Journal of Chromatography, 134 (1977) 223–229

CHROM. 9862

Note

Gel chromatography of tetracycline antibiotics

ENRICO RAGAZZI and GIOVANNI VERONESE
Istituto di Tecnica Farmaceutica dell'Università di Padova, Padua (Italy)
(Received October 19th, 1976)

In order to overcome some difficulties in the chromatographic separation of tetracycline antibiotics, we examined gel chromatography on both dextran and polyacrylamide gels. We knew of one reference[1] on the use of Sephadex in thin layers for the separation of a large number of different antibiotics, which appeared to be conditioned more by the chemical nature of the antibiotics than by their molecular size.

In our study, we used columns of Sephadex dextran gel and Bio-Gel P polyacrylamide gel, which proved to be suitable for separating certain tetracyclines and offered new analytical possibilities, particularly in some instances in which other chromatographic methods were unsatisfactory.

EXPERIMENTAL

Samples

The tetracyclines tested, all as hydrochlorides, are listed in Table I. For each substance, individual samples of 100–200 μg prepared as solutions of concentration 200 μg/ml in the appropriate eluent solution immediately before use were used.

TABLE I

TETRACYCLINES TESTED

Tetracycline	*Abbreviation*
Tetracycline	T
4-*epi*-Tetracycline	ET
Anhydrotetracycline	AT
4-*epi*-Anhydrotetracycline	EAT
Rolitetracycline (2-N-pyrrolidinomethyltetracycline)	RT
Pipacycline (N-methyl-N′-hydroxyethylpiperazinotetracycline)	PT
Lymecycline (N-methylenelysinetetracycline)	LT
Guamecycline (N′-N′-diethyleneiminobiguanidomethyltetracycline)	GT
Oxytetracycline	OT
Chlortetracycline	CT
Demethylchlortetracycline	DCT
Methacycline	MT
Doxycycline	DX
Minocycline	MN

Types of gels

The Sephadex dextran gels G-10 (40–120 μm), G-15 (40–120 μm), G-25 Fine (20–80 μm) and G-50 Fine (20–80 μm) (Pharmacia, Uppsala, Sweden), were used. The following types of Bio-Gel P polyacrylamide gels (Bio-Rad Labs., Richmond, Calif., U.S.A.) were used, all of Fine particle size (75–37 μm): P-2, P-4, P-6 and P-10.

Columns

Suitable amounts of dry gel of each type were allowed to swell in the appropriate aqueous solution (see below), following the directions recommended by the manufacturers. K 9/30 columns (30 × 0.9 cm) (Pharmacia) were packed with a 25-cm gel bed. Elution was carried out with 0.1 *M* acetic acid, 0.1 *M* sodium chloride solution and 0.1 *M* sodium chloride solution in 0.1 *M* acetic acid. The eluate was collected as 1-ml fractions by means of an Ultro-Rac LKB fraction collector, connected to an Uvicord II UV absorptiometer and these to an LKB 6520 d.c. recorder (LKB, Stockholm, Sweden).

RESULTS AND DISCUSSION

On both dextran and polyacrylamide gel columns some tetracyclines showed sufficiently different elution volumes to allow their clear separation and differentiation. Of various eluents examined in a previous series of tests (solutions with different concentrations of neutral salts, buffer solutions of various pH and of different compositions), 0.1 *M* acetic acid solution was preferred because it gave the best results in the separations without interfering with the solubility of the various tetracyclines tested.

As shown in Fig. 1 (dextran gels) and Fig. 2 (polyacrylamide gels), the separation of the various tetracyclines (and therefore the possibility of differentiating between them appeared to be dependent on the degree of cross-linking of the gel. Thus, as shown in Fig. 1, with Sephadex gels the best mutual separations of the tetracyclines tested, for identical volumes of the gel bed, were obtained with Sephadex G-10. The efficiency of separation decreased in the order Sephadex G-15, G-25 and G-50; with Sephadex G-50 all of the tetracyclines were eluted within a small elution volume.

