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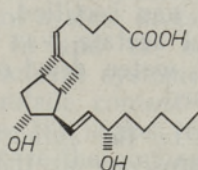
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RESOLUTION OF E- AND Z-ISOMERS OF PROSTACYCLIN CARBA-ANALOGS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Since 1976, when prostacyclin was discovered [1], its remarkable biological properties have stimulated intense research into the preparation of stable synthetic analogs for therapeutic use [2, 3]. Among them, the carba-analogs of prostacyclin are of considerable importance [4]. As both the E- and Z-isomers of different biological activity are formed in the Wittig olefination, one of the key steps in carbacyclin synthesis [3], methods for their separation are highly needed. Up to now, only R. F. Newton and A. H. Wadsworth [5] have used the HPLC method for determining the ratio of E/Z-isomers of some prostacyclin carba-analogs.

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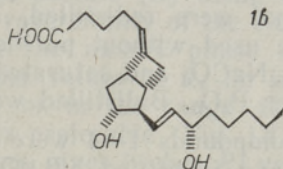
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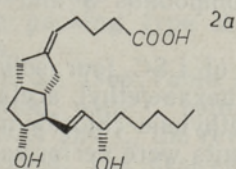
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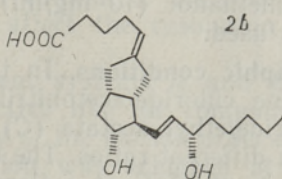
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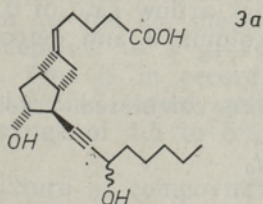
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(5Z)-9-desoxy-13,14-didehydro-
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221

4a

4b

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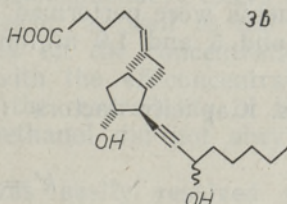
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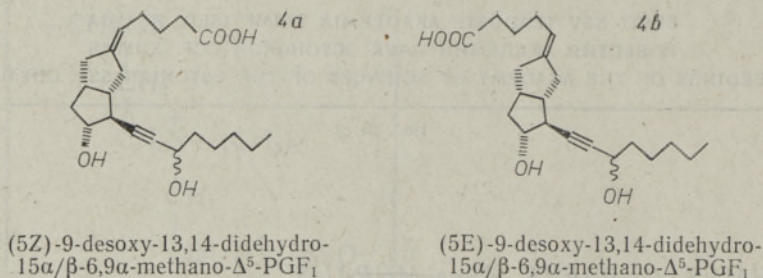
We have studied the possibilities of separating the *E/Z*-isomers of four different carba-analogs (1—4) of prostacyclin by liquid-solid (LSC) and reverse-phase mode bonded phase (BPC) chromatography.

Materials and methods

Apparatus. A DuPont HPLC system N 8845 with a UV spectrophotometrical detector was used.

Columns. Zorbax SIL, 4.6×250 mm (DuPont) 1; Zorbax SIL, 6.0×150 mm 2 and Silasorb 600 (5 μm), 6.0×150 mm 3 packed in the Special Designing Bureau (SDB), Tallinn, were used in the LSC studies.

Zorbax ODS, 4.6×250 mm (DuPont) 4; Zorbax C-8, 4.6×250 mm (DuPont) 5 and Zorbax ODS, 6.0×150 mm 6 packed in the SDB were used in the BPC studies.

Solvents. All solvents were supplied by Reakhim. Methylene chloride and methanol were redistilled, acetonitrile was rectified. Hexane and H_3PO_4 were used without purification. Ethyl acetate was washed with aqueous 5% $NaCO_3$ and saturated with $CaCl_2$ water, dried on K_2CO_3 and distilled over P_2O_5. Bidistilled water was used.

Samples. Compounds 1—4 were synthesized by the authors of the present work and identified by ¹³C-NMR spectroscopy at the Institute of Chemical Physics and Biophysics, Tallinn. By LSC, samples were converted into *p*-bromophenacyl esters according to [6, 7] and injected in methylene chloride (1 mg/ml). In the case of BPC the samples were injected in methanol (10 mg/ml). For compounds 3 and 4 the 15α/β-mixture was used.

