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Note

Use of perfluorinated carboxylic acids in the separation of aminoglycoside antibiotics by ion-pair reversed-phase high-performance liquid chromatography

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The aminoglycosides are clinically useful antibiotics exhibiting comparable antimicrobial activity against a wide range of Gram negative and Gram positive bacteria as well as mycobacterial[1]. The separation of these antibiotics is difficult because they have only slight structural differences[2].

A number of methods have been reported to achieve this goal, including paper, silica thin-layer, ion-exchange and reversed-phase chromatography[3]. The combination of rapid analysis, high resolution and elimination of a derivatization step (which involves the loss of antimicrobial activity) means that ion-pair reversed-phase high-performance liquid chromatography (HPLC) has marked advantages over other methods of separation.

Several ion-pairing systems have been described[4,5] giving satisfactory results in the analysis of aminoglycoside antibiotics. The counter ions used in these systems, however, belong to the alkanesulphonate class and are difficult to remove from the sample afterwards. Therefore they cannot be used conveniently in the preparative mode. In this paper we report the development of an HPLC method for separating aminoglycoside antibiotics using volatile perfluorocarboxylic acid buffer[6].

EXPERIMENTAL

Apparatus

A Waters M6000 A pump, equipped with a U6K liquid chromatography injector (Waters Assoc., Milford, MA, U.S.A.) and a Jobin Yvon refractive index (RI) detector Iota (Instruments S.A. Jobin, Yvon, France) were employed. An analytical reversed-phase (5 μm, C_{18}) Ultrasphere ion-pair column, 25 cm × 4.6 mm (Altex Scientific, Palo Alto, CA, U.S.A.), connected with an in-line guard column RP18 Spheri 5 (Brownlee Labs.) were used. All separations were carried out at ambient temperature.

Reagents

Lividomycin was kindly provided by Dr. R. Gregoire, Roger Bellon Laboratories (Toulouse, France), fortimicins and sagamicin by Dr. T. Nara, Kyowa Kogyo

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Company (Tokyo, Japan), gentamicins and sisomicin by Dr. G. Delonga, Cetrane Laboratories (Levallois-Perret, France), verdamicin by Dr. G. H. Wagman, Schering Corporation (Bloomfield, NJ, U.S.A.), kanamycin and streptomycin by Rhône-Poulenc Laboratories (Vitry, France) and butirosin by Parke, Davies and Co. (Detroit, MI, U.S.A.). Neomycin was obtained from Sigma (St. Louis, MO, U.S.A.).

Camphor-10-sulphonic acid, trifluoroacetic acid, pentafluoropropionic acid and heptafluorobutyric acid were purchased from Fluka (Buchs, Switzerland) and were used without further purification.

RESULTS AND DISCUSSION

Perfluorinated carboxylic acids have been widely used for the separation of peptides. Trifluoroacetic acid (TFA) was found to enhance the partition of opioid peptides into butanol during the course of aqueous–organic phase extraction[7]. In the HPLC analysis of peptides, the use of TFA buffer led to higher resolution and shorter retention times. This was explained by the elimination of adsorption of the compounds on the column matrices[6]. Heptafluorobutyric acid (HFBA) and pentafluoropropionic acid (PFPA) have also proved useful in the chromatography of pituitary hormone and peptides[8,9], providing unique selectivity.

Because of their very polar and ionic nature, the aminoglycoside antibiotics are essentially non-retained on reversed-phase columns even with a neat aqueous mobile phase. The addition of an alkanesulphonate such as camphorsulphonic acid

