ISOELECTRIC FOCUSING AND ELECTROPHORETIC TITRATION OF ANTIBIOTICS USING BIOAUTOGRAPHIC DETECTION

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Isoelectric focusing (IEF) has found wide use in protein biochemistry for measuring isoelectric points and for monitoring purification procedures[1]. Recently, the use of electrophoresis on a preformed IEF gel at right angles to the original field has been advocated as a procedure to obtain electrophoretic titration curves useful for predicting the best pH and matrix for ion exchange chromatography of proteins[2~5]. IEF techniques have been limited in use to high molecular weight materials which are detected by protein staining after fixing in acid and removal of ampholines. In the antibiotics area, the application of IEF procedures would have great potential as a diagnostic tool for determining isoelectric points and for determining the presence of charged moieties on unknown antibiotics using minimal (microgram) quantities of crude material. Such information would also be valuable for identifying the presence of known substances in an antibiotic screen. However, IEF has not been applied extensively to antibiotics because their smaller size makes detection by traditional staining techniques difficult.

Traditionally, isoelectric points are obtained for antibiotics by carrying out paper electrophoresis at a variety of pH's with bioautographic detection and extrapolating to the pH of zero mobility. Titration curves have been obtained by direct titration, a procedure requiring a minimum of 20~50 mg of material. Recently, RIGHETTI *et al.*[6] described a procedure for electrophoretic titration curve analysis of doxorubicin in polyacrylamide gels using direct visualization of the orange-red compound. In this communication, we demonstrate that IEF and electrophoretic titration analysis can be applied to other antibiotics using bioautography as the detection method. In our hands, the combination of bioautography and IEF techniques has greatly extended the usefulness of electrophoretic procedures as sensitive tools in the screening for new antibiotics and their subsequent structure elucidation.

Materials and Methods

Precast polyacrylamide gels (PAGplates, LKB) pH 3.5~9.5, were used for isoelectric focusing in conjunction with the Multiphor (LKB) isoelectric focusing unit. Doxorubicin, gentamicin, rifampicin, ristocetin and vancomycin were obtained from Sigma. Teichomycin A_2 was obtained from Gruppo Lepetit, Milan, Italy. Antibiotic A 477 was obtained from Eli Lilly, Indianapolis. Aqueous stock solutions of 0.1 mg/ml (rifampicin, A 477), 1.0 mg/ml (vancomycin, doxorubicin) and 10 mg/ml (teichomycin A_2) were used in all experiments. Appropriate concentrations to produce adequate zone sizes (with a 5 μl sample) were previously determined by direct spotting on a bioautography plate. Bioautography plates were prepared from Penn Seed agar, antibiotic Medium A (BBL, A. H. Thomas) containing 3 ml of 2% triphenyl tetrazolium chloride/liter, inoculated with 5.5×10^8 cfu/ml of *Bacillus subtilis* spores ATCC 6633, poured into Nunc plates ($245\times245\times20$, A. H. Thomas).

Isoelectric focusing was carried out according to the manufacturer's instructions using an anode solution of 1 M H_3PO_4 and a cathode solution of 1 M NaOH. The power supply was set at 30 W, 1,500 V, 50 mA, and focusing carried out for 1.5~2 hours with cooling at 10°C. Focusing was monitored visually by observing the migration of two 5 μl droplets of doxorubicin spotted at different positions in one lane of the PAG plate. Focusing was complete when the two spots overlapped. Following focusing, the pH gradient was measured using a surface electrode or by visualizing acetylated cytochrome C pI markers (United States Biochemical).

For titration curves, the pH gradient was preformed by focusing as described above without any sample on the PAGplate. After focusing, 100 μl of the sample to be analyzed was steaked across the pH gradient of the gel using a glass rod. Electrophoresis was then carried out perpendicular to the direction of focusing by switching from the analytical electrofocusing lid to the preparative lid of the Multiphor. The power supply was set at 1,000 V with power and current

settings on maximum. Electrophoresis was stopped after 60 minutes.

After isoelectic focusing or titration curve analysis, a strip of Whatman I filter paper, which had been presoaked in 1 M sodium phosphate (pH 7) buffer and air dried, was laid directly on the surface of the gel. The gel and paper were removed together from the Multiphor and placed paper-side down onto a bioautography plate for 30 minutes. The paper and gel were removed and the bioautography plate was developed overnight at 37°C. The isoelectric point was determined by the location of a clear zone of inhibition of bacterial growth.