Chromatographic conditions. In the case of LSC four solvent systems, viz. methylene chloride/acetonitrile (*A*), hexane/ethyl acetate (*B*), methylene chloride/ethyl acetate (*C*) and methylene chloride/methanol (*D*) were used in different ratios. The experiments were performed at ambient temperature or at 35 °C at a flow rate of 0.6 ml/min for column 1 and 1.0 ml/min for columns 2 and 3 and detected at 260 nm.

In the case of BPC the varied content of acetonitrile or methanol in aqueous 0.017 M H_3PO_4 was used (solvent system *E* and *F*, respectively). The experiments were performed at 35° at a flow rate of 0.8 ml/min for columns 4 and 5 and 1.0 ml/min for column 6 and detected at 208—210 nm.

Calculations. Capacity factors (k') were calculated according to the formula

$$k' = \frac{t_R - t_0}{t_0},$$

where t_R — retention time (measured from the chromatogram, nm), t_0 — retention time of the unretained compound (mm). t_0 was calculated from

operational values of the void volume of column (V_0), chart speed and flow rate. For reverse phase columns V_0 was determined as the elution volume of KNO_3 (for 4, 5 and 6 it was 2.04, 1.85 and 2.08 ml, respectively). For the column 1 pore volume was calculated as 2.41 ml according to [8]. The elution volume of the first eluted nonpolar compound was 2.68 ml. It means that the «true» void volume $2.41 < V_0 \leqslant 2.68$. Therefore, V_0 was taken as 2.68 ml, and V_0 for 2 and 3 as 2.74 ml in the same way.

The resolution factors ((a) were calculated according to the formula:

$$a = k'_b / k'_a ,$$

where k'_b — capacity factor of the compound b, k'_a — capacity factor of the respective compound a. The resolution function (R_s) was estimated according to [8].

Results and discussion

The resolution of E/Z-isomers of prostacyclin analogs as their p-bromophenacyl ester is a complicated problem due to their very similar behaviour on silica gel. It means that the differences in specific interactions between E/Z-isomers, solvents and the adsorbent are very small.

Compounds 1 and 2 were chosen as model compounds in the elucidation of the possibilities of separating the E/Z-isomers of prostacyclin analogs. The results for 1a and 1b p-bromophenacyl esters in the case of LSC are given in Table 1. According to these results 1a and 1b are fairly separable in methylene chloride-acetonitrile (A) and hexane-ethyl acetate (B), with methylene chloride-ethyl acetate (C) being ineffective. For each solvent system investigated the reversibility of the elution order of compound 1a and 1b, occurs when the mobile phase content is changed. For solvent system A, the proper values of R_s lie in the area with an acetonitrile content below 12% or in the range of 50—70%. In the first case the higher temperature is preferable (Fig. 1A), whereas in the second case the ambient temperature gives higher R_s values (Fig. 1B). For solvent system B a higher temperature (35°) decreases peak tailing and results in lower k' values (shorter analysis time) and better resolution (Fig. 1C).

The same a values were obtained for column 3. We conclude that a change in the packing type does not affect the resolution selectivity of E/Z-isomers.

For 2a and 2b (see Table 2), solvent systems A and B gave the desired resolution, with C being ineffective like for compounds 1. For solvent systems A and C, the reversibility of the elution order of 2a and 2b was also observed. The desired resolution was achieved at the acetonitrile content below 20% (Fig. 2A), but the solvent system B is preferable at a higher temperature (35°) due to shorter elution time (Fig. 2B).

Consequently, in the resolution of p-bromophenacyl esters of 1a and 1b, and also of 2a and 2b, the elution order as well as the selectivity of resolution depends to a great degree on the concentration of the mobile phases. This is in accordance with the «B-concentration rule» [8], but only for the solvent system D the reversibility of the elution order in the range of 1.5 to 5% of methanol did not obey this rule (Table 3).

