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PharmTech
International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.2, No.1, pp 682-688, Jan-Mar 2010

Rapid and Sensitive HPLC-UV method for Simultaneous Estimation of Nifedipine, Nateglinide and Lovastatin: Quantitative Application to Polypill Based Synthetic Ternary Mixture

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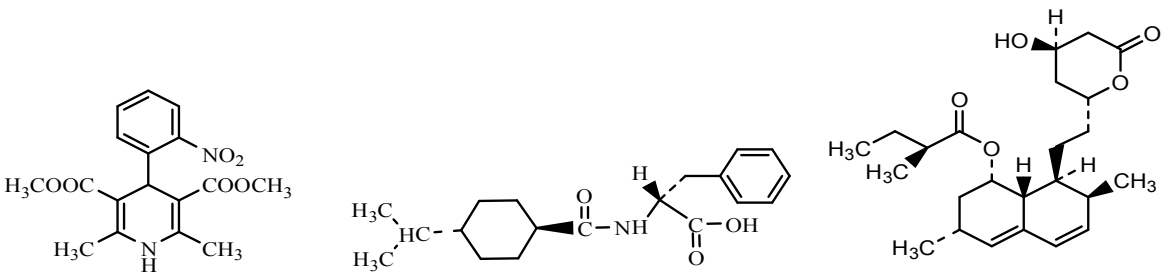
ABSTRACT: A rapid and sensitive RP-HPLC method has been developed for the purpose of analysis of antihypertensive: nifedipine (NF), antidiabetic: nateglinide (NG) and hypolipidemic: lovastatin (LT) drugs simultaneously in cardiovascular polypill based synthetic ternary mixture. Analysis was performed on C_{18} (125 × 4.6 mm id, 5-µm particle) column with acetonitrile-10 mM phosphate buffer (pH 3.5) 60:40 (v/v) as mobile phase, started at a flow rate of 1 mL min^{-1} continued for 4 min and further 6 min at a flow rate of 2 mL min^{-1}. UV detection was performed at 208 nm for NF, NG and at 236 nm for LT. The run time under these chromatographic conditions was less than 10 min. The method was linear in the range of 0.125-8.0 µg mL^{-1} for NF and 0.25-16.0 µg mL^{-1} for NG and LT. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. The sensitivity of the method, as the limits of detection (LOD) and quantification (LOQ) for each active ingredient was also determined. The validated method was successfully applied to the analysis of synthetic mixture of tablets of three drugs; the percentage recoveries obtained were 100.23% for NF, 100.35% for NG and 100.93% for LT.
Keywords: HPLC-UV, Lovastatin, Nateglinide, Nifedipine, Polypill

INTRODUCTION

Blood pressure, diabetes and LDL cholesterol are causal risk factors for cardiovascular diseases (CVD) and their combined effects make this disease common[1].A pill containing different active ingredients (polypill) to overcome these factors is more beneficial than the common pills with only one, in terms of cost and patient compliance. Nifedipine, nateglinide and lovastatin are commonly prescribed active ingredients for CVD.

Nifedipine (NF) [1,4-dihydro-2,6-dimethyl-4-(2-nitro-phenyl)-3,5-pyridinedicarboxylic acid dimethyl ester] **[Fig. 1a]** is the lead compound of the hydropyridine class of calcium-channel antagonists. It is widely used for the treatment of angina pectoris, hypertension and other vascular disorders such as Raynaud's phenomenon[2].

Nateglinide (NG) [N-(*trans*-4-isopropyl cyclohexyl carbonyl)-D-phenylalanine] **[Fig. 1b]** is a novel non-sulfonylurea oral antidiabetic agent used for the treatment of type II diabetes mellitus. NG works by stimulating the pancreas to release insulin by closing the ATP-dependent potassium channels in the β-cell membrane, which leads to opening of calcium channels. The resulting influx of calcium induces insulin secretion[3].



