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PAPER

Stability-indicating methods for the enantioselective determination of dihydropyridines by high performance liquid chromatography and capillary electrophoresis

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This paper presents simple, rapid, precise and accurate stability-indicating HPLC and CE methods, which were developed and validated for the determination of nitrendipine, nimodipine and nisoldipine. These drugs are calcium channel antagonists of the 1,4-dihydropyridine type which are used in the treatment of cardiovascular diseases. Experimental results showed a good linear correlation between the area and the concentration of drugs covering a relatively large domain of concentration in all cases. The linearity of the analytical procedures was in the range of 2.0–120.0 μg mL^{-1} for nitrendipine, 1.0–100.0 μg mL^{-1} for nimodipine and 100.0–600.0 μg mL^{-1} for nisoldipine, the regression determination coefficient being higher than 0.99 in all cases. The proposed methods were found to have good precision and accuracy. The chemical stability of these drugs was determined under various conditions and the methods have shown adequate separation for their enantiomers and degradation products. In addition, degradation products produced as a result of stress studies did not interfere with the detection of the drugs' enantiomers and the assays can thus be considered stability-indicating.

Introduction

In order to guarantee the stability of a drug there is a need to understand the nature and extent of decomposition, the mechanism, and the kinetics of the degradation reaction. The major result of decomposition is a loss of potency and/or change in the physicochemical properties.[1]

In addition, products formed during storage and dispensing may affect the efficacy and safety of drugs.[2–4] The chemical, pharmacological and toxicological properties of drugs may be changed by these products. These failures in efficiency can be caused by changes in the integrity of the chemical structure and stereochemistry or in the pharmacophore group.[5,6]

Stability-indicating methods for drugs are established and studied in order to provide evidence on the quality of drugs when exposed to different conditions such as pH, temperature, solvent system composition, solution ionic strength, and light.[7] From the data obtained in these studies it is easier to determine ideal storage conditions and re-test periods, as well as establishing shelf lives.[8] Moreover, stress testing allows the determination of the inherent stability of a molecule when subjected to adverse conditions by establishing the degradation pathways which is analogous to the established metabolic profile.[9]

By the fact that two enantiomers of a racemic drug often present different pharmacological and toxicological properties, the analytical methods should provide the enantioseparation of racemates to determine the enantiomeric purity of a chiral drug. There are several examples described in the literature on the degradation of drugs and metabolites, racemization, and configurational changes of the enantiomers, under different conditions such as pH, ionic strength, light, solvent and temperature.[10,11]

Considering the relevance of chiral drug stability to therapeutic efficacy, neutral dihydropyridines, nitrendipine (NTD), nisoldipine (NSD) and nimodipine (NMD), have been studied in this work (Fig. 1). These drugs are calcium channel antagonists of the 1,4-dihydropyridine type used in the long-term treatment of cardiovascular diseases, angina pectoris and hypertension.[12,13] These selected drugs have a chiral carbon atom in position 4 of the dihydropyridine ring, due to the presence of asymmetric ester moieties.[13] Since these drugs are used as racemates, the development of stereoselective methods is required.

The fact that no study on the stability of pure enantiomers of NMD, NTD and NSD has been reported until now, the aim of this work was to optimize simple and feasible methods for enantioselective analysis of NMD and NTD by HPLC using a chiral stationary phase, and NSD by CE, employing cyclodextrins (CD) as chiral selector to study the stability of their enantiomers under thermolytic, photolytic and acid/base hydrolytic stress conditions.

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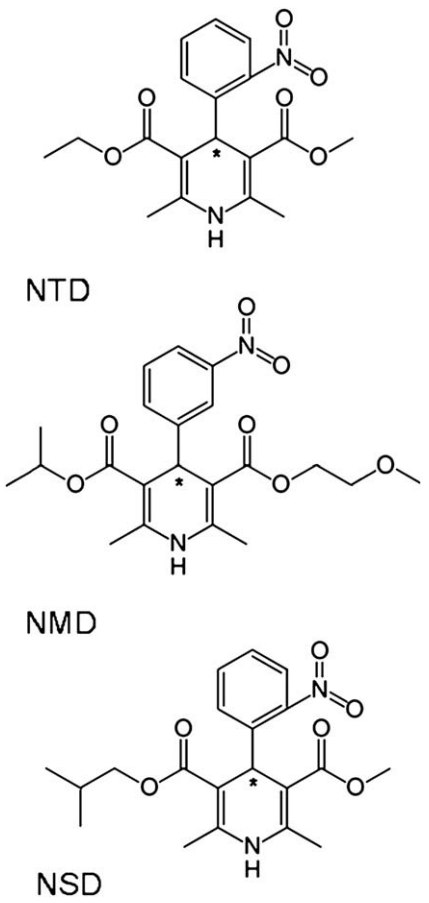

Fig. 1 Chemical structure of nitrendipine (NTD); nimodipine (MND) and nisoldipine (NSD) (* denotes the chiral center).

Experimental

Chemicals and reagents

The active pharmaceutical ingredients (API) rac-NTD and rac-NMD were kindly supplied by DEG (São Paulo, Brazil), and the rac-NSD was obtained from Bayer AG (Wupertall, Germany). HPLC grade acetonitrile, isopropanol and methanol were purchased from J.T. Baker (New Jersey, USA). Sodium chloride was obtained from Merck (Rio de Janeiro, Brazil). The other chemicals (analytical grade) were monosodium phosphate 1-hydrate and disodium phosphate 2-hydrate purchased from Synth (São Paulo, Brazil) and sodium chloride (NaCl) from Merck (Rio de Janeiro, Brazil). Water was purified with a Milli-Q PLUS system from Millipore® Corporation (Massachusetts, USA).

Preparation of standard solutions

Standard stock solutions of NTD, NMD and NSD were prepared in methanol at a concentration of 1 mg mL^{-1}. NTD, NMD and NSD working solutions were prepared in the concentration range of 2.5–100 μg mL^{-1}, 1–100 μg mL^{-1} and 50–600 μg mL^{-1} for each enantiomer respectively. All solutions were obtained by dilutions of the standard solution in the same solvent, *i.e.* methanol and all these solutions were stored at −20 °C in the absence of light.

