HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF ANTIBIOTICS OF VANCOMYCIN TYPE

COMPARATIVE STUDIES

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Comparative HPLC examination of seven antibiotics of vancomycin type has been undertaken. Investigation has shown that on column I in eluent system B, ristomycin A (ristocetin A) can be not only separated from vancomycin, but both antibiotics can be quantitatively determined. Under these conditions the lowest detectable quantities of the individual antibiotics have been also stated. By the application of this column and eluent system A, ristomycin A (ristocetin A) and the major component of the A-35512 B antibiotic complex can be readily separated from one-another and from avoparcin α and β.

Recently the vancomycin group of antibiotics (vancomycin, actinoidin, ristocetin, ristomycin, avoparcin and antibiotic complex A-35512 B) have become both theoretically and practically important[1,2].

These antibiotics, except for vancomycin, are produced as mixtures of structurally related biologically active compounds. These glycopeptide-type antibiotics of relatively high molecular weight (1,420 ~ 2,063 daltons) often suffer decomposition upon the isolation process. Therefore the exact quantitation of the active factor in the antibiotic preparations is a very important task.

The biological assay is restricted only to the determination of the total antibiotic activity of antibiotic preparations. Due to the similar chromophore system and specific optical rotation of the structurally related antibiotics and their decomposition products, ultraviolet spectroscopic[3] and polarimetric[4] methods are suitable only in a limited number of cases and primarily for homogeneous preparations.

For the detection of the vancomycin group of antibiotics and their inactivated products, paper[5] and thin-layer chromatographic[6] methods, as well as the combination of these procedures with bioautography have been used earlier with varying success. For the isolation and separation of the individual variants of the A 35512 antibiotic complex (novel members of the vancomycin-type antibiotics), MICHEL *et al.*[7] have first tried the application of HPLC by using reversed-phase ODS silica gel column and gradient elution technique. The purity of ristocetin-ψ-aglycone[8] was established by HPLC too, by means of reverse phase column (Spherisorb S 5 ODS) eluted with monobasic potassium phosphate - methanol (7: 3). According to ELLESTAD *et al.*[9] avoparcin α and β have been isolated and also separated by extensive preparative HPLC with Prep Pak-500/C_{18} cartridges for the solid support. Elution was carried out with a buffer solution consisting of 2.5% acetic acid, 0.08 M ammonium hydroxide, 0.01 M sodium heptane sulfonate, and containing of 13 ~ 17% acetonitrile.

More recently MCCLAIN *et al.*[10] have reported a HPLC method for the quantitative determination

of vancomycin in human serum on reverse phase μBondapak C_{18} column using 12% of acetonitrile and 88% of 0.01 M heptane-1-sulfonic acid as the mobile phase. However this method has not been extended as yet for the investigation of the additional members of the vancomycin group of antibiotics.

In this paper we wish to report on the comparative analytical HPLC investigation of the seven members of the vancomycin group of antibiotics. On the basis of the results, the present method is regarded superior to the previously reported methods for the control of the homogenity of the antibiotic preparations.

Materials and Equipment

The investigation was carried out with a Hewlett-Packard 1082 liquid chromatograph working in the isocratic mode, and fitted with an automatic injector and UV detector (254 nm). The separations were performed on 4.6×200 mm LiChrosorb RP-8 packed; I, particle size 10 μm, C_8-type, Hewlett-Packard, Böblingen, FRG and on 4.0×250 mm Separon SI VSK C18 packed; II, particle size 10 μm, C_{18} Laboratorni Přistroje, Prague, Cs.S.R. columns. Eluent systems:

A. Sörensen aqueous citrate buffer containing 12% of methyl cellosolve, pH 6.40,

B. 0.1 M Aqueous ammonium formate solution (free of formic acid), containing 10% of acetonitrile, pH 7.30. For the preparation of the buffers analytically pure sodium hydroxide and citric acid (Reanal), methyl cellosolve (Merck, p.a.) and acetonitrile (Merck, p.a) distilled from calcium hydride were used. Ammonium formate was prepared according to a known procedure[11].

