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THE CHROMATOGRAPHY OF ANTIBIOTICS ON SP-SEPHADEX

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SUMMARY

A rapid procedure is described that permits the quantitative separation and determination of ampicillin, dicloxacillin, methacycline, doxycycline and oxytetracycline by means of column chromatography on SP-Sephadex. The compounds are eluted from a column with sodium acetate solutions of concentration 0.005 and 0.01 *M* (pH 4.5) and assayed spectrophotometrically and microbiologically. The chromatographic method has been used to assess the purity of commercial ampicillin and oxytetracycline.

INTRODUCTION

The therapeutic importance of tetracycline and penicillin derivatives, and the fact that they need to be of extremely high purity, has led to the development of a number of analytical techniques for the separation of complex mixtures and for the identification and determination of the individual antibiotics so obtained.

The determination of penicillin derivatives is usually carried out after their conversion into the corresponding penicilloic[1] or penicillanic acids[2,3] (determined spectrophotometrically), the β-lactam ring being broken down respectively by either treatment with alkali or acid in the presence of copper or mercury ions. Tetracycline derivatives are determined spectrophotometrically[4,5], potentiometrically[6–8], microbiologically[9,10] and chromatographically[11–13].

This paper describes a technique for the isolation of some antibiotics and their subsequent direct spectrophotometric determination. It also shows that with this technique, any breakdown products of the antibiotics can be determined.

EXPERIMENTAL

Apparatus and materials

A Beckman DU-2 spectrophotometer was used, and also a Fractomat Y-3 fraction collector coupled to a Photocrom twin-ray spectrophotometer, with a Beckman 746 peristaltic pump (1–10 ml/min).

SP-Sephadex C-25 (Na^+) was obtained from Pharmacia, Uppsala, Sweden.

The antibiotics used were sodium ampicillin, trihydrate ampicillin, anhydrous ampicillin, dicloxacillin sodium, methacycline hydrochloride, doxycycline hyclate

(synthesized in the Research Laboratories of Alfa Farmaceutici S.p.A.) and commercial oxytetracycline hydrochloride (Ankerfarm, Milan, Italy).

Preparation of the resin and of the column

SP-Sephadex was suspended in water, decanted in order to remove the finer particles and equilibrated with sodium acetate solutions of concentration 0.01 and 0.005 *M* (pH 4.5). The resulting suspension was poured into a 1.5-cm-diameter glass column so as to give a depth of 20 cm after settling.

Elution

A 4-ml volume of a solution containing 4000 μg of ampicillin and dicloxacillin and 400 μg of methacycline and doxycycline was poured on to the column of cation-exchange resin. Elution was carried out first with 100 ml of 0.005 *M* sodium acetate solution and then with 150 ml of 0.01 *M* sodium acetate solution (both of pH 4.5). A constant elution rate of 0.8 ml/min was maintained, and the eluate was collected in fractions of 5.5 ml. Dicloxacillin and ampicillin were eluted separately with the first eluent, while doxycycline and methacycline were isolated by increasing the ionic strength of the eluent.

Each 5.5-ml fraction was measured spectrophotometrically against a blank of the same eluent at wavelengths corresponding to the absorption peaks of each antibiotic. Also, each fraction was subjected to microbiological analysis. Ampicillin was evaluated with the test organisms *Sarcina lutea* ATCC 9341, dicloxacillin with *Staphylococcus aureus* ATCC 209 and doxycycline and methacycline with *Bacillus cereus* ATCC 9534. At the concentrations used, the eluent did not give rise to inhibition zones.