(a) **(b)** **(c)**

Fig.1: Chemical structure of (a) Nifedipine (NF); (b) Nateglinide (NG); (c) Lovastatin (LT)

Lovastatin (LT) (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-{2-[(2*R*,4*R*)-4-hydroxy-6-oxooxan-2-yl]ethyl}-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate **[Fig. 1c]** is a drug that inhibits the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-Co A) reductase, enzyme that participates in the endogenous cholesterol synthesis, supporting its clinical use in the treatment of hypercholesterolemia[4].

A thorough literature survey revealed that numerous analytical methods such as HPLC coupled to UV detection[5-13], electrochemical detection [14-16] or mass spectrometry[17-19], GC combined with various detectors [20, 21] have been reported for estimation of NF in formulations and biological fluids. Some methods reported for the estimation of NG include HPLC with UV detection [22-26] or mass spectrometry[27-31], micellar electrokinetic chromatography [32] and HPLC using a coumarin-type fluorescent reagent[33]. Analysis of LT in formulation and biological fluids has been performed by HPLC with UV detection[34-39], mass spectrometry[40,41], GC with mass spectrometry[42], micellar electrokinetic chromatography[43], supercritical fluid chromatography[44] , charged aerosol detection[45] and UPLC with mass spectrometry[46].

Polypill concept to reduce CVD by more than 80% was firstly given by Wald and Law [47] and has been applied to pharmaceutical preparations ("Polycap" by Cadila Pharmaceuticals, India; "Red Heart Pill" by Dr Reddy's laboratories, India) and several clinical trials (TIPS, Pill Pilot study-a phase II, double-blind, randomized trial) are going on[48]. Foreseeing the need of different analytical methods for estimation of ingredients of these pills, the present paper describes a rapid and sensitive HPLC-UV method for estimation of NF, NG, and LT in one of such pill.

EXPERIMENTAL

Materials and Reagents

Pure analytical standards of NF (99.6%), NG (99.9%) and LT (99.7%) were kindly provided by Unichem Pvt. Ltd. (Goa, India). Market tablet samples of NF (Nifedine-10, Nicholas Piramal Ltd.,Mumbai, India), NG (Glinate-60, Glenmark Pharmaceuticals Ltd., Mumbai, India) and LT (Lostatin-10, Dr Reddy's laboratories, Hyderabad, India) were procured from the retail pharmacy. Potassium dihydrogen phosphate (KH_2PO_4) and 85% ortho-phosphoric acid (H_3PO_4) of analytical reagent grade were purchased from S.D. Fine Chem. Ltd. (India). HPLC grade methanol, acetonitrile (ACN) was purchased from same supplier. Water was deionized and purified by a Milli Q water purification system (Millipore, India).

Instrumentation

The HPLC system (Shimadzu, Tokyo, Japan) consisting of pump LC 10AT, a manual rheodyne injector with a 20-µL sample loop and a variable length UV-visible detector SPD 10A was used for entire analysis. Analysis was performed on a Millennium C_{18} of 125 × 4.6 mm id, 5-µm particle (India) analytical column and the guard column with same phase at ambient temperature. Chromatographic data were recorded and processed using a Spinchrom Chromatographic Station®CFR Version 1.7 (Spinchrom Pvt. Ltd., Chennai, India).

Preparation of Calibration Standards and Quality Control (QC) Solutions

Individual stock standards (1.0 mg mL^{-1} concentration) of NF, LT were prepared in methanol and that of NG in ACN. Working standard solutions of individuals and mixture were prepared by dilution with mobile phase over a concentration range of 0.125-8.0 µg mL^{-1} for NF, 0.25-16.0 µg mL^{-1} for NG and LT. Final QC concentrations of NF were 0.25, 2.0, 4.0 µg mL^{-1} and of NT, LT were 0.5, 4.0, 16.0 µg mL^{-1}. All standards were stored at 2-8°C, found to be stable during the period of study and were brought to room temperature before use.