The E/Z-mixture of compounds 3 was easily resolved using the solvent system A (separation on 15α/β-isomers also occurs). Conditions for compounds 3: column 1; methylene chloride/acetonitrile=90/10 v/v; $k'_{3a} = 4.8$ and 5.1; $k'_{3b} = 5.2$ and 5.5 (15α/β-isomers of 3 and 4 were not

Table 1

Dependence of the resolution factor (α), resolution function (R_s) of compounds 1a and 1b p-bromophenacyl esters and capacity factor (k'_{1b}) of compound 1b p-bromophenacyl ester on the mobile phase content (A — acetonitrile in methylene chloride, B — ethyl acetate in hexane, C — ethyl acetate in methylene chloride, %) by LSC.
Column Zorbax SIL (4.6×250 mm)

Mobile phase	Column temperature	Calculated parameter	Mobile phase content, %														
			100	90	80	70	60	50	45	40	35	30	25	20	15	12	11
A	ambient	k'_{1b}	0.60	0.83	0.91	0.97	1.15	1.76	1.94	2.4	3.0	3.9	5.1	7.2	16.6	22	
		α / R_s	0.91 / 0.85	0.91 / 0.95	0.91 / 1.00	0.91 / 1.00	0.91 / 1.05	0.91 / 1.20	0.93 / 1.00	0.94 / 0.90	0.96 / 0.85	0.97 / 0.70	~1	~1	1.02 / 0.70	1.06 / 1.00	
A	35°	k'_{1b}		0.63	0.68	0.81	0.97	1.28		2.0		3.3	4.4	6.0	10.1		16.7
		α / R_s		0.91 / 0.90	0.91 / 0.95	0.92 / 1.00	0.92 / 1.05	0.93 / 1.1		0.94 / 0.95		0.96 / 0.70	~1	~1	1.04 / 0.75		1.06 / 1.15
B	ambient	k'_{1b}	0.91	1.15	1.69	2.5	4.6	8.3	11.4	17.1							
		α / R_s	0.91 / 0.70	0.94 / 0.60	~1	~1	1.06 / 0.65	1.10 / 0.90	1.12 / 1.0	1.17 / 1.1							
B	35°	k'_{1b}	0.75		1.43		3.0	5.4	7.6	11.5	17.3						
		α / R_s	~1		~1		1.04 / 0.75	1.09 / 1.00	1.12 / 1.25	1.15 / 1.40	1.16 / 1.70						
C	35°	k'_{1b}	0.75	0.85	1.06	1.42	1.89	2.8		4.1		7.0		14.6			
		α / R_s	~1	0.94 / 0.70	0.95 / 0.70	0.95 / 0.70	0.96 / 0.65	~1		~1		~1		1.06 / 0.80			

identified); R_s values between these peaks are 1.05, 0.70 and 1.00, respectively. Conditions for compounds 4: column 2; methylene chloride/acetonitri-
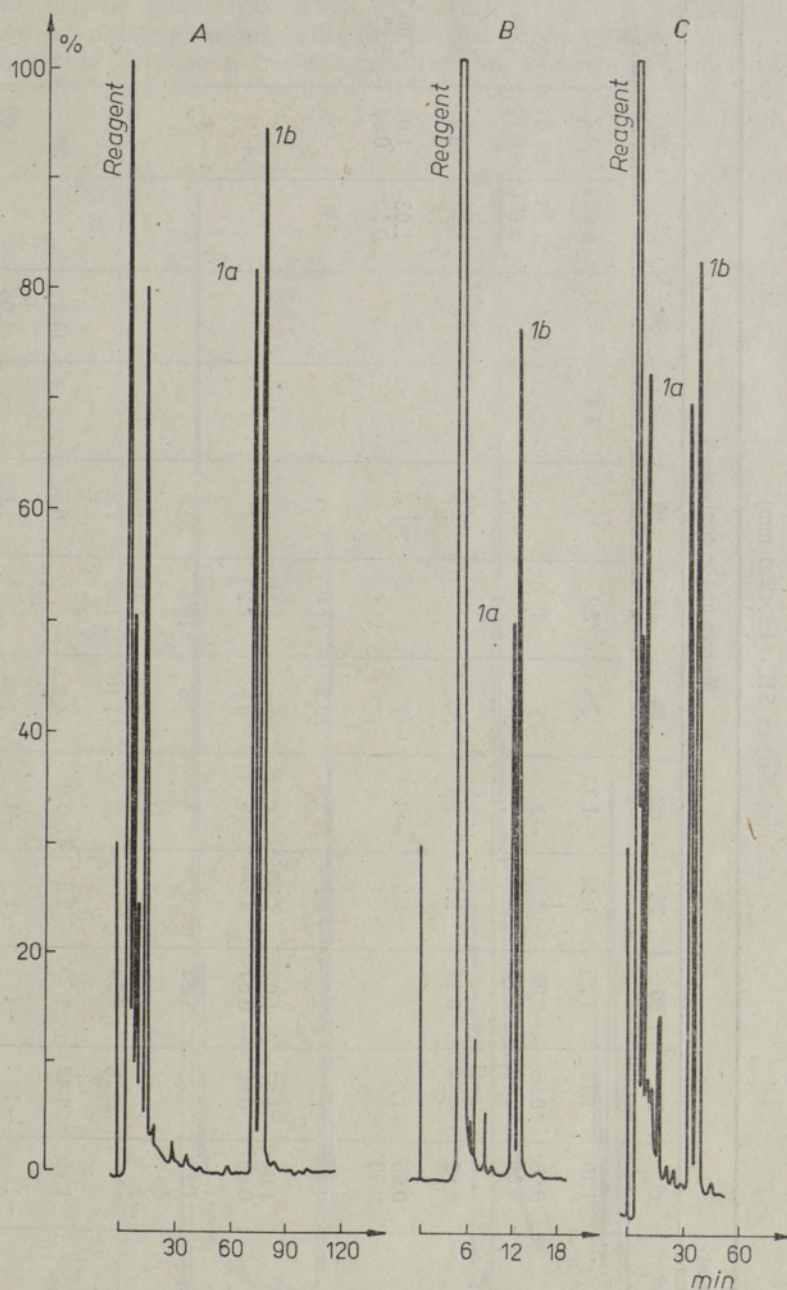