Apparatus and analytical conditions

High performance liquid chromatography. HPLC experiments were performed using liquid chromatography equipment from Shimadzu (Kyoto, Japan), equipped with an LC 10 AT model solvent pump, a Rheodyne model 7125 injector with a 20 μL loop, an SPD-10 A model variable-wavelength UV detector operating at 227 nm, and a CR6-A model integrator Shimadzu (Kyoto, Japan). The chiral resolution of the NTD and NMD enantiomers was carried out using the Chiral AGP column 150 × 4 mm internal diameter (id), 5 μm particle size (ChromTech, Hägersten, Sweden). All chromatographic procedures were conducted at 26 ± 2 °C.

Capillary electrophoresis. CE analyses for enantioseparation of NSD were performed on an Agilent Technologies CE system (Waldbronn, Germany) model G1600A consisting of an analyzer, an automatic sampler and a diode array detector operating at 208 nm. Agilent ChemStation Software was used for data acquisition. An uncoated fused-silica capillary obtained from Micro-Solv Technology Corporation (New Jersey, USA) of 50 μm id, 64.5 cm in total length, and 56.0 cm in effective length was used.

Before the first use, the capillary was conditioned by rinsing with 1.0 mol L^{-1} NaOH for 30 min, followed by water for 30 min at 25 °C. At the beginning of each working day the capillary was rinsed with 1.0 mol L^{-1} NaOH for 10 min, water for 10 min followed by background electrolyte (BGE) for 15 min. The capillary was rinsed with 0.1 mol L^{-1} NaOH for 2.0 min, water for 2.0 min and BGE for 5.0 min between consecutive analyses. After daily use, the capillary was washed with 0.1 mol L^{-1} NaOH for 15 min followed by water for 15 min. When not in use, the capillary was stored in water. All experiments were carried out at 20 °C.

Elution order

(*R*)-NTD and (*R*)-NMD are known to be dextrorotatory while (*R*)-NSD is known to be levorotatory.[14] The elution order for the NTD enantiomers was established by analyzing the pure enantiomers obtained by semipreparative analysis of the 25 μL standard methanolic solution racemates on a Chiralcel OJ column 250 × 4.6 mm id, 10 μm particle size (Daicel Chemical Industries, Tokyo, Japan) and the mobile phase consisted of *n*-hexane : isopropanol (87.5 : 12.5, v/v) at a flow rate of 1.0 mL min^{-1}.[15] The two peaks corresponding to (−)-(*S*)-NTD and (+)-(*R*)-NTD were collected at the end of the column, the solvent was evaporated and the residues were analyzed under the conditions established in the present paper and the elution order established.

The elution order for the NMD enantiomers was based on the work described by Barbato *et al.*[14] These authors employed a Chiral AGP 100 × 4.0 mm id, 5 μm particle size (Chromtech, Hägersten, Sweden) using a mobile phase which consisted of isopropanol: 0.01 mol L^{-1} phosphate-buffered saline pH 7.0 (10 : 90, v/v) and flow rate of 0.9 mL min^{-1}. In this condition the two peaks corresponding to (−)-(*S*)-NMD and (+)-(*R*)-NMD were collected separately at the end of the column, the solvent was evaporated and the residues were analyzed under the conditions established in the present paper and the elution order established.

For NSD, the elution order was based on the work described by Marques *et al.*[16] These authors employed a Chiralcel OD-H column 250 × 4.6 mm id, 5 μm particle size (Diacel Chemical Industries, California, USA) using hexane : ethanol (97.5 : 2.5, v/v) to resolve the NSD enantiomers. Under these conditions the separated enantiomers, (+)-(*S*)-NSD and (−)-(*R*)-NSD, were collected at the end of the column, the solvent was evaporated and the residues were analyzed by CE under the conditions established in the present paper.

Validation studies

Calibration curves were prepared by analyzing 25 μL working solutions at different concentrations of each enantiomer of NTD (2.5, 5.0, 10.0, 20.0, 40.0, 50.0 and 100.0 μg mL^{-1}), NMD (1.0, 2.5, 5.0, 20.0, 50.0, 60.0 and 100.0 μg mL^{-1}) and NSD (50.0, 100.0, 200.0, 300.0, 400.0, 500.0 and 600.0 μg mL^{-1}). Plots of concentrations *versus* peak area were constructed and then subjected to regression analysis by the least-squares method to calculate the linear equation and correlation coefficient (*r*).

The LOQ values were taken from the calibration line. The LOQ for each drug was considered the lowest value of the curve, provided it has adequate precision and accuracy.

The precision and accuracy of the method were evaluated by within-day and between-day assays. To assess within-day precision and accuracy, triplicate analysis of samples at three concentration levels of NTD (10.0, 50.0 and 100.0 μg mL^{-1}), NMD (5.0, 20.0 and 50.0 μg mL^{-1}) and NSD (100, 400 and 600 μg mL^{-1}) were performed. For between-day assays, triplicate samples of each concentration were analyzed for three consecutive days. Precision was expressed as relative standard deviation (RSD %) and accuracy as relative error (RE %).

Forced degradation studies

Forced degradation studies were performed on the bulk drug to provide an indication of the stability-indicating property and specificity of the proposed method to separate API enantiomers from their degradation products. Intentional degradation was carried out in stress conditions of light (visible and ultra-violet (UV)), pH and temperature.

Temperature. For NTD, a 50 μL working solution of NTD (100 μg mL^{-1}) was transferred to glass tubes and the solvent was evaporated under an air flow at room temperature. The residues of NTD were supplemented with 2 mL sodium phosphate buffer 0.1 mol L^{-1}, pH 10.0 and the tubes were incubated at different temperatures (−20, 4 and 37 °C). Aliquots (100 μL) of these spiked samples ($n = 3$) were analyzed at 0, 24, 48 and 72 hours after incubation. The concentration of these samples was determined using a calibration curve.

