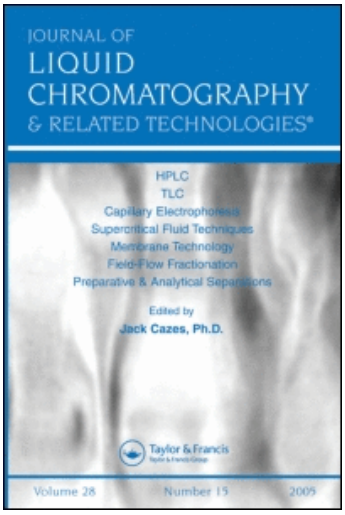

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Chromatographic Separation of Derivatives of 4-Alkoxy-6-methyl-1*H*-pyrrolo[3,4-c]pyridine-1,3(2H)-dione by TLC and HPLC

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Chromatographic Separation of Derivatives of 4-Alkoxy-6-methyl-1*H*-pyrrolo[3,4-c]pyridine-1,3(2H)-dione by TLC and HPLC

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Abstract: Five derivatives of 4-alkoxy-6-methyl-pyrrolo[3,4-c]pyridine-1,3-dione were separated by TLC and HPLC methods using silica gel 60 F_{254} plates, analytical columns: LiChrosorb® RP-8 (250 × 4 mm, 5 μm) and LiChrospher® 100 RP-18 (250 × 4 mm, 5 μm), respectively. From the seven remaining mobile phases, three were offered for use with two-dimensional TLC. The values of separation parameter (α), constant of the pair separation (R_F^{α}), and ΔR_F of two neighbouring spots on each chromatogram were defined. The mobile phase C (butan-1-ol–propan-2-ol–cyclohexane-acetic acid (1.05 kg/L); 6:3:2:1, v/v/v/v) was used in the first dimension but mobile phase D (chloroform–propan-2-ol-acetic acid (1.05 kg/L); 40:15:6, v/v/v) and/or E (chloroform–cyclohexane–propan-2-ol-acetic acid (1.05 kg/L); 20:20:15:6, v/v/v/v) were used in the second dimension. The main validation parameters (linearity, limits of quantification (LOQ) and determination (LOD), repeatability of retention times) were evaluated for the analysis of all of the investigated derivatives in their mixture, by using RP-HPLC with UV detection at 254 nm (LiChrospher® 100 RP-18 column; acetonitrile–methanol–phosphate buffer, pH 7 (45:15:40), 1.5 mL/min).

Keywords: Pyrrolo[3,4-c]pyridine-1,3-dione, TLC, HPLC, Separation

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INTRODUCTION

Synthesis of N-substituted derivatives of 4-alkoxy-6-methyl-1*H*-pyrrolo[3,4-c]pyridine-1,3(2H)-dione was described previously.[1,2] In the "*writhing syndrome*" and "*hot plate*" tests all compounds studied (I–V) (Figure 1) exhibited potent analgesic activity, which was superior to that of acetylsalicylic acid. What's more, the results of the "*writhing syndrome*" test indicate that the compounds I–IV were more potent than morphine. Furthermore, most of the investigated imides suppressed significantly spontaneous locomotor activity in mice and prolonged barbiturate sleep of these animals. Differences in the chemical structure of imides I–V did not influence the toxicity. The LD_{50} values of the investigated compounds after their i.p. administration in mice were evaluated. All these imides were not toxic ($LD_{50} > 2000$ mg/kg). The results of the preliminary radioligand binding assay suggest that these compounds display a weak affinity (in micromolar concentration) to μ-opioid receptors. They probably play a role in the mechanism of action of these imides.[1,2]

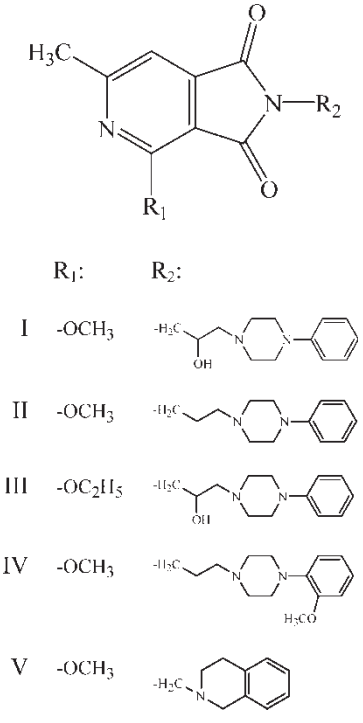

Figure 1. Chemical structure of 4-alkoxy-6-methyl-pyrrolo[3,4-c]pyridine-1,3-dione derivatives.

