Journal of Chromatography, 248 (1982) 483–487
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,147

Note

Thin-layer chromatographic identification of aminoglycoside antibiotics

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(Received June 30th, 1982)

The identification of aminoglycoside antibiotics by thin-layer chromatography (TLC) has been described in a number of papers[1,2]. Since most of these reports were published before 1970, only part of the available products was identified. For this reason we thought that it would be useful to reinvestigate these compounds by TLC. The present paper deals with the chromatographic behaviour of 21 aminoglycosides in three TLC systems. They comprise the major components of aminoglycoside antibiotics and some minor components (neomycin C, neamine, paromomycin II, paromamine and kanamycin C). The structures of the different products may be found in some recent reviews[3,4].

EXPERIMENTAL

Substances

Neomycin B, C, paromomycin I, II, kanamycin A, B and C were obtained by ion-exchange chromatography[5,6] of commercial samples of the parent antibiotics. Neamine and paromamine were prepared by methanolysis of neomycin and paromomycin[7,8]. Gentamicin C, sisomicin and netilmycin were obtained from Schering (Bloomfield, NJ, U.S.A.), amikacin and kanamycin B (kanendomycin) from Bristol (Syracuse, NY, U.S.A.), neomycin and spectinomycin from Upjohn (Kalamazoo, MI, U.S.A.), streptomycin, dihydrostreptomycin, kanamycin and dibekacin from Continental Pharma (Belgium), tobramycin and apramycin from E. Lilly (Indianapolis, IN, U.S.A.).

Thin-layer chromatography

TLC was performed on activated (1 h at 110°C) precoated plates (Kieselgel 60, E. Merck, Darmstadt, G.F.R.; Stratochrom SIF_{254}, Farmitalia-Carlo Erba, Milan, Italy; SIF Riedel-De Haën, Seelze-Hannover, G.F.R.) and also on laboratory-made silica gel G plates (0.25 mm layer) and on laboratory-made silica gel H plates containing 1% Carbomer. Carbomer (Carbopol 934®) is a polycarboxylic resin obtained from the Goodrich Chemical Company (Ohio, OH, U.S.A.).

The following procedure was followed for the preparation of the carbomer plate. Carbomer (0.25 g) was added gradually to 200 ml water with vigorous stirring. The stirred dispersion was then neutralized to pH 7 (pH-meter) with dilute potassium

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hydroxide (10%). Silica gel H (25 g) was gradually added to the carbomer dispersion and the mixture shaken vigorously. Air-bubbles were released on standing. The plates were prepared (0.75 mm layer) and air-dried.

The following solvent systems were examined: A1, chloroform–methanol–25% ammonium hydroxide (density 0.91)–water (1:4:2:1 v/v); A2, chloroform–methanol–25% ammonium hydroxide (density 0.91) (2:3:2 v/v); B1, 15% aqueous solution of potassium dihydrogen phosphate (pH 4.4); B2, 10% aqueous solution of potassium dihydrogen phosphate (pH 4.5).

A 10-μl volume of the aminoglycosides (free bases or sulphates) and 20 μl of the commercial gentamicin C (sulphate) were applied to the activated plates, which were developed to a height of 15 cm in a chamber coated with filter-paper and saturated overnight. Plates were removed from the tank, dried for 30 min at 110°C and allowed to cool.

For detection with ninhydrin, the plates were sprayed with the reagent (1 g ninhydrin in 50 ml ethanol and 10 ml glacial acetic acid) and heated for 15 min at 110°C. Streptomycin and dihydrostreptomycin are not vizualized by this reagent. For detection with naphthoresorcinol (1,3-dihydroxynaphthalene), plates were sprayed with 2% naphthoresorcinol in ethanol, followed by 9 *N* sulphuric acid and then developed by heating for 5–10 min at 120°C. The colours are dependent on the temperature and duration of heating, and vary from blue to purple for neomycin (B and C) and paromomycin (I and II), from yellow to beige for neamine, paromamine and spectinomycin, from grey to beige for dibekacin, from grey to brown for streptomycin, dihydrostreptomycin, kanamycin B, C and tobramycin, from red to reddish brown for kanamycin A, gentamycin C, sisomicin, netilmicin and apramycin.