Similarly, on Bio-Gel P gels (Fig. 2), the best separation among the tetracyclines was obtained with Bio-Gel P-2, the efficiency of separation decreasing in the order Bio-Gel P-4, P-6 and P-10 (for identical volumes of the gel bed).

In general, the width of the elution peaks of the tetracyclines, at identical concentrations, was inversely proportional to the degree of cross-linking of the gels. Thus, by proceeding from Sephadex G-10 to G-50, and from Bio-Gel P-2 to P-10, the width of the peaks became progressively smaller, while their height increased in proportion. In any event, on Bio-Gel P the elution peaks were sharper and more clear, thus allowing greater possibilities for separation and differentiation.

The V_e (elution volume) values of the tetracyclines tested were higher than the V_i (inner volume) value obtained for each column with sodium chloride, which is capable of penetrating freely the gel grains. Thus, even if gel filtration in the proper sense cannot be excluded for tetracyclines, especially with the Sephadex and Bio-Gel P gels with the highest degree of cross-linking, it seems evident that adsorption and

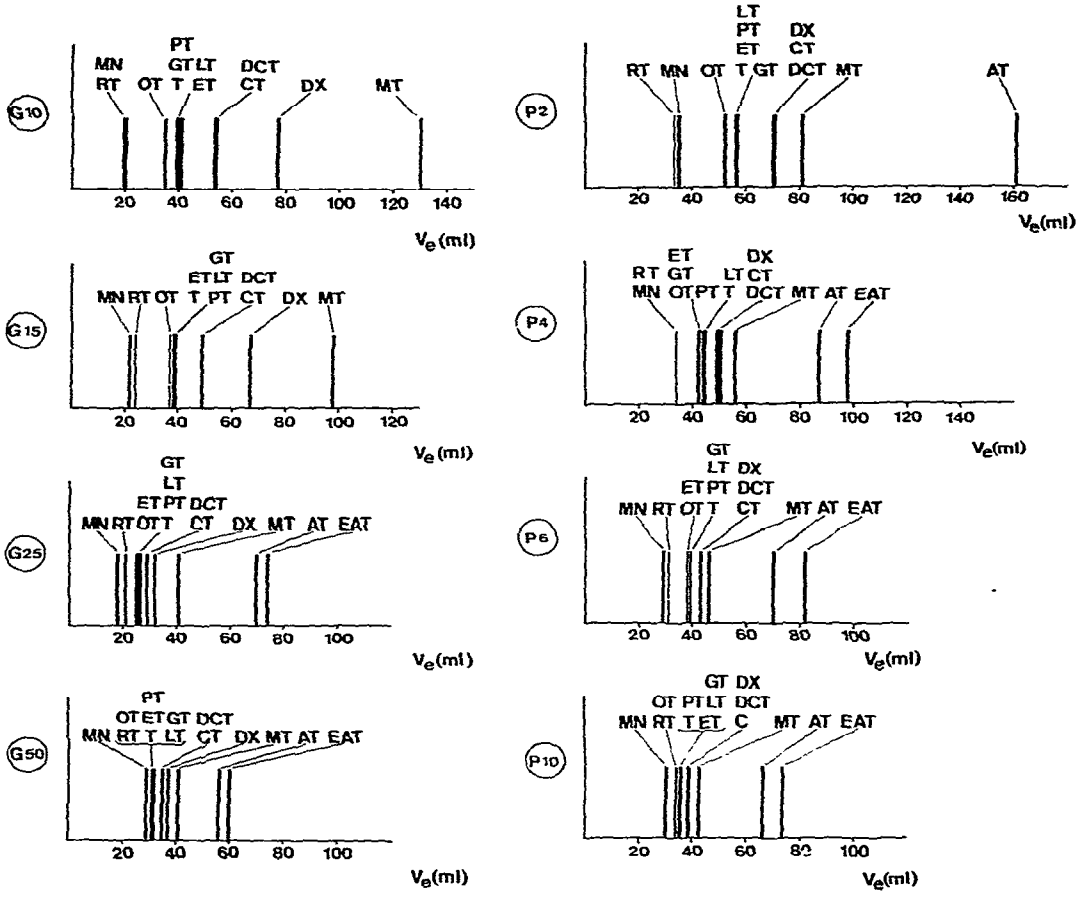

Fig. 1. Elution diagrams for tetracyclines on Sephadex columns with 0.1 *M* acetic acid as eluent. Abbreviations of tetracyclines as in Table I.

