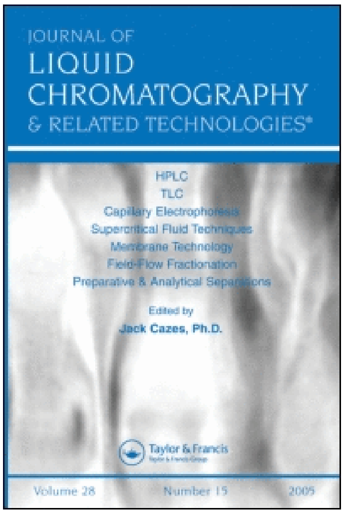

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A. Pyka[a]; W. Klimczok[a]
[a] Department of Analytical Chemistry, Faculty of Pharmacy, Silesian Academy of Medicine, Sosnowiec, Poland

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Application of Densitometry for the Evaluation of the Separation Effect of Nicotinic Acid Derivatives. Part I. Nicotinic Acid and its Amides

A. Pyka and W. Klimczok
Department of Analytical Chemistry, Faculty of Pharmacy, Silesian
Academy of Medicine, Sosnowiec, Poland

Abstract: Nicotinic acid (1) and its amides, namely nicotinamide (8), N-methylnicotinamide (9) and N,N-diethylnicotynamide (10) were investigated by NP-TLC, and RP-HPTLC. The R_F values were obtained from the densitometric analysis. The separation factors ΔR_F, R_F^{α} and selectivity α were calculated from the R_F values. The resolution R_S values obtained by use of the chromatogram were verified by use of densitometric analysis. It was affirmed, that R_S values can be correctly marked exclusively on the basis of the densitograms. The R_S values larger than 1.5 calculated on the basis of the densitograms were obtained for the pair of compounds (10)/(9) and (8)/(1) on $RP18WF_{254}$ plates, and by use of methanol + water in a volume composition of 30:70, and dioxane + water in volume compositions of 20:80 and 10:90 as mobile phases. However, these conditions did not provide for the complete separation of N-methylnicotinamide (9) from nicotinamide (8). Adsorption thin-layer chromatography (NP-TLC) in the system of a neutral aluminum oxide $60F_{254}$ and acetone + *n*-hexane mobile phase in a volume composition of 50:50 provided the optimum conditions for the separation of all the studied compounds.

Keywords: TLC, Densitometry, Separation parameters, Nicotinic acid derivatives

Address correspondence to A. Pyka, Department of Analytical Chemistry, Faculty of Pharmacy, Silesian Academy of Medicine, 4 Jagiellońska Street, PL-41-200 Sosnowiec, Poland. E-mail: apyka@slam.katowice.pl

INTRODUCTION

Nicotinic acid and its derivatives are biologically important compunds.[1–12] The term niacin is used in two different ways; it refers to both nicotinic acid and nicotinamide. Niacin is a member of the B-vitamin family. It is sometimes referred to as vitamin B_3 or vitamin PP. Nicotinic acid and nicotinamide have identical vitamin activities, but with very different pharmacological activities. Nicotinic acid, in pharmacological doses, is used as an antihyperlipidemic agent. Various studies have shown that nicotinic acid can significantly lower total cholesterol, LDL-cholesterol, triglycerides, and lipoprotein (a) levels. It can also increase HDL-cholesterol levels. Nicotinamide (vitamin PP) may have some anti–diabetogenic activity. It may also have antioxidant, anti–inflammatory, and anticarcinogenic activites.[1,2] Nicotinamide, unlike nicotinic acid, does not have significant effects on lipids, but is sometimes useful in cases of type 1 (insulin-dependent) diabetes.[3–5] PP vitamin shortage causes weakness, headaches, apathy, and pelagra. 3-Pyridylmethanol is useful in the therapy of obliterative atheromatosis. N,N-diethylnicotinamide is a central nervous system stimulator.[6] One should also take esters of nicotinic acid into consideration; some of them, for instance methyl nicotinate, ethyl nicotinate, isopropyl nicotinate, hexyl nicotinate, and benzyl nicotinate, are used as ingredients in pharmaceutical creams. They enhance the topical penetration of the active substances.[7–12]

