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Simultaneous Determination of Atenolol and Furosemide in Intestinal Perfusion Samples by Validated Reversed-Phase High-Performance Liquid Chromatography

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A reversed-phase high-performance liquid chromatographic method was developed for quantification of atenolol and furosemide in intestinal perfusion samples to assess possible drug-drug interaction during pre-absorption stage. The method utilized a simpler mobile phase [acetonitrile:50 mM pH 6.5 phosphate buffer (20:80 v/v)] than those reported previously for these drugs, with added advantage of their UV detection (λ=276 nm). The analytical method passed the validation tests recommended by ICH and United States Pharmacopeia. The method was specific for both the drugs, and able to resolve the drug peaks with no interference from components of intestinal perfusate. The method was accurate, precise and linear within the desired range. This method can be successfully employed for quantitative analysis of atenolol and furosemide, alone or in combination.

Multiple drug administration is often associated with clinically significant interactions, especially in case of narrow therapeutic index drugs, either at pre-absorption or post-absorption stage[1,2]. This can limit the desired therapeutic effect of either of the drug molecule. Present study was aimed at developing a simple and fast analytical method for simultaneous determination of atenolol (ATN), a β-adrenoreceptor antagonist, and furosemide (FUR), a diuretic, the commonly prescribed drug combination in treatment of hypertension[3].

The chemical structures of the two drugs are given in fig. 1. Both the drugs are official in Indian Pharmacopoeia (IP), British Pharmacopoeia (BP) and United States Pharmacopoeia (USP). ATN is chemically 2-[p-[2-Hydroxy-3-(isopropylamino)propoxy]-phenyl]-acetamide with molecular formula $C_{14}H_{22}N_2O_3$ and molecular weight of 266.34. FUR is chemically 4-Chloro-*N*-furfuryl-5-sulfamoylanthranilic acid with molecular formula $C_{12}H_{11}ClN_2O_5S$ and molecular weight of 330.75. The reported liquid chromatographic methods for ATN[4-7] and FUR[8,9] analysis in biological samples involve either a complex mobile phase composition, or use of fluorescence detector, which is not easily available.

The present method reports a simple and fast liquid chromatographic analytical method for ATN and FUR, using UV detector. Moreover, the method has the advantage of quantifying both the drugs either alone or in combination, with significant accuracy and precision. This method has been aptly utilized for analyzing the ATN and FUR content in intestinal perfusate samples[10,11] for investigating pre-absorption drug-drug interactions.

MATERIALS AND METHODS

ATN (99.9% purity) and FUR (99.2% purity) were received as gift samples from Panacea Biotech Ltd., Lalru. All reagents used were of analytical grade. Acetonitrile used was of high-performance liquid chromatography (HPLC) grade (J.T. Baker, Xalostoc, Mexico). Water used for HPLC was obtained after reverse osmosis (USF Elga, High Wycombe Bucks, England) of triple distilled water.

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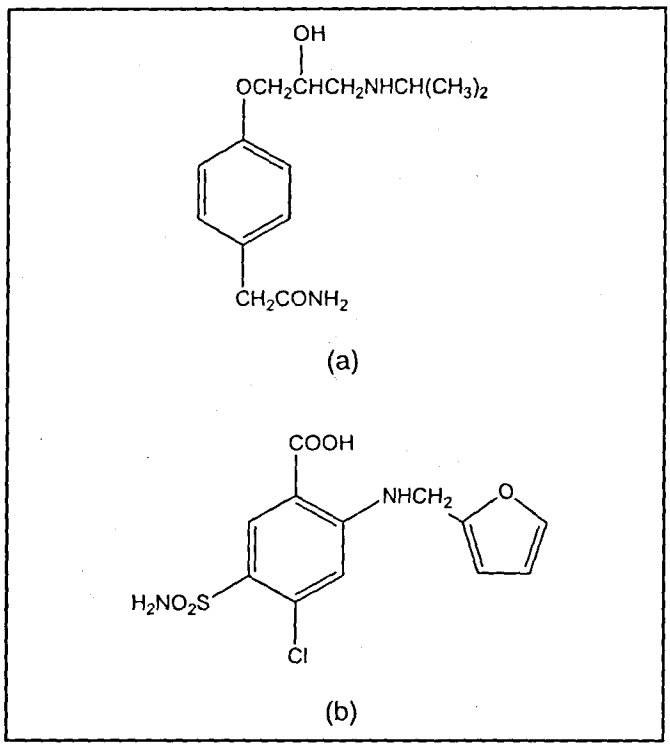

Fig. 1: Chemical structures of (a) atenolol and (b) furosemide.