Fig. 1. Chromatograms of the separation of compounds 1a and 1b p-bromophenacyl esters.
Conditions: Column — Zorbax SIL (4.6×250 mm); flow rate — 0.6 ml/min; detection — UV 260 nm.
A-mobile phase — methylene chloride/acetonitrile=89/11; column temperature — 35 °C; absorbance — 0.32 AUFS; chart speed — 2.5 cm/h; ~8 µg injected.
B-mobile phase — methylene chloride/acetonitrile=50/50; column temperature —ambient; absorbance — 0.64 AUFS; chart speed — 10 cm/h; ~4 µg injected.
C-mobile phase — hexane/ethyl acetate=55/45; column temperature — 35 °C; absorbance — 0.32 AUFS; chart speed — 2.5 cm/h; ~4 µg injected.

Table 2

Dependence of the calculated parameters (see Table 1) of compounds 2a and 2b p-bromophenacyl esters on the mobile phase content (*A*, *B* and *C* see Table 1).

Column Zorbax SIL (4.6×250 mm)

Mobile phase	Column temperature	Calculated parameter	Mobile phase content, %													
			100	90	80	70	60	50	45	40	35	30	25	20	15	14
A	ambient	k'_{2b}	1.10	1.16	1.25	1.42	1.73	2.3	3.1		4.4		9.3	10.2	23	
		α	0.96	0.96	0.96	0.97	~1	~1	~1		~1		1.03	1.04	1.06	
		R_s	0.50	0.50	0.50	0.50							0.60	0.70	1.50	
A	35°	k'_{2b}	0.82	0.88	0.95	1.11	1.37	1.80		2.6		4.5	6.4	9.2	15.6	17.4
		α	0.96	~1	~1	~1	~1	~1		~1		~1	1.03	1.04	1.06	1.06
		R_s	0.50										0.50	0.85	1.15	1.3
B	ambient	k'_{2b}	1.55	1.94	2.7	3.8	7.3	12.2	17.0							
		α	1.04	1.07	1.05	1.07	1.11	1.15	1.18							
		R_s	0.60	0.60	0.60	0.70	0.80	0.95	1.20							
B	35°	k'_{2b}	1.15		1.82		4.3	7.3	10.5	15.8						
		α	~1		1.06		1.11	1.15	1.16	1.19						
		R_s			0.50		0.85	1.10	1.30	1.8						
C	35°	k'_{2b}	1.15	1.42	1.70	2.2	3.0	4.2		6.5		11.3		24		
		α	~1	~1	~1	~1	~1	1.03		1.03		1.03		1.09		
		R_s						0.50		0.55		0.80		0.90		

le$=85/15$ v/v; $k'_{4a}=6.8$ and 7.5; $k'_{4b}=7.4$ and 7.6; R_s between the first and the second peak was 0.90. This resolution can be significantly improved by using columns with the proper plate number.

