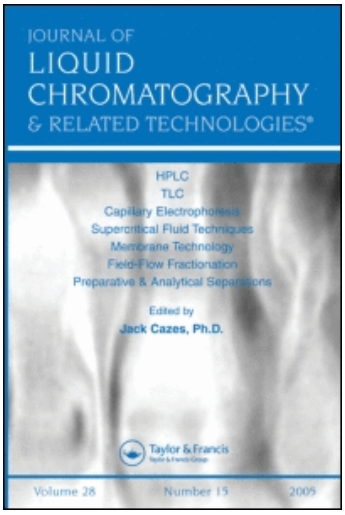

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Application of an HPTLC Densitometric Method for Quantification and Identification of Nifedipine

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To cite this Article Kowalczuk, D. , Wawrzycka, M. B. and Maj, Agnieszka Haratym(2006) 'Application of an HPTLC Densitometric Method for Quantification and Identification of Nifedipine', Journal of Liquid Chromatography & Related Technologies, 29: 19, 2863 — 2873
To link to this Article: DOI: 10.1080/10826070600960987
URL: http://dx.doi.org/10.1080/10826070600960987

Journal of Liquid Chromatography & Related Technologies®, 29: 2863–2873, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600960987

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Abstract: A high performance thin layer chromatographic method coupled with densitometric analysis has been developed for determination of nifedipine in bulk powder and their pharmaceutical dosage forms. The active substance was extracted from tablets with methanol (mean recovery: 100.4%) and chromatographed on silica gel 60 F_{254} HPTLC plates in horizontal chambers with n-hexane–ethyl acetate–acetone, 6:3:2 (*v*/*v*), as mobile phase. UV densitometric quantitation was performed at 335 nm. The calibration curve was constructed in the range from 0.025 to 0.150 $\mu g\ \mu L^{-1}$ (corresponding to 0.5–3.0 $\mu g\ spot^{-1}$) with good correlation ($r \geq 0.990$) and expressed by a second order calibration function. Determination of nifedipine in tablets was characterized by good precision ($2.69\% < RSD < 7.27\%$) and accuracy ($-3.21 < RSE < 1.12$). The HPTLC-densitometric method was successfully applied for the identification of nifedipine in the presence of its induced degradation products, in the presence of the other calcium channel blockers, and for the identification of this drug in biological matrix.

Keywords: Densitometric, HPTLC, Quantification, Identification, Nifedipine

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INTRODUCTION

Nifedipine is 3,5-pyridinedicarboxylic acid, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-, dimethyl ester belonging to a group of drugs that are termed calcium channel blockers. These drugs affect the movement of calcium into heart and blood vessel cells, and cause a relaxing effect of the muscles to allow an increased amount of blood flow into the heart. Many of these calcium channel blocking agents are used to treat angina pectoris, and they are also used to help reduce blood pressure (antihypertensives).

Chromatographic methods available for determination of nifedipine involve mostly high performance liquid chromatography. The HPLC methods have been developed for analysis of nifedipine in biological material[1–3] and in pharmaceutical preparations.[4–7] This procedure has been applied for the quantification of an individual drug,[4,5] photodegradation products,[4] or for simultaneous determination of nifedipine and other compounds, for example atenolol[6] and acebutolol.[7] Authors of the latter paper have also determined the named drugs by using UV–derivative spectroscopy and capillary gas chromatography. As concerns HPLC, the literature on pharmaceutical analysis of this drug by thin-layer chromatography is poor. One paper reports the application of a TLC method for determination of nifedipine in bulk drugs and pharmaceuticals.[8]

This report suggests the use of an HPTLC method coupled with densitometry for quantification of nifedipine in commercial tablets and extension of the method for identification of nifedipine in the biological matrix. The subsequent purpose of this work was the identification of nifedipine in the presence of its induced degradation products and in the presence of the other calcium channel blockers.