The HPLC system (Shimadzu Corporation, Kyoto, Japan) comprised of a system controller (SCL-10A), a pump (LC-10AT), a degasser (DGU-14A), an autosampler (SIL-10AD), a column oven (CTO-10AS), and a UV detector (SPD-10AP) with Class-VP (Release 6.10) software. The chromatographic conditions adopted for drug analysis are outlined in Table 1. The selection of a common analytical wavelength for ATN and FUR was based on the sufficient UV absorbance by samples of lowest drug concentration at that particular wavelength.

In addition, a pH meter (CyberScan pH 510, Eutech Instruments Pte Ltd., Ayer Rajah Crescent, Singapore), and a vapor pressure osmometer (Vapro®, 5520 XR, Wescor®,Inc., Utah, USA) were used in this study.

Preparation of stock solutions of drugs:

The composition of intestinal perfusion solution was as detailed earlier[12]. The pH and osmolality of the solution were adjusted to the value of 6.5±0.02 and 290±10 mOsmol/kg, respectively.

The stock solutions of both the drugs were prepared alone as well as in combination. An accurately weighed amount of ATN (about 30 mg) and FUR (about 10 mg) were dissolved in 50 ml of intestinal perfusion solution in a 100 ml volumetric flask by vortex mixing for 15 min. Final volume was made up to the mark with intestinal perfusion solution, so as to give concentrations of 300 µg/ml for ATN and 100 µg/ml for FUR. These solutions were labeled as 'standard stock solutions.'

TABLE 1: HPLC PARAMETERS FOR ANALYSIS OF ATENOLOL AND FUROSEMIDE

Parameter	Condition
Method	Reversed-phase high-performance liquid chromatography
Mobile phase	Isocratic elution, acetonitrile:50 mM pH 6.5 phosphate buffer (20:80 v/v)
Column	Lichrospher® 100 RP-18 e (Merck KGaA, Darmstadt, Germany) analytical column (250 mm x 4 mm, 5 µm), and guard column (4 mm x 4 mm, 5 µm)
Flow rate	0.7 ml/min
Detection	UV detector, 276 nm
Column temperature	25°
Injection volume	50 µl

Method validation:

The analytical method was validated as per the recommendations of USP and ICH for the parameters like specificity, range and linearity, accuracy and precision[13]. In addition, the test solutions were tested for drug stability during experimental time period. All tests were done for both the drugs alone and in combination.

To determine the specificity of analytical method in the presence of components of intestinal perfusion solution, the HPLC chromatograms of both the drugs in intestinal perfusion solution (ATN– 300 µg/ml and FUR– 100 µg/ml) were compared with that of blank intestinal perfusion solution.

To evaluate the linearity of analytical method, five standard solutions were made by dilution of the standard stock solutions in the working range of 60-300 µg/ml for ATN and 20-100 µg/ml for FUR using intestinal perfusion solution. The selection of upper and lower limits for concentration range was based on the expected outcome from the test samples during intestinal perfusion study.

To determine the accuracy of analytical method, working standards of both the drugs were prepared in

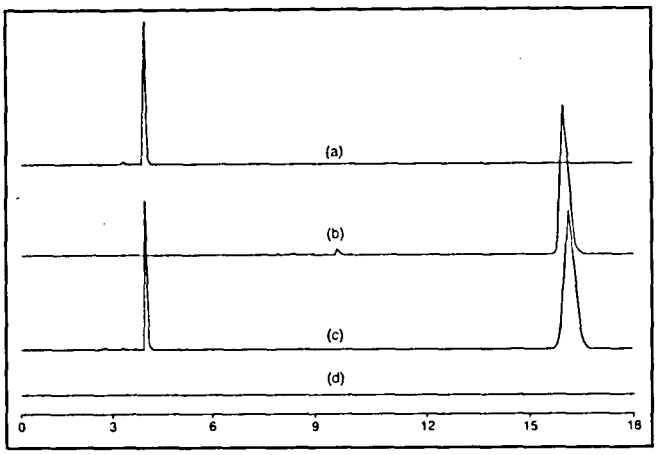

Fig. 2: Overlain chromatograms of atenolol and furosemide.

Overlain chromatograms of (a) atenolol (300 µg/ml), (b) furosemide (100 µg/ml), (c) atenolol (300 µg/ml) and furosemide (100 µg/ml), and (d) blank intestinal perfusion solution.

triplicate at three concentration levels (75, 150 and 225 µg/ml for ATN, and 25, 50 and 75 µg/ml for FUR), and analyzed.

Precision of analytical method was assessed in terms of repeatability and intermediate precision. Repeatability was checked by analyzing six independent samples of ATN and FUR at 100% concentration level, and calculating their relative standard deviation (% RSD). To determine intermediate precision, standard solutions of ATN and FUR, alone and in combination, at five concentration levels, were analyzed three times within the same day (intra-day variation) and on three different days (inter-day variation).

To check the solution state stability of drugs, standard solutions of ATN (0.83 mM ≅ 221.1 µg/ml) and FUR (0.20 mM ≅ 66.2 µg/ml) were prepared in intestinal perfusion solution, and kept in water bath (maintained at 37±0.5°). The samples were analyzed after 1.5 and 3.0 h against freshly prepared standards.

