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Journal of Pharmaceutical and Biomedical Analysis 42 (2006) 434–440
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

An efficient separation and method development for the quantifying of two basic impurities of Nicergoline by reversed-phase high performance liquid chromatography using ion-pairing counter ions

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Received 9 September 2005; received in revised form 14 April 2006; accepted 22 April 2006
Available online 12 June 2006

Abstract

A quantification method was developed for the two basic impurities, one of which is also a metabolite, of Nicergoline (NIC), by using reversed-phase high performance liquid chromatography (RP-HPLC) and diode array detector (DAD). One of these compounds,10-methoxy-6-methylergoline-8β-methanol-5-bromo-3-pyridinecarboxylate (1-DN) is the metabolite as well as the impurity whereas, the other 10-methoxy-1,6-dimethylergoline-8β-methanol-5-chloro-3-pyridinecarboxylate (5-CN) is only an impurity.

The chromatographic column was Phenomenex, Luna, 5 μm, C18 (2), 250 mm × 4.6 mm. Mobile phase was 0.1 M ammonium acetate (NH4Ac) solution containing 4 mM 1-octanesulfonicacid sodium salt (OSASS) and 6 mM tetrabutylammonium hydrogen sulphate (TBAHS) (pH: 5.9)/acetonitrile (ACN) (62:38) for 1-DN and (64:36) for 5-CN. Flow rate was 1.0 mL min^{-1}. The diode array detector was operated at 285 nm, band width: 4 nm.

Linearity was obtained in the concentration range of 0.032×10^{-5} to 3.828×10^{-5} M, $y = 116.88x + 0.2773$ ($r^2 = 0.99989$); the limit of detection (LOD) and limit of quantification (LOQ) were determined as 0.012×10^{-5} and 0.041×10^{-5} M for 1-DN, respectively. Linearity was obtained in the concentration range of 0.034×10^{-5} to 4.092×10^{-5} M, $y = 104.24x + 0.7486$ ($r^2 = 0.99996$); (LOD) and (LOQ) were determined as 0.014×10^{-5} and 0.046×10^{-5} M for 5-CN, respectively. The recovery was 100.65% for 1-DN and 100.32% for 5-CN. The amount of 1-DN in 30 mg NIC was found as 209.65 μg (0.70%) and the amount of 5-CN in 30 mg NIC was found as 27.62 μg (0.09%).

Keywords: Nicergoline; Basic impurities; Quantification; Ion pairing counter ions; Reversed-phase HPLC

1. Introduction

Nicergoline (NIC), a semisynthetic ergot alkoloid derivative, was first developed by Farmitalia Carlo Erba. 1-DN and the other four compounds have been identified as NIC's major metabolites in animal and human blood [1]. 1-DN and 5-CN occur in trace amounts as impurities in the raw material as well [2]. 1-DN (metabolite or impurity) and 5-CN (impurity) with the ester linkage were found to be more potent and toxic than all the other four ergoline-alcohol compounds of NIC [3]. Therefore, developing an efficient method has a crucial importance for these two related compounds of NIC. The molecular structures of NIC, 1-DN and 5-CN are shown in Fig. 1.

NIC itself shows vasodilating and α-receptor blocking activity [4]. It has been clinically used for improving the brain metabolism [5] and treating cerebrovascular disorders and senile mental impairment as an antioxidant [6].

However, NIC has various impurities and the related substances, the two most important ones, 1-DN and 5-CN were undertaken in the present study. A qualitative RP-HPLC-UV study was reported for NIC and some related compounds including 1-DN and 5-CN, but no quantitation was reported in this study [2]. 5-CN was used as an internal standard, but it was not quantified by the RP-HPLC (API-MS) method [7]. NIC and some metabolites were determined by a TLC-SIMS method [8]. It is also known that the TLC method is less sensitive than the HPLC method. Another quantification method by using

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doi:10.1016/j.jpba.2006.04.032