The antibiotic samples used for the investigations were as follows: 1. Ristocetin A* (commercial, Abbott Laboratories, U.S.A.); 2. ristomycin A* (commercial, National Research Institute for Antibiotics, Moscow, U.S.S.R.); 3. A-35512 B (Eli Lilly and Co., U.S.A.); 4. carboxyristomycin A[12]; 5. vancomycin·HCl (commercial, Eli Lilly and Co., U.S.A.); 6. avoparcin (American Cyanamid Co., U.S.A.); 7. actinoidin A,B (crude, Institute of New Antibiotics, Moscow, U.S.S.R.). The investigated antibiotics were dissolved in the buffer (2.0 mg/ml) and 50 μl of this solution was injected in the case of LiChrosorb RP-8 column and 100 μl was used for the analysis on the Separon SI VSK C18 column. The lowest detectable quantity of the antibiotics was determined by the serial dilution method. The measurements were carried out at ±25°C.

Results and Discussion

The chromatographic behavior of antibiotics **1~7** was investigated by using the eluents A and B on columns I and II. It was observed that the absolute retention time (t_R) of the samples was dependent on the age of the columns, but after equilibration for a few hours constant retention time values were obtained. Additionally, a 1~3% alteration of the methyl cellosolve- or acetonitrile-content of the buffers as well as a small change in the pH (±0.2) value remarkably influenced the degree of separation.

Besides the t_R and k' values, the α-values, characteristic of the separation of the major components from the minor ones were also determined on both applied columns in eluent system A and B (Table 1). It was established that, except for a few cases, the application of column I resulted in smaller retention time values with simultaneous better or equal degree of separation than that of column II.

In general, the vancomycin-type antibiotics could be eluted more slowly on the less polar C_{18} packing even upon higher flow rate of the eluent. Moreover, the t_R values of the major and minor components increased in nearly similar degree, resulting in the broadening of the peaks and worse separation.

The results of the analytical separation of antibiotics **1~7** on LiChrosorb RP-8 column (I) with eluent system A are shown on Charts 1 and 2.

* Neither pure variant B nor a mixture of the two variants A and B were available.

Table 1. The t_R, and α values of the members of vancomycin group of antibiotics.