Due to solubility problems, the thermal stability studies of NMD and NSD were carried out in methanol. For this, a 25 μL working solution of NMD (100 μg mL^{-1}) was transferred to ten glass tubes. These tubes were incubated at 37 °C for different times: 0, 1, 2 and 9 days. After each period of incubation, two tubes were removed, the solvent evaporated under a flow of compressed air, and the residues were dissolved in 50 μL mobile phase and analyzed. Considering the long period of time of the experiments, the tubes were monitored daily and, when necessary, methanol was added to prevent the complete evaporation of the solvent, thereby preventing adsorption of NMD in the glass extraction tube. For the temperatures of −20 °C and 4 °C, a 2 mL working solution (100 μg mL^{-1}) was transferred to glass tubes and incubated at the two different temperatures. After exposing the samples for 0, 1, 2 and 9 days, aliquots of 25 μL, in triplicate, were removed. The solvent was evaporated under a flow of compressed air and the residues were dissolved in a 50 μL mobile phase and analyzed. The concentration of these samples was determined using a calibration curve.

For NSD, a 25 μL working solution (500 μg mL^{-1}) was transferred to glass tubes and 500 μL of methanol was added and the tubes were incubated at 4 °C and 37 °C. After each incubation time (0, 24, 48 and 72 hours), three tubes were removed, the solvent was evaporated under a flow of compressed air and the residues were dissolved in 250 μL BGE and analyzed. In this case, the tubes were also monitored daily and, when necessary, more methanol was added to prevent the complete evaporation of the solvent. The concentration of these samples was determined using a calibration curve.

UV and visible light. For this study, a 25 μL standard solution of NTD (20 μg mL^{-1}), NMD (20 μg mL^{-1}) and NSD (500 μg mL^{-1}) in methanol was exposed to UV (254 nm) or visible light for several periods of time (energy source at 10 cm from the sample). To prevent the sample tube from drying due to evaporation of the solvent (caused by heat from the UV light), 500 μL of methanol was added to each tube when necessary. All analyses were performed in triplicate ($n = 3$).

The stability of the NTD enantiomers under UV (254 nm) light was evaluated after 0, 1, 2, 3 and 4 hours and in visible light after 0, 1, 2, 3 and 4 days. After exposure to light, the solvent was evaporated under compressed air at room temperature. The residues were dissolved in 100 μL of the mobile phase and 20 μL were injected into the HPLC equipment in the conditions previously established.

For NMD, the study was performed at time intervals of 0, 1, 2, 3 and 4 hours and 0, 2, 3, 4 and 5 days for UV (254 nm) and visible light, respectively. After exposure to light the solvent was evaporated and the residues were dissolved in 100 μL of the mobile phase and 20 μL was injected into the HPLC equipment under the conditions previously established.

For NSD, this study was performed only under UV light (254 nm). The tubes were removed at time intervals of 0, 10, 20 and 40 minutes. After exposing the samples to the action of UV light, the solvent was evaporated under a flow of compressed air and the residues were dissolved in 250 μL of BGE and analyzed.

To determine the concentration of the drugs, calibration curves of the racemic mixture were prepared and analyzed for the NTD, NMD and NSD enantiomers.

pH. For the stability study of NTD at different pH values, a 0.1 mol L^{-1} sodium phosphate buffer was used and the pH was adjusted to 4.0, 7.0 and 10.0 with a NaOH solution. For this study a 50 μL standard solution of NTD (100 μg mL^{-1}) was dried and dissolved by adding 2 mL of 0.1 mol L^{-1} sodium phosphate buffer solution. The solutions were then stored at 4 °C and aliquots of these spiked sample solutions ($n = 3$) were

withdrawn and analyzed after 0, 3, 24, 48 and 72 hours of incubation. For the stability study of NMD (100 μg mL^{-1}) the experimental procedure was identical to that of NTD.

For NSD, this study was performed in two pH values only, 4.0 and 9.0; a sodium acetate buffer and sodium phosphate buffer were used respectively. In this case a 250 μL standard solution of NSD (1000 μg mL^{-1}) in methanol was mixed with 250 μL of buffer solution (sodium acetate buffer pH 4.0 or sodium phosphate buffer pH 9.0). Aliquots (25 μL) of spiked sample solutions ($n = 3$) were withdrawn and 225 μL of buffer were added. The samples were analyzed at 0, 24, 48 and 72 hours after incubation.

The concentration of these spiked samples was determined using a calibration curve for each analyte.

Statistical analysis

All values were expressed as mean ± S.E.M. Differences between incubation times were compared by one-way ANOVA and Dunnet's Test was used for comparison with the control time ($\alpha = 0.05$).

Results and discussion

Method development

Nitrendipine and nimodipine. The optimization of analyses conditions of NTD and NMD were based on the method described by Barbato *et al.*[14] Some modifications were preformed to improve the resolution of enantiomers. Several mobile phases consisting of different proportions of buffer and organic solvent were tested (Table 1). In addition, the ionic strength using sodium chloride was also tested: the same conditions above were analyzed using 0.016, 0.026 or 0.066 mol L^{-1} NaCl. The organic and aqueous phase proportions were adjusted to obtain a simple NTD and NMD assay method with a reproducible and rapid run time.

The best analytical conditions for NTD were the mobile phase n° 8 (Table 1) with ionic strength (NaCl) 0.026 mol L^{-1} at a flow rate of 0.65 mL min^{-1}; and for NMD were the mobile phase no. 4 (Table 1) with ionic strength (NaCl) 0.066 mol L^{-1} at a flow rate of 0.65 mL min^{-1}.

Fig. 2 shows the typical chromatograms of separation of NTD (Fig. 2A) and NMD (Fig. 2B) enantiomers.