EXPERIMENTAL

Chemicals, Reagents, and Samples

Nifedipine, nitrendipine, nicardipine hydrochloride, and nimodipine pure substances were purchased from Sigma (St. Louis, USA). Cordafen tablets containing 10 mg of nifedipine per tablet were obtained commercially. Methanol, ethyl acetate, acetone, n-hexane, and dichloromethane from Merck (Darmstadt, Germany) and sodium hydroxide, hydrochloric acid, 30% hydrogen peroxide from POCh (Gliwice, Poland), were of analytical reagent grade. Drug-free human pooled serum was from ICN Biomedicals. Inc., lot no 2502 H. Human whole blood was obtained from the Gynaecological and Obstetric Hospital in Lublin.

Standard substances, tablets, and the prepared solutions were stored in the dark at 4°C. Serum samples were stored in the dark at −20°C. The analyses

were carried out in a dark room to avoid light induced decomposition of drug samples.

Standard Solutions for Separation Analysis

Stock solutions (1.0 mg mL^{-1}) of the compounds were prepared by dissolving 10.0 mg nicardipine hydrochloride, nifedipine, nimodipine, or nitrendipine pure substances in 10.0 mL methanol. Working standard solutions (0.1 mg mL^{-1}) of individual compounds were obtained by diluting the stock solutions 1:10 with methanol. A mixture of the drugs was obtained by transferring 1.0 mL of stock solution of each compound to a 10-mL volumetric flask and filled to volume with methanol (concentration of each substance was 0.1 mg mL^{-1}).

Standard Solution for Degradation Analysis

A standard solution (1.0 mg mL^{-1}) of nifedipine was prepared by dissolving 10.0 mg pure substance in 10.0 mL methanol.

Calibration Solutions

Calibration solutions containing concentrations of nifedipine from 0.025 to 0.150 mg mL^{-1} were prepared by appropriate diluting of nifedipine stock solutions (1.0 mg mL^{-1}) with methanol.

Tablet Sample

The average mass of 20 tablets of nifedipine was determined and the tablets were accurately ground to a fine powder.

An amount of power equivalent to 10 mg of nifedipine (approx. 103.17 mg) was placed in a 10 mL volumetric flask containing approximately 7 mL of methanol. The mixture was shaken mechanically for 20 min, diluted to volume with methanol, and filtered. An aliquot of 0.75 mL of the filtrate was transferred to a 10 mL volumetric flask and diluted to volume with methanol to give the resulting solution of nifedipine (0.075 mg mL^{-1}), which was used for chromatographic analysis.

Serum Standard Sample

An 0.1 mL of 1.8 μg mL^{-1} nifedipine solution, prepared by appropriately diluting its stock solution (1.8 mg mL^{-1}) with methanol, was made up to a volume of 3.0 mL with drug free human serum.

Extraction Procedure

To the serum sample was added 0.5 mL of 20% trichloroacetic acid, mixed, and centrifuged for 5 min at 10000 rpm. The aqueous phase was transferred to a funnel, 0.6 mL of 1 mol L^{-1} NaOH was added, and extracted with 8.0 mL of the organic solvent mixture hexane-dichloromethane (70:30, *v*/*v*) for 15 min. Next, the organic layer was evaporated to dryness under a stream of nitrogen. The residue of the sample was reconstituted in 0.18 mL of methanol.

Patient Sample

Human whole blood samples (about 7 mL) were centrifuged for 10 min at 5000 rpm, and 3.0 mL of the obtained serum were processed in the same manner as the extraction procedure for standard serum.

Degradation Procedure

To 1.0 mL of nifedipine standard solution (1.0 mg mL^{-1}) or tablet solution (1.0 mg mL^{-1}) was added 6.0 mL methanol, and these solutions were subjected to the photo- and UV-degradation by exposure to direct sunlight and UV radiation for 4 h.

The forced degradation in acidic, basic, and oxidative media were performed by adding, separately, 6.0 mL each of 1 mol L^{-1} HCl, 1 mol L^{-1} NaOH, and 3% H_2O_2 to 1.0 mL of nifedipine standard solution (1.0 mg mL^{-1}) or tablet solution (1.0 mg mL^{-1}), and shaking these mixtures for 1 h.

Before spotting on an HPTLC plate, the degraded solutions were diluted to a volume of 10 mL with methanol (0.1 mg mL^{-1}).