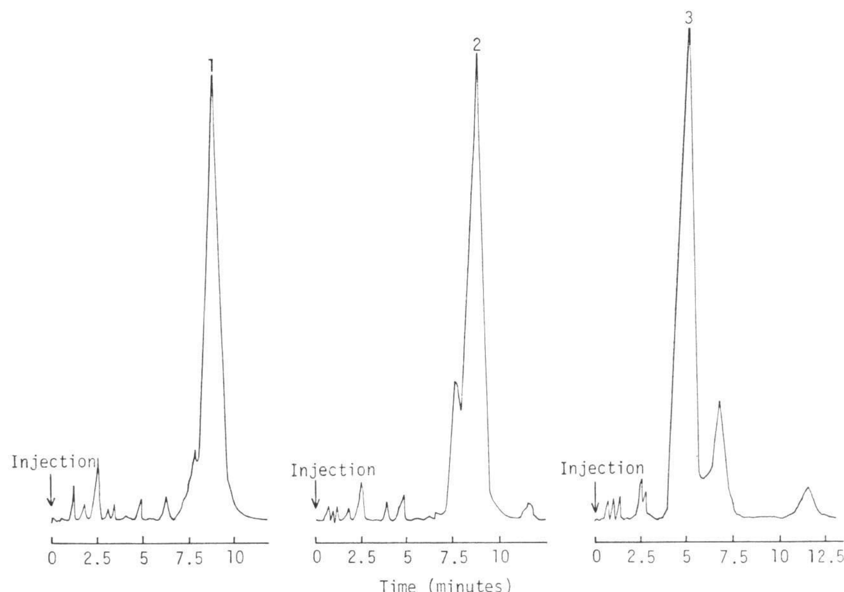
	Compound	I (C_8)			II (C_{18})		
		t_R (minutes)	k′	α	t_R (minutes)	k′	α
Eluent system A	**1**	(2.37) 8.65	(2.38) 11.35	4.77	(3.22) 7.70	(1.68) 5.42	3.22
	2	(2.40, 7.67) 8.65	(2.43, 9.95) 11.35	4.67, 1.14	(3.26) 7.70	(1.72) 5.42	3.15
	3	(5.13) 7.33, 11.80	(6.33) 9.47, 15.85	1.50, 2.50	(5.47) 7.63 (11.0)	(3.56) 5.36 (8.17)	1.50, 1.52
	4	(1.80) 2.33 (3.04)	(1.57) 2.33 (3.34)	1.48, 1.43	3.16 (4.76)	1.63 (2.97)	1.82
	5	(3.42) 7.34	(3.88) 9.48	2.44	(4.50) 12.0	(2.75) 9.00	3.27
	6	(10.45) 17.15	(13.90) 23.50	1.69	(16.5) 31.2	(12.75) 25.00	1.96
	7	8.50, 15.25	11.14, 20.78	1.86	14.7, 19.8	11.25, 15.50	1.38
Eluent system B	**1**	(1.46, 3.18)	(0.54, 2.35)	7.65, 17.5	(2.21, 4.71)	(0.64, 2.54)	6.74, 1.75
		4.87 (6.20)	4.13 (5.52)	1.33	7.25 (9.35)	4.45 (6.03)	1.35
	2	(1.46, 3.37, 4.01)	(0.54, 2.55, 3.22)	7.65, 1.62	(2.25, 4.86, 6.25)	(0.69, 2.65, 3.69)	6.45, 1.68
		4.87	4.13	1.28	7.25	4.45	1.23
	3	(4.19) 4.91 (6.32)	(3.41) 4.17 (5.65)	1.22, 1.35	(3.26, 4.53)	(1.45, 2.41)	3.24, 1.95
					7.60 (9.30)	4.71 (5.99)	1.27
	4	(1.10) 1.45 (1.89)	(0.16) 0.53 (0.99)	3.31, 1.87	(1.75) 2.15 (3.34)	(0.31) 0.62 (1.51)	2.00, 2.43
	5	(2.67) 6.34	(1.81) 5.67	3.13	(4.70) 11.8	(2.53) 7.87	3.11
	6	(5.31, 6.50) 8.05	(4.59, 5.84) 7.47	1.63, 1.28	(11.2, 15.9) 20.75	(7.42, 10.95) 14.60	1.97, 1.33
	7	4.08, 4.73	3.29, 3.98	1.20, 1.34	11.0, 14.4	7.27, 9.83	1.35
		6.03, 6.95	5.34, 6.31	1.18			

Flow rate System A packing C_8 Column I 2.0 ml/minute $t_0=0.70$
C_{18} Column II 2.5 ml/minute $t_0=1.20$
System B packing C_8 Column I 3.0 ml/minute $t_0=0.95$
C_{18} Column II 3.5 ml/minute $t_0=1.33$

The parameters of the minor components are indicated in parentheses. With the exception of **7**, the α values are characteristic of the separation of the major component from the minor components.

Chart 1. Chromatograms of ristocetin A (**1**), ristomycin A (**2**) and A-35512 B (**3**).

UV Range: 254 nm, chart speed: 3 mm/minute, flow rate: 2.0 ml/minute, column size: 4.6× 200 mm, packing: LiChrosorb RP-8, 10 μm (Hewlett-Packard), solvent: aqueous citrate buffer - methyl cellosolve (88: 12), pH 6.40, pressure: 82.6 kg/cm^2.



One can see, that the chromatograms of ristocetin A (**1**) and ristomycin A (**2**) are identical indicating, again, the identity[13] of these two antibiotics. The only difference can be observed in the accompanying contaminations. Of these latter substances the one, appearing with $t_R = 2.37 \sim 2.40$ was found to be identical with the major component of carboxyristomycin A (**4**), isolated after the alkaline treatment (0.5 N NaOH, 20°C, 30 minutes) of ristomycin A. At the same time, no even traces of the parent antibiotic could be detected in the sample of **4**.

The formation of **4** can be explained by the hydrolysis of the *C*-terminal methoxy-carbonyl group of the antibiotic, and enrichment of **4** in the antibiotic preparation results in an undesired decrease of the antibiotic activity.