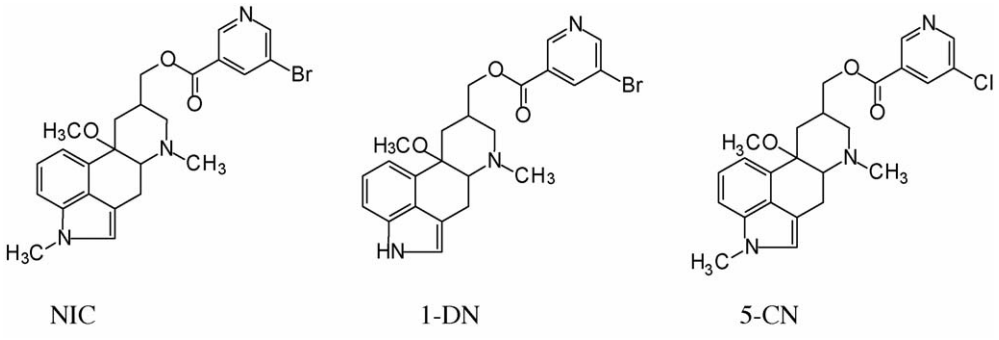

Fig. 1. Nicergoline and its two impurities.

HPLC-API-MS for 1-DN and 5-CN was reported by Banno and Horimoto [9], but this method was not validated. However, API-MS detector is expensive and is not being used extensively in the analysis laboratories. Another study was recently reported about the determination of NIC in the presence of its hydrolysis products by using first derivative ratio spectrophotometric, HPTLC-densitometric and HPLC methods [10]. However, this study was not included 1-DN and 5-CN.

In the reversed-phase chromatography of nitrogen containing bases on chemically bonded ODS-silica; peak tailing, prolonged retention, selectivity [11], poor separation and reproducibility [12,13] are often considerable problems. These problems are probably due to the presence of residual silanol groups on the surface of the column material [14]. To solve these problems triethylamine and ion-pairing agents were added to the mobile phases and the end-capped columns were suggested [15].

Although there are many reported methods on the separate usage of the ion-pairing agents; the simultaneous usage of both anionic and cationic modifiers are rare in the literature. Reproducible results with high resolving power were reported by simultaneous usage of them in the study of quaternary ammonium drugs [16] and some basic drugs [17–19].

It is known that the cationic additives improve the peak shape and alter the selectivity by blocking silanol groups on the stationary phase [20–22]; anionic ones adjust the retention and improve the resolution [23–29]. A synergistic effect is observed when both are used; the tailing, resolution and selectivity problems of the quantitative analysis of 1-DN and 5-CN were overcome in the present study. The present RP-HPLC-DAD method is efficient, cheaper and validated as well.

2. Experimental

2.1. Materials and reagents

NIC, 1-DN and 5-CN were kindly obtained from Pharmacia & Upjohn (Italy) and Deva Pharmaceutical Company (Turkey) as a gift. Ammonium acetate (Riedel deHaen), sodium acetate (Merck), dimethylformamide (Merck), acetic acid (Merck), tetrabutylammonium hydrogen sulphate (Merck), 1-octanesulfonic acid sodium salt (Sigma) were of reagent grade. Acetonitrile (Lab Scan) was of HPLC grade.

2.2. Apparatus

The HPLC (Agilent Technologies) was a combination of a Model G1311A quaternary pump, a Model G1322A vacuum degasser and a Model G1315A diode array detector. Sample solutions were injected with a syringe through a Rheodyne Model 7725i loop type injector. Loop volume was 50 and 20 μL. The separation was performed on Phenomenex, Luna, 5 μm, C18 (2) 250 mm × 4.6 mm column. Data were processed through the Agilent ChemStation. Genex beta 50 μL and Eppendorf 1000 μL automatic pipettes were used.

2.3. Chromatographic conditions

The column used was Phenomenex, Luna, 5 μm, C18 (2), 250 mm × 4.6 mm. The following mobile phases are used: mobile phase 1: 0.1 M NH_4Ac solution (pH: 6.0)/acetonitrile (ACN) (62:38); mobile phase 2: 0.1 M NH_4Ac solution containing 10 mM OSASS (pH: 6.0)/ACN (62:38); mobile phase 3a: 0.1 M NH_4Ac solution containing 4 mM OSASS and 6 mM TBAHS (pH: 5.9)/ACN (62:38); mobile phase 3b: 0.1 M NH_4Ac solution containing 4 mM OSASS and 6 mM TBAHS (pH: 5.9)/ACN (64:36). Flow rate was 1.0 mL min^{-1}. Detection was performed with an Agilent 1100 Model diode array detector at λ: 285 nm, band width: 4 nm. Ambient temperature was used. Injection volume was 50 or 20 μL. Dimethylformamide (DMF) was used for void volume (2.717 min). DMF was detected at 254 nm.