Chromatographic Procedure

For tablet analysis, the calibration solutions (20 μL, corresponding to 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 μg $spot^{-1}$) and six tablet solutions (20 μL, corresponding to 1.5 μg $spot^{-1}$) were spotted, alternately, as 5 mm bands (10 mm apart) on 200 × 100 mm silica gel 60 F_{254} HPTLC plates by means of a Desaga AS 30 applicator equipped with a 100 μL microsyringe (Hamilton, Switzerland).

The chromatogram was developed to a distance of 50 mm in an unsaturated horizontal DS chamber (Chromdes, Lublin, Poland) with n-hexane–ethyl acetate–acetone, 6:3:2 (*v*/*v*), as mobile phase. After development, the plate was dried at room temperature and subjected to densitometric analysis.

For separation analysis, solutions of the calcium channel blockers (20 μL, corresponding to 3.0 μg $spot^{-1}$) were spotted as 5 mm bands (10 mm apart)

on 100 × 200 mm silica gel 60 F_{254} HPTLC plates and the chromatogram was developed to a distance of 100 mm with the mobile phase.

For the degradation test, the reference standard solution of nifedipine (20 μL, corresponding to 2.0 μg spot^{-1}), the degraded standard solutions, and degraded tablet solutions (20 μL) were spotted as 5 mm bands (10 mm apart) on 200 × 100 mm silica gel 60 F_{254} HPTLC plates and the chromatogram was developed to a distance of 50 mm with the mobile phase.

For biological analysis, serum standard samples of nifedipine (90 μL, corresponding to 90 ng spot^{-1}) and patient samples (90 μL) were spotted as 5 mm bands (10 mm apart) on 100 × 200 mm silica gel 60 F_{254} HPTLC plates and the chromatogram was developed to a distance of 50 mm with the mobile phase.

The analysed compounds on the silica gel layer were visualised under UV irradiation and identified densitometrically at $\lambda = 335$ nm.

Densitometric Procedure

Densitometric evaluation was performed with a Desaga CD 60 densitometer (Heidelberg, Germany) controlled by means of Desaga ProQuant software. The chromatograms were scanned at $\lambda = 335$ nm with slit dimensions of 0.2 × 4.0 mm.

Analyses of standard and sample solutions were repeated three times in the same day and five times on five consecutive days. The amount of the compound chromatographed was determined from changes in the intensity of diffusely reflected light. The mean peak areas were calculated and regression analysis on standard data was performed. The concentration was calculated from the calibration curve after multiplying by the dilution factor.

RESULTS AND DISCUSSION

Chromatographic separation of nitrendipine, nicardipine, nitrendipine, and nimodipine, structural analogues belonging to a class of pharmacological agents named the calcium channel blockers, was performed in a normal-phase high performance thin layer chromatographic (NP-HPTLC) system using a horizontal technique and the mixture of n-hexane–ethyl acetate–acetone, 6:3:2 (v/v) as the optimum mobile phase.

Figure 1 presents the separation of the drug mixture after chromatography with this mobile phase. The calcium channel blockers in the mixture were identified by comparing the retention of the individual compounds with those of the respective standards.

Under these conditions, the chromatographed compounds were eluted as well shaped, symmetrical spots with hR_F values of 47.10, 56.46, 61.00, and 64.50 for nicardipine, nitrendipine, nimodipine, and nitrendipine, respectively.

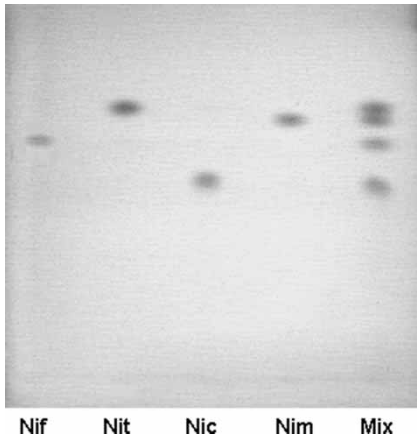

Figure 1. Chromatogram presenting of separation of the mixture (Mix) of nifedipine (Nif), nitrendipine (Nit), nicardipine (Nic), and nimodipine (Nim) on a silica gel HPTLC plate with n-hexane–ethyl acetate–acetone, 6:3:2 (v/v).

