.

Amount added µg/mL	Amount found µg/mL, mean, *n*=6	Recovery %	Accuracy %	RSD %
Atenolol				
3.0	2.999	99.96	-0.033	0.117
6.0	6.000	100.003	0.003	0.027
9.0	8.998	99.98	-0.017	0.056
Indapamide				
2.0	1.998	99.93	-0.070	0.144
4.0	3.999	99.98	-0.015	0.051
6.0	5.998	99.97	-0.026	0.082

Human milk is a unique, complex, nutritionally natural food containing proteins, lipids, carbohydrates, vitamins and minerals [37]. Although this composition can sometimes be troublesome for sample preparation, the validation data, for example precision, obtained by use of the experimental conditions described above show no problems were encountered and validation data were satisfactory. Results obtained for the method recovery and precision are listed in Table 5 for the free drugs atenolol and indapamide in spiked human milk. Recovery was approximately 72% for direct injection of the clear supernatant and there were no interferences from the milk matrix. Human milk contain quite different components from plasma, for example lipids and protein in high and variable quantities, it can, therefore, be more difficult to handle than plasma. There have been reports of factors affecting drug concentrations in milk [37]. First, they can be affected by protein binding. Second milk in slightly more acidic than maternal blood so weak bases will ionize to a greater extent in milk than in plasma. Third, fat soluble drugs will dissolve preferentially in the lipid component of milk and may, therefore, not be available for diffusion back into plasma [36-39]. Low recoveries of atenolol and indapamide from human milk could be a result of these factors, especially possible solubility in fat and protein binding.

The results show that determination of atenolol and indapamide in human milk can be achieved successfully after use of a simple, selective and rapid sample-preparation technique.

Table 5. Precision of atenolol and indapamide in dosage forms, serum and milk samples.

Atenolol			Indapamide		
Conc. spiked	Inter-day	Intra-day	Conc. spiked	Inter-day	Intra-day
	Recovery ± RSD%	Recovery ± RSD%		Recovery ± RSD%	Recovery ± RSD%
	Formulations				
1.0	99.98±0.381	99.92±0.277	0.4	99.95±0.447	100.10±0.335
2.0	99.94±0.159	99.81±0.222	0.8	100.15±0.240	100.12±0.197
4.0	99.98±0.041	99.90±0.174	1.2	99.91±0.144	99.95±0.172
	Serum				
1.0	99.86±0.313	99.60±0.158	0.4	99.95±0.410	100.00±0.395
1.5	99.92±0.098	99.92±0.098	0.8	100.00±0.197	99.98±0.186
2.0	99.98±0.115	99.76±0.233	1.2	99.91±0.108	99.98±0.180
	Milk				
0.5	99.76±0.297	100.02±0.340	0.6	99.90±0.401	100.06±0.383
1.0	99.80±0.381	99.92±0.277	0.8	100.00±0.197	100.07±0.259
1.5	99.96±0.138	99.69±0.223	1.0	99.98±0.303	100.02±0.268

4. Conclusion

A simple and rapid RP-HPLC method for the simultaneous determination of atenolol and indapamide in pharmaceuticals, human serum and human milk has been developed and validated in this study. The retention times observed (2.29 and 3.83 min) enable rapid determination of the drugs, which is important for routine analysis. The linearity range, limits of detection and quantifications, precision and accuracy were determined to asses the suitability of the method and satisfactory results were obtained. This HPLC method with UV detection has several advantages over other methods. In the proposed method the analysis time is quite short and a simple mobile phase is used, for this reason consumption of organic solvent is very low. The method is rapid, specific, reliable and cost-effective and can be recommended for routine analysis and for quality control.

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