SEPARATION AND QUANTITATIVE DETERMINATION OF AMINO SUGAR ANTIBIOTICS AND THEIR DEGRADATION PRODUCTS BY MEANS OF AN IMPROVED METHOD OF CHROMATOGRAPHY ON RESIN

SHIGEHARU INOUYE AND HIROSHI OGAWA

Central Research Laboratories, Meiji Seika Kaisha Ltd., Yokohama (Japan)

(Received June 20th, 1963)

Amino sugar antibiotics, such as kanamycin, neomycin, and paromomycin, are usually produced as a mixture of closely related antibiotics, whose separation and differentiation have always been difficult problems. In the present study, an ion-exchange method of chromatography, developed originally to separate kanamycins A and B[1], has been somewhat improved and applied successfully to the separation and quantitative determination of eight amino sugar antibiotics and their degradation products.

The technique consists of separation on strongly basic anion-exchange resins, preferably Dowex 1 X 2 in the hydroxyl form, the column being developed with distilled water. The modifications that have been introduced are: (1) the use of a shorter column (resin volume, 25–50 ml), (2) slower rates of elution (20–30 ml/h), (3) a finer particle size for the resin (200–400 mesh instead of 50–100 mesh), and (4) a ninhydrin method for the determination of the amino sugars.

The use of finer particles coupled with slower rates of elution resulted in a considerably improved separation and an increased sharpness of the elution peaks; these become primary advantages when more complex mixtures are analyzed. Introduction of the photometric ninhydrin method permits quantitative analysis of the amino sugar antibiotics on a milligram scale. The majority of the antibiotics examined gave 40 to 50% color yield (diketohydrindylidene-diketohydrindamine) when the ninhydrin procedure of MOORE AND STEIN for amino acids[2] was used. The modified system can be used either with a fraction collector or with an automatic recording equipment.

EXPERIMENTAL

Quantitative determination of a mixture of kanamycins A, B and C by means of an automatic amino acid analyzer

Chromatography on resin combined with an automatic amino acid analyzer provided an adequate method for the quantitative determination of kanamycins in a mixture. The procedure is essentially the same as that described for amino acid determination[3] except for the chromatographic column and developing solvent. The results obtained by this procedure when a synthetic mixture of kanamycins A[4], B[5] and C[6] was chroma-

tographed are superior to those previously published[1] and are illustrated in Fig. 1 by the satisfactorily sharp peak for kanamycin A together with complete separation of B and C. The size of a peak, which was integrated by the convenient height–time–width method[8], was linear with respect to the concentration of kanamycin A, B, or C within the respective ranges so far examined: 1–3.5 mg for A, 0.1–0.35 mg for