2.4. Coating procedure

A commercial RP-column from Phenomenex was dynamically coated by a solution of 0.1 M NH_4Ac solution containing 4 mM OSASS and 6 mM TBAHS (pH: 5.9)/ACN (mobile phase 3a or 3b) at 0.4 mL min^{-1}. The analytical column was equilibrated with the eluent for 16–17 h until the baseline was stabilized. Sample injection was made at this point.

The capacity factor (k') was calculated according to the expression: $k' = (T_R - T_0)/T_0$ where T_R is retention time of peak (min). The dead time of the system (T_0) which is used to calculate the capacity factor (k') was determined by injecting DMF into the system. The tailing factor (T) was calculated according to the expression: $T = W_{5.0}/2t_w$, where $W_{5.0}$ is the width of

the peak at 5% of the peak height (min); t_w is the distance in min between the peak front and the T_R, measured at 5% of the peak height. The resolution (R) was calculated according to the expression: (pertaining to peaks a and b, T_R of a peak a < T_R of a peak b; T_R in min) $R = 2(T_{R(b)} - T_{R(a)})/W_{B(b)} + W_{B(a)}$ where $W_{B(x)}$ is the base width for peak x (min).

2.5. Preparation of the standard solutions

2.5.1. Stock standard solutions

30.0 mg NIC, 1-DN and 5-CN were dissolved separately in 100.0 mL of the mobile phase 1. They were prepared daily.

2.5.2. Calibration curves

5.0, 15.0, 50.0, 150.0, 300.0, 600.0 μL of 1-DN and 5-CN stock standard solutions were transferred separately to a 10.0 mL of volumetric flask and 40.0 μL DMF was added. Each solution diluted to the volume with the mobile phase 1. 20.0 μL of each diluted solution was injected into the column three times. This procedure repeated three times in different days.

2.6. Preparation of samples

90.0 mg NIC was weighted and transferred to a 100.0 mL volumetric flask. 40.0 μg DMF was added. It was diluted to volume with the mobile phase 1 and shaken for 30 min. This solution was filtered by a 0.2 μm nylon filter. This procedure was done three times. 20.0 μL of each solution was injected into the HPLC column three times.

2.7. Recovery procedure

The amount of placebo tablet powder equal to 10 tablets and appropriate amounts of 1-DN and 5-CN were weighted separately and each mixed uniformly in a porcelain vessel for 10 min. An amount of powdered mixture equal to one tablet was weighted and transferred to a 100.0 mL volumetric flask. It was diluted to volume with the mobile phase 1 and shaken for 30 min. This solution was filtered by 0.2 μm nylon filter. 150 μL for 1-DN and 300.0 μL for 5-CN of the filtrates were taken into a 10.0 mL volumetric flasks. 40 μL DMF was added and diluted to volume with the mobile phase 1. This procedure was done three times. 20.0 μL of each solution was injected into the HPLC column three times.

The peak purity is based on the comparison of spectra recorded during the elution of the peak. Five spectra per peak are used to assess the purity. Two spectra on each of the up and down the slopes and one at the top. The five spectra are averaged and compared with all spectra recorded in the peak. Similarity curve technique was used to judge the peak's purity. This technique calculates a purity factor representing the degree of similarity between the spectra. Values above the 990 indicate the spectra is similar. The peak purity curves obtained for the peaks corresponding to 1-DN and 5-CN which present in the major substance of NIC.

3. Results and discussion

3.1. Detection wavelength and the mobile phase ratio

The preliminary experiments were started with the study of Banno and Horimoto [9]. The detection wavelength and the mobile phase ratio were changed to 285 nm and 62:38 (0.1 M NH_4Ac/ACN, v/v), respectively.

3.2. Mobile phase additives

3.2.1. Adding of the anionic exchanger

By adding of 10 mM OSASS to 0.1 M NH_4Ac solution of the mobile phase the k' values were increased, T values were surprisingly decreased. However, the total analysis time was quite long with 24 min. The R of 5-CN to the closest peak of the major compound NIC was 3.723 (Table 1). An interfering unknown peak appeared next to the 5-CN peak (related chromatogram was not shown in the text).

3.2.2. Simultaneous adding of the two counter ion exchangers

OSASS (4 mM) and TBAHS (6 mM) were added to 0.1 M NH_4Ac solution of the mobile phase [30,31]. The analysis time was drastically shortened and the T values were decreased (Fig. 2a; Table 2). The R was found as 2.920 for 5-CN/NIC in these conditions.