Table 1 Mobile phases used on optimization of analyses conditions of NTD and NMD

Mobile Phase (n°)	Sodium phosphate buffer 4 mM (%)		Solvents (%)		
	pH 7.0	pH 7.1	2-Propanol	Acetonitrile	Methanol
1	87	—	13	—	—
2	86	—	12	2	—
3	86	—	12	—	2
4	—	87	13	—	—
5	—	86	12	2	—
6	—	86	12	—	2
7	—	85	7	—	8
8	85	—	7	—	8

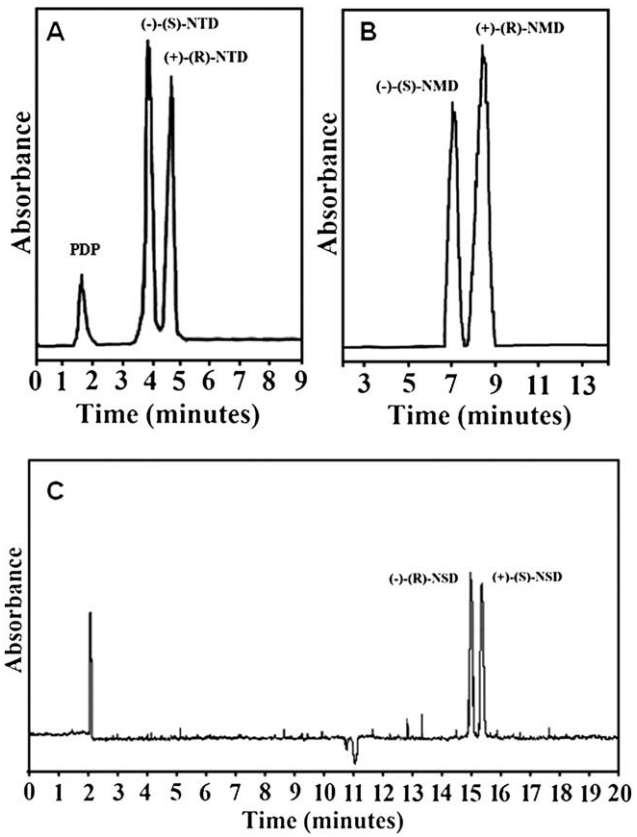

Fig. 2 Typical chromatogram showing the resolution of NTD (A) and NMD (B) enantiomers, and typical electropherogram showing the resolution of NSD enantiomers (C). For analysis conditions see the text.

Nisoldipine. The optimization of analysis conditions of NSD was based on the CE method described by Van Eeckhaut *et al.*[17] These authors used 60 mmol L^{-1} boric acid, pH 5.5 with methanol 15% (v/v) as BGE, and carboxymethyl-β-cyclodextrin (CM-β-CD) as chiral selector. But with these conditions it was not possible to detect the drug before 60 minutes had elapsed. For this reason some modifications were preformed to achieve the resolution of enantiomers, such as boric acid substitution by sodium borate buffer solution as BGE.

So, the selected electrophoretic conditions were: 50 mmol L^{-1} sodium borate buffer, pH 9.0, 15% methanol, 5 mmol L^{-1} CM-β-CD, voltage at 20 kV, temperature at 20 °C, hydrodynamic injection (10 seconds at 40 mbar) and detection at 227 nm. Fig. 2C shows the electropherogram corresponding to a separation of NSD enantiomers.

Elution order

To establish the elution order, the pure stereoisomers (obtained by semi-preparative analysis under the conditions presented in the present paper) were analyzed using the techniques of high performance liquid chromatography and capillary electrophoresis. The retention and migration times of the stereoisomers from both studies were then compared and the elution order was established as follows: the first enantiomer was (−)-(*S*)-NTD followed by (+)-(*R*)-NTD; in the case of NMD the first was (−)-(*S*)-NMD followed by (+)-(*R*)-NMD and for NSD the first

Table 2 Linearity, limits of detection and quantification of the methods

Parameters	Nitrendipine		Nimodipine		Nisoldipine	
Linear equation[a]	(−)-(*S*)	$y = 354.98x - 73.316$	(−)-(*S*)	$y = 10\,918x - 50.745$	(−)-(*R*)	$y = 0.0897x - 1.7201$
	(+)-(*R*)	$y = 326.52x - 214.05$	(+)-(*R*)	$y = 19\,991x - 7171.1$	(+)-(*S*)	$y = 0.0805x - 0.5267$
Correlation coefficient (*r*)	(−)-(*S*)	0.9992	(−)-(*S*)	0.9985	(−)-(*R*)	0.9989
	(+)-(*R*)	0.9997	(+)-(*R*)	0.9983	(+)-(*S*)	0.9984
Range (μg mL^{-1})	(−)-(*S*)	2.0–120	(−)-(*S*)	1.0–100	(−)-(*R*)	100–600
	(+)-(*R*)	2.0–120	(+)-(*R*)	1.0–100	(+)-(*S*)	100–600
LOQ (μg mL^{-1})	(−)-(*S*)	2.0	(−)-(*S*)	1.0	(−)-(*R*)	100
	(+)-(*R*)	2.0	(+)-(*R*)	1.0	(+)-(*S*)	100

[a] Calibration curves were analyzed in triplicate ($n = 3$) for each concentration: $y = Ax + B$; where y is the peak area of analyte, A is the slope, B is the intercept, and x is the concentration of the measured solution in μg mL^{-1}.

enantiomer was (−)-(*R*)-NSD followed by (+)-(*S*)-NSD, as shown in Fig. 2.

Method validations

The chromatograms and the electropherogram appearing in Fig. 2 indicate no interference. The calibration curve was prepared by plotting the peak area of analytes against drug concentration. Peak area and concentration were subjected to least-squares linear regression analysis to calculate the calibration equation and correlation coefficient (*r*). The linear equations, correlation coefficient and RSD (%) are shown in Table 2. All *r* values were ≥ 0.99, showing excellent linearity, and the points in residual plots are randomly dispersed around the horizontal axis showing that the linear regression model is appropriate for the data.

For the purpose of calculating the LOQ the lowest concentration on the analytical curve linearity was considered. The values for the LOQ are also shown in Table 2.

The precision and accuracy of the methods were evaluated by calculating RSD (%) and RE (%), respectively, for three determinations of analytes at three different concentrations over the course of three days under the same experimental conditions. Table 3 shows within-day ($n = 3$, three replicates for each concentration) and between-day ($n = 3$, on three different days) assays. These results confirm the precision and accuracy of the method within the desired range.

Forced degradation studies

Investigations on drug-stability are gaining great pharmaceutical relevance because they are important factors to determine both efficiency and toxicity, since changes in the structure induce changes in the drug's pharmacological properties with a lack of therapeutic effect. For this reason the stability of the compounds of interest was tested under different conditions.

