CHROM. 4349

The separation of neomycin sulphate, polymyxin B sulphate and zinc bacitracin

There are several pharmaceutical formulations which contain neomycin, polymyxin B and zinc bacitracin in various admixtures and dosage forms[1] and the separation of these into their components could facilitate their routine estimation.

The separation of antibiotics by paper chromatography has been reviewed in detail by BETINA[2]. Other chromatographic techniques have been widely used for the separation of antibiotics in general. FOPPIANI AND BROWN[3] used a thin-layer technique for the separation and potency determination of neomycin sulphate. GUVEN AND OZSARI[4] separated neomycin, polymyxin B and bacitracin using thin-layer chromatography on silica gel using a mixture of benzene, water and acetic acid. BRAMMER AND HEMSON[5] were unable to separate polymyxin B and neomycin, using electrophoresis on cellulose acetate with 0.07 *M* barbital buffer (pH 8.6). They used Ponceau S for the detection of the antibiotics. However, LIGHTBOWN AND DE RISSI[6] were able to separate bacitracin, polymyxin B and neomycin using agar gel electrophoresis with Tris–maleate buffer at pH 5.6. In this case the antibiotics were located using the production of inhibition zones after inoculation with the appropriate organism and subsequent incubation. The production of colour with ninhydrin has been used extensively for the detection of peptide antibiotics and for the quantitative estimation of neomycin by MAEHR AND SCHAFFNER[7] and THORBURN BURNS, LLOYD AND WATSON-WALKER[8].

Experimental

Antibiotics and reagents. Polymyxin B sulphate (690 μg/mg), neomycin sulphate (810.6 units/mg), and zinc bacitracin (60 units/mg) were dissolved in 1% sodium ethylenediamine tetracetate to give a final concentration per millilitre of: neomycin sulphate B.P., 483 units; zinc bacitracin B.P., 360 units and Polymyxin B sulphate, 146 units, approximating the commercial aerosol spray under investigation.

All reagents were of analytical reagent grade. Ninhydrin, 0.2 g dissolved in 100 ml *n*-butanol, was used as spray reagent. Ninhydrin stabilising reagent was according to Merck[9] and the ninhydrin quantitative reagent was prepared according to the method of JACOBS[10]. Of Ponceau S 200 mg were dissolved in 100 ml of 3% trichloroacetic acid and excess dye was removed with 5% acetic acid.

Paper chromatography. All separations were carried out on Whatman No. 3 paper, using the following solvents: S_1 = *n*-butanol–water–glacial acetic acid (30:13:8); S_2 = *n*-butanol–glacial acetic acid–pyridine–water–ethanol (60:15:6:5:5); S_3 = *n*-butanol–water–glacial acetic acid–pyridine–NaCl (30:12:7:2:0.1).

Thin-layer chromatography. For thin-layer chromatography 5 cm × 20 cm Kieselgel G (Merck) plates were used. The following solvents were used: S_4 = *n*-butanol–glacial acetic acid–water–pyridine (30:22:38:6) and S_5 = *n*-butanol–water–pyridine–glacial acetic acid–ethanol (60:10:6:15:5).

Electrophoresis. For electrophoresis a Shandon (Kohn Mark II) apparatus was used. Cellulose acetate (Oxoid) strips of 2.5 × 20 cm or Whatman No. 1 paper, strips of 5 × 20 cm were used. For paper the solvent S_6 = glacial acetic acid–formic acid–

water (60:30:910) and for cellulose acetate the solvent S_7 = pyridine–glacial acetic acid–water (75:2.5:922.5), pH 6.6, was taken.

Procedure

Paper chromatography was carried out by the ascending technique in the normal way. The antibiotic solutions were applied in 5-μl aliquots, using a micro syringe. The resulting chromatograms were developed using the ninhydrin spray reagent followed by heating at 105° for 5 min. The developed chromatograms were stabilised using the stabilising spray reagent.

Thin layers of Kieselgel G, 250 μ thick, were prepared in the normal way, activated by heating at 110° for 45 min, and stored over silica gel. Antibiotic samples were applied in 1-μl aliquots. The chromatograms were developed and visualised as above.

Electrophoretic separations were carried out on either paper strips, using solvent S_6, at a constant voltage of 700 V for 40 min, or on cellulose acetate strips using 400 V, constant voltage, for 60 min. In each case the temperature of the electrophoretic solutions were 12°. The samples were applied in 3.5-μl aliquots as a band across the strip. The antibiotic bands were visualised using either ninhydrin, which stained all three antibiotics, or by staining with Ponceau S reagent for 10 min and removing excess stain by soaking for 15 min in 5% acetic acid. In the latter case only polymyxin B and neomycin sulphate retained the dye, giving red and orange bands, respectively.

