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Development of a Sensitive HPLC Method for Determination of Plasma Levels of Nifedipine

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As part of an ongoing programme on the development of sustained release formulation of nifedipine (20 mg tablet) and determination of bioequivalence, a specific, sensitive and simple HPLC method with UV detection for estimation of nanogram levels of nifedipine in plasma has been developed. Nitrendipine is used as an internal standard. With a simple one-step extraction of nifedipine from plasma, the method has a linearity range of 6 to 200 ng/ml and an average recovery of 77.48%. Nifedipine plasma concentration versus time profile is presented for the sustained release formulation under development (formulation T) and compared with the standard formulation (formulation S).

Nifedipine, a dihydropyridine class of drug, is a widely used calcium channel blocker in the treatment of hypertension and angina pectoris due to its coronary vasodilatory effect[1,2]. It is administered at very low doses (maximum daily dose of 180 mg and minimum therapeutic concentration of 10 to 20 ng/ml)[3]. Therefore it is very important to assure that the minimum therapeutic concentration is maintained in the body over a suitable period. In case of sustained release formulations maintenance of therapeutic levels of drug in the body is very critical for safety and efficacy of such dosage form. To determine the lower nanogram levels of nifedipine in plasma, a sensitive, precise and accurate analytical method is required.

Several analytical methods such as spectrofluorimetry[4], GC[5-7] and HPLC[8-12], involving various sample preparation methods (extraction procedures), for quantitation of nifedipine in biological fluids (mainly plasma and serum) have been reported. As a part of an ongoing programme on the development of sustained release formulation of nifedipine (20 mg tablet), a simple, rapid, specific and sensitive HPLC method with UV detection for determination of plasma levels of nifedipine has been developed and is described here. Nitrendipine is used as an internal standard.

EXPERIMENTAL

Analytically pure samples of nifedipine and nitrendipine were gifted by Torrent Pharmaceuticals Ltd. Methanol, acetonitrile and n-hexane (HPLC grade, S.D. Fine Chem.), dichloromethane (Extra pure, S.D. Fine Chem.), disodium hydrogen phosphate (AR, S.D. Fine Chem.), sodium hydroxide (LR, S.D. Fine Chem.) and triple glass distilled water filtered through 0.45 µm filter were used.

Chromatographic conditions

The chromatographic system consisted of a Spectraphysics HPLC system with an isocratic solvent delivery system (Model Spectraphysics 8810), a universal injector, Rheodyne 7125 model with 100 µl loop and a UV/VIS detector (Spectra 100 multiwavelength UV/VIS detector). Ultraviolet absorption was measured at 238 nm. Integration of data was performed using Spectraphysics 4270 integrator model (AUFS = 0.02; PT = 150; CS = 0.25 cm/min; attenuation=16). Forty microlitres of sample was injected into the analytical

column. The analytical column used was ET 250/4 Nucleosil® 100-5 C18 (5µm; 25 cm X 4 mm) supplied by Macherey Nagel, Germany. [A guard column filled with C18 (Perkin Elmer) was also used].

The mobile phase was prepared by mixing 0.05 M disodium hydrogen phosphate buffer (pH 3, adjusted with 50% v/v phosphoric acid), methanol and acetonitrile in the proportion of 37, 40 and 23% v/v (final pH of the mobile phase is about 6). It was filtered through a 0.45 µm cellulose nitrate filter and was degassed by ultrasonication. The mobile phase was passed through the column at the rate of 0.8 ml/min.

Preparation of the solutions: Standard stock solutions (1 mg/ml) of nifedipine and nitrendipine were prepared in methanol. They were further diluted with methanol to obtain the concentration of 10 µg/ml. Standard aqueous solution of nifedipine (1 µg/ml) was also prepared by diluting the stock solution with water. Nitrendipine was used as an internal standard. Twenty microlitres of nitrendipine solution (10 µg/ml) was added to 1 ml of sample (untreated plasma) separately. A mixture of dicholoromethane:n-hexane (3:7) was used as an extraction solvent to extract nifedipine and nitrendipine from the plasma.