The most recently isolated member of this group A-35512 B (**3**)[7] is structurally very similar[1,2] to ristocetin (ristomycin) A. According to the present chromatographic studies the sample of **3**, believed to be preparatively homogeneous, contains two minor components with higher t_R values, which can be readily separated from ristocetin (ristomycin) A.

The additional three members of the vancomycin-type antibiotics, vancomycin (**5**), avoparcin (**6**), and actinoidin (**7**) are structurally very similar[1,2], as well. Chromatogram 5 on Chart 2 demonstrates the homogenity and good quality of the commercial vancomycin preparation. The variants α and β in the avoparcin preparation can be also readily separated under the applied chromatographic conditions (chromatogram 6 on Chart 2). At the same time, this chromatographic system is not sufficient for the examination of the multi-component actinoidin preparation (**7**). According to the above observations compounds **1**$\sim$**3** can be eluted from column I always with lower t_R value than compounds **5**$\sim$**7**. The advantage of the application of this column is that ristocetin (ristomycin) A and A-35512 B (major component), as well as the variants α and β of avoparcin can be readily separated (Chart 3).

Using the Separon SI VSK C18 column (II) and eluent system A, ristocetin (ristomycin) A could

Chart 2. HPLC examination of carboxyristomycin A (**4**), vancomycin (**5**), avoparcin (**6**) and actinoidin (**7**).

UV Range: 254 nm, chart speed: 3 mm/minute, flow rate: 2.0 ml/minute, column size: 4.6× 200 mm, packing: LiChrosorb RP-8, 10 μm (Hewlett-Packard), solvent: aqueous citrate buffer - methyl cellosolve (88: 12), pH 6.40, pressure: 82.6 kg/cm^2.

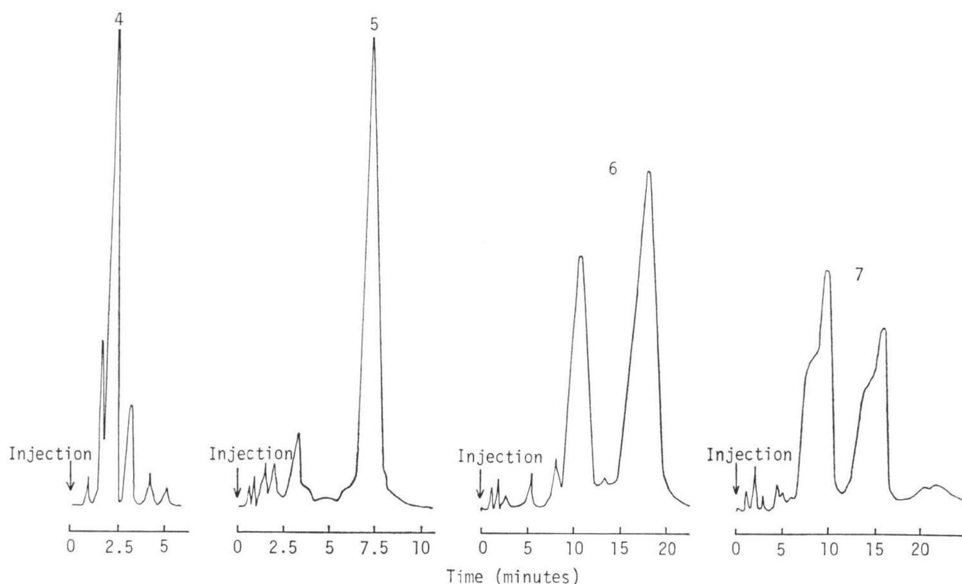


Chart 3. Separation of A-35512 B (**3**), ristomycin A (**2**) and avoparcin (**6**) on adsorbent C_8.

UV Range: 254 nm, chart speed: 3 mm/minute, flow rate: 2.0 ml/minute, column size: 4.6× 200 mm, packing: LiChrosorb RP-8, 10 μm (Hewlett-Packard), solvent: aqueous citrate buffer - methyl cellosolve (88: 12), pH 6.40, pressure: 82.6 kg/cm^2.