We have previously used reversed-phase high performance thin layer chromatography on $RP18WF_{254}$ plates for the evaluation of lipophilicity of the nicotinic acid (1), methyl nicotinate (2), ethyl nicotinate (3), isopropyl nicotinate (4), butyl nicotinate (5), hexyl nicotinate (6), benzyl nicotinate (7), nicotinamide (8), N-methylnicotinamide (9), N,N-diethylnicotinamide (10), 3-pyridinecarbaldehyde (11), 3-pyridinecarbonitrile (12), 3-pyridylmethanol (13), and methyl 3-pyridyl ketone (14).[13] Lipophilic parameters (R_{MW} and φ_0) were compared, both with measured ($\log P_{exp}$), and calculated partition coefficients ($A\log P_S$, IAlogP, $\log P_{Kowwin,}$ xlogP, ClogP, miLogP). We have also used the selected structural descriptors to estimate the lipohilic properties of vitamin PP and its derivatives.[13]

The aim of this study was to use densitometry for the evaluation of the separation effects of nicotinic acid and its amides, investigated by adsorption and reversed-phase thin layer chromatography.

EXPERIMENTAL

Chemicals and Sample Preparation

The components of the mobile phases: acetone, dioxane (POCh, Poland; analytical grade), methanol (Merck, Germany; for liquid chromatography), *n*-hexane (AnalaR, UK; analytical grade), and redistilled water were used for TLC analysis. The commercial samples of nicotinic acid, nicotinamide, N-methylnicotinamide, and N,N-dietylnicotinamide (Sigma-Aldrich, USA) were used as test solutes. The purities of the studied standard samples were at least 98%. The above-mentioned compounds (about a concentration of 1 mg mL^{-1} of each standard) were dissolved in ethanol (POCh, Poland; 96%; analytical grade).

Thin Layer Chromatography

Reversed-Phase High Performance Thin-Layer Chromatography

Reversed-phase high performance thin-layer chromatography (RP-HPTLC) was performed on 10 × 10 cm glass HPTLC plates, coated with RP-$18WF_{254}$ (Merck, #1.13124). The plates were prewashed with methanol and dried for 24 h at room temperature (18 ± 1°C). The mixture solution of the nicotinic acid and its amides (2 μL) was spotted manually using a microcapillary (Camag, Switzerland) on the chromatographic plate. The methanol-water and dioxane-water in volume compositions of 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100 were used as mobile phases. Plates were developed to a distance of 7.5 cm at room temperature (18 ± 1°C) in a classical bottom chamber (Camag, Switzerland) previously saturated with the mobile phase for 30 min. After development, the plates were dried for 24 h at room temperature (18 ± 1°C).

Adsorption Thin-Layer Chromatography

Adsorption thin-layer chromatography (NP-TLC) was performed on 20 × 20 cm aluminium plates precoated with 0.2 mm layer of a neutral aluminium oxide $60F_{254}$ (Type E) (E.Merck, #1.05550). The plates were prewashed with methanol and dried for 24 h at room temperature. The plates were then activated at 120°C for 30 min. The mixture solution of the nicotinic acid and its amides (2 μL) was spotted manually using a microcapillary (Camag, Switzerland) on the chromatographic plate. The mixture of nicotinic acid and its amides was separated using acetone + *n*-hexane in volume compositions of 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100 as mobile phases. The mobile phase (50 mL) was placed in a classical chamber (Camag, Switzerland) and the chamber was saturated with the mobile phase for 30 min. The plates were developed to a distance 14 cm at room temperature (18 ± 1°C). The plates were dried for 24 h at room temperature (18 ± 1°C) in a fume cupboard.

Visualization of Spots by Use of UV Lamp

The spots on a plate were visualized using a UV lamp (Cobrabid, Poland) at $\lambda = 254$ nm.

Visualization of Spots by Use of a Camag Densitometer

Densitometric scanning was then performed at $\lambda = 254$ nm with a Camag Scanner TLC 3 operated in the absorbance mode and controlled by winCATS 1.4.1 software. The radiation source was a deuterium lamp emitting a continuous UV spectrum between 190 and 450 nm. The slit dimensions were 6.00 × 0.30 mm, Micro for HPTLC plates, and 8.00 × 0.30 mm, Macro for TLC plates; the optimized optical system was light; the scanning speed was 20 mm s^{-1}; the data resolution was 100 μm $step^{-1}$; the measurement type was remission; and the measurement mode was absorption; the optical filter was second order. Each track was scanned three times and baseline correction (lowest slope) was used.

Separation Factors

The chromatograms were done in triplicate and each track was scanned three times; the mean R_F values were calculated.

The separation factors, namely: ΔR_F values, selectivity (α),[14] and the constant of the pair separation (R_F^α)[15] were calculated for all the densitograms.

ΔR_F was calculated according to the formula:

$$\Delta R_{F(1,2)} = R_{F1} - R_{F2} \tag{1}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} > R_{F2}$.