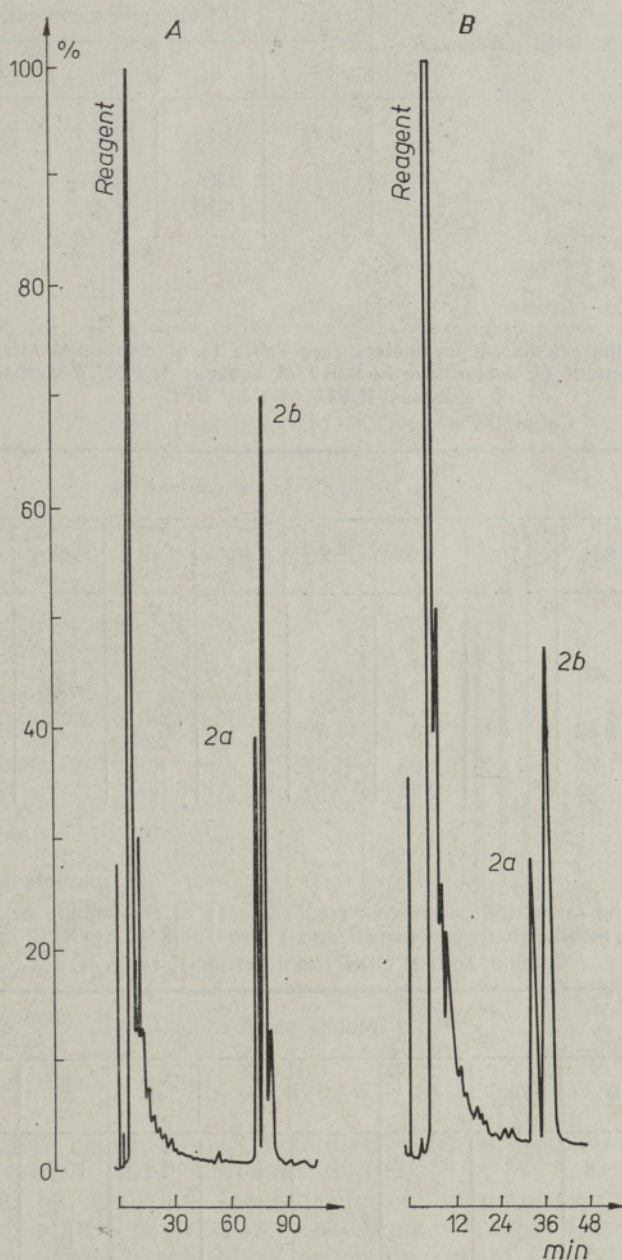In order to achieve a better resolution of E/Z-isomers of 1—4, BPC

Fig. 2. Chromatograms of the separation of compounds 2a and 2b p-bromophenacyl esters.
Conditions: Column — Zorbax SIL (4.6×250 mm); flow rate — 0.6 ml/min; column temperature — 35 °C; detection — UV 260 nm; absorbance — 0.32 AUFS.
A-mobile phase — methylene chloride/acetonitrile=86/14; chart speed — 2.5 cm/h; ~12 µg injected.
B-mobile phase — hexane/ethyl acetate=50/50; chart speed — 5 cm/h; ~10 µg injected.

Table 3

Dependence of the calculated parameters (see Table 1) of compounds 1a and 1b p-bromophenacyl esters on the mobile phase content (D—methanol in methylene chloride, %) by LSC.
Column Zorbax SIL (6.0×150 mm), t=35 °C

Mobile phase	Calculated parameter	Mobile phase content, %				
		10	5	3	2	1.5
D	k'_{1b}	0.43	1.02	3.3	7.6	14.1
	α	~1	0.94	0.97	~1	1.02
	R_s		0.70	0.60		0.55

Table 4

Dependence of the calculated parameters (see Table 1) of compounds 1a and 1b on the mobile phase content (E-acetonitrile in 0.017 M aqueous H_3PO_4, F-methanol in 0.017 M aqueous H_3PO_4, %) by BPC.
Column Zorbax ODS (4.6×250 mm), t=35 °C