RESULTS AND DISCUSSION

Solvent system A1, which consists of chloroform, methanol, ammonium hydroxide and water, was originally reported by Maehr and Schaffner[9] for identification of gentamicins. The R_F values for the 21 aminoglycosides are listed in Table I and schematically represented in Fig. 1a. The figure shows that ten of the sixteen major components can be separated by this system. Poor separation or no separation at all was observed for sisomicin–gentamicin C_1, C_{1a}, for dibekacin–spectinomycin, for kanamycin A–kanamycin B–apramycin and for streptomycin–dihydrostreptomycin. If one also considers the minor components, the following pairs are also not separated: neamine–apramycin, kanamycin C–dibekacin and paromamine–spetinomycin. These interferences however will not be encountered during analyses of commercial samples, since it is known that the amounts of kanamycin C, neamine and paromamine found in the parent antibiotics are low (less than 1 %). It should be noted that separation is not obtained for neomycin B and C or for paromomycin I and II. The separation of these components however is not essential (and even not desired) for identification of commercial aminoglycoside antibiotics.

The sequence of R_F values observed in the system A1 is very similar to that obtained in another system (system A2)[10], made up from the same components but in a different ratio. One exception is the order of gentamicin C_1 and C_2, which is inverted in this system. The results obtained are summarized in Table I and Fig. 1b. The figure shows that resolution is better for aminoglycosides located between specti-

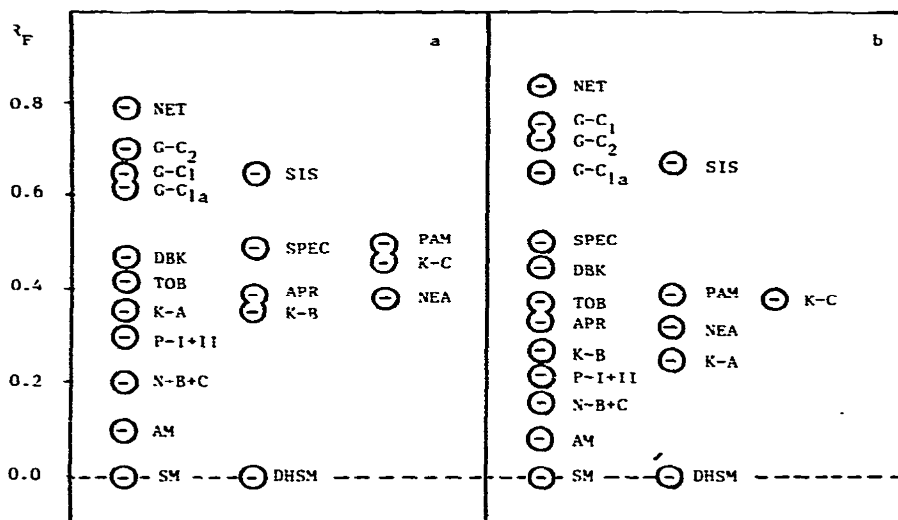

Fig. 1. Thin-layer chromatography of aminoglycosides: (a) in the system A1; (b) in the system A2.

TABLE I

R_F VALUES* OF AMINOGLYCOSIDE ANTIBIOTICS

Aminoglycoside antibiotic	*TLC system*			
	*A1***	*A2***	*B1***	*B2****
Streptomycin (SM)	0.00	0.00	0.66	0.56
Dihydrostreptomycin (DHSM)	0.00	0.00	0.64	0.54
Neomycin B (N-B)	0.20	0.16	0.27	0.17
Neomycin C (N-C)	0.22	0.16	0.27	0.17
Neamine (NEA)	0.38	0.32	0.42	0.37
Paromomycin I (P-I)	0.30	0.22	0.38	0.33
Paromomycin II (P-II)	0.31	0.22	0.38	0.33
Paromamine (PAM)	0.49	0.39	0.61	0.57
Kanamycin A (K-A)	0.35	0.25	0.47	0.42
Kanamycin B (K-B)	0.35	0.27	0.34	0.27
Kanamycin C (K-C)	0.46	0.38	0.47	0.42
Tobramycin (TOB)	0.42	0.37	0.36	0.30
Dibekacin (DBK)	0.47	0.45	0.31	0.27
Amikacin (AM)	0.10	0.08	0.57	0.51
Gentamicin C_1 (G-C_1)	0.65	0.75	0.21	0.20
Gentamicin C_2 (G-C_2)	0.70	0.72	0.28	0.24
Gentamicin C_{1a} (G-C_{1a})	0.62	0.65	0.28	0.24
Sisomicin (SIS)	0.65	0.67	0.29	0.26
Netilmicin (NET)	0.79	0.84	0.25	0.23
Apramycin (APR)	0.38	0.33	0.37	0.32
Spectinomycin (SPEC)	0.49	0.50	0.48	0.54

* Means of two values.