Results and Discussion

Fig. 1 shows the results of isoelectric focusing followed by bioautography of rifampicin, doxorubicin, and the glycopeptides vancomycin[7,8], A 477[9] and teichomycin A_2[10]. The final point of focusing of each antibiotic was independent of the initial position of the spotting droplet. The antibiotic could also be applied as a streak parallel to the direction of focusing with no effect on the final position of the focused spot (data not shown). The migration of rifampicin and doxorubicin could be observed visually during focusing due to the color of these antibiotics. The homogeneous overlap of two doxorubicin spots applied initially at different positions in the same lane of the gel was an accurate gauge of the completion of focusing. Rifampicin was revealed to separate into two overlapping species. Table 1 shows the apparent pI's obtained for seven such antibiotics by isoelectric focusing and bioautography, and their corresponding literature values. For those cases where an isoelectric point is not available, an estimated pI value was calculated as the midway point between the two relevant *pKa* values. For most of the compounds studied, there is reasonable agreement with literature values. The value for teichomycin A_2 deviates from the reported value by two pH units. This difference may be caused by limited solubility of the antibiotic at acid pH as evidenced by the titration curve data (see below). However, when samples of teichomycin A_2 were spotted on the gel at the extreme ends of the plate, a clear symmetrical zone could be obtained, implying that normal focusing was occurring. The value of 6.5 was determined by extrapolation of mobility on electrophoresis and may be in error.

Fig. 1. Isoelectric focusing patterns, pH 3 to 9.5.
A; Doxorubicin, B; rifampicin, C; vancomycin, D; A 477, E; teichomycin A_2.
Detection: Bioautography on *B. subtilis*.

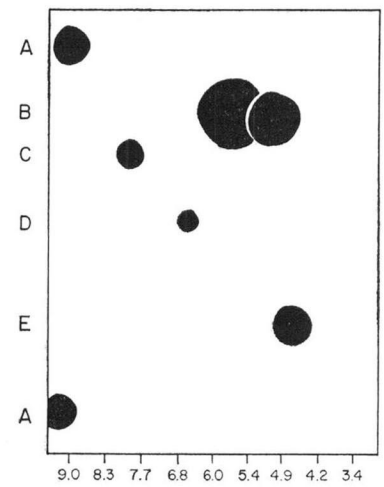


Table 1. Isoelectric points of various antibiotics.

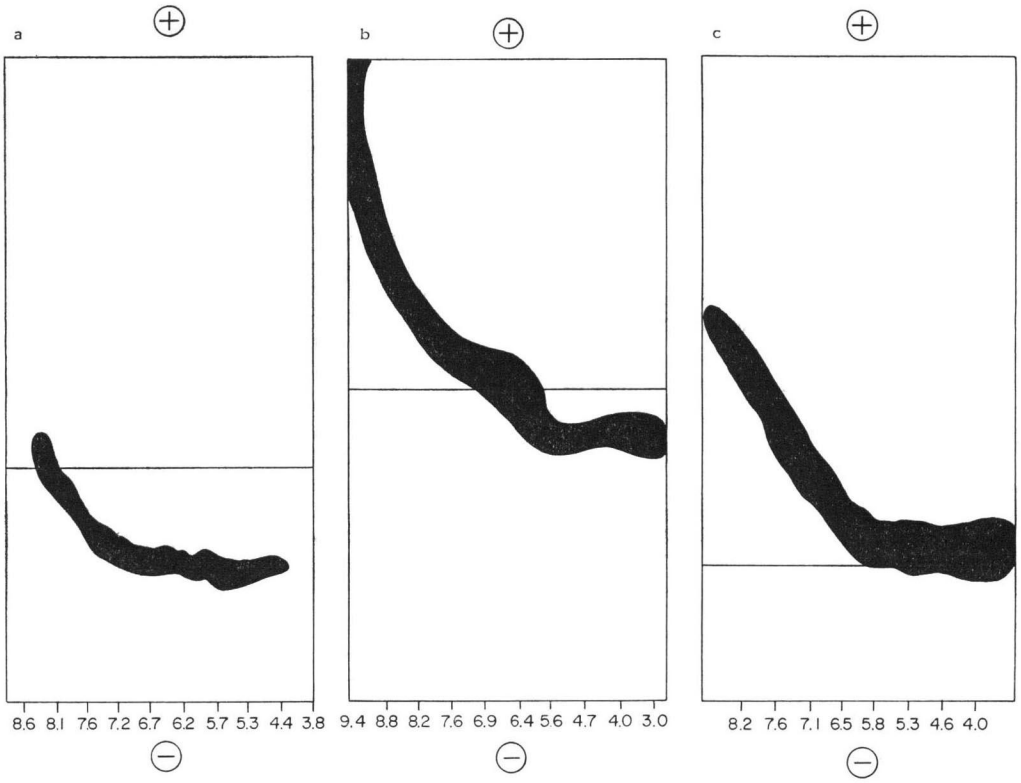
Antibiotic	pI	Literature value*
A 477	6.6	7.1 (estimate)[9]
Doxorubicin	9.2	8.7~9.3[8]
Gentamicin	9.5	
Rifampicin	4.7 (5.6 minor)	4.8 (estimate)[11]
Ristocetin	7.9	7.8 (estimate)[12]
Teichomycin A_2	4.5	6.5[10]
Vancomycin	8.1	7.7 (estimate)[13]