Fig. 2. Elution diagrams of tetracyclines on Bio-Gel P columns with 0.1 *M* acetic acid as eluent. Abbreviations of tetracyclines as in Table I.

partition are the fundamental mechanisms involved in the separation of the antibiotics. This fact appeared to be confirmed even by the observations on the degree of separation of the tetracyclines with respect to the gel porosity, the separation improving in proportion to the fineness of the porosity and the specific surface area of the gel.

As can be seen by comparison of Figs. 1 and 2, the chemical nature of the gels did not appear to exert any influence on the order of elution of the tetracyclines.

The composition of the eluent, for a particular type of gel, also influenced the mobility of the tetracyclines through the column. As shown in Table II, a variation of the mutual separation of the tetracyclines was observed on carrying out the elution with 0.1 *M* sodium acetate solution (pH 6.9) in comparison with 0.1 *M* acetic acid (pH 2.9); a similar effect was observed with 0.1 *M* sodium chloride solution (pH 5.3). No variations in the V_e values of the tetracyclines were observed on increasing the concentrations of these three eluents. However, on using 0.1 *M* sodium chloride

TABLE II

V_e VALUES OF THE TETRACYCLINES TESTED ON COLUMNS OF BIO-GEL P-2 ON ELUTION WITH DIFFERENT SOLUTIONS

Tetracycline	*Eluent*			
	Acetic acid (0.1 M)	*Sodium acetate (0.1 M)*	*Sodium chloride (0.1 M)*	*0.1 M sodium chloride in 0.1 M acetic acid*
Tetracycline	56	46	40	66
Rolitetracycline	33	40	43	39
Pipacycline	56	37	42	64
Lymecycline	56	45	42	65
Guamecycline	57	40	43	69
Oxytetracycline	52	38	37	61
Chlortetracycline	66	45	43	77
Demethylchlortetracycline	65	44	45	77
Methacycline	81	58	52	90
Doxycycline	66	50	48	74
Minocycline	35	45	41	48

solution in 0.1 *M* acetic acid (pH 2.9), a constant retardation of the elution of the antibiotics from the column in comparison with the use of 0.1 *M* acetic acid alone was observed, although their mutual separation was unchanged.

This result suggested that, in the separation of tetracyclines, ion exchange also occurred to some extent, as both the pH and the type of ions of the eluent could exert an influence.

Salts that are known to form complexes with tetracyclines[2,3] (magnesium chloride, calcium chloride, aluminium chloride), when tested as 0.2 *M* solutions, yielded results analogous to those of the above two solutions of sodium acetate and sodium chloride, indicating that the formation of such complexes of the tetracyclines did not influence their behaviour on the two types of gels tested.

In the practical utilization of the method, the most suitable gels were those with the greatest degree of cross-linking, such as Sephadex G-10 and G-15 and Bio-Gel P-2 and P-4, which allowed greater separation among the bands of the tetracyclines and therefore clearer differentiation.

By eluting columns of Sephadex G-10 and G-15 with 0.1 *M* acetic acid (see Fig. 1), minocycline could be separated from the other tetracyclines except rolitetracycline; the pairs oxytetracycline–tetracycline and chlortetracycline–demethylchlortetracycline appeared to be separated from each other and also from doxycycline and methacycline. Three of the derivatives substituted at the carboxamide function of tetracycline, namely pipacycline, lymecycline and guamecycline (see Table I), showed the same mobility as the tetracycline and therefore could not be separated. Rolitetracycline, however, was well separated from the tetracycline from which it is derived (Fig. 3), and could also be separated from the above three derivatives of tetracycline. Of the three best known degradation products of tetracycline, 4-*epi*-tetracycline appeared to be eluted together with the parent tetracycline, while anhydrotetracycline and 4-*epi*-anhydrotetracycline were eluted as greatly expanded bands that were sub-