Our investigations were based on our previous kinetic studies on stability and degradation of compounds I–III[3–8] and the knowledge of other aryldicarboximides, such as thalidomide,[9–12] buspirone,[13–17] a new derivative of phthalimide,[18] their analogues, pro-drugs, and metabolites.[9–17] Separation was carried out using either an octadecyl (RP-18)[10–12,14,16–18] or octyl (RP-8)[9,13] column, and a mixture of methanol or acetonitrile with phosphate buffer (pH range from 2.5 to 6.5) as a mobile phase. Detection was performed spectrophotometrically; only in one case, coulometric detection[17] was used.

This study developed effective methods (TLC and RP-HPLC) for simultaneous identification and determination of five 4-alkoxy-6-methyl-1*H*-pyrrolo[3,4-c]pyridine-1,3(2H)-diones (I–V) from their mixtures.

EXPERIMENTAL

Chemicals and Reagents

All of the derivatives of 4-alkoxy-6-methyl-1*H*-pyrrolo[3,4-c]pyridine-1,3(2H)-dione (I-V) were synthesized by published methods[1,2] and supplied by the Department of Chemistry of Drugs at the Wrocław University of Medicine. The crude products were purified with crystallization. The identification of the obtained compounds was performed using the following parameters: melting points, microanalysis, IR, and ^{1}H-NMR spectral data. Acetonitrile (Merck, Germany) was of HPLC grade and other solvents or chemicals (POCh, Gliwice, Poland) were of analytical reagent grade.

Apparatus

A thin layer chromatographic tank with a flat bottom of transparent glass and size 27 × 25 × 7 cm was suitable for TLC plastic sheets coated with an 0.2 mm thick layer of silica gel 60 F_{254} (size: 10 × 20 cm). A 10 μL microsyringe (Hamilton) was used to apply the standard solutions to the plates.

The high performance liquid chromatographic (HPLC) consisting of a Rheodyne 7120, 20 μL fixed–loop injector, an LC 3–UV detector (Pye Unicam, England), an L-6000 A pump (Merck–Hitachi, Germany), and an A/C transmitter with Chromed software (Medson, Poland), was used for the HPLC separation.

Thin-Layer Chromatography

Standard solutions of each of the pyrrolo[3,4-c]pyridine-1,3-dione derivatives (ca. 5 mg/mL) and their mixture (*ca*. 5 mg/mL) were prepared in a solution of

chloroform–methanol (1:3), and 5 μL of each solution was applied to the plates.

Plates were developed with the following mixtures of solvents: a mobile phase A: butan-1-ol–propan-2-ol–acetic acid (1.05 kg/L) (8:3:1, v/v/v); B: butan-1-ol–chloroform–cyclohexane–propan-2-ol-acetic acid (1.05 kg/L) (3:2:3:3:1, v/v/v/v/v); C: butan-1-ol–propan-2-ol–cyclohexane-acetic acid (1.05 kg/L) (6:3:2:1, v/v/v/v); D: chloroform–propan-2-ol-acetic acid (1.05 kg/L) (40:15:6, v/v/v) and (8:3:1, v/v/v); E: chloroform–cyclohexane–propan-2-ol-acetic acid (1.05 kg/L) (20:20:15:6, v/v/v/v) and (5:2:4:1, v/v/v/v) (Tables 1 and 2). Mobile phase of 40 mL was placed in the chromatographic tank and the chamber was saturated for 1 hour. The solvent system was advanced approximately 15 cm, the plates were dried at room temperature, and an ultraviolet light at 254 nm was used for detection.