The selectivity (α) was calculated using the equation:

$$\alpha = \frac{(1/R_{F1})-1}{(1/R_{F2})-1} \tag{2}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} < R_{F2}$.

The constant of the pair separation (R_F^α) was calculated for the investigated compounds as the ratio of the R_F values of the two adjacent peaks on the densitogram:

$$R^{\alpha}_{F(1,2)} = \frac{R_{F1}}{R_{F2}} \tag{3}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} > R_{F2}$

Resolution Factors

R_S Calculation on the Basis of Chromatogram

The resolution of two spots ($R_{S(c)}$) was calculated using the formula:[14]

$$R_{S(c)} = 2 \times \frac{d}{s} \tag{4}$$

where d is the distance between the centers of two adjacent spots on the chromatogram, and s is the sum of the widths of the two spots in the direction of flow of mobile phase.

R_S Calculation on the Basis of Densitometric Analysis

The peak resolution ($R_{S(b)}$) was calculated using the equation:[16]

$$R_{S(b)} = \frac{2d}{w_{b1} + w_{b2}} \tag{5}$$

where d is the distance between the centers of two adjacent peaks on the densitogram, whereas w_{b1} and w_{b2} are the peaks-width at the base.

The peak resolution ($R_{S(h)}$) was also calculated using the equation:[17]

$$R_{S(h)} = \frac{d}{w_{h1} + w_{h2}} \sqrt{\ln 4} \tag{6}$$

where d is the distance between the centers of two adjacent peaks on the densitogram, whereas w_{h1} and w_{h2} are the peaks-width at half height.

The average values of peak resolution ($R_{S(a)}$) were also calculated according to the formula:

$$R_{S(a)} = \frac{R_{S(b)} + R_{S(h)}}{2} \tag{7}$$

RESULTS AND DISCUSSION

RP-HPTLC and NP-TLC techniques were used for the separation of nicotinic acid (1), nicotinamide (8), N-methylnicotinamide (9), and N,N-diethylnicotinamide (10). $RP18WF_{254}$ plates with spotted compounds were separated using a methanol + water and dioxane + water mobile phase in different volume compositions. It was affirmed that the R_F values of the studied compounds decrease with an increase in water content in the mobile phase. Nicotinic acid (1) is an exception because its R_F values were changing in the narrow range. The chromatograms were analyzed by use of UV light at $\lambda = 254$ nm, after development and dried. Next, the resolutions of chromatographic spots $R_{S(c)}$ were calculated using the Equation (4).

The $R_{S(c)}$ values greater than 1 for all pair compounds (NN-diethylnicotinamide (10) – N-methylnicotinamide (9); N-methylnicotinamide (9) – nicotinamide (8); nicotinamide (8) – nicotinic acid (1)) on the chromatogram were obtained by use of methanol + water in volume compositions 40:60, 30:70, 20:80 and 10:90 as well as dioxane + water in volume compositions 20:80 and 10:90 as mobile phases. The $R_{S(c)}$ values obtained by use of this method were verified using densitometric analysis. The plates developed by the use of methanol + water and dioxane + water mobile phases in the above-mentioned volume compositions were densitometrically analyzed at $\lambda = 254$ nm. The R_F values were obtained from the densitometric analysis. The separation factors ΔR_F, $R_{F\alpha}$, and selectivity α were calculated from the R_F values. Moreover, the resolutions of peaks $R_{S(b)}$ and $R_{S(h)}$ were calculated from the Equations (5) and (6) by the use of the obtained densitometric bands for the studied compounds. The obtained data are presented in Table 1. The average $R_{S(a)}$ values calculated by use of Equation (7) and the characteristic densitometric peaks are also presented in Table 1. The characteristics of the densitometric peaks concern their height, area, and the angle (β) between the tangents at the inflection points to the curves of the densitometric peaks. It was affirmed, that $R_{S(b)}$, $R_{S(h)}$, and $R_{S(a)}$ values calculated on the basis of the densitograms are considerably lower than the $R_{S(c)}$ values calculated on the basis of the chromatograms. This shows that R_S values can be correctly marked exclusively on the basis of the densitograms. The scientific literature data indicate that, at R_S values smaller than 0.8, we cannot expect any good separations. However, the R_S value is required to be larger than 1.5 to obtain the complete separation of the neighboring compounds on the densitogram. R_S values larger than 1.5 calculated on the basis of the densitograms were obtained for the pair of compounds (10)/(9) and (8)/(1) by use of methanol + water in a volume composition 30:70, and dioxane +water in volume compositions of 20:80 and 10:90 as mobile phases. From the presented comparison (Table 1) it is apparent that the best separation of the studied substances was obtained by use of the methanol + water mobile phase in a volume composition of 30:70. For these conditions, the $R_{S(a)}$ value for pair of compounds (9)/(8) is equal to 1.07. The densitogram of the studied compounds on RP18WF$_{254}$ plates and the use of the methanol + water mobile phase in a volume composition 30:70 is presented in Figure 1. However, these conditions do not provide for the complete separation of N-methylnicotinamide (9) from nicotinamide (8). The studied compounds were then separated on neutral aluminum oxide 60F$_{254}$ and by use of an acetone + *n*-hexane mobile phase in various volume compositions. The R_F value of nicotinic acid was equal to 0 or 0.01 with the applied chromatographic conditions using an acetone + *n*-hexane mobile phase with particular volume compositions. It was affirmed, on the basis of $R_{S(c)}$ values calculated from the chromatograms, that the complete separation of the studied compounds should be obtained on a neutral aluminum oxide 60F$_{254}$ and by use of an acetone + *n*-hexane mobile phase with a volume composition of 50:50, 60:40, 70:30 and 80:20.