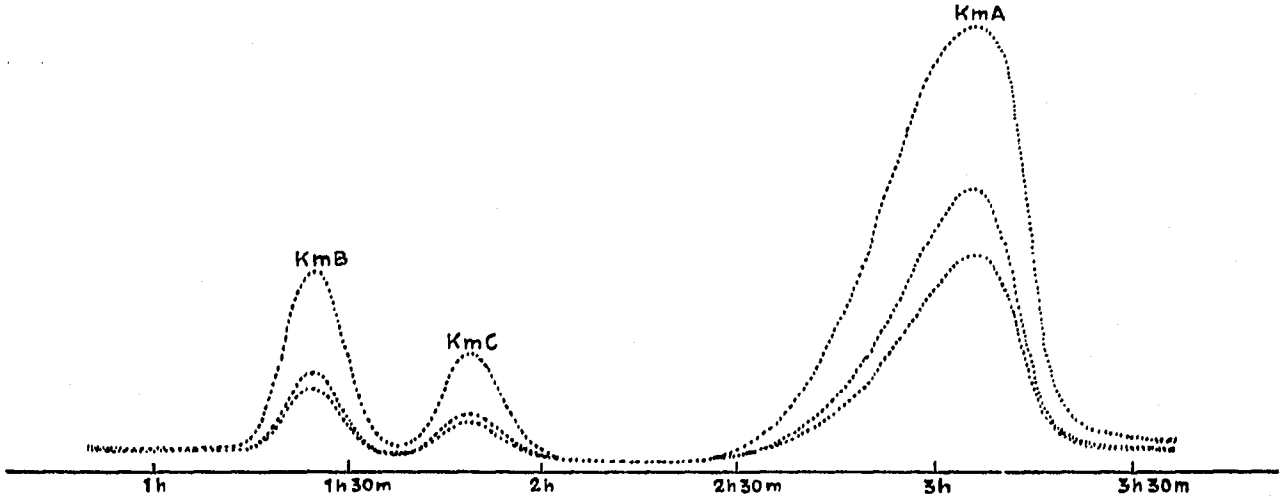

Fig. 1. Tracing of the chromatographic separation of a kanamycin mixture automatically recorded by Hitachi Amino Acid Analyzer (Type KLA-2). Column, Dowex 1 X 2 0.9 × 39 cm. Flow rate, 30 ml/h. KmA 2.08 mg; KmB 0.207 mg; KmC 0.143 mg.

B, and 0.05–0.25 mg for C. A sample may be analyzed within 3.5 h by this method, which is particularly suitable for the determination of kanamycin B in the presence of A and C, as it was indicated in the present investigation that the differential bioassay[7] of B from A gives an abnormally high estimate of B in samples which are contaminated with C.

Separation of a mixture of other amino sugar antibiotics and isolation of paromomycin II

The modified procedure, with its good resolving power, was then applied to the study of other amino sugar antibiotics and related compounds.

Fig. 2 illustrates a typical chromatogram obtained with a mixture of kanamycins A, B and C, neomycins A (neamine)[8], B[9] and C[9], and paromomycins I[10] and II[9],

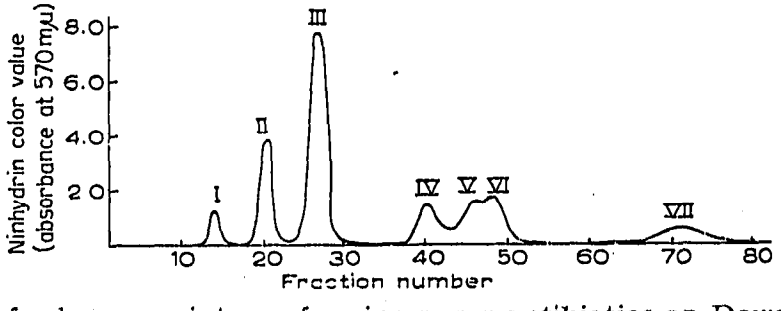

Fig. 2. Separation of a known mixture of amino sugar antibiotics on Dowex 1 X 2 (50 ml). I = Neamine (0.5 mg); II = Kanamycin B (2.2 mg); III = Kanamycin C (2.2 mg) and neomycin C (2.2 mg); IV = Paromomycin II (2.2 mg); V = Kanamycin A (2.2 mg); VI = Neomycin B (2.2 mg); VII = Paromomycin I (4.4 mg).

while their relative peak effluent volumes are shown in Table I*. With the exception of the overlapping of neomycin C and kanamycin C, separation of the other six amino sugar antibiotics is satisfactory. Since the antibiotics belonging to the neomycin group or paromomycin group are separated completely, it is possible, as in the case of