The following separation parameters were calculated according to the formula:

$$\Delta R_F = R_{F1} - R_{F2}$$

where: R_{F1} and R_{F2} are the values of two adjacent spots ($R_{F1} > R_{F2}$)

$$\alpha = \frac{1/R_{F1} - 1}{1/R_{F2} - 1}$$

where: $R_{F1} < R_{F2}$

$$R_F^{\alpha} = \frac{R_{F1}}{R_{F2}}$$

where: $R_{F1} > R_{F2}$ (Table 1).[19]

Table 1. The average values (n = 5) of separation parameters (α), constant of the pair separation (R_F^{α}) and ΔR_F of two neighbouring spots of investigated compounds on TLC chromatogram

Neighbouring spots	Mobile phase A			Mobile phase B			Mobile phase C		
	α	R_F^{α}	ΔR_F	α	R_F^{α}	ΔR_F	α	R_F^{α}	ΔR_F
IV/(I, II)	1.687	1.440	0.11	1.280	1.154	0.06	1.714	1.500	0.10
(I, II)/III	1.455	1.250	0.09	1.378	1.178	0.08	1.621	1.367	0.11
III/V	9.855	1.978	0.44	11.824	1.755	0.40	12.964	2.195	0.49

A: butan-1-ol–propan-2-ol–acetic acid (1.05 kg/L) (8:3:1, v/v/v).

B: butan-1-ol–chloroform–cyclohexane–propan-2-ol - acetic acid (1.05 kg/L) (3:2:3:3:1, v/v/v/v/v).

C: butan-1-ol - propan-2-ol - cyclohexane - acetic acid (1.05 kg/L) (6:3:2:1, v/v/v/v).

Table 2. The average values (n = 5) of separation parameters (α), constant of the pair separation (R_F^{α}) and ΔR_F of two neighbouring spots of investigated compounds on TLC chromatogram

	Mobile phase D					
	40:15:6, v/v/v			8:3:1, v/v/v		
Neighbouring spots	α	R_F^{α}	ΔR_F	α	R_F^{α}	ΔR_F
I/III	1.556	1.111	0.08	1.246	1.054	0.04
III/(II, IV)	1.220	1.038	0.03	1.282	1.051	0.04
(II, IV)/V	20.482	1.193	0.16	5.226	1.171	0.14
	Mobile phase E					
	20:20:15:6, v/v/v/v			5:2:4:1, v/v/v/v		
	α	R_F^{α}	ΔR_F	α	R_F^{α}	ΔR_F
I/IV	1.225	1.094	0.05	1.250	1.079	0.05
IV/(II, III)	1.234	1.086	0.05	1.210	1.059	0.04
(II, III)/V	6.750	1.460	0.29	3.928	1.264	0.19

D: chloroform - propan-2-ol - acetic acid (1.05 kg/L).
E: chloroform - cyclohexane - propan-2-ol - acetic acid (1.05 kg/L).

HPLC Conditions

The separations were performed using a reversed phase technique on analytical columns: LiChrosorb® RP-8 (LiChroCART, dimensions 250 × 4 mm, 5 μm particle diameter) and LiChrospher® 100 RP-18 (LiChroCART, 250 × 4 mm, 5 μm). The isocratic elution systems were developed utilizing different mobile phases consisting of acetonitrile, propan-2-ol and/or methanol with phosphate buffer pH 2 or 7 (Tables 3 and 4). The flow rate was 1.0 mL/min or 1.5 mL/min and the chromatograms were acquired at a wavelength of 240 nm. All analyses were carried out at laboratory temperature. The effectiveness of the HPLC separation was evaluated using a standard solution containing five derivatives of 4-alkoxy-6-methyl-pyrrolo[3,4-c]pyridine-1,3-dione I–V (ca. 0.1 mg/mL) in the mixture of acetonitrile–chloroform (3:1).

RESULTS AND DISCUSSION

Thin layer chromatography is a widely used chromatography technique used to separate chemical compounds. All derivatives of 4-alkoxy-6-methyl-1*H*-pyrrolo[3,4-c]pyridine-1,3(2H)-dione (I–V) were separated on silica gel

Table 3. Retention times (t_r) and retention factors (k') for the four investigated compounds (I, II, III, V) on column LiChrosorb® RP-8 (250 × 4 mm, 5 μm)