Although, the peak parameters were more efficient and the analysis time was shortened, the interference of the unknown peak was still relevant as shown in the Fig. 2a. For resolving the interfering unknown peak and 5-CN peak, the mobile phase ratio was changed to 64:36 from 62:38. As it can be seen in the Fig. 3a, the separation of 5-CN peak was successful and

Table 1
The capacity factors, resolutions and tailing factors of NIC and its impurities

Mobile phase	Ratio	1-DN		5-CN		NIC		R (5-CN/NIC)
		k'	T	k'	T	k'	T	
0.1 M NH_4Ac (pH: 6.0)/ACN	62:38	2.234	1.359	3.934	1.387	4.509	1.396	3.191
Theoretical plate number (N^*)		12270		14325		14258		
0.1 M NH_4Ac cont. 10mM OSASS (pH: 6.0)/ACN	62:38	3.580	1.229	6.044	1.266	6.916	1.248	3.723
N		14871		17184		17255		

Standart substances were used for the data.
* N: according to half width method.

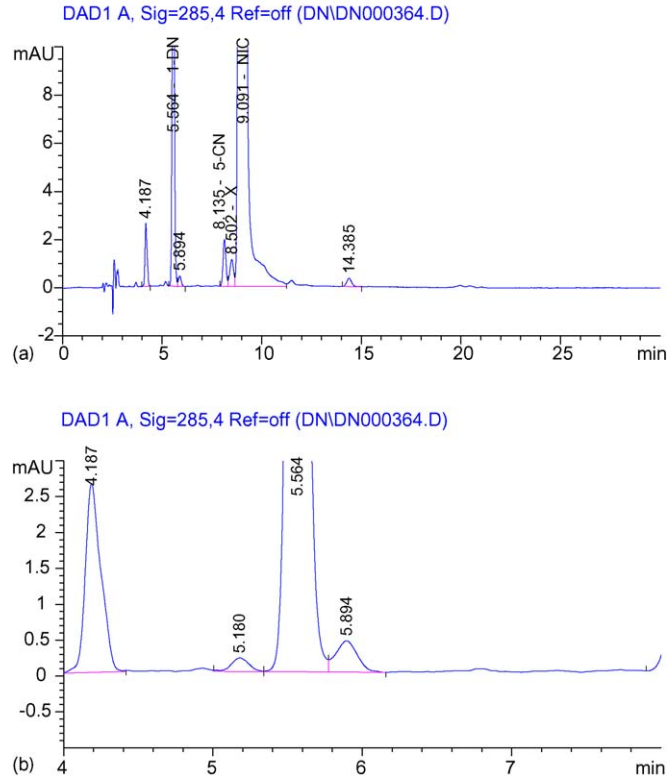

Fig. 2. (a) Chromatogram of overloaded NIC. Column: Phenomenex, Luna 5 μm, C18 (2) 250 mm × 4.6 mm, mobile phase, 0.1 M NH_4Ac solution containing 4 mM OSASS and 6 mM TBAHS (pH: 5.9)/ACN (62:38); flow rate, 1.0 mL min^{-1}; detector, Agilent 1100 Model DAD λ: 285 nm; band width, 4 nm; sample concentration, 0.90 mg mL^{-1} for NIC; sample volume, 20 μL. (b) Enlarged chromatogram of overloaded NIC. Other chromatographic conditions are same in a.