Preparation of Sample Solutions

20 tablets of each drug were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 10 mg each of NF, NG and LT was transferred separately into 10 mL volumetric flasks containing 5 mL of methanol,

acetonitrile and methanol, respectively, sonicated for 15 min and diluted to 10 mL with same solvent. The resulting solutions were centrifuged at 2000 rpm for 10 min. Clear supernatant (1mL) containing suitable amount of drug was taken from the above filtered solutions and diluted up to10 mL with the mobile phase (100 µg mL^{-1}). Ternary mixture was prepared by mixing solutions of NF, NG and LT in ratio of 1:2:2.

Optimization of Chromatographic Conditions

In order to select optimum conditions for the simultaneous isocratic elution of the three components, a set of preliminary trials were conducted with NF, NG and LT. Parameters such as detection wavelength, different combinations of different organic solvents and buffers at various pH and compositions, and flow rate were studied. Feasibility of various mobile phase compositions such as ACN and methanol using buffer (KH_2PO_4) with various concentrations and pH range of 3.0-4.5, along with altered flow rates (in the range of 1.0-2.5 mL min^{-1}) was tested for complete resolution of NF, NG and LT. Rapidity of the method was further checked with two C_{18} columns viz., Luna Su C_{18} (250 x 4.6 mm id, 5µm, Phenomenex, USA) and Sil C_{18} (125 x 4.6 mm id, 5µm, Millennium, India). It was found that chromatographic resolution, selectivity, sensitivity and rapidity were good with Millennium Sil C_{18} column. For quantification purpose individual dilutions of NF, NT and LT in same linear range as that of mixture were used as external standards.

Validation Procedure

Validation of the developed and optimized HPLC method was carried out in the light of ICH Guidelines [49, 50] with respect to validation parameters-linearity, specificity, selectivity, Limits of detection (LoD) and quantification (LoQ), Intra and inter-day precision, accuracy.

RESULTS AND DISCUSSION

Method Development

The UV spectra of NF, NG, and LT in mobile phase show they have absorption maxima at 208, 205, and 236 nm, respectively. The chromatographic peaks obtained for NF and NG at 208 nm are more intense than those obtained at 236 nm. Because an intense peak is obtained for LT only at 236 nm, HPLC analysis for simultaneous determination of the active components was performed at two wavelengths, 208 nm was set for quantification of NF, NG and 236 nm for LT.

The chromatographic separation was best carried out on reversed phase Millennium C_{18} (125 x 4.6 mm id, 5µm) column. Various mobile phase (ACN:PBS) compositions at different pH were studied for the simultaneous isocratic elution of the three components, finally acetonitrile and 10 mM KH_2PO_4 buffer solution (PBS) (adjusted to pH 3.5 with 10% H_3PO_4) (60:40, v/v) found to provide adequate peak separation. Analysis of the mixture at a flow rate of 1 mL min^{-1} was taken a long time of 14 min and at a flow rate of 2 mL min^{-1} inadequate separation of peaks was found. For achievement of shorter run time and good resolution flow rate was programmed (started at 1 mL min^{-1}, continued for 4 min and a further 6 min at a flow rate of 2 mL min^{-1}) and as a result, all the components were eluted in less than 10 min.

Method Validation

Calibration Curve

Calibration plots were constructed by plotting the peak area (y) versus analyte concentration (x) in µg mL^{-1}, with a weighting factor $1/C^2$. Plots were generated by replicate analysis (n =3) at seven concentration levels and the linear regression equations were calculated using the least square method within Microsoft Excel® program. The curves followed Beer's law in the range 0.125-8.0 µg mL^{-1} for NF and 0.25-16.0 µg mL^{-1} for NG and LT **[Table1]**. The linearity of the method was good; the values obtained for the correlation coefficients were 0.9998 for NF, 0.9986 for NG, and 0.9997 for LT.