Table 1. R_F values, separation factors, resolutions, and characteristic of denstitometric bands of investigated compounds by RP-HPTLC and NP-TLC techniques

		Separation factors			R_S values calculated from Eqs.				Characteristic of densitometric band		
Comp. no	R_F	ΔR_F	α	R_F^{α}	(4)	(5)	(6)	(7)	Height [AU]	Area [AU]	β [°]
RP18WF$_{254}$, Methanol + water, 40:60 (v/v)											
10	0.31								239	12678	12
9	0.47	0.16	1.97	1.52	3.07	1.54	1.59	1.56	213	10338	14
8	0.56	0.09	1.44	1.19	2.00	0.91	0.93	0.92	186	8849	15
1	0.67	0.11	1.60	1.20	2.29	1.12	1.12	1.12	182	9210	16
RP18WF$_{254}$, Methanol + water, 30:70 (v/v)											
10	0.19								270	11959	10
9	0.34	0.15	2.20	1.79	2.32	1.56	1.59	1.58	238	10783	11
8	0.44	0.10	1.52	1.29	2.00	1.06	1.08	1.07	206	9344	13
1	0.68	0.22	2.70	1.54	4.38	2.23	2.24	2.24	168	9418	20
RP18WF$_{254}$, Methanol + water, 20:80 (v/v)											
10	0.09								285	12292	10
9	0.22	0.13	2.85	2.44	2.00	1.33	1.27	1.30	252	11530	13
8	0.32	0.10	1.67	1.45	1.47	1.03	1.00	1.02	219	10126	16
1	0.63	0.31	3.62	1.97	5.65	2.69	2.70	2.70	171	10104	27
RP18WF$_{254}$, Methanol + water, 10:90 (v/v)											
10	0.04								284	11833	8
9	0.14	0.10	3.91	3.50	1.56	1.03	0.98	1.00	256	10906	13
8	0.23	0.09	1.83	1.64	1.75	1.05	1.02	1.04	215	9436	15
1	0.61	0.38	5.24	2.65	7.00	3.55	3.53	3.54	149	9029	29

(*continued*)

Table 1. Continued

		Separation factors			R_S values calculated from Eqs.				Characteristic of densitometric band		
Comp. no	R_F	ΔR_F	α	R_F^{α}	(4)	(5)	(6)	(7)	Height [AU]	Area [AU]	β [°]
$RP18WF_{254}$, Dioxane + water, 20:80 (v/v)											
10	0.30								240	10699	11
9	0.45	0.15	2.00	1.55	3.20	1.67	1.73	1.70	208	9773	14
8	0.52	0.07	1.32	1.16	1.43	0.67	0.70	0.68	188	8542	15
1	0.75	0.23	2.77	1.44	4.53	2.06	2.18	2.12	155	8472	21
$RP18WF_{254}$, Dioxane + water, 10:90 (v/v)											
10	0.16								270	11005	10
9	0.30	0.14	2.25	1.88	2.32	1.50	1.52	1.51	224	10390	13
8	0.39	0.09	1.49	1.30	1.47	0.88	0.90	0.89	192	8605	15
1	0.75	0.36	4.69	1.92	4.95	2.99	3.02	3.00	129	8180	30
Neutral aluminum oxide $60F_{254}$, Acetone + *n*-hexane, 50:50 (v/v)											
1	0.01								158	3402	5
8	0.10	0.09	11.0	10.00	2.67	2.06	2.02	2.04	115	8267	26
9	0.38	0.28	5.12	3.80	6.00	3.07	3.01	3.04	140	12185	30
10	0.72	0.34	4.20	1.89	8.55	3.93	3.92	3.92	190	11224	15