The obtained hR_F values were characterized by good repeatability (RSDs; 0.21% for Nic, 0.80% for Nif, 0.58% for Nim and 0.78% for Nit, n = 3).

For assessing specificity of the NP-HPTLC-densitometric method, the standard and tablet samples of nifedipine were deliberately degraded and the appearance of other spots representing degraded products of these samples was observed. The resulting chromatogram under stressed conditions such as acidic−, alkaline-, oxidative-medium, sunlight, and UV irradiation are shown in Figure 2. It can be observed, that other spots appear with the

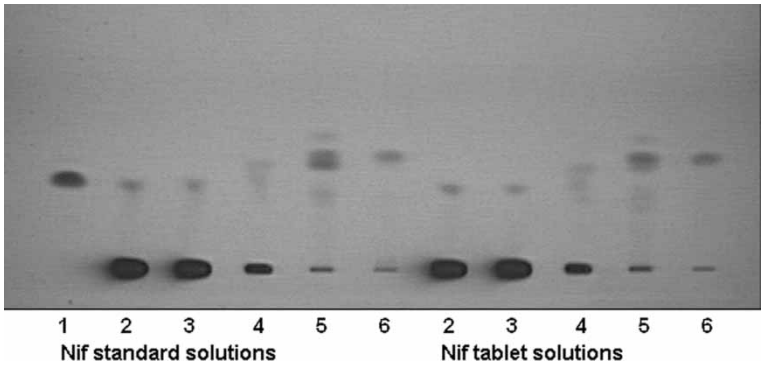

Figure 2. Specificity for unknown degradation products of nifedipine standard and tablet solutions; 1-untreated nifedipine, 2-Nif degraded by base, 3-Nif degraded by acid, 4-Nif degraded by hydrogen peroxide, 5-Nif degraded by sunlight, 6-Nif degraded by UV-light.

nifedipine sample degradation, but they are of no concern for assay of those samples because they are well resolved from the spot of nifedipine. The percentage of degradation was evaluated by comparing the area of the nifedipine degraded peak with the area of the nifedipine standard peak, which appeared at hR_F 45. Photodegradation (sunlight and UV irradiation) and oxidation (3% hydrogen peroxide) almost caused the whole decomposition of nifedipine samples (∼99%). Chemical degradation under acidic and base conditions caused the significant decomposition of nifedipine (∼90% and ∼85%, respectively), but it was less as compared to the photodegradation. Besides, these experiments indicate that nifedipine is susceptible to acid and base hydrolysis, oxidation, and photo and UV degradation.

Because nifedipine is sensitive to light, the stability of its methanolic solution was evaluated by the UV spectrophotometric measurement of the absorbance at the maximum wavelength of 238.0 nm, in order to be sure that this step does not influence the assay. This study was performed by comparing the concentrations of the same solutions at regular intervals of 60 min for 12 h and, next, one time daily for a month under non-actinic light (stored at 4°C in a refrigerator). The analytical signal, as a function of drug concentration was stable for at least one month and no significant difference was observed in the peak area and shape recorded densitometrically.

The procedure was unaffected by a small but deliberate variation of the following method conditions: mobile phase composition (±2%), amount of mobile phase (±5%), temperature (22 ± 2°C), chamber geometry of (10 cm × 20 cm or 20 cm × 20 cm), and measurement wavelength (335 ± 2 nm). As a result, the method was sufficiently robust for routine laboratory use.

The calibration plot of peak areas against the corresponding concentrations was constructed over the range of 0.5–3.0 μg spot^{-1} with good coefficient correlation ($r \geq 0.990$). Each concentration within this range was analyzed repeatedly (three times on the first day and once on each of next four days). The relative standard deviations expressing the precision of measurement varied from 2.15% to 5.06%.