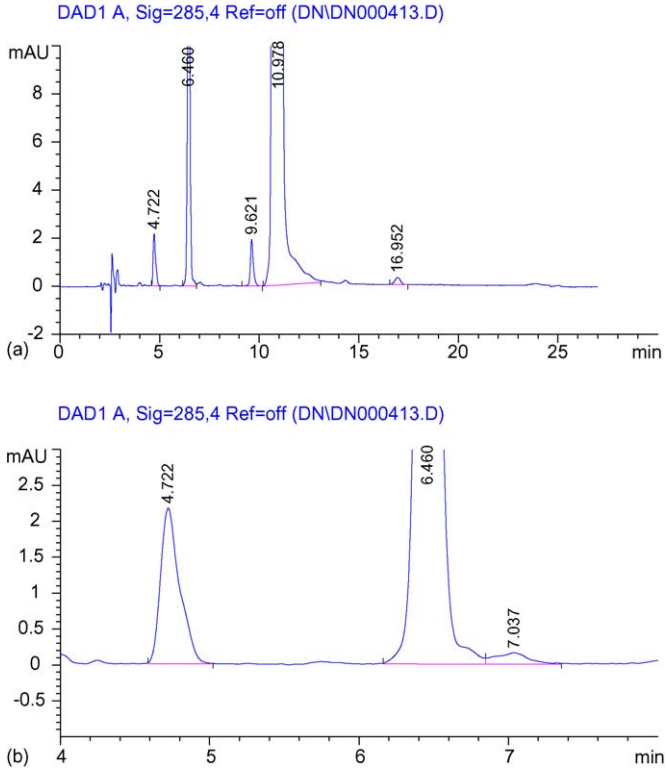

Fig. 3. (a) Chromatogram of overloaded NIC. Mobile phase: 0.1 M NH_4Ac solution containing 4 mM OSASS and 6 mM TBAHS (pH: 5.9)/ACN (64:36). Other chromatographic conditions are same in Fig. 2a. (b) Enlarged chromatogram overloaded NIC. Other chromatographic conditions are same in a.

the unknown peak (X) co-eluted with the major compound of NIC. Therefore, we decided to use the mobile phase ratio of 62:38 for the quantification of 1-DN (mobile phase 3a) and 64:36 (mobile phase 3b) for 5-CN. The well-resolved minor unknown impurities could clearly be seen in the front and back of the major peak of 1-DN in Fig. 2b (mobile phase 3a) comparatively in Fig. 3b (mobile phase 3b).

3.3. Selectivity

The selectivity was checked by injecting two other related compounds of NIC. One was 1-MMDL, a degradation product and/or metabolite and the other, NIC-10-amethoxylated which is the most apolar of all the related compounds [9]. No interference was detected, as shown in Fig. 4.

Table 2
The capacity factors, resolutions and tailing factors of NIC, 1-DN and 5-CN in 0.1 M NH_4Ac containing 4 mM OSASS vs. 6 mM TBAHS (pH: 5.9)/ACN

Mobile phase ratio[a]	1-DN		5-CN		NIC		*R* (5-CN/NIC)
	k′	*T*	*k′*	*T*	*k′*	*T*	
62:38	1.114	1.174	2.038	1.120	2.350	1.139	2.920
N	11212		16044		14488		
64:36	1.458	1.158	2.643	1.075	3.090	1.106	3.854
N	12137		24410		15232		

Standart substances were used for the data.
[a] 0.1 M NH_4Ac containing 4 mM OSASS vs. 6 mM TBAHS (pH: 5.9)/ACN.

3.4. Linearity

3.4.1. 1-DN

Linearity was obtained in the concentration range of 0.032×10^{-5} to 3.828×10^{-5} M, $y = 116.88x + 0.2773$ ($r^2 = 0.99989$); the limit of detection (LOD) was determined as 0.012×10^{-5} M, the limit of quantification (LOQ) was determined as 0.041×10^{-5} M.

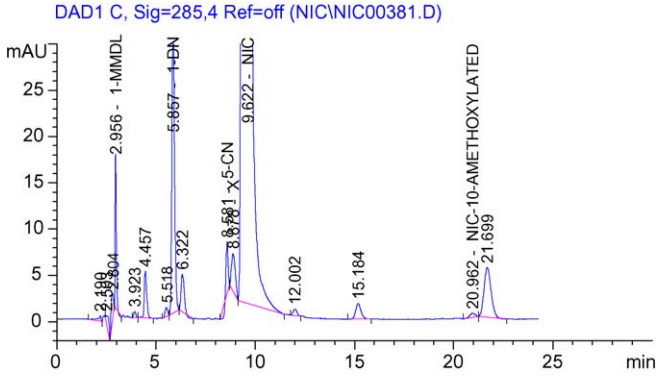

Fig. 4. Selective separation of 1-DN, 5-CN, possible degradation products and the metabolites of NIC. Other chromatographic conditions are same in Fig. 2a.

Table 3a
Statistics of calibration curve of 1-DN

	1st day	2nd day	3rd day	Mean	S.D.
a	0.6731	1.6421	−1.4832	0.2773	1.60
b	116.49	116.66	117.50	116.88	0.54
r^2	0.99993	0.99977	0.99996	0.99989	

a, intercept; *b*, slope.