* Estimated pI values were obtained by calculating the midpoint between the relevant literature *pKa* values.

The IEF procedure is not limited to substances normally considered "amphoteric". For example, the basic aminoglycoside gentamicin is actually amphoteric above pH 10 presumably due to ionization of a hydroxyl group to produce an alkoxide anion. If it were not amphoteric, it would migrate off the gel into the electrode solution. However in our hands, gentamicin is focused on the gel in front of the high pH electrode.

Figs. 2a, 2b and 2c show electrophoretic titration curves obtained for vancomycin, A 477 and teichomycin A_2, respectively. These patterns, observed as a clear band of bacterial growth inhibition, were highly reproducible and had a characteristic shape for each antibiotic tested.

Fig. 2. Electrophoretic titration curves.
Detection: Bioautography on *B. subtilis*.
a; Vancomycin, b; A 477, c; teichomycin A_2.



The point where the curve crosses the application line for vancomycin and A 477 were within a half pH unit of the isoelectric points determined in Fig. 1. This is to be expected since at their pI's, they should have no net charge and therefore no net mobility in an electric field. Vancomycin is reported to have *pKa*'s of 2.9 (carboxyl), 7.2 (amine), 8.6, 9.6, 10.5 and 11.7 (phenols and another amine[11]). The observed electrophoretic titration curve is consistent with these *pKa* values, with a clear plateau of mobility occurring from pH 7.2 to 5.3 where the predicted charge is +1. Above its isoelectric point, vancomycin should have a negative charge, and consistent with this, some movement towards the cathode is observed. The other two glycopeptide antibiotics, A 477 (Fig. 2b) and teichomycin A_2 (Fig. 2c), show profiles clearly different from that of vancomycin, being anionic above pH 6 as suggesting by their literature data[9,10]. These figures clearly highlight the charge characteristics of these three antibiotics.

The discontinuity in the titration curves at pH 5.9~6.1 was observed in all cases and probably results from a discontinuity in ampholine population. Teichomycin A_2 showed no mobility below pH 6. Similar results were obtained with rifampicin, which smeared below pH 6. Some of this effect is seen with A 477, and to a lesser extent with vancomycin. Doxorubicin gave similar results in our hands and in the cited reference[6]. The lack of mobility at these pH's probably resulted from limited solubility coupled with a hydrophobic interaction with the lipophilic polyacrylamide backbone. Because of the possibility of hydrophobic interactions and the lack of a correction factor for electro-osmosis, these figures should be considered as semi-qualitative only. Since bioautographic detection yields a larger spot than traditional protein staining techniques, the values for isoelectric points for antibiotics determined by this procedure are by necessity less precise, probably ± 0.3 pH units. The use of vancomycin, teichomycin A_2 and doxorubicin as internal standards facilitates the interpretation of

incubated bioautogram plates. For further precision we have found the use of narrow range gels to be helpful.

The use of filter paper pretreated with 1 M sodium phosphate (pH 7) buffer for bioautography was found to minimize transfer of the electrode solutions, thus eliminating the large background inhibition zones otherwise present at either edge of the pH gradient. Since water solubility and therefore transfer into the aqueous agar is minimal for an antibiotic at its isoelectric point, the buffered paper is necessary for enhancing transfer of acidic or basic antibiotics. This enables lower loading levels to be used and minimizes streaking.

We have found the above procedures to be useful for determining isoelectric points and charge characteristics of antibiotic preparations.

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