Mandel's fitting test was applied for mathematical verification of linearity. The second order calibration function showed significantly better fit than first order calibration function ($TV = 37.34 > F = 8.68$, $n = 18$, $P = 99\%$). Consequently, the appointed calibration curve was non-linear. Besides, residual analysis showed that the vertical distances of the observations from the regression plot, calculated by linear regression, were distributed around the 0-line abnormally with a non-linear trend (Figure 3). Consequently, the second order regression model was chosen (Table 1).

The limit of detection (*LD*) defined as three times the signal-to-noise ratio was evaluated by analysis of a series of solutions containing decreasing concentrations of the drug.

In order to determine the limit of quantitation (*LOQ*), solutions of decreasing quantities over the range of 0.05–0.4 μg spot^{-1} were analyzed

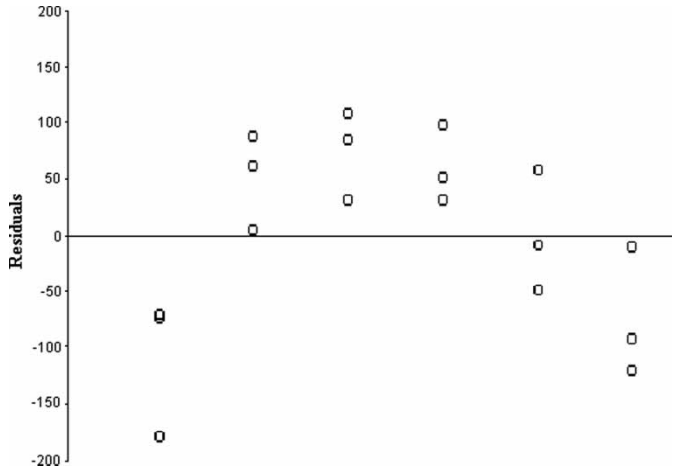

Figure 3. Residual distribution around the 0-line (evaluation of linearity).

and the RSD of the peak areas were plotted against the concentrations. *LOQ* was calculated from the resulting graph as the lowest amount for which RSD was 10%. *LD* and *LOQ* values were found to be 0.05 μg spot^{-1} and 0.1 μg spot^{-1}, respectively.

The developed HPTLC-densitometric method was successfully applied to the determination of nifedipine in the commercial tablets. To ensure identical conditions during TLC analysis, calibration, and sample solutions were chromatographed on the same plate. A typical chromatogram is illustrated in Figure 4. A single spot at hR_F 45 ± 0.57% (mean ± *RSD*, $n = 6$) was observed in the chromatogram of the drug samples extracted from tablets. There was no interference from the excipients present in the formulation. The drug content was found to be 10.11 mg ± 1.12% (±*RSE*, $n = 6$).

Table 1. Regression equations obtained from analysis of nifedipine calibration solutions

	Regression equations $y = ax^2 + bx + c$	Correlation coefficient
1 day	$y_1 = -121.787x^2 + 919.688x - 92.572$	0.9987
	$y_2 = -98.898x^2 + 844.798x + 43.527$	0.9990
	$y_3 = -101.281x^2 + 820.422x + 54.807$	0.9992
2 day	$y_4 = -112.403x^2 + 886.043x + 43.002$	0.9977
3 day	$y_5 = -90.591x^2 + 753.779x + 66.915$	0.9982
4 day	$y_6 = -52.939x^2 + 712.464x + 59.816$	0.9908
5 day	$y_7 = -67.175x^2 + 462.418x + 21.929$	0.9889
	$y_{(1-7)} = -71.601x^2 + 731.901x + 86.038$	0.9929

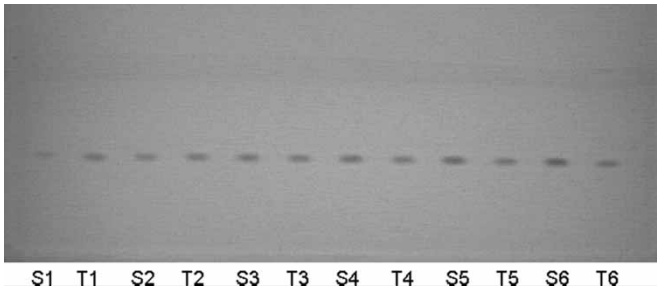

Figure 4. Chromatogram obtained from analysis of nifedipine calibration solutions (S1-S6) and tablet samples (T1-T6) on a silica gel HPTLC plate developed with n-hexane–ethyl acetate–acetone, 6:3:2 (v/v).

