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Method Development of Verapamil in Presence of NSAIDs using RP-HPLC Technique

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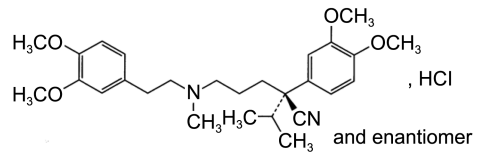
Verapamil is a calcium channel blocker and is classified as a class IV *anti*-arrhythmic agent. It is used in the control of supra ventricular tachyarrhythmias, and in the management of classical and variant angina pectoris. It is also used in the treatment of hypertension and used as an important therapeutic agent for angina pectoris, ischemic heart disease, hypertension and hypertrophic cardiomyopathy. Verapamil commonly co-administered with NSAIDs (non-steroidal *anti*-inflammatory drugs) i.e. diclofenac sodium, flurbiprofen, Ibuprofen, mefanamic acid and meloxicam. A simple and rapid RP-HPLC method for simultaneous determination and quantification of verapamil and NSAIDs was developed and validated. The mobile phase constituted of acetonitrile: water (55:45) whose pH was adjusted at 2.7 and pumped at a flow rate of 2.0 mL min^{-1} at 230 nm. The proposed method is simple, precise, accurate, low cost and least time consuming for the simultaneous determination of verapamil and NSAIDs which can be effectively applied for the analysis of human serum.

Key Words : Verapamil, *anti*-arrhythmic agent, NSAIDs

Introduction

Verapamil is 5-[*N*-(3,4-dimethoxyphenethyl)-*N*-methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride. It is a synthetic papaverine derivative, which belongs to phenylalkylamine class. Verapamil is a calcium blocker and is classified as a class IV *anti*-arrhythmic agent.[1] It is used in the control of supra ventricular tachyarrhythmias, and in the management of classical and variant angina pectoris. It is also used in the treatment of hypertension.[2] It has been used an important therapeutic agent for angina pectoris, ischemic heart disease, hypertension and hypertrophic cardiomyopathy.[3]



Verapamil HCl

The molecule contains an asymmetric carbon and is clinically administered as a racemic mixture of the (+)-R- and (−)-S-enantiomers.

Verapamil has been determined by spectrophotometry,[4,5] gas chromatography[6,7] capillary electrophoresis.[8] Several high-performance liquid chromatographic (HPLC) method have been reported for the determination of verapamil in biological samples by UV detection[9-11] or more frequently fluorescence detection[12-17] owing to the native fluorescence properties of this compound.

More sensitive and specific approaches to measure plasma verapamil are mass spectrometry (MS) with isotope dilution (mass fragmentography)[18] and HPLC/MS.[19] But, these techniques have limitations including requirement of expensive instruments and spacious laboratory.

Hypertension and musculoskeletal are two common co-existing problems for which antihypertensive and analgesics as nonsteroidal *anti*-inflammatory drugs (NSAIDs) are most commonly prescribed together.[20-23]

Over the last two decades, concern about probable drug interaction between NSAIDs and antihypertensive agents has been grown. Cases with hypertensive emergency have been reported after taking NSAIDs in patients with previously well-controlled hypertension.[24,25]

In the current study a RP HPLC method has been develop for the simultaneous determination of verapamil with different NSAIDs like diclofenac sodium, flurbiprofen, ibuprofen, mefanamic acid and meloxicam in raw material and serum.

Experimental

Material and Reagents. Verapamil was a kind gift from Searle Pakistan Limited, diclofenac sodium from Novartis Pharma Pakistan Limited, Ibuprofen from Abbott Laboratories Pakistan Limited, flurbiprofen and mefanamic acid from Pfizer Laboratories Limited Pakistan and meloxicam from Hilton Pharma Private Limited Pakistan were used without further purification. HPLC grade acetonitrile (Merck) and water were used. However, phosphoric acid (Merck) was of analytical grade.

Apparatus. Electrical Balance (Mettler Toledo # AB54), pH meter (Mettler Toledo MP 220), UV-visible 1601 Shimadzu double beam spectrophotometer, 1 cm rectangular quartz cells, Deionizer (Stedec CSW-300), distillation unit (GFL Type 2001/2).