Temperature. Marciniec and Ogrodowczyk[18] studied the effect of temperature and moisture on the stability of seven 1,4-dihydropyridine derivatives and they showed there was degradation of all dihydropyridines at high temperatures of 70, 80 and 90 °C and 76% humidity. Therefore it is necessary to obtain results at different temperatures which might be encountered in storage and use.

Table 3 Precision and accuracy of the methods for the determination of NTD, NMD and NSD enantiomers

Drug	Nominal concentration (μg mL^{-1})	Within-day ($n = 3$)[a]			Between-day ($n = 3$)[b]		
		Obtained (μg mL^{-1})	RE (%)[c]	RSD (%)[d]	Obtained (μg mL^{-1})	RE (%)[c]	RSD (%)[d]
(−)-(*S*)-NTD	10	9.75	−2.55	5.62	9.83	−1.73	5.00
	50	50.26	0.52	6.55	50.24	0.49	4.25
	100	102.44	2.44	2.97	100.89	0.89	2.89
(+)-(*R*)-NTD	10	10.08	2.82	3.25	10.03	0.28	2.80
	50	51.53	3.05	6.7	51.01	2.01	4.91
	100	100.14	0.14	6.44	99.55	−0.45	4.98
(−)-(*S*)-NMD	5	4.60	−8.10	4.24	4.87	−2.70	5.76
	20	19.74	−1.31	3.84	21.33	6.94	9.53
	50	46.33	−7.30	1.84	46.35	−7.30	6.13
(+)-(*R*)-NMD	5	4.85	−2.93	5.04	4.67	−6.60	5.92
	20	21.37	6.85	3.27	20.70	3.51	8.76
	50	46.69	−6.62	1.70	51.11	2.22	7.72
(−)-(*R*)-NSD	100	98.88	−1.12	0.72	94.98	−5.02	4.64
	400	381.70	−4.57	4.61	390.13	−2.46	3.85
	600	600.75	0.12	3.10	602.15	0.36	2.85
(+)-(*S*)-NSD	100	103.16	3.16	2.49	97.12	−2.88	6.92
	400	382.98	−4.25	1.65	319.95	−2.01	3.29
	600	641.12	6.85	2.55	606.24	1.04	2.39

[a] n = Number of determinations. [b] n = Number of days. [c] RE, expressed as relative error in percentage (%). [d] RSD, expressed as relative standard deviation in percentage (%).

Both enantiomers, (−)-(*S*)-NTD and (+)-(*R*)-NTD, were found not to decompose at −20 °C during thermodegradation studies. However (−)-(*S*)-NTD showed a small degradation which proved to be statistically significant ($P < 0.05$), after 72 hours at both 4 and 37 °C (Fig. 3A).

In the case of NMD (Fig. 3B), under the same conditions but for a longer time, it was observed that at 37 °C, both enantiomers (−)-(*S*)-NMD and (+)-(*R*)-NMD were degraded after nine days of study ($P < 0.001$), while at −20 °C and 4 °C, no statistically significant degradation was observed for both enantiomers.

For NSD, no degradation during the test was observed for both enantiomers (Fig. 3C).

UV and visible light. Many articles show that the dihydropyridines when exposed to light lead to decomposition.[19–22] The chemical changes in irradiated molecules include the oxidation of the dihydropyridine ring of pyridine and the reduction of the aromatic nitro group to the nitroso group. Depending on the source of irradiation, two different products can be obtained: nitrophenylpyridine and nitrosophenylpyridine, resulting from exposure to UV and visible light respectively.[16] These pyridine analogues may not have a therapeutic effect.[21]

NTD was found to be photosensitive for both enantiomers (Fig. 4A). Exposure to UV light caused 51.64% of (−)-(*S*)-NTD and 54.82% of (+)-(*R*)-NTD degradation of the sample within one hour and 82.52% of (−)-(*S*)-NTD and 85.83% of (+)-(*R*)-NTD within four hours ($P < 0.05$). In the present study it was observed that the degradation product was already present in the standard powder, but in a small amount. This fact was confirmed by the increase of peak of the degradation product after exposure to UV light (Fig. 5A). On the other hand, there was no statistically significant difference in up to three days of study ($P > 0.05$), in visible light. This behavior can be explained by the lower intensity of energy supplied by visible light.

In the case of NMD under UV light conditions, its enantiomers were found to be more stable than NTD enantiomers. Exposure to UV light caused a degradation of NMD enantiomers of 40.57% for (−)-(*S*)-NMD and 38.66% for (+)-(*R*)-NMD after one hour while the exposure to UV light for four hours caused 63.13% of (−)-(*S*)-NMD and 60.10% of (+)-(*R*)-NMD