Quantitative estimations of the antibiotics present in the electrophoresis strips were made by eluting the Ponceau S stained material with 2 ml of 0.1 *N* NaOH and estimating the colour produced at 510 nm. The results were in agreement with those obtained by BRAMMER AND HEMSON[5]. The system was found to be suitable for quantitative estimation of polymyxin B.

Alternatively two strips were run in parallel, the presence of the antibiotics located on one and the corresponding section removed from the other and the antibiotic eluted from this with 2 ml of pH 5.0 acetate buffer. To this solution 1 ml of quantitative ninhydrin reagent was added and the colour was developed by heating at 98° for 10 min. The colour produced was estimated at 570 nm and by comparison with calibration curves of the pure antibiotics the amount in each sample could be determined for all three antibiotics in the same manner as has been described for neomycin[7,8].

The calibration curves obtained for the ninhydrin method were linear over the range of 10 to 800 μg. Beyond these limits there was considerable departure from linearity.

Results

The results obtained are represented by Tables I–III.

Discussion

The presence of the antibiotics polymyxin B, neomycin and zinc bacitracin may be detected using either paper or thin-layer chromatography. In the case of paper chromatography the addition of small amounts of sodium chloride to the solvent system was found to reduce the tailing and to give discrete spots.

The electrophoretic separation was rapid and allowed for quantitative elution

TABLE I

R_F VALUES FOR THE PAPER CHROMATOGRAPHIC SEPARATION OF THE ANTIBIOTICS

Solvent system	*Neomycin*	*Polymyxin B*	*Bacitracin*
S_1	0.02	0.16	0.30
S_2	0.04	0.70	0.97
S_3	0.05	0.56	0.75

TABLE II

R_F VALUES FOR THE THIN-LAYER CHROMATOGRAPHIC SEPARATION OF THE ANTIBIOTICS

Solvent system	*Neomycin*	*Polymyxin B*	*Bacitracin*
S_4	0.14	0.50	0.61
S_5	0.05	0.34	0.66

TABLE III

DISTANCE TRAVELLED (CM) FROM POINT OF APPLICATION TOWARDS THE CATHODE BY THE ANTIBIOTICS ON ELECTROPHORESIS

Medium	*Neomycin*	*Polymyxin B*	*Bacitracin*
Cellulose acetate	11.2	8.5	5.5
Paper	17.7	14.0	11.0

TABLE IV

CORRELATION BETWEEN THE AMOUNT OF ANTIBIOTIC PRESENT ON AN ELECTROPHORETIC STRIP AND THE AMOUNT ELUTED ESTIMATED BY THE NINHYDRIN METHOD

Amount present (μg)	*Amount detected after elution (μg)*		
	Neomycin	*Bacitracin*	*Polymyxin B*
10	9	8	10
20	18	17	19
30	29	29	30
40	35	35	38
50	48	50	51
100	98	101	100
200	205	200	202
400	400	404	395
500	505	501	498

of the antibiotics for estimation (Table IV). The use of Ponceau S for quantitative work is limited by the narrow range of compounds giving a stained product. The ninhydrin reaction is more versatile. Whilst it has been widely studied for the estimation of neomycin[7,8], it can be used to estimate other peptide antibiotics. The elution of the antibiotics was carried out in pH 5 acetate buffer in preference to the normal 0.1 *N* NaOH[5] because it was found that zinc bacitracin decomposed rapidly in alkaline solution.

The electrophoretic separation allows a rapid and quantitative estimation for routine quality control of the antibiotics present in the aerosol preparation investigated in comparison to the normal microbiological methods of assay.

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Dünnschichtchromatographie von 4-Acetyl-2-nitrophenyl-Derivaten phenolischer Verbindungen

In Analogie zum 2,4-Dinitrofluorbenzol ist 4-Fluor-3-nitroacetophenon zur Abscheidung phenolischer Verbindungen geeignet[1,2], wobei die Reaktionsfähigkeit durch deren Nucleophilie und sterischen Bau bestimmt wird. Enthält die phenolische Komponente Aminogruppen, so treten auch diese in Reaktion. Im Falle des *o*-Aminophenols erfolgt lediglich Substitution am Stickstoff. Die entstehenden 4-Acetyl-2-nitrophenyl-Derivate (ANP-Derivate) liefern bei der UV-spektroskopischen Charakterisierung im Gegensatz zu den entsprechenden 2,4-Dinitrophenyläthern wertvolle Informationen zur Struktur der ursprünglichen Phenole[3]. Aus diesem Grund ist ihr dünnschichtchromatographisches Verhalten von Interesse. Von einer Vielzahl an Sorbentien erwies sich Kieselgel am geeignetsten, das als manuell gefertigte Dünnschicht sowie