The liquid chromatographic system consisted of Shimadzu model LC-20 AT VP pump, Rheodyne manual injector fitted with a 20 μL loop, a Shimadzu model SPD-20AV variable wavelength UV-visible detector (Shimadzu Corporation, Kyoto, Japan). Chromatographic system was integrated *via* Shimadzu model CBM-102 Communication Bus Module. Analysis was conducted on a Shim-pack CLC-ODS (6.0 × 150 mm) analytical reverse phased column.

HPLC.

Optimization of Mobile Phase: In order to develop an RP-HPLC method initially, different ratios of acetonitrile: water was tried for simultaneous estimation of verapamil and NSAIDs (diclofenac sodium, flurbiprofen, ibuprofen, mefanamic acid and meloxicam). Individual drug solutions were injected into the column at the concentration of 100 μgmL^{-1} and both elution pattern and resolution parameters were studied as a function of pH. The best separation was obtained in acetonitrile: water (55:45) mobile phase. The pH effect showed that optimized conditions are reached when the pH of mobile phase is 2.6, producing well resolved and symmetrical peaks for all drugs assayed.

Wavelength Selection: In addition, the UV spectra of individual drugs were recorded in the wavelength range from 200 to 400 nm and compared. The choice to use a common wavelength set at 230 nm was considered satisfactory, permitting the detection of all drugs with adequate sensitivity.

Chromatographic Conditions: The mobile phase consisted of acetonitrile-water (55:45) whose pH was adjusted to 2.6 with phosphoric acid (85%). Prior to delivering into the system it was filtered through 0.45 μm filter and degassed using an ultrasonic bath. The analysis was carried out under isocratic conditions using a flow rate of 2.0 mL min^{-1} at room temperature. The samples were introduced by injector with a 20-μL sample loop. Chromatograms were recorded at 230 nm using a detector SPD-20AV Shimadzu UV visible.

Analytical Procedure.

Sample Preparations: Stock solutions (100 μg mL^{-1}) of Verapamil and all NSAIDs (diclofenac sodium, flurbiprofen, ibuprofen, mefanamic acid and meloxicam were prepared by dissolving 10 mg of each drug in 100 mL of acetonitrile. The stocks solutions were sequentially diluted with 50% acetonitrile (diluent) to yield 5, 10, 15, 20 and 25 μg mL^{-1} working standard solutions for preparation of calibration curves.

Serum Drug Analysis. Multiple blood samples (10 mL) of ten healthy non-smoker volunteers (age ranging from 22-25 years) not involved in any strenuous activity and not taking any other medicaments were collected in evacuated glass tubes. The blood was then centrifuged at 3000 rpm for 10 minutes and the plasma separated and deprotinated by acetonitrile. The supernatant obtained was filtered through a 0.45 μm filter. Serum thus obtained was mixed in ratio of 1:1 with drug solutions; these were stored at –20 °C pending drugs analysis.

Result and Discussion

Method Validation (HPLC). The use of HPLC methods for simultaneous determination and quantitation of drugs has received considerable attention in the recent past and its importance in the quality control of drugs and drug products is unquestioned. Several problems were encountered in the simultaneous determination of compounds investigated. The first was the selection of separation conditions to ensure efficient extraction of the two or more drugs from human serum with minimum interference from serum endogenous compounds. The second was the choice of proper chromatographic conditions to obtain separation of the different components from the endogenous compounds. Thirdly, the method had to be sufficiently sensitive to measure concentrations of all the investigated drugs in serum within their therapeutic range.[26]