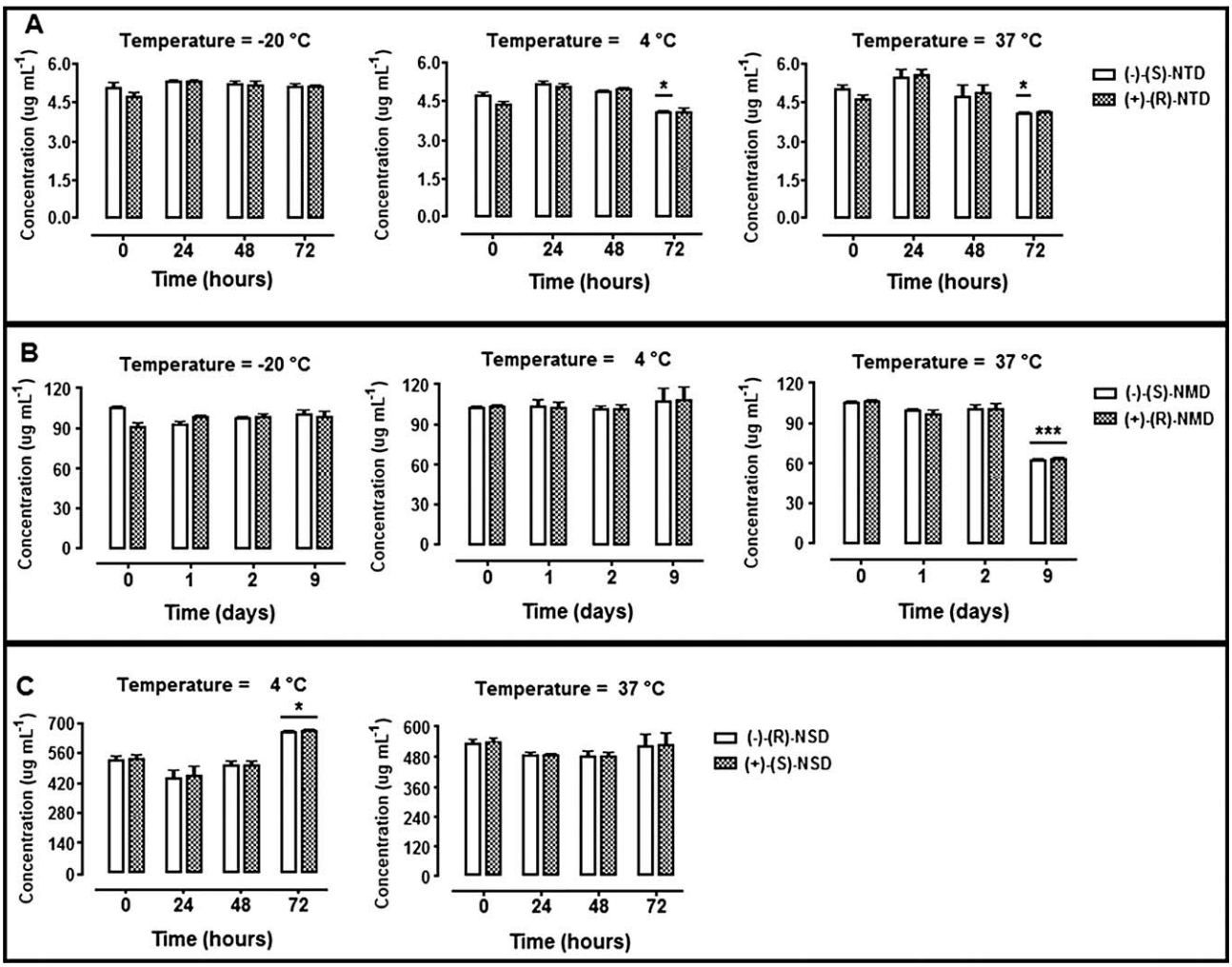

Fig. 3 Influence of temperature on the stability of the drugs NTD (A); NMD (B) and NSD (C). Each bar represents the mean ± S.E.M. The symbols adjacent to the bars represent the *P* value, with a level of significance of 95% (one-way ANOVA followed by Dunnet's post-test), when compared with the fresh sample. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

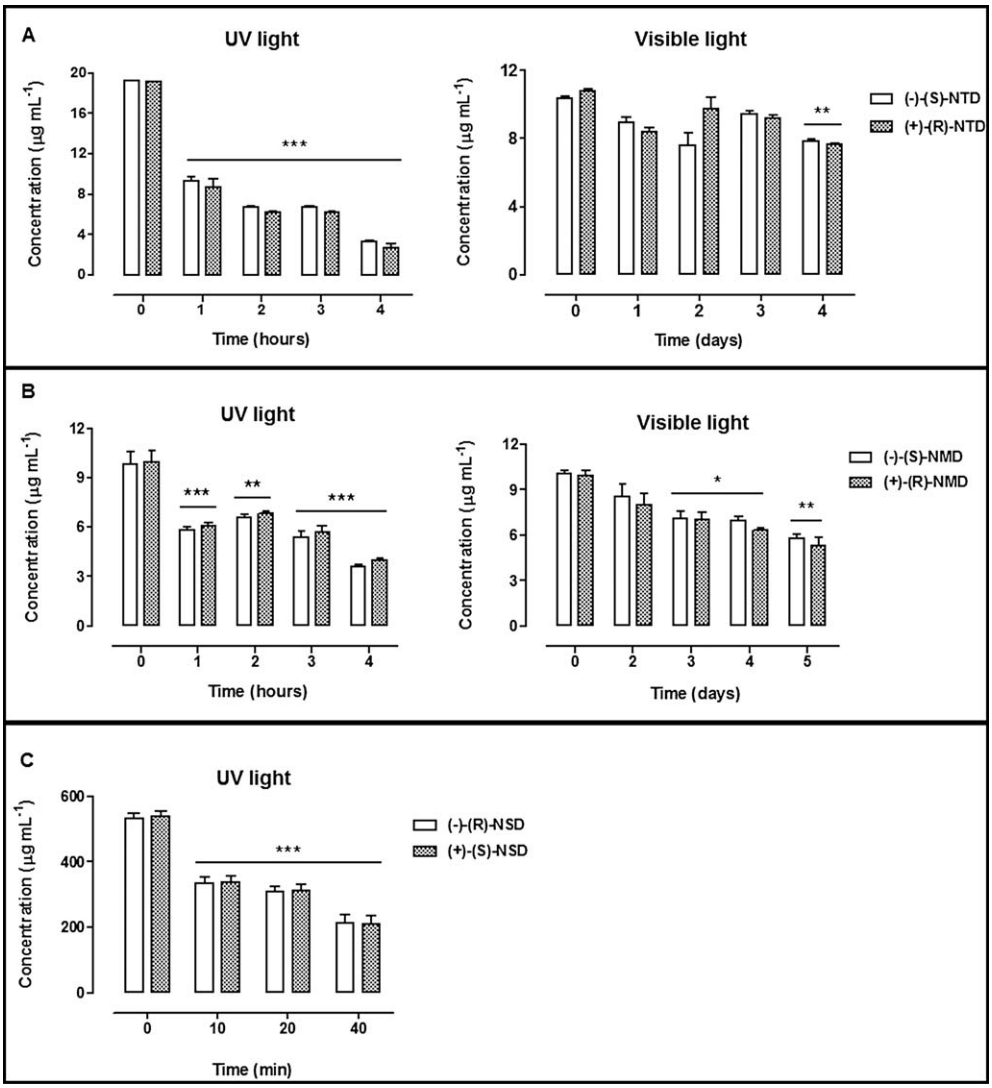

Fig. 4 Influence of UV and visible light on the stability of the drugs NTD (A); NMD (B) and NSD (C). Each bar represents the mean ± S.E.M. The symbols adjacent to the bars represent the *P* value, with a level of significance of 95% (one-way ANOVA followed by Dunnet's post-test), when compared with the fresh sample. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

degradation ($P < 0.05$) (Fig. 4B). In the same way as shown for NTD, the formation of a degradation product was also observed for NMD (Fig. 5B). Both enantiomers, (−)-(*S*)-NMD and (+)-(*R*)-NMD, when exposed to visible light were degraded after two days of exposure ($P < 0.05$), but the formation of any degradation product was not observed, such as when the enantiomers were exposed to UV light.