		I		II		III		V	
Mobile phase quality	quantity	t_r, (min)	k'	t_r, (min)	k'	t_r, (min)	k'	t_r, (min)	k'
Acetonitrile–phosphate buffer pH 2 (0.01 mol/L); 1.0 mL/min	60:40	12.4	6.7	17.3	9.8	17.7	10.1	3.0	0.9
	40:60	37.4	22.4	59.5	36.2	59.5	36.2	4.5	1.8
Propan-2-ol- phosphate buffer pH 2 (0.01 mol/L); 1.0 mL/min	30:70	9.5	4.9	13.0	7.7	16.4	9.2	4.4	1.7
	45:55	6.0	2.7	7.0	3.4	7.5	3.7	3.0	0.9
	50:50	4.5	1.8	4.9	2.1	4.9	2.1	2.6	0.6
Acetonitrile–propan-2-ol–phosphate buffer pH 2 (0.01 mol/L); 1.0 mL/min	35:10:55	19.1	10.9	25.6	15.0	29.8	17.6	3.8	1.4
	30:20:50	10.7	5.7	12.8	7.0	15.2	8.5	3.3	1.1

($60F_{254}$) plates using mobile phases consisting of triple, four- or five components such as acetic acid (1.05 kg/L), butan-1-ol, chloroform, cyclohexane, and propan-2-ol, in the different volume compositions (A–E) (Table 1 and 2). The binary eluent systems turned out to be non-suitable because four (I–IV) from five investigated compound (compound V is a Mannich base) has very similar properties in polarity (Figure 1). Tables 1–2 show only the best selected results, characterizing the selectivity in relation to separated compounds. The compounds studied were separated with the mobile phase A, B, and C in the R_F range of 0.20 to 0.93. However, in these conditions, the pair of investigated compounds I and II was not separated. The values of separation parameter (α), constant of the pair separation (R_F^{α}) and ΔR_F of two neighbouring spots on chromatogram are listed in Table 1. Analysis of data contained in Table 1, indicate that the mobile phase C: butan-1-ol–propan-2-ol–cyclohexane-acetic acid (1.05 kg/L) in a volume composition 6:3:2:1 are optimal for separation of compound I (or II), III, IV, and V. However, in the mobile phase D: chloroform–propan-2-ol-acetic acid (1.05 kg/L) in volume compositions 40:15:6, as well as 8:3:1, the pair of investigated compounds II and IV was not separated. In these conditions, the R_F values of all observed spots were in the range of 0.72 to 0.96. The values of separation parameter (α), constant of the pair separation (R_F^{α}) and ΔR_F of two neighbouring spots on the chromatogram (Table 2), indicate that the mobile phase D in a volume composition 40:15:6 is optimal for separation of compound I, II (or IV), III, and V. Analysis of data contained in Table 2, indicate that in the mobile phase E: chloroform–cyclohexane–propan-2-ol-acetic acid (1.05 kg/L) in

Table 4. Retention times (t_r) and retention factors (k') for the five investigated compounds (I, II, III, IV, V) on column LiChrospher® 100 RP-18 (250 × 4 mm, 5 μm)

	I		II		III		IV		V	
Mobile phase	t_r, (min)	k'	t_r, (min)	k'	t_r, (min)	k'	t_r, (min)	k'	t_r, (min)	k'
Acetonitrile–propan-2-ol–phosphate buffer pH 2 (0.02 mol/L) (30:20:50); 1.0 mL/min	5.6	1.8	4.4	1.2	6.7	2.3	4.4	1.2	3.3	0.6
Acetonitrile–methanol–phosphate buffer pH 2 (0.02 mol/L) (30:20:50); 1.0 mL/min	10.2	4.1	8.0	3.0	11.2	4.6	8.8	3.4	4.5	1.2
Acetonitrile–methanol–phosphate buffer pH 7 (0.02 mol/L) (30:20:50); 1.5 mL/min	14.7	6.6	32.5	20.4	25.0	15.4	26.0	16.1	3.0	1.0
Acetonitrile–methanol–phosphate buffer pH 7 (0.02 mol/L) (45:15:40); 1.5 mL/min	5.6	2.7	10.9	6.2	8.1	4.4	9.1	5.0	2.2	0.5

volume compositions 20:20:15:6, as well as 5:2:4:1, the pair of investigated compounds II and III was not separated. In these conditions, the R_F values of all observed spots were in the range of 0.53 to 0.92. This mobile phase in a volume composition 20:20:15:6 is optimal for separation of compound I, II (or III), IV, and V. From the seven remaining mobile phases, three were offered for use in two dimensions of TLC on silica gel ($60F_{254}$) plates. The mobile phase C was used in the first dimension but mobile phases D (40:15:6) and/or E (20:20:15:6) were used in the second dimension. In these conditions, the separation of all of the investigated compounds was possible, but the best selected result was observed with eluents C and E (Figure 2).