Plasma spiking studies

A definite volume (6, 10, 20, 50, 100 and 200 µl) of standard aqueous solution of nifedipine (1 µg/ml) was spiked into individual pyrex glass test tubes containing 1 ml of untreated human plasma and was shaken for 10 seconds on a vortex mixer. It was allowed to stand for 5 min, 50 µl of 1 M NaOH solution was added to each test tube and mixed by vortexing for 10 seconds (plasma pH about 12). Twenty microlitres of nitrendipine solution (10 µg/ml) was added to individual test tubes and mixed well. The plasma was treated with 5 ml of the extraction solvent and agitated by vortexing for 1 min. The organic layer was separated by centrifugation (2000 rpm, 10 min). The separated organic layer was transferred into amber colored vial and evaporated to dryness on a water bath at 60° under a gentle stream of nitrogen. The residue after evaporation was reconsituted with 100 µl of mobile phase. Forty microlitres of the resulting solution was injected into the HPLC system.

Above procedure was carried out under red light to avoid photodegradation of nifedipine and nitrendipine. The tubes were also covered with aluminum foil to protect the samples from light. The calibration curve was prepared by plotting the peak height ratios (ratio of the height of peak corresponding to nifedipine to the height of peak corresponding to nitrendipine) against the concentration of nifedipine (6, 10, 20, 50, 100 and 200 ng/ml of plasma).

Recovery Studies

Drug free plasma samples spiked with nifedipine in the range 6-200 ng/ml were subjected to the procedure described above and the peak heights for nifedipine were compared to the peak height obtained from its corresponding standard solution, containing the internal standard (IS) prepared in mobile phase.

In vivo evaluation of the SR formulations of nifedipine

A preliminary *in vivo* (bioequivalence) study for the formulation under development (Formulation T) and reference formulation (Formulation S) was carried out. Formulation T and Formulation S were SR tablets containing 20 mg of nifedipine. Three healthy male volunteers aged 20 to 25 years weighing 50 to 55 kg participated in the study. The written informed consent was taken from all volunteers and the study protocol was approved by the local ethical committee. None of the volunteers received any other drug one week prior to study and during the study. The volunteers were fasted overnight and fasting was continued until 4 h post dose, but water intake was not restricted. Each volunteer received single dose of Formulation T on the day one of the study and after a wash-out period of eight days, each of them received Formulation S. Blood samples were withdrawn before administration of the tablet and at 1, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h after the administration. The samples were collected in tubes containing an anticoagulant (CPDA solution) and centrifuged (3500 rpm, 20 min) to separate plasma. Separated plasma samples were then stored at-20° until analysis and were protected from exposure to light. These samples were then subjected to analysis as described earlier.

RESULTS AND DISCUSSION

Fluorimetric analysis of samples of biological origin has the limitation that the endogenous components may interfere during the analysis and hamper specificity of the method. Inspite of various GC methods reported in the

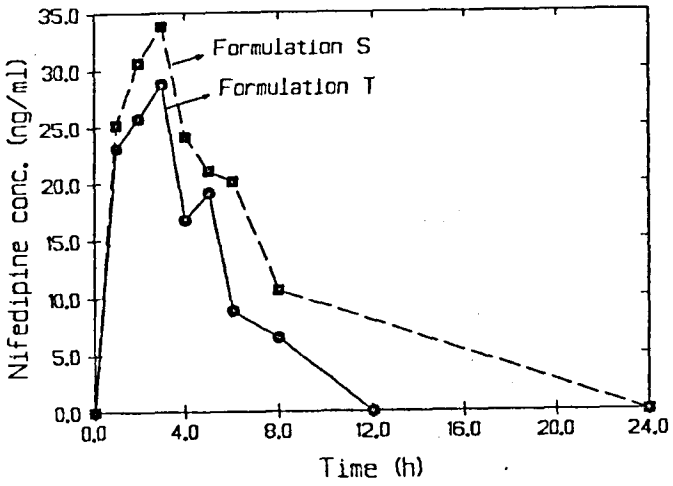

Fig. 1 : Plasma Concentration-time plot Average plasma concentration (ng/ml) v/s time (h) profiles after administration of sustained ralease tablet formulations (20 mg) of nifedipine (FormulationT and Formulation S) to three healthy human volunteers