Mobile phase	Calculated parameter	Mobile phase content, %								
		80	70	60	52	50	48	40	36	32
E	k'_{1b}					2.6		7.5	11.4	25
	α					~1		1.05	1.07	1.07
	R_s							0.80	0.90	1.00
F	k'_{1b}	0.82	1.22	5.3	15.5		26			
	α	~1	~1	1.06	1.09		1.09			
	R_s			0.85	0.95		1.00			

Table 5

Dependence of the calculated parameters (see Table 1) of compounds 2a and 2b on the mobile phase content (E and F see Table 4) by BPC.
Column Zorbax ODS (4.6×250 mm), t=35 °C

Mobile phase	Calculated parameter	Mobile phase content, %								
		80	70	60	50	47	45	40	36	34
E	k'_{2b}				3.3	4.3	5.3	10.0	15.6	24
	α				1.03	1.05	1.11	1.12	1.14	1.15
	R_s				0.60	0.80	0.90	1.10	2.20	2.50
F	k'_{2b}	1.51	2.4	7.9						
	α	1.08	1.08	1.15						
	R_s	0.50	0.90	1.50						

was studied, using 1 and 2 as model compounds. These data are given in Tables 4 and 5, respectively. It is rather difficult to obtain acceptable resolution of 1a and 1b on columns Zorbax ODS, but 2a and 2b are
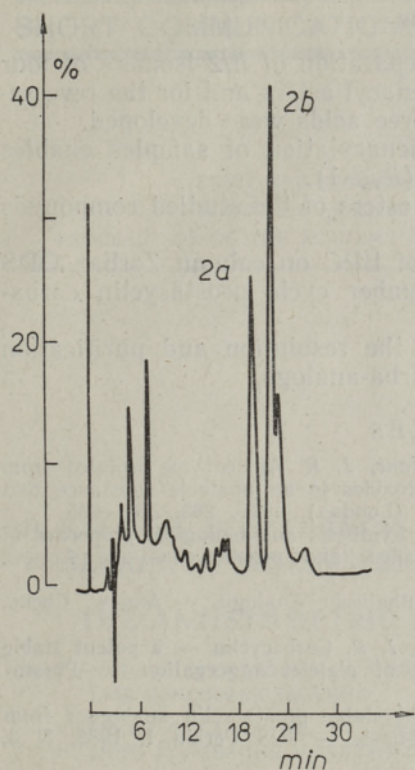
Fig. 3. Chromatogram of the separation of compounds 2a and 2b. Conditions: Column — Zorbax ODS (4.6×250 mm); mobile phase — 0.017 M aqueous H_3PO_4/methanole=40/60; flow rate — 0.8 ml/min; column temperature — 35 °C; detection — UV 210 nm; absorbance — 0.32 AUFS; chart speed — 10 cm/h; ∼40 μg injected.
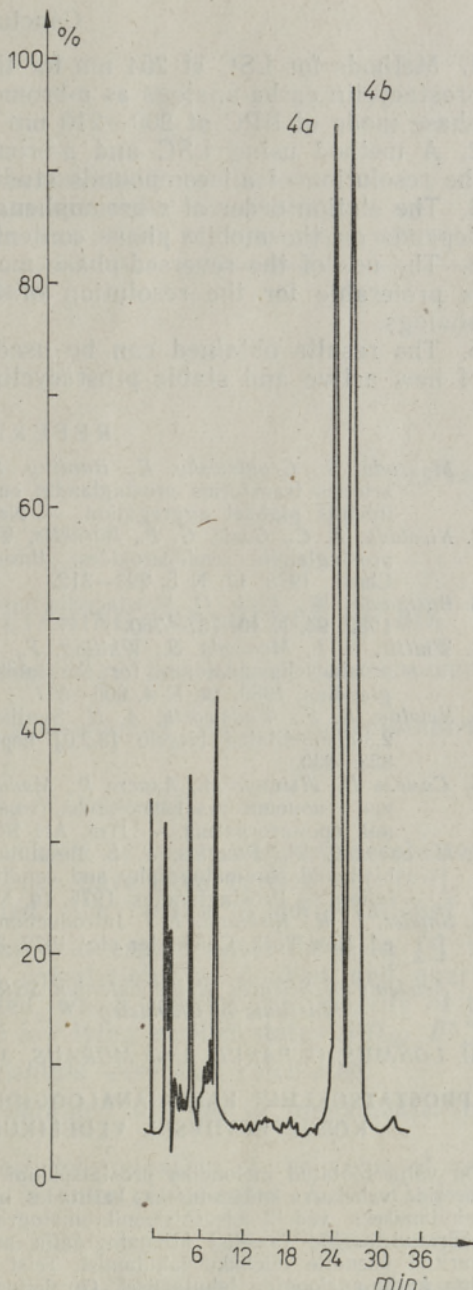
Fig. 4. Chromatogram of the separation of compounds 4a and 4b. Conditions: Column — Zorbax ODS (6.0×150 mm); mobile phase — 0.017 M aqueous H_3PO_4/acetonitrile=60/40; flow rate — 1.0 ml/min; column temperature — 35 °C; detection — UV 208 nm; absorbance — 0.32 AUFS; chart speed — 10 cm/h; ∼80 μg injected.