High performance chromatography, especially with reversed phase, is a form of chromatography used frequently in analytical chemistry. It is used to separate components of a mixture by using a variety of chemical interactions between the substance being analyzed and the chromatography column. Two reversed-phase (C_8 and C_{18}) columns with similar parameters (internal diameter, particle size) were tested for separation of 4-alkoxy-6-methyl-1*H*-pyrrolo[3,4-c]pyridine-1,3(2H)-dione derivatives (I-V). The base properties and the stability of compounds I-III in solutions at pH about 2[3–5] suggested the addition of phosphate buffer pH 2 as the component of mobile phase. The complete separation of five compounds (I–V) was not achieved using the stationary phase LiChrosorb® RP-8. In this condition, the influence of mobile phases consisting of acetonitrile and/or propan-2-ol in phosphate buffer pH 2 (0.01 mol/L) were observed (Figure 3). The values of the retention times (t_r) and the retention factors (k') are listed

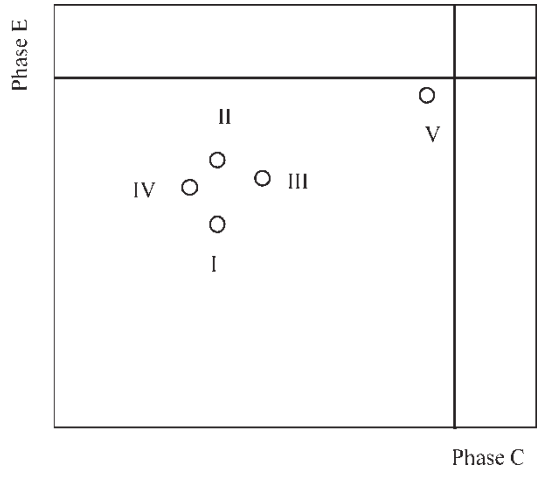

Figure 2. Chromatographic separation of compounds I–V using bidirections TLC (silica gel $60F_{254}$; mobile phase C: butan-1-ol–propan-2-ol–cyclohexane-acetic acid (1.05 kg/L) (6:3:2:1, v/v/v/v) and E: chloroform–cyclohexane–propan-2-ol-acetic acid (1.05 kg/L) (20:20:15:6, v/v/v/v).

in Table 3. Chromatograms show that the resolution for compounds II and III is not achieved in the binary eluent system (Figure 3a, 3b, 3c). In the case of the mobile phase consisting of propan-2-ol–phosphate buffer pH 2 (30:70), peaks are separate but are not symmetric (Figure 3d). Therefore, mobile phases consisting of three components: acetonitrile, propan-2-ol and phosphate buffer pH 2 (0.01 mol/L) were selected for the study (Table 3). As can be seen in Figures 3e and 3f, peaks of compounds I, II, III, and V