For NSD, shorter analysis times were required due to its high sensitivity to UV light. The percent of decomposition for NSD enantiomers was 37.21% for (−)-(*R*)-NSD and 37.11% for (+)-(*S*)-NMD after 10 minutes and 59.99% for (−)-(*R*)-NSD and 60.72% for (+)-(*S*)-NMD after 40 minutes of UV light exposure (Fig. 4C). These results are corroborated by Baranda *et al.*,[13] in which the authors did not find any detectable compound after 2 h of light exposure. In this case a chiral degradation product is observed at a retention time of 12.5 minutes (Fig. 5C).

As explained above, special precautions against light exposure must be taken when working with dihydropyridines; this is due to the fact that dihydropyridines are easily photodegraded when exposed to light.

pH. Among the factors that can alter the solubility and stability of drugs, we can cite pH. pH has great importance in the drug effect, since most drugs are weak acids or bases, and pH is still closely related to the absorption and stability of the drugs.[23] For this reason the stability study of drugs in different pH values is essential.

For ionizable drugs, such as NTD, which present a weakly basic character, the fraction of a drug present in any particular form will depend on the pH solution. So, changes in the pH of the medium altered the solubility of NTD enantiomers (data not shown). For this reason, if the reactivity of the NTD enantiomers depends on their form (ionized), in a high pH value, lower degradation will be observed, as shown in Fig. 6A. The effect of pH on the degradation rate of NTD enantiomers can be explained by the catalytic effects that hydronium or hydroxide

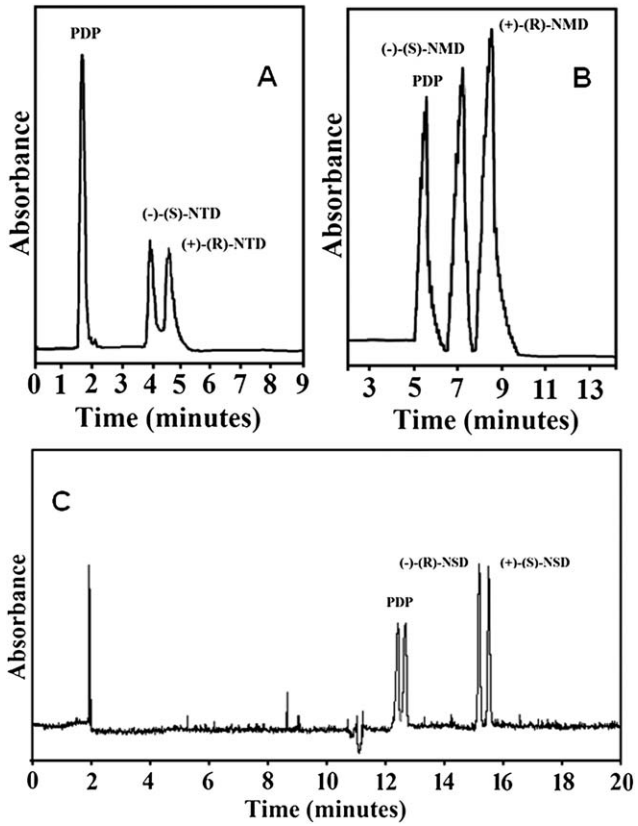

Fig. 5 Typical chromatograms of NTD (A) and NMD (B), and a typical electropherogram of NSD (C) after exposure to UV light. *PDP – principal degradation product. For analysis conditions see the text.

ions may have on the chemical reactions. Degradation rates of drug substances are generally affected by pH because most degradation pathways are catalyzed by hydronium and/or hydroxide ions.[24]

For NMD some difficulties in solubilization during the stability study at different pH values were found. Here, some modifications to the solution were made such as: changes in the volume of resuspension buffer, addition of surfactants, and addition of standard solution without drying the solvent. After these modifications the same improvements in solubility were achieved. On the other hand, the worst resolution was achieved between NMD enantiomers. Thus, due to the difficulty found in the solubilization of the drug in aqueous medium, the stability study in this condition cannot be undertaken.

In the case of NSD, a neutral drug, no degradation during the stability study was observed for both enantiomers at both pH values (Fig. 6B). This fact can be explained by low and/or no ionization of the drug, which reduces the reactivity of the groups to acid or basic hydrolysis.

Conclusion

Efficient stereoselective methods were developed and validated. The proposed methods have the ability to separate these drugs from their degradation products and can be applied to the analysis of samples obtained during accelerated stability experiments. From the stability tests it can be concluded that temperature and pH had little influence on the stability of drugs during the studies, but precautions against light exposure must be taken when working with dihydropyridines, since this class of drugs has a high rate of degradation under light.