TABLE I

RELATIVE PEAK EFFLUENT VOLUMES (*Rv*) OF VARIOUS AMINO SUGARS AND RELATED COMPOUNDS ON DOWEX 1 X 2

Compound	*M/N*[a]	*Rv*[b]	
Tetrasaccharide			
Neomycin B	102	1.04	
Neomycin C	102	0.59	
Paromomycin I	123	1.54	
Paromomycin II	123	0.87	
Trisaccharide			
Kanamycin A	121	1.00	(1.00)[c]
Kanamycin B	97[d]	0.45	(0.33)
Kanamycin C	121	0.59	
Disaccharide			
Neamine	81	0.26	(0.22)
Paromamine	108	0.32	
6-O-α-D-3-Amino-3-deoxy-glucopyranosyl-2-deoxystreptamine	108	0.43	
Methyl (3-O-neosaminido-B)-α-D-ribofuranoside	162	0.50	
Methyl (3-O-neosaminido-B)-α-D-ribopyranoside	162	0.64	
Methyl (3-O-neosaminido-B)-β-D-ribofuranoside	162	0.96	
Methyl (3-O-neosaminido-B)-β-D-ribopyranoside	162	1.24	
Methyl (3-O-neosaminido-C)-α-D-ribofuranoside	162	0.38	(0.28)
Methyl (3-O-neosaminido-C)-α-D-ribopyranoside	162	0.44	(0.36)
Methyl (3-O-neosaminido-C)-β-D-ribofuranoside	162	0.64	(0.61)
Methyl (3-O-neosaminido-C)-β-D-ribopyranoside	162	0.88	(0.76)
Monosaccharide			
Methyl 3-amino-3-deoxy-α-D-glucopyranoside	193	0.48	(0.47)
Methyl 6-amino-6-deoxy-α-D-glucopyranoside	193	0.38	(0.32)
Methyl 2-amino-2-deoxy-α-D-glucopyranoside	193	0.38	
2-Deoxystreptamine	81	0.275	(0.22)
Streptamine	89	0.38	
Streptidine	—	0.13	
Amines			
Benzylamine	107	0.50	(0.40)
Histamine	56	0.38	(0.21)

[a] Molecular weight divided by the number of amino groups.
[b] Peak effluent volume of a substance divided by the peak effluent volume of kanamycin A.
[c] *Rv* on a column of Dowex 1 X 4 resin.
[d] *M/N* was calculated on the basis of the revised molecular formula $C_{18}H_{37}N_5O_{10}$ with five amino groups[16].

kanamycin, to estimate the amount of each antibiotic produced in a mixture by chromatography in combination with ninhydrin photometry. Such a chromatographic analysis when applied to a fradiomycin preparation obtained from the fer-

* For this procedure a fraction collector was employed.

mentation broth of *Streptomyces fradiae* No. 260, indicated that it consisted of 74 % of neomycin B and 26 % of neomycin C. Application of the procedure to a commercial paromomycin preparation revealed the presence of 91 % paromomycin I, admixed with a minor amount of paromomycin II (9.0 %).

In addition to analytical uses, resin chromatography can be used for preparative purposes and has the advantage of yielding pure antibiotics, as evaporation of the aqueous alkaline portions of the effluent usually affords the free bases. Thus, the free base of paromomycin II was easily isolated* when a column overloaded with a paromomycin mixture was used. The product had an $[\alpha]_D^{20}$ of + 99° (in water), and gave an almost identical ultraviolet absorption curve to that of neomycin C, but differed from that of neomycin B and paromomycin I when heated with 50 % sulfuric acid. Its biological activity against many micro-organisms was in general weaker than that of neomycins B, C and paromomycin I. On methanolysis with 2 *N* HCl it yielded equimolar amounts of paromamine and methyl neobiosaminide C, the main component of the latter being methyl (neosaminido-C)-β-D-ribopyranoside. These results are consistent with the proposed structure for paromomycin II[9] and zygomycin A_2[11].

Separation of methanolysis products of amino sugar antibiotics and isolation of new ribofuranoside forms of neobiosaminides B and C

Methanolysis of neomycins B and C in dilute HCl gave neamine and the respective methyl neobiosaminides, the latter usually being isolated as a mixture of their anomeric α- and β-glycosides. A crude methyl neobiosaminide B, when subjected to chromatographic analysis, afforded five ninhydrin positive peaks (B_1, B_2, B_3, B_4 and B_5 in Fig. 3); B_1 was identified as neamine. The four remaining peaks showed a

