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## Note

# Use of the image analyser Optomax for the quantitative evaluation of antibiotics separated by gel electrophoresis and by thin-layer chromatography

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Many antibiotics in current use are complex mixtures of biologically active components and possibly some degradation products. The composition of the same antibiotic produced by different manufacturers can vary greatly, *e.g.* neomycin[1]. A knowledge of an antibiotic's composition is essential when considering the replacement of a microbiological assay by a chemical or physical assay. Both gel electrophoresis and thin-layer chromatography (TLC) have proved invaluable in demonstrating the heterogeneous nature of many antibiotics. Quantitative *in situ* bioautographic determinations have been described for biologically active components separated by gel electrophoresis[2] and by TLC[3] and several quantitative densitometric determinations of antibiotic complexes have been reported[4–6].

The Optomax image analyser has been used in the Division of Antibiotics for measuring the area of zones of inhibition of growth in antibiotic diffusion assays. This report describes the use of the Optomax for the determination of the amount of benzylpenicillin in carbenicillin by an *in situ* microbiological assay after the electrophoretic separation of the antibiotics in an agar gel. The content of kanamycin B in kanamycin was estimated using the Optomax to measure the areas of zones produced by the ninhydrin reaction after separation by TLC. The relative composition of samples of polymyxin B have been determined using biological assay and colorimetric assay after separation of the components by TLC.

## EXPERIMENTAL

The Optomax (Micro Measurements, Saffron Walden, Great Britain) is a modular image analysis system which utilises television scanning techniques to make measurements on any image which can be received by a television camera. The basic system measures areas on features in the image which can be differentiated from the background by sufficient "grey level" difference (*i.e.* contrast).

The television scanner had a Vivitar 135-mm lens fitted with a gelatin filter (Spectrum Red, 608, Ilford, Great Britain) to improve the image contrast. The objects were illuminated from below by four small fluorescent tubes (Gro-lux, Sylvania, Great Britain), dark ground illumination was used for the measurement of zones of inhibition of growth. For the measurement of the ninhydrin zones, direct transmitted light was used; a clear precoated silica gel plate was placed in the base below the light source to ensure a uniformly illuminated background.

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Benzylpenicillin sodium B.C.R.S.* and a sample of carbenicillin sodium were used for the determination of the benzylpenicillin in carbenicillin. Details of the method used are described in the British Pharmacopoeia[7]. The zones of inhibition of growth were measured using the Optomax. The conventional statistical method of parallel line assay for two + two dose assay was used for the analysis of the results. The area of the zone of inhibition of growth was taken as the response metameter and was analysed in relation to the logarithm of the dose.

Kanamycin B was separated from kanamycin by TLC[8] using precoated silica gel G plates, layer thickness 0.25 mm (E. Merck, Darmstadt, G.F.R.; Art. 5721). The plates were heat activated at 100°C for 60 min; solutions of 1 $\mu$l were applied to the plates, which were then developed in an equilibrated chromatography tank containing an aqueous solution of 10% w/v $KH_2PO_4$. The plates were chromatographed over a 15-cm path, removed, air-dried and dipped into ninhydrin reagent (300 mg ninhydrin in 5 ml 2,4,6-trimethylpyridine and 95 ml ethanol), air-dried, then heated at 100°C for 10 min. The dark blue zones were measured with the Optomax using direct transmitted light without dark ground illumination. To estimate the content of kanamycin B in a sample of kanamycin, three duplicate doses of the standard (the International Reference Preparation of Kanamycin B) 1.5, 3.0, 6.0 $\mu$g, were placed on the chromatographic plate, together with duplicate doses of 100 $\mu$g of the samples. The amount of kanamycin B in the samples was calculated from the standard curve.

The TLC separation of polymyxin B was as previously described[9]. For the colorimetric assay the developed plates were treated with ninhydrin reagent and measured as for kanamycin B. Biological activity was estimated using *Bordetella bronchiseptica* (NCTC 8344). After incubation the seeded agar was floated off the chromatographic plate and transferred to a clean glass plate; excess water was drained off and the areas of inhibition of growth were measured with the Optomax. The results of both assays were expressed as percentages of the two main components: polymyxin $B_1$ and $B_2$ in the total $B_1 + B_2$.

Because the purified individual components were unavailable, no calibration curves could be obtained for response metameter against known amounts of each component. Regression coefficients ($b$) for polymyxin $B_1$ and $B_2$ were obtained by measuring the response metameters of the two components after separation for several concentrations of a sample of polymyxin B. The regression coefficients of the two components were almost identical and the means were used for each assay ($b$ = 12853 biological assay and $b$ = 3018 ninhydrin response).

The relative concentrations of the two main components were calculated from the following:

$$\log \frac{\text{concn. } B_1}{\text{concn. } B_2} = \frac{\text{area } B_1 - \text{area } B_2}{b} = \frac{4341 - 2503}{3018} = 0.609$$

$$\frac{\text{concn. } B_1}{\text{concn. } B_2} = 4.064$$

Relative concentrations; polymyxin $B_1$ = 80.26 and polymyxin $B_2$ = 19.74.