** Obtained by chromatography on E. Merck precoated silica gel plates.

*** Obtained by chromatography on Carbomer–silica gel H plates.

nomycin and kanamycin B, but less for those between kanamycin B and neomycin. It should be mentioned that the R_F values given for both systems were obtained on E. Merck precoated silica gel plates. The same sequence of R_F values was observed on laboratory-made silica gel G plates and on precoated silica gel plates from other manufacturers (see Experimental section).

These results show that identification of the fourteen antibiotics requires a second TLC system with a different selectivity. Thus, chromatography was performed in the system B1, originally described by Dubost *et al.*[11] for detection and semi-quantitative analysis of the B component in kanamycin. E. Merck precoated silica gel plates, with 15% aqueous potassium dihydrogen phosphate as mobile phase, have to be employed. It was found that the separation is due to the presence of a poly-carboxylic binder, which acts as an ion-exchange resin. The results obtained with this system are in Table I and Fig. 2a. It can be seen that the aminoglycosides, which are not separated in the previous systems, are well resolved in the Dubost system. The exceptions are neomycin B and C, paromomycin I and II, dihydrostreptomycin and streptomycin and sisomycin and one of the components of gentamicin C. The separation of the components of gentamicin C reported by Wilson *et al.*[12] in the lower layer of methanol–chloroform–con. ammonium hydroxide (1:1:1) is superior to that obtained in the present systems. It should be noted that neomycin, paromomycin and the kanamycins do not migrate in the Wilson system.

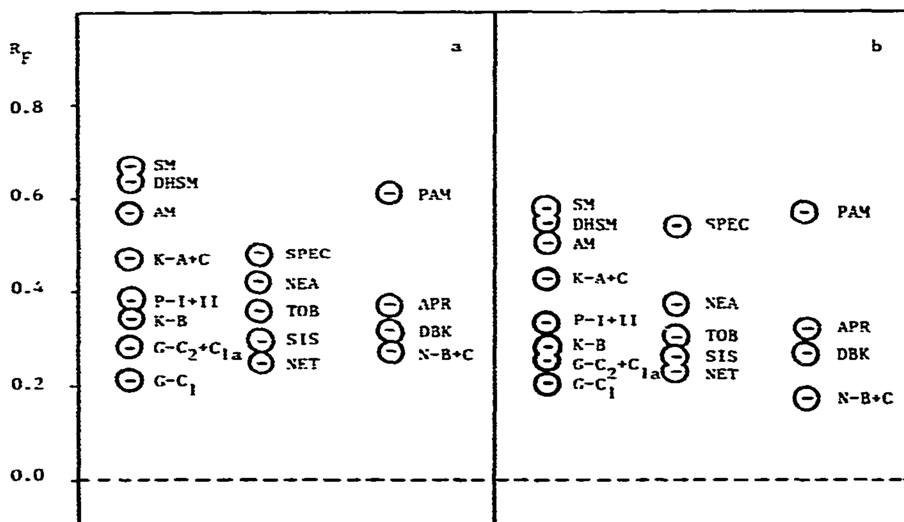

Fig. 2. Thin-layer chromatography of aminoglycosides: (a) in the system B1; (b) in the system B2.

If the Merck precoated silica gel plates are not available, a separation similar to that achieved in system B1 can be obtained on silica gel H plates containing 1% Carbomer (Carbopol 934®), a polycarboxylate resin. The concentration of the potassium dihydrogen phosphate is then lowered to 10% (system B2). The use of Carbomer–silica gel plates is also described in the *European Pharmacopoeia*[13] for a semi-quantitative determination of the B component in commercial kanamycin. Results obtained with the system B2 are also given in Table I and Fig. 2b.

It can be concluded that a combination of the two systems (A1 or A2 and B1 or B2) permits identification of almost all major components of aminoglycoside antibiotics. In the systems A1 and A2 the migration depends on the lipophilic character of the products. Replacement of hydroxyl groups by hydrogen (tobramycin and dibekacin *vs.* kanamycin B) and the presence of methylamino (gentamicin, sisomicin) and ethylamino groups (nethilmicin = 1-N-ethylsisomicin) increases the R_F value. In the B1 and B2 systems the aminoglycosides are classified according to the number of basic groups: six in neomycin; five in paromomycin, kanamycin B, tobramycin and dibekacin; four in neamine, kanamycin A and C; three in streptomycin, dihydrostreptomycin and paromamine. Aminoglycosides with methyl- or ethylamino groups do not migrate as far as one would expect from the number of basic groups. The sequence of the aminoglycoside antibiotics on Dowex 50-X8 type resin-coated plates[14] is similar to that observed in the B1 and B2 systems.

ACKNOWLEDGEMENTS

The authors wish to thank S. Eerdekens and L. Kerremans for technical assistance.

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