Specificity and Selectivity

The specificity and selectivity of the method was determined by comparing the chromatograms obtained from the samples containing NF, NG and LT in mixture with those obtained from individuals. Adequate chromatographic separation was obtained using the method described above. **Fig.2**

Precision and Accuracy

An acceptable intra- and inter-day assessment was reached when the mean calculated accuracy at each standard concentration was 100 ± 5%, the theoretical value and precision was not to exceed 5%[50]. For assessment of intra-day variability standard solutions were analyzed within one day (morning, afternoon and evening) and among nine days for inter-day variability. Intra- and inter-day data are summarized in **Table [2]** for NF, NG and LT enables the conclusion that the high precision and excellent accuracy was obtained. The precision and accuracy were given in terms of relative standard deviations (RSD) and percent of amount found of analyte to that of actual, respectively.

Sensitivity

Limits of detection *(LoD)* and quantification *(LoQ)* represent the concentration of the analyte that would yield signal-to-noise ratios of 3:1 for *LoD* and 10:1 for *LoQ*.[51] *LoDs* were 11.81 ng mL^{-1} for NF, 4.89 ng mL^{-1} for NG and 18.24 ng mL^{-1} for LT. *LoQ*s were 39.37 ng

mL^{-1} for NF, 16.32 ng mL^{-1} for NG and 60.79 ng mL^{-1} for LT. Under these chromatographic conditions sensitivity was best for NG.

System Suitability

A system suitability test according to USP was performed on the chromatograms obtained from standard and test solutions to check different above mentioned parameters and the results obtained from six replicate injections of the standard solution are summarized in the **Table [3]**.

Method Application

The validated HPLC method was successfully applied for simultaneous determination of NF, NG and LT in their proposed combined dosage form. The mean assay results, expressed as a percentage of the label claim, are shown in **[Table 4]** and indicate satisfactory accuracy and precision of the method. Tablet excipients did not interfere with the assay.

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CONCLUSIONS

The assay developed is specific and reproducible for the quantitative determination of NF, NG and LT simultaneously with good resolution in short run time (10 min) and high sensitivity. The flow rate programming of isocratic mobile phase, cut down on overall time of sample analysis and thereby made the method more cost effective and rapid. Detection of analyte at their maximal absorbance wavelength made the method more sensitive. The proposed method is simple, relatively rapid, and sufficiently precise for routine analysis of the active ingredients (NF, NG and LT) in bulk and combined dosage forms

Table 1: Spectral and statistical data for determination of NF, NG and LT by proposed HPLC method

Parameters	NF	NG	LT
UV wavelength (nm)	208	208	236
Linearity range (µg mL^{-1})	0.125-8.0	0.25-16	0.25-16
Correlation coefficient (r^2)	0.9998	0.9986	0.9997
Regression equation	0.1325+35.447x	0.2325+9.193x	1.0749+23.029x
Slope (b)	35.447	9.193	23.029
Intercept (a)	0.1325	0.2325	1.0749
Limit of detection, LoD (ng mL^{-1})	11.81	4.89	18.24
Limit of quantitation, LoQ (ng mL^{-1})	39.37	16.32	60.79

** $y = mx + b$, where, y = peak response, m = slope, concentration (µg mL^{-1}), b = intercept.*

Table 2: Intra- and inter-day precision and accuracy data of the quantitation of NF, NG and LT

Active ingredient	Individual			Mixture		
	SD	% RSD	% Accuracy	SD	% RSD	% Accuracy
Intra-day variation (n = 3)						
Nifedipine	0.1026	1.41	99.44	0.0163	1.10	99.99
Nateglinide	0.1103	1.38	99.05	0.0348	1.42	99.25
Lovastatin	0.0418	1.12	98.98	0.0451	1.28	98.93
Inter-day variation (n = 3)						
Nifedipine	0.0173	1.33	99.87	0.0276	1.21	99.73
Nateglinide	0.0621	1.32	99.75	0.0165	1.53	98.73
Lovastatin	0.0361	1.09	99.63	0.083	1.30	99.42

Table 3: System suitability parameters

Parameters	NF	NG	LT
Retention time, R_t (min)	3.12	4.45	7.23
Area (mv-s)	35.42	18.53	44.89
Capacity factor (k')	2.11	3.45	6.22
Theoretical Plates	4461	10980	11291
HETP (h, mm)	0.0282	0.0114	0.0111
Resolution (R_s)	2.09	7.51	12.54
Asymmetry (A_s)	1.33	1.57	1.39