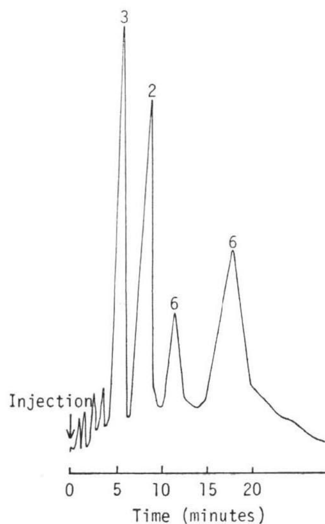
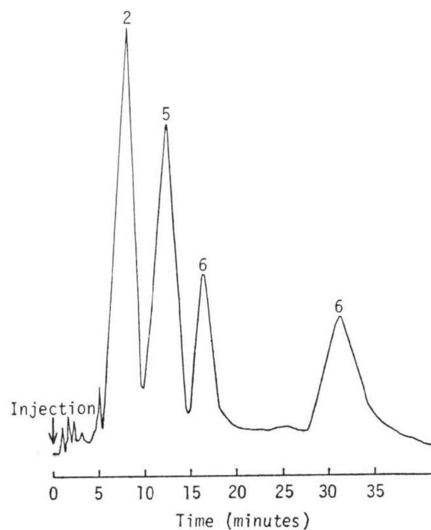


Chart 4. Separation of ristomycin A (**2**), vancomycin (**5**) and avoparcin (**6**) on adsorbent C_{18}.

UV Range: 254 nm, chart speed: 1 mm/minute, flow rate: 2.5 ml/minute, column size: 4.0×250 mm, packing: Separon SI VSK C18, 10 μm (Laboratorni Přistroje, Prague), solvent: aqueous citrate buffer - methyl cellosolve (88: 12), pH 6.40, pressure: 68.2 kg/cm^2.



not be separated from A-35512 B, whereas ristomycin A could be successfully separated from vancomycin and from the avoparcin variants α and β (Chart 4). The chromatograms obtained with column I and eluent B are shown on Charts 5 and 6. Comparing chromatograms 1 and 2 with the respective ones on

Chart 5. Chromatograms of ristocetin A (**1**), ristomycin A (**2**), A-35512 B (**3**) and carboxyristomycin A (**4**).
UV Range: 254 nm, chart speed: 3 mm/minute, flow rate: 3.0 ml/minute, column size: 4.6× 200 mm, packing: LiChrosorb RP-8, 10 μm (Hewlett-Packard), solvent: 0.1 M aqueous ammonium formate - acetonitrile (9: 1), pH 7.30, pressure: 92.8 kg/cm^2.