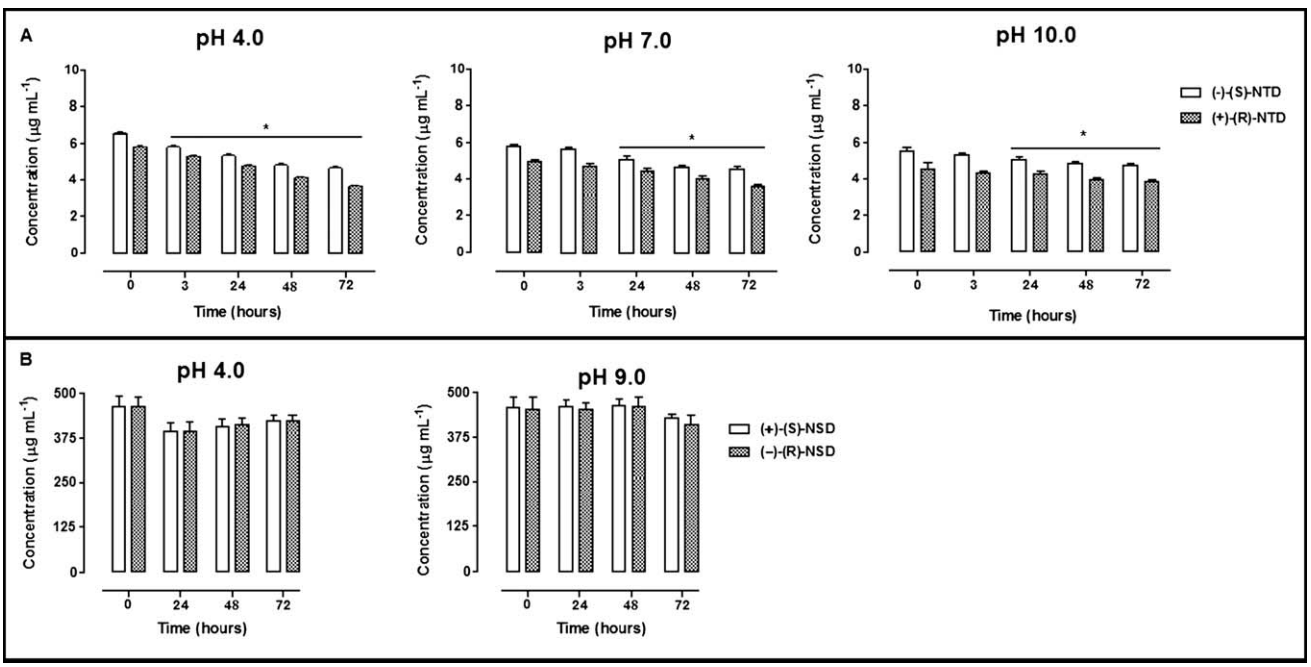

Fig. 6 Influence of pH on the stability of the drugs. Each bar represents the mean ± S.E.M. The symbols adjacent to the bars represent the *P* value, with a level of significance of 95% (one-way ANOVA followed by Dunnet's post-test), when compared with the fresh sample. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

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References

1 H. H. Tonnesen, *Int. J. Pharm.*, 2001, **225**, 1–14.
2 FDA, *Guidance for Industry: Impurities in Drug Product, Draft Guidance*, Center for drug evaluation and research (CDER), 1998.
3 A. Alnajjar, A. M. Idris and H. H. Abu Seada, *Microchem. J.*, 2007, **87**, 35–40.
4 Z. Fu, L. Wang and Y. Wang, *Anal. Chim. Acta*, 2009, **638**, 220–224.
5 S. Goodman and J. Stoltefuss, *Angew. Chem., Int. Ed. Engl.*, 1991, **30**, 1559–1578.
6 A. Álvarez-Lueje, J. Sturm, J. A. Squella and L. J. Núnez-Vergara, *J. Pharm. Biomed. Anal.*, 2002, **28**, 887–895.
7 A. W. Wong and A. Datla, *Sep. Sci. Technol.*, 2005, **6**, 335–358.
8 K. M. Al Azzam, B. Saad, C. Y. Tat, Y. Mat and H. Y. Aboul-Enein, *J. Pharm. Biomed. Anal.*, 2011, **56**, 937–943.
9 X. Xu, M. G. Bartlett and J. T. Stewart, *J. Pharm. Biomed. Anal.*, 2001, **26**, 367–377.
10 J. Ducharme, C. Fernandez, F. Gimenez and R. Farinotti, *J. Chromatogr., Biomed. Appl.*, 1996, **686**, 65–75.
11 C. M. de Gaitani, P. S. Bonato and A. S. Martinez, *Electrophoresis*, 2003, **24**, 2723–2730.
12 B. B. Samuelsson, M. Norlander and P. Toren, PCT Int Appl WO 94, 14441; *Chem. Abstr.*, 1994, **121**, 545.
13 A. B. Baranda, R. M. Alonso, R. M. Jiménez and W. Weinmann, *Forensic Sci. Int.*, 2006, **156**, 23–34.
14 F. Barbato, F. Quaglia, M. T. Quercia and M. I. La Rotonda, *Helv. Chim. Acta*, 2000, **83**, 767–776.
15 P. A. Soons, M. C. M. Roosemalen and D. D. Breimer, *J. Chromatogr., Biomed. Appl.*, 1990, **528**, 343–3564.
16 M. P. Marques, N. A. G. Santos, E. B. Coelho, P. S. Bonato and V. L. Lanchote, *J. Chromatogr., Biomed. Appl.*, 2001, **762**, 87–95.
17 A. Van Eeckhaut, M. R. Detaevernier and Y. Michotte, *J. Pharm. Biomed. Anal.*, 2004, **36**, 799–805.
18 B. Marciniec and M. Ogrodowczyk, *Acta Pol. Pharm. Drug Res.*, 2006, **63**, 477–484.
19 M. Jadwiga, W. Augustyniak, P. Grobelny and G. Nowacka, *Int. J. Pharm.*, 2005, **304**, 145–151.
20 K. Javidnia, B. Hemmateenejad, R. Miri and M. S. Boroujeni, *J. Pharm. Biomed. Anal.*, 2008, **46**, 597–602.
21 A. R. Fakhari, S. Nojavan, S. Haghgoo and A. Mohammadi, *Electrophoresis*, 2008, **29**, 4583–4592.
22 Y. Kawabe, H. Nakamura, E. Hino and S. Suzuki, *J. Pharm. Biomed. Anal.*, 2008, **47**, 618–624.
23 A. G. Gilman, T. W. Rall, A. S. Nies and P. Taylor, *The Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, 8th edn, 1992.
24 S. Yoshioka and V. J. Stella, in *Chemical Stability of Drug Substances*, ed. S. Yoshioka and V. J. Stella, Kluwer Academic Publishers, New York, 2002, ch. 2.