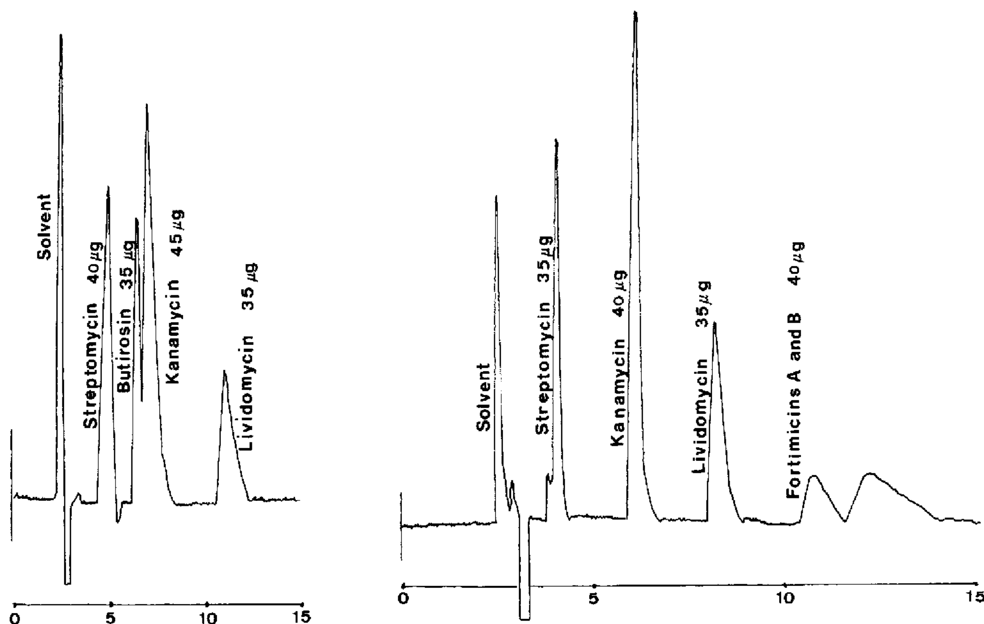

Fig. 1. Ion-pair separation of aminoglycosides. Mobile phase: methanol–0.05 *M* pentafluoropropionic acid, pH 2.1 (42:58); flow-rate 1.0 ml/min. Sensitivity: × 4.

Fig. 2. Ion-pair separation of aminoglycosides. Mobile phase: methanol–0.05 *M* heptafluorobutyric acid, pH 2.1 (60:40); flow-rate 1.0 ml/min. Sensitivity: × 4.

or hexanesulphonate causes the antibiotics to be retained to extents depending on the concentration of the counter ion. We have found that, in spite of their short chain lengths, PFPA and HFBA can be used satisfactorily for the ion-pair chromatography of aminoglycosides. Representative HPLC chromatograms of a few common antibiotics are presented in Figs. 1 and 2. Baseline separation of these compounds is achieved with both acids. The efficiency of the separation in terms of the number of theoretical plates compares favourably with the results obtained with camphorsulphonic acid[5], Fig. 3. No dramatic change in selectivity was observed between these three acids.

After separation, the recovery of micro quantities of ultrapure antibiotic is easily accomplished by simple lyophilization of the eluate.

Most aminoglycosides among those tested are essentially unretained with an aqueous TFA mobile phase. However, retention is observed for some gentamicins and for fortimicins A and B. This retention is dependent upon the concentration of TFA, while the retention times of other aminoglycosides are completely unaffected (Table I). In addition, in contrast to PFPA and HFBA which provide only a slight separation of the gentamicins, use of aqueous TFA results in a baseline separation of the most common antibiotics of this family (Fig. 4). This separation compares favourably with the results described by Anhalt *et al.*[10] with pentanesulphonate as

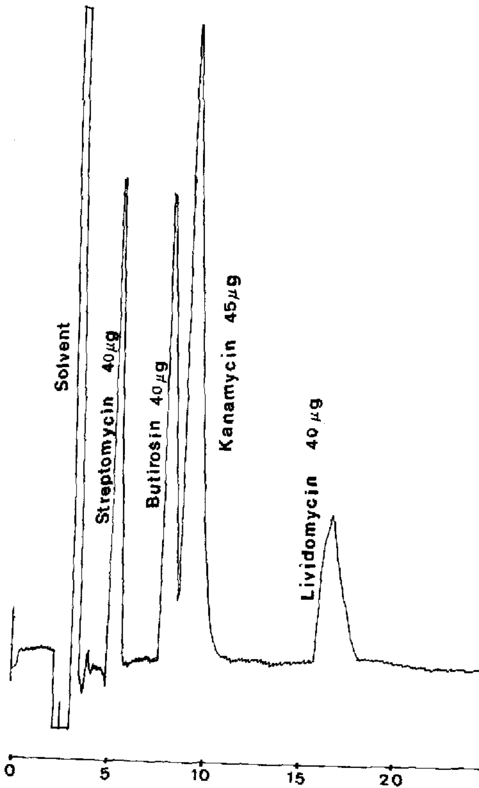

Fig. 3. Ion-pair separation of aminoglycosides. Mobile phase: methanol–0.05 *M* camphorsulphonic acid, pH 2.05 (52:48); flow-rate 1.0 ml/min. Sensitivity: × 2.

TABLE I

DEPENDENCE OF THE CAPACITY FACTOR, k', OF SOME AMINOGLYCOSIDE ANTIBIOTICS UPON THE TRIFLUOROACETIC ACID CONCENTRATION

Mobile phase: aqueous TFA. Flow-rate: 1.0 ml/min.