All the results are average of six determinations

Table 4: Determination of NF, NG and LT in proposed pharmaceutical preparation by developed method

Tablet mixture	Labeled Amount (mg)	Found Amount (mg)[a]	% Recovery [a]
Nifedine-10	10	9.98±0.17	99.83±0.53
Glinate-60	60	59.78±0.26	99.64 ± 1.34
Lostatin-10	10	9.97±0.21	99.78 ± 1.03

[a] *Mean value ± standard deviation of six determinations*

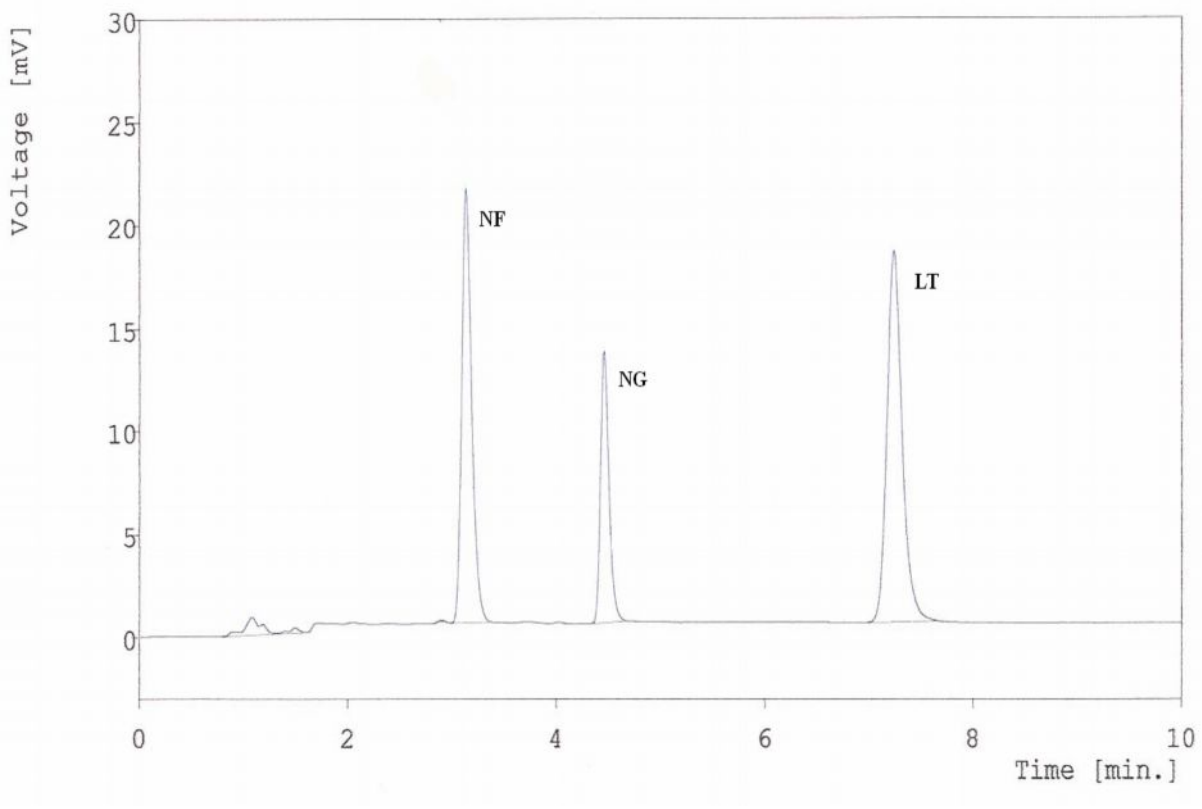

Fig.2: Typical chromatogram of standard solution of NF, NG and LT.

ACKOWLEDGEMENT
The author would like to thank Dr. R.C. Gupta, Emeritus scientist, Central Drug Research Institute, Lucknow, India for valuable guidance.

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