Table 3b
Statistics of calibration curve of 5-CN

	1st day	2nd day	3rd day	Mean	S.D.
a	0.4240	0.8840	0.9379	0.7486	0.28
b	104.44	104.75	103.52	104.24	0.64
r^2	0.99994	0.99999	0.99995	0.99996	

a, intercept; *b*, slope.

3.4.2. 5-CN

Linearity was obtained in the concentration range of 0.034×10^{-5} to 4.092×10^{-5} M, $y = 104.24x + 0.7486$ ($r^2 = 0.99996$); LOD was determined as 0.014×10^{-5} M, LOQ was determined as 0.046×10^{-5} M. $LOD = y_b + 3S_B$ and $LOQ = y_b + 10S_B$ were used. (y_b = blank signal; S_B = random errors in the *y*-direction; $S_B = \sqrt{\Sigma (y_i - \tilde{y}_i)^2/n - 2}$; y_i = the values of peak areas of the minimum concentration of analyte; $\tilde{y}_i$ = the average of peak areas of the minimum concentration of analyte; $(n - 2)$ = the number of degrees of freedom in the linear regression calculations.) The statistical results of the calibration curve were given in Tables 3a and 3b.

3.5. Recovery

3.5.1. 1-DN

The accuracy of the method was determined and the mean recovery was found to be 100.65% (Table 4a). The RSD of the founded values was 0.38% (Table 4b).

Table 4a
Experimental values obtained in the recovery test for 1-DN in 30 mg 1-DN/placebo tablet

n	1-DN (*x*) (mg/placebo tablet)	Recovery (%) of 1-DN in placebo tablet
1	30.13	100.43
2	30.33	101.10
3	30.13	100.43
Mean	30.20	100.65

Table 4b
Statistical evaluation of the results in Table 4a

$\bar{x}$	30.20
S	0.12
RSD	0.38
n	3.00
$\pm t \times S/(n)^{1/2}$	±0.29
Confidence interval (%95)	29.91–30.48

Table 5a
Experimental values obtained in the recovery test for 5-CN in 30 mg 5-CN/placebo tablet

n	5-CN (*x*) (mg/placebo tablet)	Recovery (%) of 5-CN in placebo tablet
1	30.61	102.03
2	29.68	98.93
3	30.00	100.00
Mean	30.10	100.32

Table 5b
Statistical evaluation of the results in Table 5a

$\bar{x}$	30.10
S	0.47
RSD	1.57
n	3.00
$\pm t \times S/(n)^{1/2}$	±1.17
Confidence interval (%95)	28.92–31.27

3.5.2. 5-CN

The accuracy of the method was determined and the mean recovery was found to be 100.32% (Table 5a). The RSD of the founded values was 1.57% (Table 5b).

The peak purity curves were given in Figs. 5 and 6; the purity factors were above 990 with the values of 999.996 and 999.942 for 1-DN and 5-CN, respectively. The RSD's were with the value of 0.980 for 1-DN and 0.709 for 5-CN ($n = 3$). These values indicated that the purities of the peaks in the different samples were satisfactory.

3.6. Repeatability

3.6.1. 1-DN

The precision of the method was determined by repeatability (intra-day) and was expressed as RSD (%) of a series of measurement. The result obtained for 1-DN is shown in Table 6a. The RSD of 0.97% for 1-DN is indicating a good intra-day precision (Table 6b).

3.6.2. 5-CN

The result obtained for 5-CN is shown in Table 7a. The RSD of 1.40% is indicating good intra-day precision (Table 7b).

Table 6a
Results of the determination of 1-DN in 30 mg NIC

n	Amount of 1-DN in NIC (*x*) (μg/30 mg NIC)	1-DN (mg)/NIC(100 mg)
1	207.78	0.69
2	211.82	0.71
3	209.34	0.70
Mean	209.65	0.70

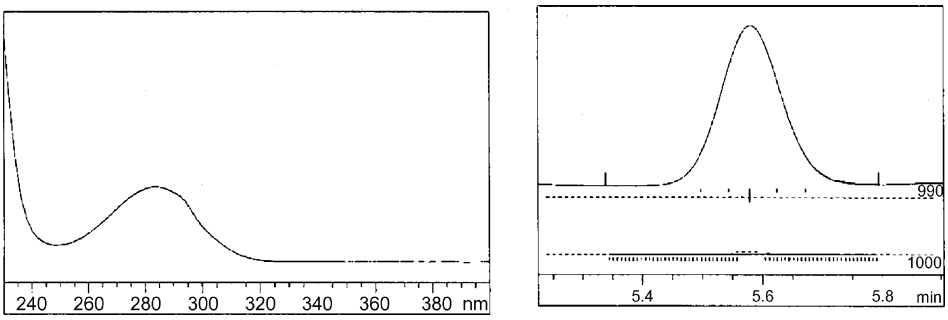

Fig. 5. Absorption spectra and peak purity curve for 1-DN.