Compound	k'	
	TFA concentration (mol/l)	
	0.1	*0.2*
Streptomycin	0.4	0.4
Kanamycin	0.1	0.1
Lividomycin	0.6	0.6
Neomycin	0.8	0.8
Sisomicin	1.9	2.3
Gentamicin		
C_{1a}	2.1	3.2
C_2	4.2	6.5
C_1	8.9	14.8
Verdamicin	3.6	3.8
Sagamicin	7.1	10.8

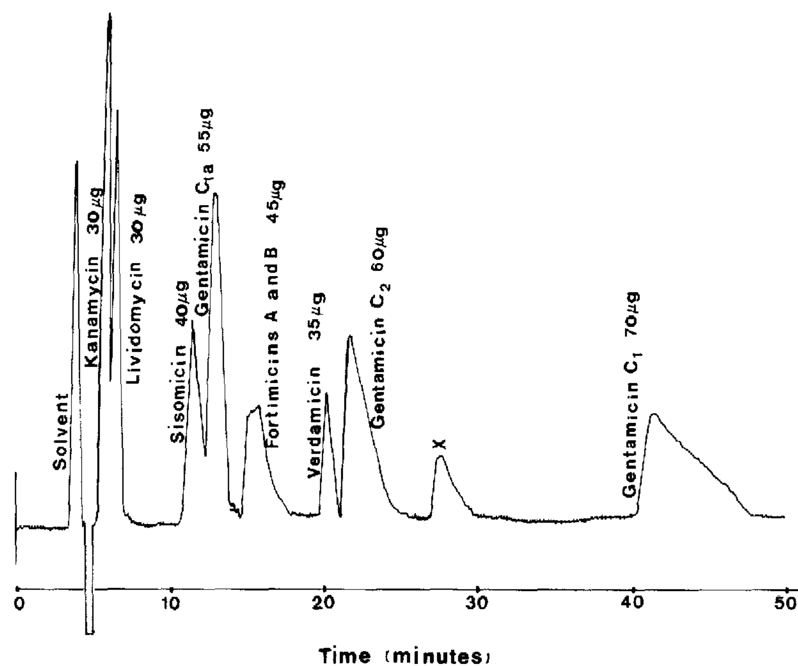

Fig. 4. Ion-pair separation of aminoglycosides. Mobile phase: 0.2 M trifluoroacetic acid, pH 1.4; flow-rate 1.0 ml/min. Sensitivity: × 4. X = Uncharacterized gentamicin.

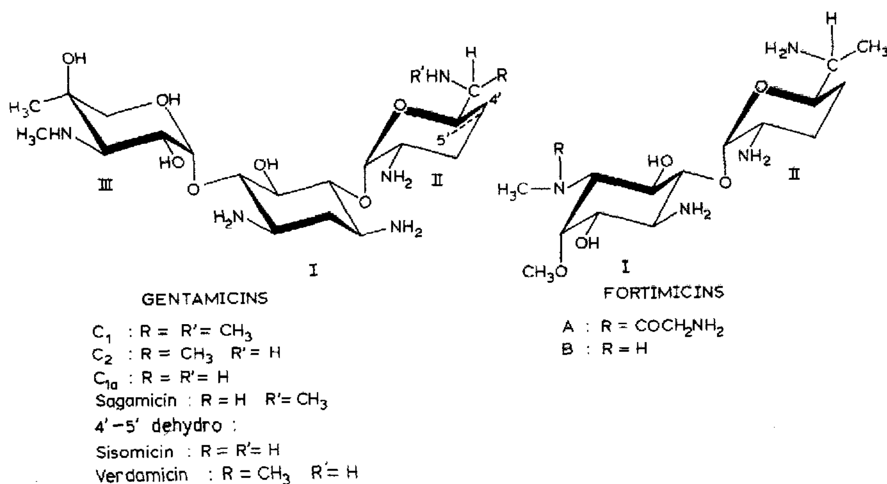

Fig. 5. Structure of gentamicins and fortimicins.

buffer. Fortimicins A and B, however, are eluted as a broad peak even though they are baseline separated with PFPA and HFBA.

It is interesting that the retained antibiotics are each characterized by the presence of a 2,3,4,6-tetradeoxy-2,6-diaminopyranoside (aminohexose II) (Fig. 5). Compared to the structural units usually present in the aminoglycosides, these sugars are relatively aliphatic, although basic and this may account for the specificity of the retention in an ion-pair mechanism, implying a very short chain counter ion.

TFA appears then to be uniquely suited to the chromatography of the gentamicin antibiotics tested in this work, providing specific retention and impressive separation. These properties could be profitably used to design a screening test aimed at the discovery and characterization of new gentamicins. They may also be applied to the clinically important determination of gentamicins in serum.

CONCLUSION

The results presented here show that short chain perfluorinated carboxylic acids can be used advantageously for the analysis and recovery of pure aminoglycoside antibiotics. These volatile buffers would be of special interest in the study of new antibiotics produced by soil bacteria. Further work is in progress to extend the range of application of these counter ions and to understand better the mechanism of the separation obtained, particularly with TFA.

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