literature for estimation of plasma concentrations of nifedipine, the controversy regarding the thermostability of nifedipine at higher temperature conditions exists[4]. HPLC method was selected owing to its attributes like simplicity, versatility, specificity, sensitivity, reliability and reproducibility. It also provides rapid analysis with minimum amount of sample.

Nifedipine shows very high protein binding (92-98%)[3,13]. Therefore, extraction procedure remains quite crucial to obtain good recoveries. Out of the various extraction methods reported, the method reported by Kleinbloesem et al[8] seemed to be rapid, having minimum sample preparation steps and is suitable for photosensitive drugs. The solvents like toluene having very high boiling point were rejected. Ethyl acetate and diethylether were not suitable as they show lesser extraction capability for single extraction and inadequate clean-up of the plasma. The dichloromethane:n-pentane (3:7) mixture suggested by Kleinbloesem et al., was replaced by dichloromethane:n-hexane (3:7) mixture due to higher cost of n-pentane. The plasma was alkalinized to a pH of about 12 with 1 M NaOH to achieve maximum recovery (to release the protein-bound drug). In alkaline conditions the drug and internal standard remain in the free form and get extracted in the organic phase. Addition of higher amount of NaOH led to formation of emulsion on vortex mixing. Thus, a simple single step extraction procedure was developed for the extraction of drug and nitrendipine from alkalinized plasma (pH 12) with 5 ml dichloromethane:n-hexane (3:7) mixture.

An internal standard was, added to determine any losses that may occur during extraction. Nitrendipine was selected as an internal standard due to its close similarity with the structure of the drug of interest and certain physicochemical properties. RP-HPLC on an octadecyl column was a successful approach to achieve well resolved peaks of nifedipine and nitrendipine. The column with 5 μm particle size gave better resolution and increased peak response to detect nifedipine in lower concentration range.

The mobile phase containing methanol : 0.05 M disodium hydrogen phosphate (pH 3) butter (70:30), failed to resolve nifedipine from endogenous components present in plasma samples. Based on the eluotropic strength of this mobile phase a ternary mixture of methanol, acetonitrile and 0.05 M disodium hydrogen phosphate (pH 3) buffer (40:23:37) was tried [Elutropic strengths; MeOH (5.5); CN (7.3); Water (or buffer solutions) (0.0)][14]. The higher salt concentration and lower pH of the buffer assisted to obtain sharper peaks and hence, to increase the chromatographic efficiency. This eluent showed good resolution of nifedipine and nitrendipine. It was found that nifedipine and the IS were well separated from the interfering endogenous plasma components in this eluent at a flow rate of 0.8 ml/min. The detection was carried out at 238 nm, the wavelength maxima of nifedipine in the mobile phase. The retention times for nifedipine and nitrendipine were about 7.8 min and 13.3 min, respectively.

The least square linear regression evaluation of the peak height ratio (y) versus concentration (x) obtained by assaying plasma samples spiked with nifedipine (6, 10, 20, 50, 100 and 200 ng/ml) and nitrendipine (200 ng/ml in each sample) gave equation of the straight line "x=143.12 y - 5.3116", with a correlation coefficient of 0.9995. Similarly solutions containing known quantity of nifedipine and nitrendipine prepared in mobile phase were assayed. The least square linear regression evalution of peak height ratio (y) versus concentration (x) gave equation of the line "x 161.14 y - 4.5573" and the correlation coefficient was 0.9998. The linearity range in both the cases was found to be 6 to 200 ng/ml for nifedipine (Table 1). The limit of quantitation and minimum detect-