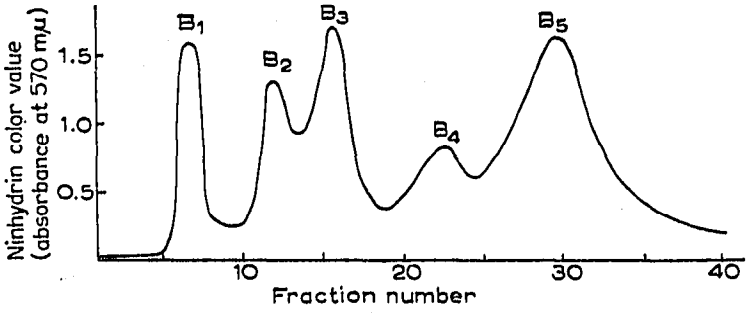

Fig. 3. Separation of crude methyl neobiosaminide B derived from mild methanolysis of neomycin B on Dowex 1 X 2 (25 ml).

positive furfural assay and must be anomeric glycoside isomers in the ribose moiety of the disaccharide methyl neobiosaminide B, since all the components could be hydrolyzed in *N* HCl, at 90°, to products with the same constant rotation ($[\alpha]_D^{20}$, + 30°) as that of neobiosamine B[12] and retreatment of the hydrolyzate of B_5 with 0.3 *N* methanolic HCl at 4° yielded the same four components (B_2, B_3, B_4 and B_5) as before. A ring structure was assigned to the ribose moiety in these components (furanoside for B_2 and B_4, pyranoside for B_3 and B_5) on the basis of the relative rates of formation

* After completion of this work, a paper on the isolation of zygomycin A_2 which was identical with paromomycin II was published[15].

and data from hydrolysis under various conditions[13]. Assignment of an anomeric configuration (α-form for B_2 and B_3, β-form for B_4 and B_5) was made from a comparison of their optical rotations with those of anomeric methyl D-ribosides as shown in Table II. Similar evidence allowed assignment of a ribose structure to the four isomers* of methyl neobiosaminide C which were isolated in the same manner (see Table II). Methyl α- and β-neobiosaminides B and C containing ribofuranoside moieties have not been isolated in previous studies[7,9,12].

TABLE II

OPTICAL ROTATIONS OF ANOMERIC RIBOGLYCOSIDE ISOMERS OF METHYL NEOBIOSAMINIDES AND METHYL RIBOSIDE

Compound	α-D- *Ribofuranoside*	α-D- *Ribopyranoside*	β-D- *Ribofuranoside*	β-D- *Ribopyranoside*
Methyl neobiosaminide B[a]	+ 109	+ 69	+ 25	— 10
Methyl neobiosaminide C[a]	+ 168	+ 124	+ 88	+ 41
Methyl D-riboside[b]	+ 147	+ 103	— 62	—107

[a] $[\alpha]_D^{20}$ in water (concn. 0.6–1.2 %).
[b] $[\alpha]_D^{20}$ in methanol (concn. 1.0 %)[14].

Paromomycin I, when treated in methanol with 0.3 *N* HCl, afforded five components, of which four were identified as the anomeric riboglycoside isomers of methyl neobiosaminide B as would be expected from the structure[9,10]. The first emerging peak was identified as paromamine, which is eluted more slowly than neamine. 6-O-α-D-3-amino-3-deoxy-glucopyranosyl-2-deoxystreptamine, a partial degradation product of kanamycin A[4], emerges immediately after paromamine with complete separation.

As for the monosaccharide amino sugars, chromatography on Dowex 1 X 2 separated a mixture of 2-deoxystreptamine, methyl 6-amino-6-deoxy-α-D-glucopyranoside and methyl 3-amino-3-deoxy-α-D-glucopyranoside, which are the main methanolysis products of kanamycin A[4]. Further application of the method indicated the resolution of streptidine, streptamine and 2-deoxystreptamine, the former two being the degradation products of streptomycin.

The above results together with data from studies still in progress, emphasise the usefulness of chromatography on resins for the fractionation and quantitative estimation of various types of amino sugar glycosides.

Scope of chromatography on resin

The strongly alkaline and adsorptive properties of Dowex 1 X 2 limit the number of substances which can be effectively used on such columns. Neobiosamines, 2-amino-2-deoxy-D-glucose, isonicotinic acid hydrazide, homosulfamine and lysine, when examined, were adsorbed strongly on the resin with decomposition in some cases. On the other hand, a mixture of histamine and benzylamine was successfully separated, the former being eluted faster than the latter.