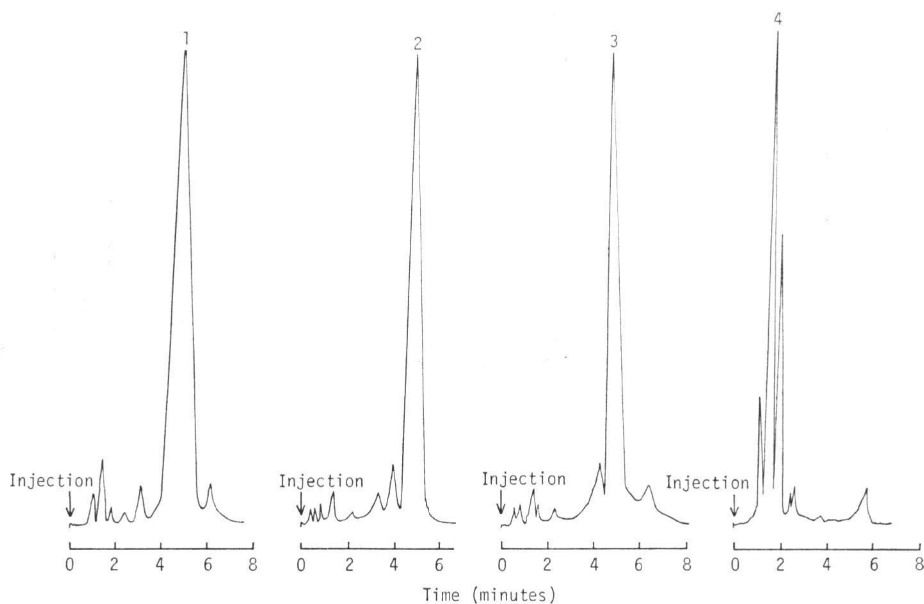


Chart 6. HPLC examination of vancomycin (**5**), avoparcin (**6**) and actinoidin (**7**).
UV Range: 254 nm, chart speed: 3 mm/minute, flow rate: 3.0 ml/minute, column size: 4.6× 200 mm, packing: LiChrosorb RP-8, 10 μm (Hewlett-Packard), solvent: 0.1 M aqueous ammonium formate - acetonitrile (9: 1), pH 7.30, pressure: 92.8 kg/cm^2.

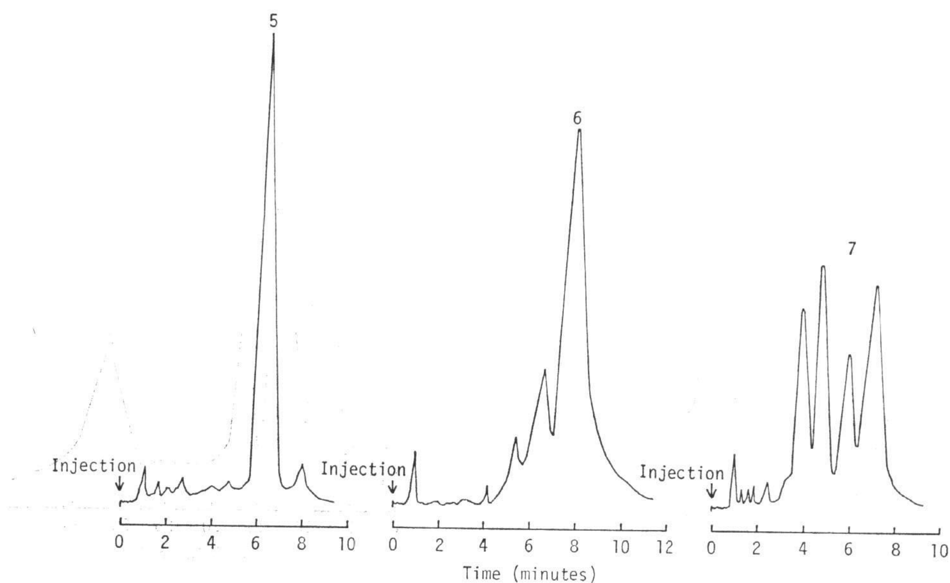


Chart 1 it can be established that the peaks belonging to the antibiotics and to the accompanying contaminations are even more separated under these conditions and the peak with $t_R=1.45$ minutes was assigned, again, to compound **4**. At the same time, the two minor components of sample **3** — appeared before and after the major peak assigned to A-35512 B — could not be separated from ristocetin (ristomycin) A. Nevertheless, this latter chromatographic method was found to be servicable not only for the identification of vancomycin, and separation thereof from ristocetin (ristomycin) A (chromatogram 5), but also for the quantitative determination of the active factor in these antibiotic preparations. In the case of the multi-component **6** and **7** this method allowed the convenient qualitative separation of the mixtures. In particular, it can be distinguish between the variants A and B and decomposition products of the crude actinoidin preparation (chromatogram 7). Using eluent B the investigated antibiotics were more poorly eluted from packing C_{18} (II). Due to the remarkable broadening of the peaks and the decrease of the lowest detectable quantity, these chromatographic conditions were found to be insufficient for the separation of the vancomycin-type antibiotics.

The lowest detectable quantity of the antibiotics was determined on the more advantageous packing C_8 (I) using eluent B. A value of 60 ng was obtained for the homogeneous samples of **1**, **2** and **3**, while a value of 140 ng was measured for **5**. In the case of the two-component **6** and multi-component **7** the value of the lowest detectable quantity was 300 ng and 100 ng, respectively.

The investigation of this group of antibiotics has not been finished as yet, and extensive research work is being done for discovering additional representatives. As the quick and convenient qualification of the products introduced in therapy or diagnosis is of primary importance, the analytical HPLC method may further contribute to the solution of this problem.

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