* B.C.R.S. = British Chemical Reference Substance.

## RESULTS AND DISCUSSION

The reproducibility of the Optomax for measuring the zones of inhibition of growth produced by benzylpenicillin and the coloured zones resulting from the reaction of kanamycin B with ninhydrin is demonstrated in Table I. The variability associated with the application of the solutions is shown in Table II. Where the area measured was small, the variability was greatest, as shown with kanamycin B. In both assay methods the coefficients of variation were greater for the repeated application of the solutions than for the repeated measurement of a single zone. Variation in the application of the solutions was a greater source of error than instrumental parameters in the quantification of biological response of colorimetric response.

TABLE I

REPRODUCIBILITY AS A FUNCTION OF MEASUREMENT

Zones of inhibition of growth produced by benzylpenicillin in an agar-gel after electrophoresis and zones produced by kanamycin B after TLC and visualisation with ninhydrin reagent, each single zone measured ten times.

| *Substance* | *Amount (μg)* | *Mean area (arbitrary units)* | *Coefficient of variation (%)* |
|---|---|---|---|
| Benzylpenicillin | 0.025 | 22730 | 0.15 |
| | 0.05 | 27798 | 0.16 |
| | 0.1 | 34117 | 0.11 |
| Kanamycin B | 1.0 | 1051.6 | 2.05 |
| | 2.0 | 1720.3 | 1.58 |
| | 4.0 | 2274.7 | 0.84 |
| | 8.0 | 3554.5 | 0.58 |

TABLE II

REPRODUCIBILITY AS A FUNCTION OF APPLICATION SOLUTIONS

Zones of inhibition of growth produced by benzylpenicillin, 5 μl volumes, in an agar-gel after electrophoresis and zones produced by kanamycin B, 1 μl volumes, after TLC and visualisation with ninhydrin reagent, ten replicate zones each measured once.

| *Substance* | *Amount (μg)* | *Mean area (arbitrary units)* | *Coefficient of variation (%)* |
|---|---|---|---|
| Benzylpenicillin | 0.0125 | 6009 | 2.10 |
| | 0.025 | 10415 | 0.99 |
| | 0.05 | 14924 | 1.43 |
| | 0.1 | 20067 | 1.12 |
| Kanamycin B | 1.0 | 1042.1 | 7.00 |
| | 2.0 | 1964.8 | 5.24 |
| | 4.0 | 2546.2 | 4.53 |
| | 8.0 | 4181.1 | 6.24 |

There was a linear relationship between the area of the zone of inhibition of growth and the logarithm of the dose of benzylpenicillin over the range 0.0125–0.1 μg. Similarly, a direct relationship was shown for the colorimetric response and the logarithm of the dose of kanamycin B over the range 0.5–8.0 μg. Correlation co-

efficients from separate experiments ranged from 0.9919 to 0.9999 for benzylpenicillin and 0.9919 to 1.000 for kanamycin B, confirming that the logarithm of the dose–response relationships were linear. The actual response recorded for the same dose varied from experiment to experiment, as indicated in Tables I and II. Therefore, the estimation of unknown quantities must be made by comparison with the known concentration of a standard or reference substance included in the same experiment. This is the basis of most biological assays and therefore the experimental design included standards for the biological assay of benzylpenicillin in carbenicillin. For the estimation of kanamycin B in kanamycin, reference was made to the calibration graph obtained with known concentrations of the reference substance included in each experiment.

The benzylpenicillin content of a sample of carbenicillin was estimated on three separate occasions. The precision of each estimate was within $\pm$ 5% and there was good agreement between the three independent estimates (Table III). The weighted mean estimate was 36.67 mg of benzylpenicillin sodium per 1000 mg of carbenicillin. Even after an electrophoretic separation the Optomax can be used with confidence to measure the zones of inhibition of growth produced by benzylpenicillin.

TABLE III

ESTIMATE OF THE BENZYLPENICILLIN (mg/1000 mg) CONTENT IN A SAMPLE OF CARBENICILLIN DETERMINED BY BIOASSAY AFTER ELECTROPHORETIC SEPARATION

| *Experiment* | *Benzylpenicillin (mg/1000 mg)* | *Confidence limits (%) (P = 0.95)* |
|---|---|---|
| 1 | 35.23 | $\pm$ 3.9 |
| 2 | 38.51 | $\pm$ 3.6 |
| 3 | 36.36 | $\pm$ 4.2 |

To determine the accuracy of estimating the amount of kanamycin B in kanamycin, known amounts of kanamycin B were added to a sample of kanamycin containing no detectable kanamycin B; the estimates and the percentage recovery are shown in Table IV. Quantification of kanamycin B with the Optomax was found to be simple and accurate, after chromatographic separation.

Estimates of kanamycin B in samples of kanamycin, obtained using the current chromatographic system and measuring the areas with the Optomax, were compared with previous results obtained by TLC and visually matching the zones against known standards. The results are shown in Table V. The agreement between the two estimates of kanamycin B after TLC was very good.