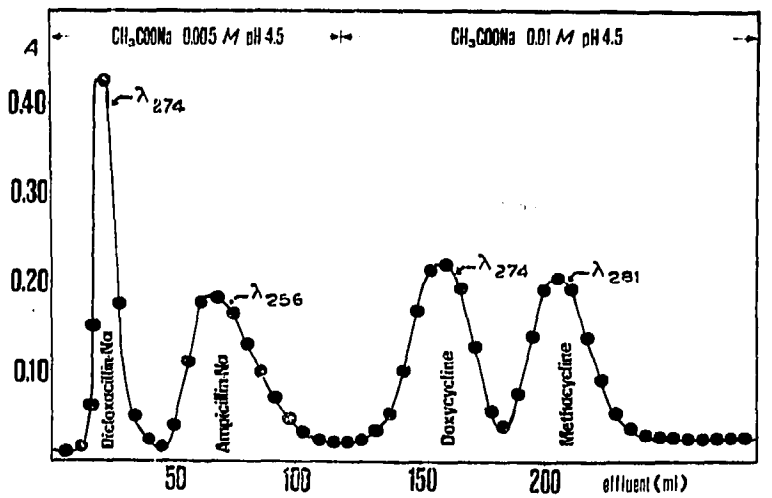

Fig. 1. Chromatographic separation of antibiotics on a 1.5×20 cm column of SP-Sephadex C-25 (Na^+). Dicloxacillin and ampicillin eluted with 0.005 *M* acetate buffer (pH 4.5) at a flow-rate of 48 ml/h. Doxycycline and methacycline eluted with 0.01 *M* acetate buffer (pH 4.5). The volume of each fraction is 5.5 ml. Absorbance measurements were made with the Photocrom instrument at the wavelengths indicated for each antibiotic.

RESULTS

Fig. 1 shows the separation of ampicillin, dicloxacillin, methacycline and doxycycline. The elution took about 5 h.

Quantitative analysis

In order to perform a quantitative spectrophotometric analysis on ampicillin and dicloxacillin, we calculated their respective percentage extinction values in 0.005 *M* sodium acetate (pH 4.5) at characteristic wavelengths and verified that the Lambert–Beer Law was obeyed in the concentration range 200–1000 μg/ml (Figs. 2 and 3).

Table I shows the calculated percentage extinction values and percentage recovery of the antibiotics. They were obtained by the spectrophotometric analysis of each of the eluted fractions, the following equation being used for the calculation:

$$\text{Antibiotic } (\mu g) = \frac{\Sigma E_{(\lambda_i)} \cdot V_i}{E^{1\%}_{1\,cm(\lambda_i)} \cdot 10^{-3}}$$