Table 1 - Determination of Calibration Range

S.No.	Nifedipine (ng/ml)	Drug+I.S. in Mobile Phase		Drug+I.S. in Plasma	
		Height ratio Mean ± S.D. (n=4)	S.E.M.	Height ratio Mean ± S.D. (n=4)	S.E.M.
1.	6	0.0634 ± 0.0090	0.00450	0.0613 ± 0.0083	0.00415
2.	10	0.0872 ± 0.0065	0.00325	0.1222 ± 0.0175	0.00875
3.	20	0.1497 ± 0.0078	0.00390	0.1857 ± 0.0159	0.00795
4.	50	0.3401 ± 0.0130	0.00650	0.4038 ± 0.0229	0.01145
5.	100	0.6622 ± 0.0303	0.01515	0.7685 ± 0.1073	0.05865
6.	200	1.2628 ± 0.0549	0.02745	1.4412 ± 0.1020	0.05100

Peak height ratios (Drug : IS) obtained for different concentrations of nifedipine in the standard solutions prepared in mobile phase and spiked in plasma (6-200 ng/ml) using nitrendipine as an Internal Standard (200 ng/ml) by the proposed HPLC method

Table 2 - Precision of the developed HPLC method

S.No.	Nifedipine (ng/ml)	Nifedipine spiked in plasma % C.V. (n=4)		Nifedipine in mobile phase % C.V. (n=4)	
		Interday	Intraday	Interday	Intraday
1.	6	14.32	5.0387	14.06	1.1055
2.	10	15.58	4.3010	7.45	0.8560
3.	20	8.59	3.7389	5.21	5.793
4.	50	5.67	4.8478	3.80	2.5207
5.	100	13.96	2.7096	4.87	1.3284
6.	200	7.08	0.8992	4.35	1.7278

Interday and intraday precision for the peak height ratios (nifedipine : nitrendipine) determined by proposed HPLC method for different concentrations of nifedipine on four days in a week and for four times on the same day, respectively.

able concentration of nifedipine were found to be 6 ng/ml and 3 ng/ml of plasma respectively.

The method was validated by determining reproducibility and accuracy for four spiked plasma samples (n=4) with respect to calibration curve. Intra-day (intra-assay) coefficients of variation and the day-to-day (inter-assay) coefficients of variation for analysis of plasma samples on the same day and on four days over a period of one week varied from 0.8992% to 5.0387% and 5.67% to 15.58% (n=4), respectively (Table 2).

The accuracy of the method given as mean percent deviation of all concentations from the theoretical value i.e. percent bias, averaged from 0.476 to 21.77%. The overall average recovery was found to be 77.48% for nifedipine (Table 3).

Table 3 - Recovery of Nifedipine Spiked in Plasma

S.No.	Nifedipine (ng/ml)	Mean % Recovery (n=4)
1.	6	64.54
2.	10	95.66
3.	20	81.54
4.	50	76.93
5.	100	72.80
6.	200	73.49
Average% recovery		77.48

Extraction efficiency of the sample preparation method for extraction of nifedipine from plasma

The selectivity of the method was ascertained by the fact that no endogenous plasma components interfere with the drug peak.

The plasma concentration of nifedipine versus time curves are displayed in Fig. 1. From these curves it is evident that formulation T releases nifedipine in the therapeutic range over a period of about 6 h while formulations S shows better sustained release to maintain the therapeutic concentration for about 8 h. The maximum concentration achieved after administration of formulation T and formulation S (C_{max}) were found to be 28.7 ng/ml and 33.8 ng/ml respectively. The time to reach maximum concentration (T_{max}) was found to be 3 h for both the formulations.

The applicability of this HPLC method is, thus, established by the preliminary *in vivo* study for sustained release formulation of nifedipine and hence, the proposed simple, sensitive, specific, accurate and precise method can be used in the determination of nifedipine in biological fluids and bioavailability and bioequivalence studies of various nifedipine formulations.

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