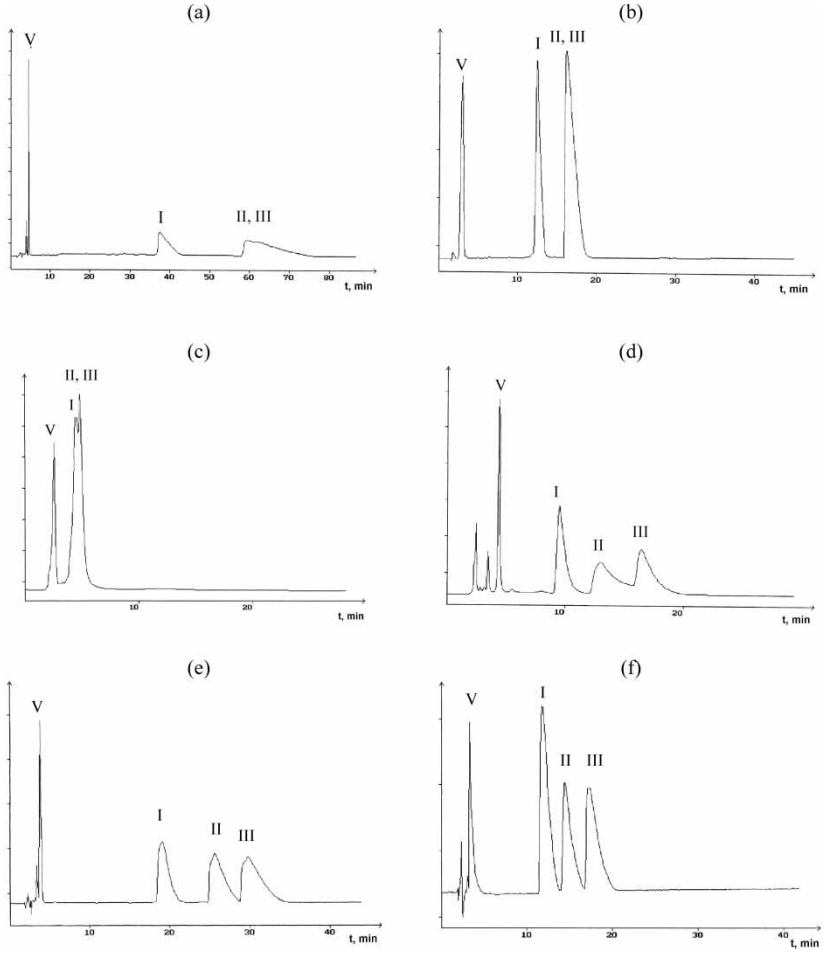

Figure 3. Chromatographic separation of compounds I, II, III, and V using analytical column LiChrosorb® RP-8 (250 × 4 mm, 5 μm) and mobile phases: acetonitrile–phosphate buffer pH 2 (0.01 mol/L) (a–40:60; b–60:40; 1.0 mL/min), propan-2-ol-phosphate buffer pH 2 (0.01 mol/L) (c–50:50; d–30:70; 1.0 mL/min), acetonitrile–propan-2-ol–phosphate buffer pH 2 (0.01 mol/L) (e–35:10:55; f–30:20:50; 1.0 mL/min).

are separated with satisfactory results in volume composition 30:20:50. Unfortunately, retention times for compound II and IV turned out to be the same.

Analysis of data contained in Table 4, indicate that fast separation can be performed using the similar mobile phase (acetonitrile-propan-2-ol-phosphate buffer pH 2 (0.02 mol/L); 30:20:50) and LiChrospher® 100 RP-18 column instead of LiChrosorb® RP-8 column. The separation of compounds II and IV was not observed in this condition (Table 4). Modifications of eluent systems by changing propan-2-ol to methanol and phosphate buffer pH from 2 to 7 were studied for the most satisfactory resolution of all of the investigated compounds (I–V) (Table 4, Figure 4). As can be seen in Figures 3b, the complete separation was achieved by the isocratic elution with the mobile phase composed of acetonotrile–methanol–phosphate buffer pH 7 (45:15:40) on the LiChrospher® 100 RP-18 column and a flow rate of 1.5 mL/min. The chromatographic characteristic for LiChrosorb® RP-8 and LiChrospher® 100 RP-18 columns for the best chromatography conditions are summarized in Table 5. The main validation parameters for the analysis of all of the investigated derivatives in the mixture by using RP-HPLC (LiChrospher® 100 RP-18 column; acetonotrile–methanol–phosphate buffer pH 7 (45:15:40)) were evaluated (Table 6). The repeatability of retention times were determined from 8 injections of standards in different concentrations, and it was under 1.2% for all standards. The calibration lines were constructed by plotting the peak area as a function of standard concentration values in the range of 1.4×10^{-6} to 6.9×10^{-4} mol/L for each analyte (8 concentrations, each measured in triplicate) in the mobile phase. The linearity was calculated by the least squares method ($y = ax$ because values of intercept (b) were statistically insignificant for all derivatives), giving a correlation coefficient $r^2 > 0.99$ for all compounds. In order to establish the limits of detection (LOD) and quantification (LOQ), calibration was performed in the low concentration region

