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Differential Analysis of Amikacin and Butirosin

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Abstract ☐ In order to develop an analytical method for amikacin and butirosin in presence of their parent antibiotics, kanamycin A and ribostamycin, high-performance liquid chromatographic technique and microbioassay method were evaluated and compared. Using high performance liquid chromatography, two acylated antibiotics, amikacin and butirosin was partially separated from their parent antibiotics, to provide a qualitative analytical method. In microbioassay using *Pseudomonas aeruginosa* TI-13, a producer of aminoglycoside-3′-phosphotransferase I, only acylated antibiotics were selectively analyzed when paper disc-susceptibility assay was used. The standard curve showed a good correlation between the response and dose in semilogarithmic plot with correlation coefficients above 0.96, and analytical deviation from expected dose was within 10%.

Keywords ☐ Amikacin, Butirosin, Microbiassay method.

Butirosin complex produced by *Bacillus circulans* is a unique aminoglycoside antibiotic having (S)-4-amino-2-hydroxybutyric acid attached to ribostamycin or xylostatin at C-1-amino group. It gives an improved antibiotic activity against some aminoglycoside resistant organisms.[2,3] Based on the structures of butirosin, 1-N-[(S)-4-amino-2-hydroxybutyryl]-kanamycin A (amikacin) have been synthesized and confirmed to be active against kanamycin resistant organisms including *Pseudomonas* species.[4,6]

The quantitative assay for amikacin commonly used are radioimmunoassay[8] enzymatic method[7] and microbioassay.[1,10] However, these analytical techniques have not been able to provide any differential analysis between amikacin and kanamycin A.

Aminoglycoside-3′-phosphotransferase I [APH (3′)-I] can not phosphorylate C-1-N-acyl aminoglycoside antibiotics such as butirosin and amikacin but can phosphorylate their parent antibiotics such as ribostamycin and kanamycin A.[5,9] An attempt was made to analyze the former antibiotics in presence of the latter by microbioassay using *Pseudomonas aeruginosa*, an APH (3′)-I producer. High performance liquid chromatographic (HPLC) technique has also been employed for the differential analysis of these two antibiotics, and the results were compared with microbioassay method.

EXPERIMENTAL METHODS

Materials

The authentic sample of ribostamycin sulfate was kindly supplied from Meiji Seika Kaisha, Ltd., Yokohama, Japan, and the standard sample of butirosin sulfate was kindly provided by Warner-Lambert Company, Michigan, U.S.A. The standard samples of kanamycin A and amikacin (free bases) were also obtained from Schering Corporation, New Jersey, U.S.A.

Microbioassay

Pseudomonas aeruginosa TI-13 obtained from Institute of Microbial Chemistry, Tokyo, Japan, was used as a test organism. The paper-disc susceptibility assay was employed as a micro-

bioassay using this strain by spreading 0.2ml of heavy culture broth on an agar plate containing 1.3% of nutrient broth, 0.3% of sodium chloride, and 0.2% of bacto-peptone after cultivation in a sumberged culture broth having the same composition at 30°C for 18-24 hrs. Filter paper disc impregnated with a measured amount of antibiotics and then dried at the room temperature, was placed on the agar plate, and it was incubated at 36°C for 12-24 hrs. The diameters of clear inhibition zones were measured and the average inhibition zone was calculated by taking a logarithmic average.

High Performiance Liquid Chromatographic Assay

For separation of ribostamycin and butirosin, or kanamybin A and amikacin, high-performance liquid chromatography (HPLC) was employed (Waters Associated Inc., Massachussetts, U.S. A.). The column used was μBondpak/Carbohydrate, and the elution solvent was 0.1M phosphate buffer (pH 7.8) containing 10-20% of methanol. The eluted antibiotics were detected using differential refractometer.