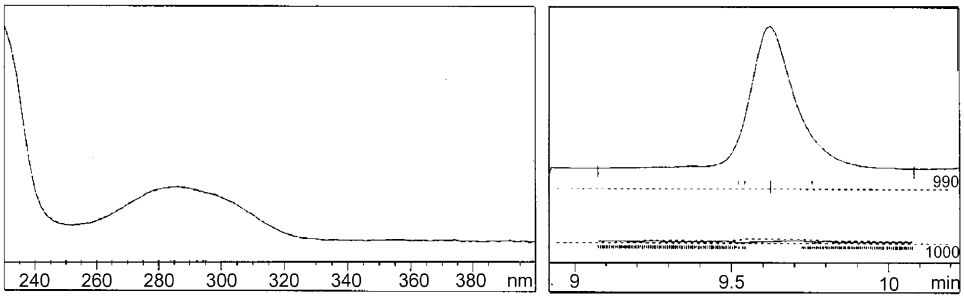

Fig. 6. Absorption spectra and peak purity curve for 5-CN.

Table 6b
Statistical evaluation of the results in Table 6a

$\bar{x}$	209.65
S	2.04
RSD	0.97
n	3.00
$\pm t \times S/(n)^{1/2}$	±5.06
Confidence interval (%95)	204.59–214.70

Table 7a
Results of the determination of 5-CN in 30 mg NIC

n	Amount of 5-CN in NIC (x) (μg/30 mg NIC)	5-CN (mg)/NIC(100 mg)
1	27.33	0.09
2	28.06	0.09
3	27.48	0.09
Mean	27.62	0.09

Table 7b
Statistical evaluation of the results in Table 7a

$\bar{x}$	27.62
S	0.39
RSD	1.40
n	3.00
$\pm t \times S/(n)^{1/2}$	±0.96
Confidence interval (%95)	26.67–28.58

4. Conclusion

Nitrogen containing basic compounds constitute a major proportion of drugs and their related substances (impurities, degradation products, etc.). Improving the peak parameters of their HPLC analyses has a particular importance in separation sciences.

The pK_a values of 1-DN and 5-CN are unknown; however, they both contain three nitrogens in their molecular structures. Probably because of these nitrogens, pronounced tailing had been investigated by the mobile phase used without any ion-pairing agents (Table 1). Whereas, by adding 10 mM OSASS (anionic ion pairing agent) to the mobile phase, the tailing factors decreased and the retention times increased. The efficiencies of the peaks were better with OSASS because of the ionic interactions. N values were higher with OSASS than they were with the mobile phases not containing additives (Table 1). When 4 mM OSASS and 6 mM TBAHS were used simultaneously as additives, a drastic reduction was obtained in retentions and tailing factors. N values were reasonable if compared to the previous conditions used (Table 2). Besides, the resolving power was high (Figs. 2 and 3) and the repeatability was good (Tables 6a and 6b and Tables 7a and 7b).

There are a few reported studies on the analyses of basic drugs by using counter ions in RP-HPLC method [17–19]. Apart from the basic drugs, a few more studies were reported [30–32].

In the RP-HPLC system, the mechanism of the interaction of the counter ions and the analytes are not yet fully understood. Our opinion is that both hydrophobic and ionic interactions between the analytes and the system are effective on the efficiency and high resolving power. Besides, the steric effects of the apolar structures might have a role in the separation. We highly propose this method for substances that have potential tailing and resolution problems. As a result, the present method can be used safely for the quantification of 5-CN and 1-DN in the quality control of

them and the quantification of the metabolite of 1-DN in blood.

Acknowledgments

We would like to thank to the Pharmacia Upjohn and Deva İlaç San. A. Ş. for their giving the impurities of Nicergoline as a gift and Marmara University Research Fund, İstanbul, Turkey for its financial support.

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