TABLE 2: REGRESSION STATISTICS FOR HPLC ANALYSIS OF ATENOLOL AND FUROSEMIDE

Sample	Range (µg/ml)	Goodness-of-fit (r^2)	Slope	Intercept
Alone				
Atenolol	60-300	0.9984	17940	99554
Furosemide	20-100	0.9991	269859	128880
Combination				
Atenolol	60-300	0.9995	17572	107448
Furosemide	20-100	0.9997	269060	128012

Acceptance criteria: r^2>0.99

RESULTS AND DISCUSSION

Validation of an analytical method assures the confidence in generated data for its purported results against method, instrument, analyst or any other variation[14]. An analytical method is said to be specific when it can detect the analyte in the presence of expected impurities or additives in test sample[14]. The representative chromatograms for blank intestinal perfusion solution as well as drug solutions, alone and in combination, are presented in fig. 2. The components of intestinal perfusion solution did not interfered with the drug peaks, when tested alone as well as in combination. Retention times for ATN and FUR were found to be ≅ 4 and 17 min, respectively, when tested alone. During analysis of drugs in combination, the drug peaks were well resolved, and eluted at same retention times as when analyzed alone. Thus, the analytical method is capable of analyzing both the drugs alone and in combination. Also, the method is specific for both the drugs, when tested for possible interference from intestinal perfusion solution.

The linearity of an analytical method signifies direct proportionality between a quantifiable response and the concentration of analyte within a given range (bounded by upper and lower limits)[14]. The linearity of the analytical method for ATN and FUR, alone and in combination, was observed in the tested concentration range, demonstrating its suitability for analysis. The regression statistics are shown in Table 2. The goodness-of-fit (r^2) was found to be > 0.99 in all the cases, indicating functional linear relationship between the concentration of analytes and area under the peak.

TABLE 3: ACCURACY DATA FOR HPLC ANALYSIS OF ATENOLOL AND FUROSEMIDE

	Concentration (µg/ml)	% Agreement (% RSD)	
		Alone	Combination
Atenolol	75	100.5 (1.1)	102.9 (1.1)
	150	101.3 (1.3)	103.1 (1.0)
	225	102.8 (0.8)	101.0 (1.2)
Furosemide	25	95.6 (1.8)	100.0 (0.3)
	50	98.5 (1.5)	100.5 (0.2)
	75	99.6 (1.0)	99.9 (0.6)

Acceptance criteria: % agreement = 100±2 %; % RSD <2 %

TABLE 4: INTERMEDIATE PRECISION DATA FOR HPLC ANALYSIS OF ATENOLOL AND FUROSEMIDE

Concentration (µg/ml)	Intra-day variation (% RSD)		Inter-day variation (% RSD)	
	Alone	Combination	Alone	Combination
Atenolol				
60	1.1	0.6	1.9	1.6
120	0.8	1.4	1.2	0.7
180	0.3	0.5	0.3	0.6
240	0.5	0.2	0.7	0.8
300	1.0	0.3	0.3	0.5
Furosemide				
20	0.7	1.1	0.5	0.9
40	0.2	1.7	1.3	0.1
60	0.5	0.4	0.7	0.7
80	0.6	0.8	0.3	0.4
100	1.5	0.6	1.6	0.8

Acceptance criteria: % RSD <2 %

The accuracy of an analytical method bestows the exactness in analytical response of the analyte to its true value[14]. The results of accuracy studies are shown in Table 3, and it is evident that the method is accurate within the desired range.

The precision of an analytical method certifies the exactness of analytical response when tested for multiple samplings of a homogeneous sample[14]. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment, and is expressed as the % RSD. The % RSD values for six independent injections of ATN solution at 100% test concentration were 0.12% and 0.39%, when analyzed alone and in combination, respectively. The corresponding values for FUR were 0.30% and 0.45%, respectively. The results of intra- and inter-day variation are shown in Table 4. Thus, the analytical method passed the tests for repeatability and intermediate precision for both the drugs, alone and in combination, with % RSD values within the acceptable limits of <2%.

To demonstrate the stability of ATN and FUR in intestinal perfusion solution during the course of experiment, solution state stability of these drugs was carried out at 37±0.5° for 3 h. The drug content ranged from 100.22 to 100.33% for ATN, and 100.58 to 100.83% for FUR. These results demonstrated that there was no significant change in the drug content, and the solutions were stable for 3 h. During the stability study, no additional peaks developed, and no changes in the chromatographic pattern were observed for either of the drugs.

The developed and validated analytical method was applied for the determination of ATN and FUR content in intestinal perfusate samples[10,11]. The method was found to demonstrate the interactions between the two drugs during absorption. Thus, the developed analytical method can be suitably used for simultaneous determination of ATN and FUR in test samples.

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