RESULTS

Microboassay

Based on the fact that *P. aeruginosa* TI-13 capable of producing APH (3′)-I is sensitive to amikacin and butirosin while resistant to kanamycin A and ribostamycin, a new analytical microbioassay methods was developed for differential analysis of former antibiotics in presence of the latter As shown in Fig. 1, kanamycin A and ribostamycin did not give any inhibition zone to this strain, but a distinct clear inhibition zone was formed against their acylated antibiotics, amikacin and butirosin. Typical standard dose-response curves of these acylated antibiotics for *P. aeruginosa* in paper-disc susceptibility

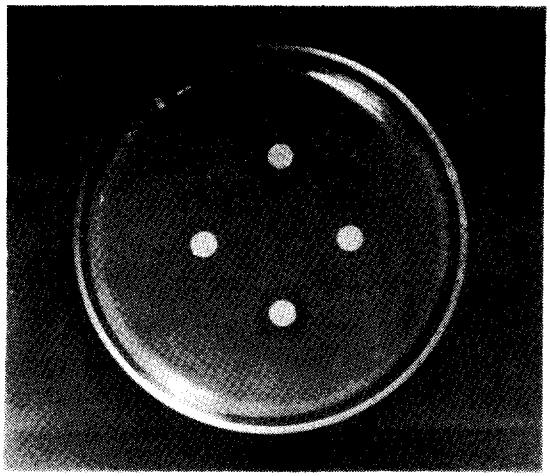

Fig. 1: Growth inhibition of *P. aeruginosa* TI-13 by four different aminoglycoside antibiotics; 10μg of kanamycin A(left), 10μg of amikacin (above), 25μg of ribostamycin sulfate(right), and 25μg of butirosin sulfate (below).

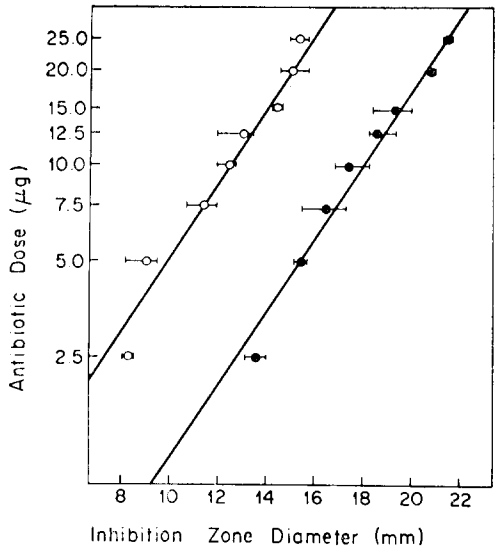

Fig. 2: Standard curve for amikacin (●) and butirosin sulfate (○) in paper disc susceptibility assay using *P. aeruginosa* TI-13.

assay are seen in Fig. 2. The correlation coefficients for amikacin and butirosin were 0.96 and 0.97, and these linear relationships showed regression coefficient 0.116. In the range of pH 5 to 10, no difference could be found by this

Table I: Estimate of assay for amikacin and butirosin in the presence of their parent antibiotics in microbioassay*.

Amikacin				Butirosin sulfate			
Per cent of standard	Per cent of measured amount	Deviation (%)	Coefficient of variation (%)	Per cent of standard	Per cent of measured amoujt	Deviation (%)	Coefficient of variation (%)
20	21.5±3.2	+7.5	14.5	20	18.9±3.5	−5.5	18.5
40	44.4±8.8	+11.0	19.8	40	37.5±4.0	−6.3	10.7
60	64.8±7.0	+8.0	10.8	60	56.5±5.6	−5.8	9.9
80	84.5±6.8	+5.6	8.1	80	76.2±6.7	−4.8	8.8

* Total antibiotic concentration was same in all experiments.

assay. An estimate of sample precision for this analysis was obtained from the results of four different concentration of amikacin and butirosin in the mixed solution of their parent antibiotics, kanamycin A and ribostamycin(Table I). The observed coefficient of variation was within 20% of a mean potency and the deviation of calculated mean potency was in the range of 10%.