Chemical structure and chemical properties are the most important facts that predict chromatographic behavior. In the present investigation the best separation of verapamil from its internal standard was achieved using a Shim-pack CLC-ODS (6.0 × 150 mm) column. Using other type of column under similar experimental condition, the separation lasted about 15 minutes. For the determination of verapamil and group of NSAIDs, best results were obtained using mobile phase acetonitrile/water (55:45 v/v). The lower percentage of acetonitrile in mobile phase results in peak tailing of both components and long analysis duration while higher percentage of acetonitrile in mobile phase results in merging of different peaks and resolution is also affected. Optimal retention times (verapamil-1.80, meloxicam-4.37, flurbiprofen-6.02, diclofenac sodium-7.14, ibuprofen-8.01 and mefanamic acid-11.08 minutes) were achieved when the pH of mobile phase was adjusted to 2.6 with 85% phosphoric acid. Small changes in pH of the mobile phase had a great influence to the chromatographic behavior of these substances. The higher pH of the mobile phase also results in peak tailing of verapamil and other NSAIDs. The method has successfully determined verapamil in serum in concentrations, as low as 2.5 μg mL^{-1}.

Specificity: Specificity was demonstrated by injecting

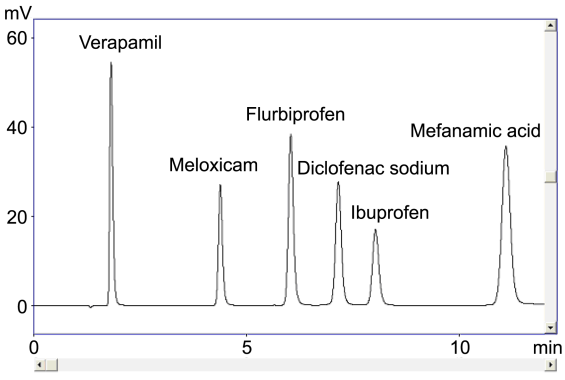


Figure 1. A typical chromatogram showing verapamil and NSAIDs peaks.

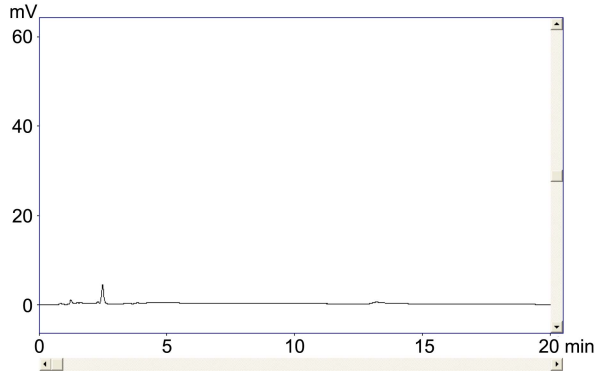


Figure 2. A typical chromatogram showing blank serum peaks.

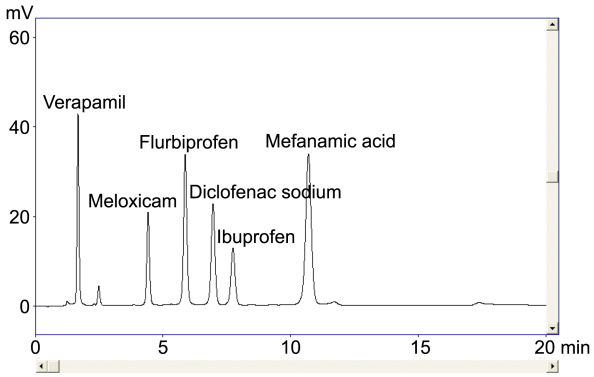


Figure 3. A typical chromatogram showing verapamil and NSAIDs peaks in serum.

specificity samples in triplicate (n=3). Representative chromatograms were generated to show that all NSAIDs which were present in the sample matrix are resolved without interfering the parent analyte verapamil which clearly indicated the specificity of the method (Figure 1-3).

Range and Linearity: For linearity studies 5 different concentrations (5, 10, 15, 20, 25 μg mL^{-1}) of a mixture of each drug. Linearity was demonstrated at five concentrations over the range of 5-25 μg mL^{-1} for four consecutive days. The linearity study was also carried out in serum over the concentration range of 2.5-25 μg mL^{-1}. Standard curve, slope, intercept and the correlation coefficient were determined. For calculation of the standard curve plots of peak areas against concentration were used. The regression statistics are shown in Table 1.