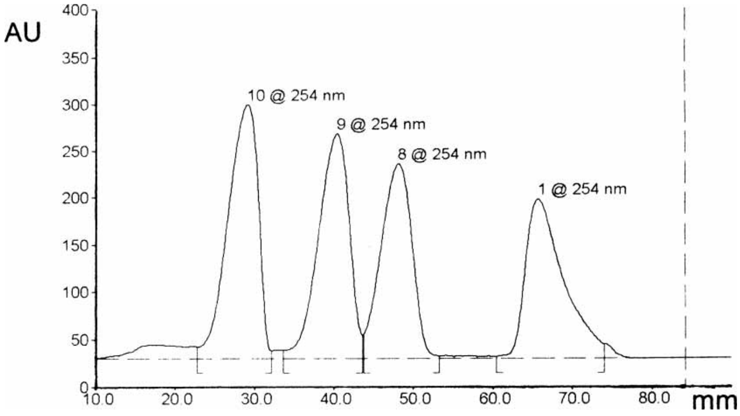

Figure 1. The densitogram of the studied compounds on RP18WF$_{254}$ plates using a methanol + water mobile phase with a volume composition of 30:70.

The R$_F$ value of N,N-diethylnicotinamide on a neutral aluminum oxide 60F$_{254}$ and with the use of an acetone + *n*-hexane mobile phase in the volume composition of 50:50 is equal to 0.72. However, the R$_F$ values of N,N-diethylnicotinamide are larger than 0.85 using an acetone + *n*-hexane mobile phase with volume compositions of 60:40, 70:30, and 80:20. That is why only the plate with the studied compounds on a neutral aluminum oxide 60F$_{254}$ and using an acetone + *n*-hexane mobile phase with a volume composition 50:50 was densitometry applied. The obtained results are presented in Table 1. It was affirmed, that all the studied substances are completely separated under these conditions. The obtained R$_{S(b)}$, R$_{S(h)}$, and R$_{S(a)}$ values are larger than 1.5.

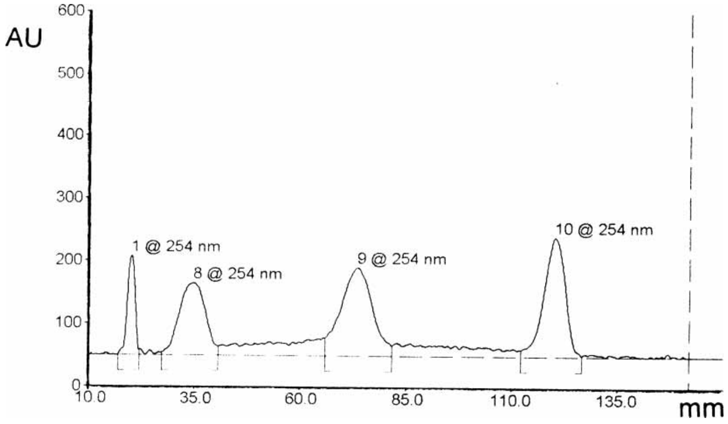

Figure 2. The densitogram of the studied compounds on a neutral aluminum oxide 60F$_{254}$ using an acetone + *n*-hexane mobile phase with a volume composition of 50:50.

The densitogram of the studied compounds on a neutral aluminum oxide $60F_{254}$ and using an acetone + *n*-hexane mobile phase with a volume composition of 50:50 is presented in Figure 2.

In the case of the investigation of the studied substances by the RP-HPTLC technique, the angle value (β) is large for nicotinic acid in relation to the remaining compounds. It results from the fact of spot diffusion of nicotinic acid under the influence of methanol + water and dioxane + water mobile phases. The numerical value of angle (β) for nicotinic acid increases with the increase of water content in the applied mobile phases. However, the values of heights and areas of the densitometric peaks depend on the type of studied compound and applied mobile phase.

The obtained results show, unambiguously, that adsorption thin-layer chromatography (NP-TLC) in the system of a neutral aluminum oxide $60F_{254}$ and acetone + *n*-hexane mobile phase with a volume composition of 50:50 provides the optimum conditions for the separation of the studied compounds.

Further investigations are in progress and concern the separations of nicotinic acid and its esters and other derivatives.

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