High Performance Liquid Chromatographic Assay

Separation of kanamycin A, amikacin, ribostamycin, or butirosin by HPLC was carried out using μBondapak/Carbohydrate column, 0.1M phosphate buffer (pH 7.8) and methanol as an eluent. The residence time for kanamycin A, amikacin, ribostamycin sulfate, and butirosin sulfate were 5.7, 5.1, 5.1 and 5.7 min. when eluted with 0.1M phosphate buffer (pH 7.8)—methanol (9:1), and 6.9, 6.2, 6.3, and 7.0 min. when eluted with 0.1M phosphate buffer (pH 7.8)—methanol (8:2) (Figs. 3 and 4). However, this chromatographic assay method could provide only a qualitative analysis of the acylated antibiotics.

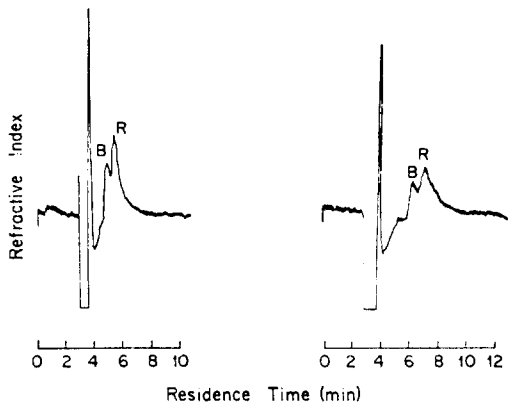

Fig. 3: Separation of ribostamycin sulfate (R) and butirosin sulfate (B) by HPLC when eluted with 0.1M phosphate buffer-methanol (9:1) (left) and 0.1M phosphate buffer-methanol (8:2) (right).

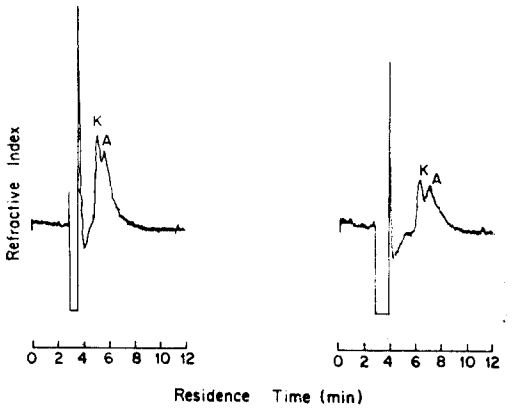

Fig. 4: Separation of kanamycin A (K) and amikacin (A) by HPLC when eluted with 0.1M phosphate buffer-methanol (9:1) (left) and 0.1M phosphate buffer-methanol(8:2) (right).

DISCUSSION

In the chemical or biological synthesis of

amikacin from kanamycin, or butirosin from ribostamycin, it is necessary to establish differential analytical method for these two groups of antibiotics. But no method has been developed or available for this purpose as yet.

These two antibiotics in each group did not show any difference in HPLC when phosphate buffer was not used as an eluent. Using the difference in pK values of C-1-amino function, phosphate buffer (pH 7.8) was employed and relatively good results were obtained. But by this technique full separation of two antibiotics with a high resolution could not be achieved, although a good qualitative analysis could be performed.

By utilizing the different resistance of *P. aeruginosa* TI-13 against the above antibiotics in each group, microbioassay was developed by agar disc susceptibility test. The growth of this strain on agar plate was completely inhibited by acylated antibiotics but not by their parent antibiotics. The diameter of clear inhibition zone showed a linear correlation with the logarithmic value of the amount of acylated antibiotics on disc. This technique also suffered from a large variation and deviation, nearly 20%, in spite of its convenience and ease. In order to increase the analytical precision, an enzymatic analysis could be developed using purified APH (3′)-I.

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