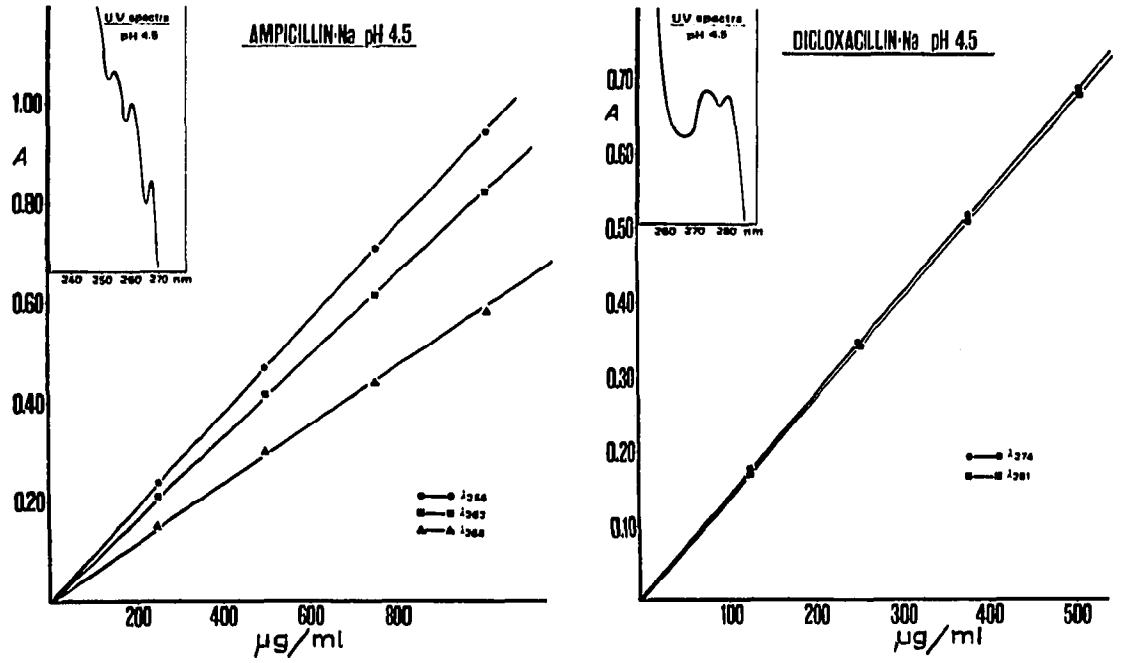

Fig. 2. Ultraviolet absorption spectrum of ampicillin and the relationship between absorbance and concentration at 256, 262 and 268 nm in 0.005 *M* sodium acetate (pH 4.5). Temperature 20°. Measurements were made in the Beckman DU-2 spectrophotometer with quartz cuvettes with a light path of 1 cm.

Fig. 3. Ultraviolet absorption spectrum of dicloxacillin and the relationship between absorbance and concentration at 274 and 281 nm in 0.005 *M* sodium acetate (pH 4.5). Temperature 20°. Measurements were made in the Beckman DU-2 spectrophotometer with quartz cuvettes with a light path of 1 cm.

where

$\Sigma E_{(\lambda i)}$ = the sum of extinctions relative to the single eluted fractions;
V_t = the number of millilitres (5.5) of the single fractions;
10^{-3} = factor to convert percentage extinction from 1 g per 100 ml to 10 μg/ml;
$E^{1\%}_{1\,cm(\lambda i)}$ = percentage extinction of each substance.

The fractions corresponding to the tetracyclines were determined after combining them in a volumetric flask. The spectrophotometric determination was carried out after decreasing the pH to 2.0 with 0.01 *N* methanol–HCl.

TABLE I

SPECTROPHOTOMETRIC CHARACTERISTICS AND RECOVERY OF ANTIBIOTICS SEPARATED ON SP-SEPHADEX C-25 (Na^+)

Antibiotic	*pH*	λ_{min} (*nm*)	λ_{max} (*nm*)	$E^{1\%}_{1\,cm(\lambda_{max})}$	*Recovery* (% ± S.D.) *
Ampicillin sodium	4.5	254	256	9.5	97.6 ± 0.7
		259	262	8.4	98.1 ± 0.6
		265	268	6.0	–
Ampicillin	4.5		256	9.8	99.4 ± 0.8
			262	8.7	–
			268	6.2	–
Ampicillin trihydrate	4.5		256	8.7	–
			262	7.8	98.3 ± 1.1
			268	5.6	–
Dicloxacillin sodium	4.5	265	274	14.5	100.1 ± 0.3
		278	281	14.4	–
Oxytetracycline hydrochloride	2.0		353	284	101.3 ± 0.3
Doxycycline hyclate	2.0**		267	343	92.3 ± 1.2
			351	309	99.5 ± 0.4
Methacycline hydrochloride	2.0**		253	450	–
			348	310	–

* Standard deviation based on nine determinations.
** Methanolic hydrochloric acid, 0.01 *N*.

Assessment of purity

The chromatographic technique described here can also be used to assess the purity of the individual raw materials.

An aqueous solution of ampicillin sodium of pH 10 was left at room temperature for 30 min and was then eluted from an SP-Sephadex column, giving a chromatogram of the type shown in Fig. 4. The breakdown products isolated during chromatography had no antibacterial activity.

Some samples of commercial oxytetracycline gave a chromatogram of the type shown in Fig. 5. With oxytetracycline, the microbiological method in current use (B.P. 1968)[9] is not specific in that the impurities present in oxytetracycline are also responsible for the inhibition of growth observed in the test organism and are therefore determined together with the oxytetracycline.