The relative proportions of polymyxin $B_1$ and $B_2$ were determined in samples of polymyxin after TLC separation on the basis of antimicrobial activity and ninhydrin reactivity, (Table VI). If, on the basis of the known composition of the two polymyxin components, it is assumed that they have identical ninhydrin reactivity, then the differences in the results obtained biologically indicate that polymyxin $B_2$ is about 3.5 times as active against the test organism as polymyxin $B_1$. The correlation between the biological and the chemical assays for the estimates of polymyxin $B_1$ and $B_2$ was very good—correlation coefficients $r = 0.93$ were obtained for both components when

TABLE IV

ESTIMATION OF KANAMYCIN B IN SAMPLES OF KANAMYCIN A TO WHICH KNOWN AMOUNTS OF KANAMYCIN B HAD BEEN ADDED

The results are the mean of duplicate determinations calculated from a three point logarithm dose–response graph (μg kanamycin B per 100 μg kanamycin A).

| *Kanamycin B* | | *Recovery (%)* |
|---|---|---|
| *Amount added (μg)* | *Amount estimated (μg)* | |
| 1.0 | 1.018 | 101.84 |
| 1.5 | 1.477 | 98.50 |
| 2.0 | 2.007 | 100.35 |
| 2.5 | 2.544 | 101.74 |
| 3.0 | 3.039 | 101.32 |
| 4.0 | 4.204 | 105.09 |
| 5.0 | 4.963 | 99.26 |
| 6.0 | 5.926 | 98.76 |

TABLE V

ESTIMATION OF KANAMYCIN B (AS A PERCENTAGE) IN SAMPLES OF KANAMYCIN

Determinations were made after TLC using the Optomax and by visual matching.

| *Sample* | *Kanamycin B (%)* | |
|---|---|---|
| | *Optomax* | *Matching* |
| 1 | 2.3 | 3 |
| 2 | 4.2 | 5 |
| 3 | 0.0 | None detected |
| 4 | 2.4 | 3 |
| 5 | 3.2 | 4 |

TABLE VI

THE RELATION PROPORTIONS OF POLYMYXINS $B_1$ AND $B_2$ IN SAMPLES OF POLYMYXIN B

The proportions were determined by biological and colorimetric assay after TLC.

| *Sample* | *Bioassay* | | *Colorimetric assay* | |
|---|---|---|---|---|
| | $B_1$ | $B_2$ | $B_1$ | $B_2$ |
| A | 53.39 | 46.59 | 80.26 | 19.74 |
| B | 54.59 | 45.41 | 84.24 | 15.76 |
| C | 45.78 | 54.21 | 63.80 | 36.20 |
| D | 51.51 | 48.49 | 82.92 | 17.08 |
| E | 54.76 | 45.24 | 84.95 | 15.05 |
| F | 55.45 | 44.55 | 83.00 | 17.00 |
| G | 50.35 | 49.65 | 72.05 | 27.95 |

comparing the two methods. The incomplete separation of polymyxin and the lack of sensitivity for the minor components meant that only the two major components could be quantified. Nevertheless, a method which enables the antimicrobial activities of constituents of complex antibiotic mixtures to be compared is extremely useful.

The Optomax has already proved to be satisfactory for the measurement of areas of inhibition of growth obtained in a microbiological assay. It followed that it could be used for the measurement of areas of inhibition of growth produced by antibiotics after separation by either gel electrophoresis or TLC. The ability of the Optomax to measure areas of any shape make it particularly useful, as separation techniques often produce distorted zones.

The Optomax purchased by The National Institute for Biological Standards and Control was intended to be used for measuring areas of inhibition of growth. Thus, the results of the estimation of kanamycin B were very encouraging; the use of the Optomax to measure areas produced by reaction with ninhydrin on chromatographic plates is a valuable bonus. The examination of polymyxin B demonstrates its application to quantification of the chemical composition and antimicrobial activity of a heterogeneous antibiotic complex, a facility of value in laboratories engaged in the correlation of biological activity and chemical composition.

REFERENCES

1 J. W. Lightbown, P. de Rossi and P. Isaacson, *J. Biol. Standardization*, 7 (1979) 221.
2 J. W. Lightbown and P. de Rossi, *Analyst (London)*, 90 (1965) 89.
3 T. F. Brodasky, C. Lewis and T. E. Eble, *J. Chromatogr.*, 123 (1976) 33.
4 H. Fricke, P. Muhlig and Ch. Kühne, *J. Chromatogr.*, 153 (1978) 495.
5 J. F. Martin and L. E. McDaniel, *J. Chromatogr.*, 104 (1975) 151.
6 W. L. Wilson, G. Richards and D. W. Hughes, *J. Pharm. Sci.*, 62 (1973) 282.
7 *British Pharmacopoeia*, HMSO, London, 1973, p. A97.
8 M. Dubost, C. Pascal, B. Terlain and J. P. Thomas, *J. Chromatogr.*, 86 (1973) 274.
9 A. H. Thomas and I. Holloway, *J. Chromatogr.*, 161 (1978) 417.