The above limitation, however, offers a convenient purification procedure for an

* Thin-layer and paper chromatography using various solvent systems was unsuccessful in resolving these isomers.

appropriate basic substance when it is contaminated with substances which are highly adsorbed or have alkaline unstability. In fact, the separation of methyl 2-amino-2-deoxy-D-glucoside from unreacted 2-amino-2-deoxy-D-glucose was easily accomplished by passing a crude methylation product through a short column of the resin.

As indicated by ROTHROCK *et al.*[1], chromatography on Dowex 1 X 2 is a typical example of adsorption chromatography on ion-exchange resins. Although the data available are relatively few, consideration of the data shown in Table I reveals some correlation between structure and relative peak effluent volume (*Rv*): (1) Among the amino sugars having similar molecular weights, *Rv* in many cases increases with increasing *M/N* value (molecular weight divided by the number of amino groups); (2) with regard to the relationship of molecular size to pore size of resin, it is noted that, although Dowex 1 X 2 is preferable for oligosaccharide amino sugars, the 4% cross-linked resin gave a much improved resolution with monosaccharide amino sugars and the alkyl amines so far examined.

Full experimental will be published elsewhere.

SUMMARY

Chromatography on Dowex 1 X 2 resin was modified to improve the sharpness of the effluent peaks and applied to the separation and quantitative determination of amino sugar antibiotics and their degradation products. A photometric ninhydrin method was used to determine the amino sugars.

The usefulness of the method was further demonstrated by the isolation of paromomycin II and methyl neobiosaminides B and C containing a ribofuranoside moiety. Finally, limitations of the technique arising from the strong alkaline and adsorptive properties of the resin were indicated.

REFERENCES

1 J. W. ROTHROCK, R. T. GOEGELMAN AND F. J. WALF, *Antibiotic Ann.*, (1958/59) 796.
2 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
3 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
4 H. OGAWA, T. ITO, S. KONDO AND S. INOUYE, *Bull. Agr. Chem. Soc. Japan*, 23 (1959) 289.
5 T. WAKAZAWA, Y. SUGANO, M. ABE, S. FUKATSU AND S. KAWAJI, *J. Antibiotics, Ser. A*, 14 (1961) 180.
6 M. MURASE, *J. Antibiotics, Ser. A*, 14 (1961) 367.
7 T. WAKAZAWA, M. ABE, Y. SUGANO AND S. KAWAJI, *J. Antibiotics, Ser. A*, 14 (1961) 187.
8 H. E. CARTER, J. R. DYER, P. D. SHAW, K. L. RINEHART, Jr. AND M. HICHENS, *J. Am. Chem. Soc.*, 83 (1961) 3723.
9 K. L. RINEHART, Jr., M. HICHENS, A. D. ARGOUDELIS, W. S. CHILTON, H. E. CARTER, M. P. GOERGIOUS, C. P. SCHAFFNER AND R. T. SCHILLINGS, *J. Am. Chem. Soc.*, 81 (1962) 3218.
10 T. H. HASKELL, J. C. FRENCH AND Q. R. BARTZ, *J. Am. Chem. Soc.*, 81 (1959) 3482.
11 S. HORII, *J. Antibiotics, Ser. A*, 15 (1962) 187.
12 K. L. RINEHART, Jr., A. D. ARGOUDELIS, W. A. GOSS, A. SOHLER AND C. P. SCHAFFNER, *J. Am. Chem. Soc.*, 82 (1960) 3938.
13 P. A. LEVENE, A. L. RAYMOND AND R. T. DILLON, *J. Biol. Chem.*, 95 (1932) 699.
14 G. R. BARKER AND D. C. C. SMITH, *J. Chem. Soc.*, (1954) 2151.
15 S. HORII, H. HITOMI AND A. MIYAKE, *J. Antibiotics, Ser. A*, 16 (1963) 144.
16 T. ITO, M. NISHIO AND H. OGAWA, to be published.