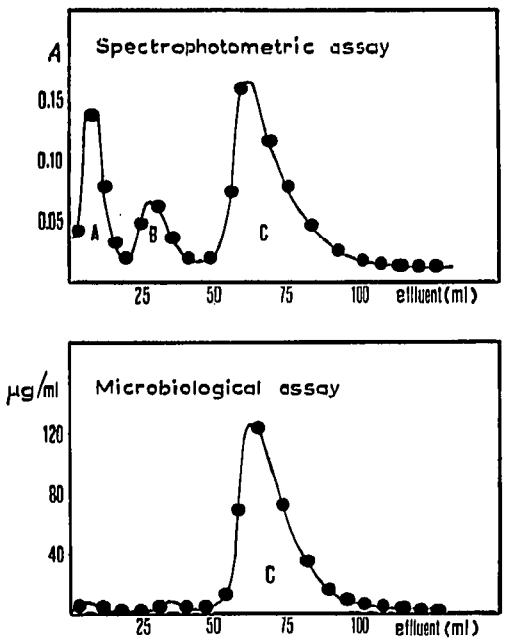

Fig. 4. Elution profile of ampicillin solution of pH 10, from SP-Sephadex ion-exchange column chromatography 30 min after preparation. Column: SP-Sephadex C-25 (Na^+), 1.5×20 cm. Elution with 0.005 *M* acetate buffer (pH 4.5). Measurements were made by both spectrophotometric and microbiological assays. A and B, uncharacterized substances; C, ampicillin.

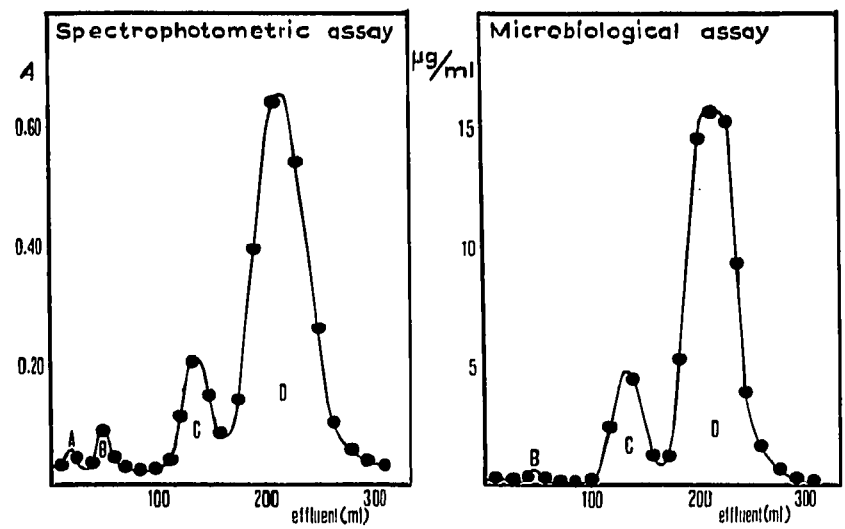

Fig. 5. Elution profile of commercial oxytetracycline solution. Deposition of 1 ml of aqueous solution containing 2000 μg of oxytetracycline hydrochloride. Column: SP-Sephadex C-25 (Na^+), 1.5×20 cm. Elution with NaCl–HCl, 0.05 *M* (pH 2.0), at a flow-rate of 30 ml/h. The volume of each fraction is 5.0 ml. Absorbance measurements were made at 353 nm. Measurements were made by both spectrophotometric and microbiological assays. A, B and C, uncharacterized substances; D, oxytetracycline hydrochloride.

DISCUSSION

The chromatogram shown in Fig. 1 illustrates the clear separation of the individual antibiotics. SP-Sephadex, owing to its high capacity, proved particularly effective; it has a high capacity for ampicillin and dicloxacillin (*ca.* 4 mg) and gave symmetrical, unflattened peaks even with eluents of low ionic strength. Consequently, it was possible to carry out direct spectrophotometric determinations of those antibiotics that have very low $E^{1\%}_{1\,cm}$ values. The Sephadex proved to be particularly useful in the determination of the breakdown products of the antibiotics, because as these products are often also biologically active, they cannot be isolated by the microbiological techniques described in the U.S.P.[10] and B.P.[9].

The technique described in this paper is particularly useful for obtaining and maintaining high standards of purity in the antibiotics used in pharmaceutical formulations. Furthermore, the ease with which the column can be prepared and re-used in successive chromatographic runs and the rapidity of analysis make the technique particularly interesting for routine determinations also.

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