Precision and Accuracy (Recovery): The precision of the assay was determined by (repeatability) intra-day and inter-day (intermediate precision) analysis. Accuracy was determined by analyzing independently prepared solutions of verapamil at different concentration levels covering the entire linearity range. In serum the relative recoveries of verapamil and all NSAIDs were calculated by comparing the concentrations obtained from drug supplemented plasma to the actual added concentrations. Precision and accuracy were expressed in %RSD as shown in Table 2-4.

Table 2. Precision and recovery of verapamil and NSAIDs in raw material

Drugs	Conc. Injected (μg mL^{-1})	Conc. Found (μg mL^{-1})	% RSD	% Recovery
Verapamil	5.0	5.09	0.5193	101.83
	10.0	10.13	0.1498	101.33
	15.0	15.03	0.0272	100.20
	20.0	20.03	0.1090	100.15
	25.0	24.99	0.1182	99.94
Diclofenac Sodium	5.0	5.09	0.3171	101.88
	10.0	10.10	0.1245	100.96
	15.0	15.01	0.1177	100.04
	20.0	20.10	0.2538	100.51
	25.0	25.15	0.1034	100.62
Flurbiprofen	5.0	5.02	0.6619	100.33
	10.0	10.06	0.3415	100.57
	15.0	14.90	0.1884	99.36
	20.0	19.90	0.3478	99.49
	25.0	24.67	1.5466	98.69
Ibuprofen	5.0	5.11	0.2835	102.11
	10.0	10.10	0.1254	101.03
	15.0	14.78	0.1633	98.56
	20.0	19.83	0.3001	99.16
	25.0	24.93	0.5209	99.74
Mefanamic acid	5.0	5.12	0.5355	102.36
	10.0	10.06	0.3355	100.58
	15.0	14.91	0.9126	99.38
	20.0	19.83	0.1095	99.16
	25.0	24.85	1.5509	99.39
Meloxicam	5.0	4.97	0.7853	99.39
	10.0	10.20	0.4937	101.97
	15.0	15.12	0.1098	100.81
	20.0	20.06	0.2164	100.32
	25.0	25.12	0.4734	100.46

Table 1. Regression statistics

	Raw Material		Serum	
Drugs	Regression equation	R^2	Regression equation	R^2
Verapamil	11148x + 2106.2	0.9999	10164x + 3905.2	0.9994
Diclofenac sodium	10718x + 562.7	0.9999	11277x + 4683.7	0.9991
Flurbiprofen	13376x + 2218.9	0.9989	14872x + 5672.9	0.9992
Ibuprofen	7026.5x + 967.76	0.9999	6860.3x + 3288.4	0.9992
Mefanamic acid	21151x + 3869.2	0.9999	24973x + 10136	0.9990
Meloxicam	7323.4x + 336.56	0.9999	7371.3x + 2610.8	0.9995

Table 3. Precision and recovery of verapamil and NSAIDs in Serum

Drugs	Conc. Injected ($\mu g\ mL^{-1}$)	Conc. Found ($\mu g\ mL^{-1}$)	% RSD	% Recovery
Verapamil	5.0	5.15	0.1356	102.93
	10.0	9.96	0.5256	99.64
	15.0	14.98	0.4545	99.86
Diclofenac Sodium	5.0	5.02	0.3984	100.45
	10.0	9.92	1.1057	99.22
	15.0	14.85	0.6832	98.99
Flurbiprofen	5.0	5.00	0.2703	100.03
	10.0	9.94	0.8667	99.39
	15.0	15.06	1.1426	100.37
Ibuprofen	5.0	5.00	0.2202	99.92
	10.0	9.87	1.8885	98.68
	15.0	14.85	1.8757	98.97
Mefanamic acid	5.0	5.05	0.6185	100.99
	10.0	9.99	0.2080	99.85
	15.0	15.48	0.3657	103.23
Meloxicam	5.0	5.00	0.2048	99.96
	10.0	10.00	0.0223	100.02
	15.0	14.91	0.5810	99.40

Table 5. Limit of detection (LOD) and limit of quantification (LOQ) of Verapamil and different NSAIDs in raw material and serum