Accuracy of this method was confirmed after application of the Student's t-test. There was no significant difference between the mean recovery of the tested drug (100.4%) and 100% ($TV = 0.05 < t_{95\%} = 2.05$, $n = 30$). It can be concluded, that degradation of nifedipine in commercial preparation has not occurred.

To assess the precision of quantification of tablet samples, several aspects were considered. The variability of spotting was tested by analysis of six spots of the same tablet sample. The variability of the sample preparation was evaluated by analysis of six tablet samples prepared separately. The variability of the chromatographic procedure was investigated by the repeated analysis of tablet samples on three plates within one day (within-day precision) and after five days (day-to-day precision). From the results summarised in Table 2, it can be inferred that the method is satisfactorily precise (*RSD*% from 2.69 to 7.27).

In order to demonstrate another applicability of the developed method, the work was extended for the identification of nifedipine in the biological matrix. Nifedipine in the serum of patients after administration of Cordafen

Table 2. Statistical evaluation of results from determination of nifedipine in Cordafen tablets (10 mg tablet^{-1})

Parameter	Spotting ($n = 6$)	Sample preparation ($n = 6$)	Within-day precision ($n = 18$)	Day-to-day precision ($n = 30$)
Mean amount found (mg)	10.1118	9.9938	9.6795	10.0397
Standard deviation	0.2722	0.5956	0.3437	0.7301
Standard error	0.1111	0.2432	0.0834	0.1356
Variance	0.0741	0.3548	0.1181	0.5331
Relative standard deviation (%)	2.69	5.96	3.55	7.27
95% Confidence interval	±0.2857	±0.6252	±0.1767	±0.2777
Relative standard error (%)	1.12	−0.06	−3.21	0.40

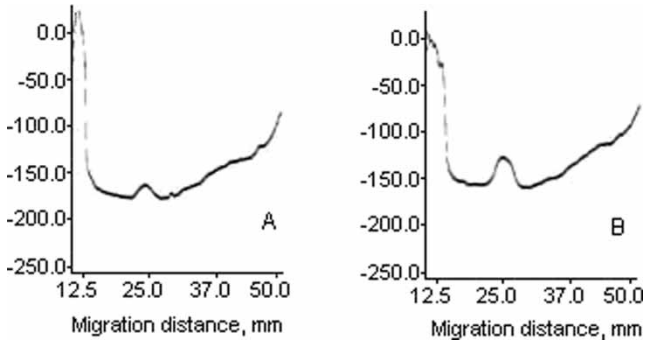

Figure 5. Typical densitogram obtained from analysis of nifedipine spiked serum (60 ng mL^{-1}) (A) and of nifedipine patient sample (B) after liquid-liquid extraction with hexane-dichloromethane, 70:30 (v/v) and chromatography on a silica gel HPTLC plate developed with n-hexane–ethyl acetate–acetone, 6:3:2 (v/v).

tables (3×10 mg daily, after five days) was identified by comparing the retention of the patient sample with that of the standard serum sample.

After application of the liquid-liquid extraction and the HPTLC chromatography with densitometric detection, the peaks at hR_F $46.73 \pm 2.85\%$ (mean $\pm$ *RSD*, $n = 5$) were obtained for the spiked serum sample (60 ng mL^{-1}) and patient sample (Figure 5).

In conclusion, the developed HPTLC-densitometric method is simple in performance, specific and stability indicating, and can be recommended for quantitative determination of nifedipine in pharmaceutical preparations. This method can be used for identification of nifedipine in the serum and in the presence of the other calcium channel antagonists. Besides, this method can be extended for the analysis of nifedipine and its degradation products in stability samples in industry.

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Received October 31, 2005
Accepted July 25, 2006
Manuscript 6751