resolved easily, using either acetonitrile (E) or methanol (F) as a «strong» solvent in 0.017 M aqueous H_3PO_4 (Fig. 3). A better resolution was achieved by methanol (peak tailing is diminished), but the «lifetime» of Zorbax ODS and Zorbax C-8 columns in this system is shorter. The E/Z-isomers of 3 are resolved poorly on Zorbax C-8 column (column 5; 0.017 M aqueous H_3PO_4/acetonitrile=68/32 v/v; $k'_{3a}=22$; $k'_{3b}=21$; $R_s=0.95$) and similarly to 1 on Zorbax ODS column. Likewise, the resolution of 5-member cycle carba-analogs 4a and 4b is easily obtained on Zorbax ODS column (see conditions in Fig. 4). Zorbax C-8 column gives poorer results. The 15α/β-isomers of these compounds were inseparable.

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Conclusions

1. Methods for LSC at 254 nm for the separation of E/Z-isomers of four prostacyclin carba-analogs as p-bromophenacyl esters and for the reverse-phase mode of BPC at 200—210 nm as free acids were developed.
2. A method using LSC and p-bromophenacylation of samples enables the resolution of all compounds studied ($R_s \geqslant 1$).
3. The elution order of p-bromophenacyl esters of the studied compounds depends on the mobile phase content.
4. The use of the reversed-phase mode of BPC on column Zorbax ODS is preferable for the resolution of 5-member cycle prostacyclin carba-analogs.
5. The results obtained can be used in the resolution and purification of new active and stable prostacyclin carba-analogs.

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PROSTATSÜKLIINI KARBA-ANALOOGIDE E- ja Z-ISOMEERIDE LAHUTAMINE KÕRGEFEKTIIVSEL VEDELIKUKROMATOGRAAFIAMEETODIL

On välja töötatud metoodika prostatsükliini nelja erineva karba-analoogi E- ja Z-isomeeride vahekorra määramiseks, kasutades nende isomeeride 1) p-bromofenatsüülestrite lahutamiseks vedelik-adsorbtsioonikromatograafia ja 2) vabade hapete lahutamiseks pööratud seotud faasilist kromatograafia meetodit. Esimene meetod võimaldas kõigi uuritud ühendite täielikku lahutamist. Teist meetodit tuleb eelistada 5-liikmelise tsükliga karba-analoogide lahutamisel. On leitud, et uuritud isomeeride p-bromofenatsüülestrite elueerumisjärjekord silikageelil sõltub liikuva faasi koosseisust.

М. ЛЫХМУС, О. ПАРВЕ, Анне МЮРАУС, М. ЛОПП, Ю. ЛИЛЛЕ

РАЗДЕЛЕНИЕ E- И Z-ИЗОМЕРОВ КАРБА-АНАЛОГОВ ПРОСТАЦИКЛИНА МЕТОДОМ ВЫСОКОЭФФЕКТИВНОЙ ЖИДКОСТНОЙ ХРОМАТОГРАФИИ

Разработана методика для анализа смесей E- и Z-изомеров четырех карба-аналогов простациклина, заключающаяся в разделении их в виде n-бромофенациловых эфиров с помощью жидкостно-адсорбционной хроматографии и в виде свободных кислот с помощью химически связанной обращенно-фазовой хроматографии. Первый способ обеспечил полное разделение всех изученных соединений. Второй способ предпочтительнее для разделения карба-аналогов с 5-членным циклом. Найдено, что порядок элюирования n-бромофенациловых эфиров исследованных изомеров на силикагеле зависит от состава подвижной фазы.