Drugs		LOD $\mu g\ mL^{-1}$	LOQ $\mu g\ mL^{-1}$
Verapamil	Raw material	0.023	0.069
	Serum	0.024	0.073
Diclofenac sodium	Raw material	0.053	0.10
	Serum	0.069	0.209
Flurbiprofen	Raw material	0.062	0.187
	Serum	0.046	0.141
Ibuprofen	Raw material	0.048	0.146
	Serum	0.038	0.117
Mefanamic acid	Raw material	0.067	0.204
	Serum	0.053	0.161
Meloxicam	Raw material	0.062	0.189
	Serum	0.035	0.107

Limit of Detection (LOD) and Quantitation (LOQ): LOD and LOQ for each standard were determined from the calibration curves, by using following formulas,

Table 4. Intraday precision of verapamil and NSAIDs

Drugs	Conc. Injected ($\mu g\ mL^{-1}$)	Day 1 %RSD	Day 2 %RSD	Day 3 %RSD	Day 4 %RSD	Average %RSD
Verapamil	5	0.1557	0.1970	0.0516	1.6729	0.519
	10	0.0486	0.1009	0.4460	0.0037	0.150
	15	0.0008	0.0147	0.0046	0.0887	0.027
	20	0.0243	0.0176	0.1657	0.2282	0.109
	25	0.1267	0.2609	0.0095	0.0755	0.118
Diclofenac sodium	5	0.748	0.3045	0.1966	0.0192	0.317
	10	0.1616	0.0871	0.1731	0.076	0.124
	15	0.0669	0.1079	0.0477	0.2484	0.118
	20	0.0597	0.2237	0.5878	0.1439	0.254
	25	0.0459	0.0933	0.1779	0.0964	0.103
Flurbiprofen	5	1.5872	0.2239	0.2076	0.6289	0.662
	10	0.1172	0.3328	0.1695	0.7464	0.341
	15	0.407	0.0907	0.2081	0.0479	0.188
	20	0.1568	0.0119	0.4612	0.7611	0.348
	25	0.0108	0.2166	0.4872	1.0864	0.450
Ibuprofen	5	0.3681	0.0855	0.2894	0.3907	0.283
	10	0.306	0.0937	0.0059	0.0959	0.125
	15	0.1091	0.0243	0.4900	0.0297	0.163
	20	0.216	0.5868	0.1986	0.1988	0.300
	25	0.0673	1.3848	0.4585	0.1729	0.521
Mefanamic acid	5	0.215	0.5784	0.7483	0.6002	0.535
	10	0.0154	0.4161	0.3836	0.5269	0.336
	15	0.0991	3.2568	0.0128	0.2817	0.913
	20	0.0852	0.2783	0.0395	0.0350	0.110
	25	1.3425	1.1397	1.1849	0.2536	0.980
Meloxicam	5	0.1325	2.0380	0.8305	0.1403	0.785
	10	0.2248	0.1596	1.3885	0.2021	0.494
	15	0.1656	0.0915	0.0745	0.1078	0.110
	20	0.1623	0.2383	0.3911	0.0739	0.216
	25	0.5058	0.0635	1.2998	0.0244	0.473

$$LOD = (3.3 \times \sigma)/m$$
$$LOQ = (10 \times \sigma)/m$$

Where σ the standard deviation of the response and m is the slope of the linear regression equation. The LOD & LOQ for verapamil and different quinolones in raw material and serum are given in Table 5.

Ruggedness & Robustness: Ruggedness of this method was evaluated in two different labs with two different instruments. Lab 1 was in the Research Institute of Pharmaceutical Sciences, Faculty of Pharmacy University of Karachi, while Lab 2 was in the Department of Chemistry, Faculty of Science, and University of Karachi. The method did not show any notable deviations in results from acceptable limits. Robustness was evaluated by slight changes in pH levels of mobile phase and it was found that the %R.S.D. values did not exceed more than 2%.

Conclusion

A rapid, precise, accurate, low cost and least time consuming RP-HPLC method for the simultaneous determination of verapamil and NSAIDs has been successfully developed which was also applied effectively on human serum. These studies were beneficial to determine the drug in therapeutic concentrations inside human body. Results are accurate and precise and are confirmed by the statistical parameters.

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