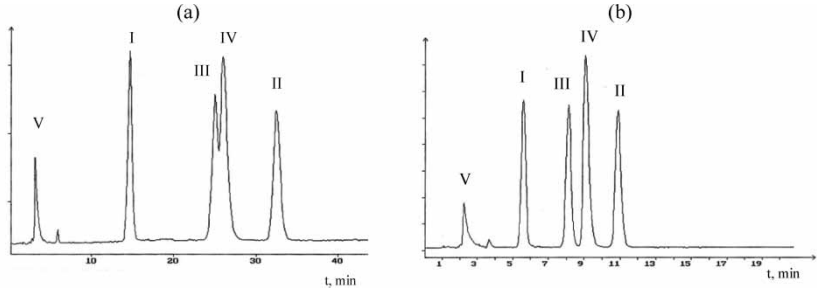

Figure 4. Chromatographic separation of compounds I-V using analytical column LiChrospher® 100 RP-18 (250 × 4 mm, 5 μm) and mobile phase: acetonitrile–methanol-phosphate buffer pH 7 (0.02 mol/L) a) 30:20:50; b) 45:15:40; 1.5 mL/min.

Table 5. Capacity factor (k′), chromatographic resolution (R) and asymmetry factor (A_s) for all analytes using two different analytical columns and mobile phases

HPLC conditions/analytes	k′	R	A_s
Column: LiChrosorb® RP-8 (250 × 4 mm, 5 μm)			
Mobile phase: acetonitrile–propan-2-ol–phosphate buffer			
pH 2 (0.01 mol/L) (30:20:50); 1.0 mL/min	1.1	8.73	1.0
V	5.7	1.28	1.5
I	7.0	1.33	1.3
II	7.0		1.3
IV	8.5		1.5
III			
Column: LiChrosorb® 100 RP-18 (250 × 4 mm, 5 μm)			
Mobile phase: acetonitrile–methanol–phosphate buffer	0.5	8.85	1.4
pH 7 (0.02 mol/L) (45:15:40); 1.5 mL/min	2.7	5.64	1.1
V	4.4	2.27	1.1
I	5.0	3.63	1.1
III	6.2		1.1
IV			
II			

($10^{-6} - 10^{-5}$ mol/L) and the statistical data (parameters of calibration lines, LOD, LOQ, repeatability of retention times) are given in Table 6. The validation parameters were satisfactory, so the proposed HPLC method can be used for quantitative analysis of each of the studied compounds in their mixture.

In conclusion, bidimensional TLC on silica gel ($60F_{254}$) plates and RP-HPLC analysis on LiChrospher® 100 RP-18 columns can be suitable for identification and simultaneous determination of derivatives of 4-alkoxy-6-

Table 6. Parameters of calibration lines $y = ax$, limit of detection LOD, limit of quantification LOQ and repatability of retention times (column: LiChrospher® 100 RP-18 (250 × 4 mm, 5 μm); mobile phase: acetonitrile–methanol–phosphate buffer pH 7 (0.02 mol/L) (45:15:40); 1.5 mL/min)

Compounds	Slop (a)	Correlation coefficient (r^2)	LOD (mol/L)	LOQ (mol/L)	Repeatability of retention times (%)
I	$2.439 \cdot 10^4$	0.9998	$2.07 \cdot 10^{-6}$	$6.88 \cdot 10^{-6}$	1.1
II	$2.552 \cdot 10^4$	0.9996	$2.17 \cdot 10^{-6}$	$7.23 \cdot 10^{-6}$	1.0
III	$2.651 \cdot 10^4$	0.9994	$1.70 \cdot 10^{-6}$	$5.65 \cdot 10^{-6}$	1.2
IV	$2.068 \cdot 10^4$	0.9994	$2.17 \cdot 10^{-6}$	$7.24 \cdot 10^{-6}$	1.1
V	$9.522 \cdot 10^3$	0.9982	$2.60 \cdot 10^{-6}$	$8.67 \cdot 10^{-6}$	1.2

methyl-1*H*-pyrrolo[3,4-c]pyridine-1,3(2H)-dione (I-V). These proposed methods give the opportunity for monitoring synthesis results and differences in properties of all of the investigated compounds.

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