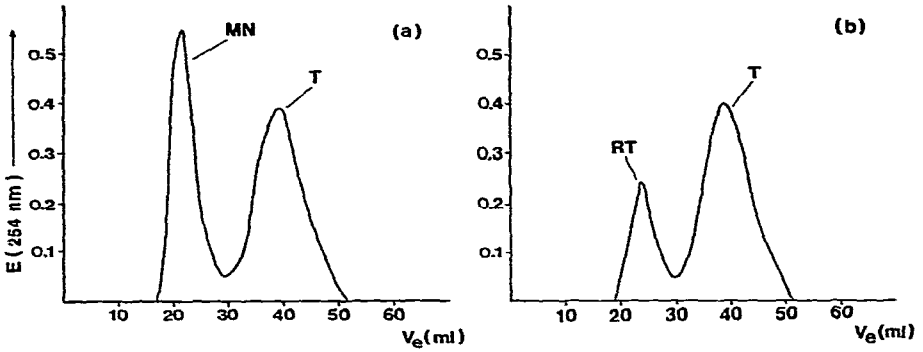

Fig. 3. Elution diagrams of mixtures of tetracyclines (200 μg each) on a Sephadex G-15 column with 0.1 *M* acetic acid as eluent. (a) Mixture of minocycline (MN) and tetracycline (T); (b) mixture of rolitetracycline (RT) and tetracycline (T).

stantially retarded in comparison with the other substances tested, with V_e values between about 200 and 300 ml (for tetracycline $V_e = 56$ ml).

On Bio-Gel P-2 and P-4, similar but clearer separations were effected as a result of the sharper bands that could be obtained. As in the preceding case, minocycline could be clearly separated from the other tetracyclines (see Figs. 2 and 4), always with the exception of rolitetracycline, which had a similar V_e value. Tetracycline and oxytetracycline were always eluted at the same elution volume, as well the group consisting of chlortetracycline, demethylchlortetracycline and doxycycline. Methacycline was well separated (Fig. 2). In this instance also rolitetracycline alone was separated from tetracycline and the other carboxamide derivatives (see Figs. 2 and 4). Of the degradation products of tetracycline, the 4-*epi*-tetracycline appeared not to be separated from tetracycline; anhydrotetracycline and 4-*epi*-anhydrotetracycline, on the other hand, were clearly separated from the other tetracyclines (see Fig. 2) and, moreover, could also be separated from each other (Fig. 5).

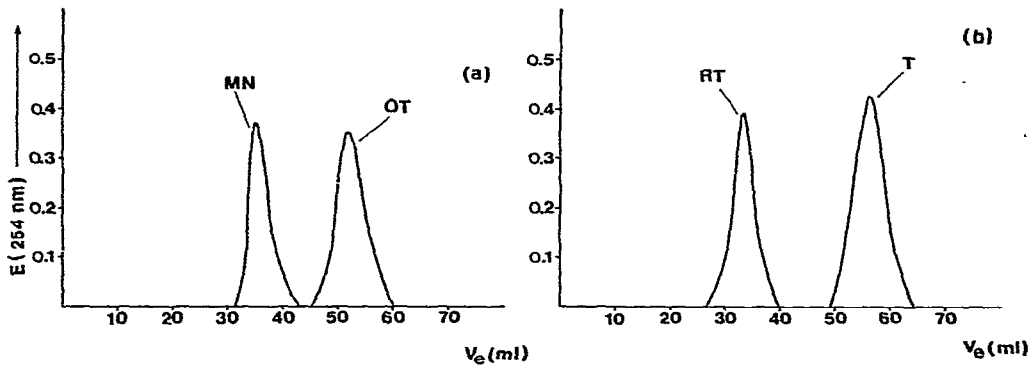

Fig. 4. Elution diagrams of mixtures of tetracyclines (200 μg each) on a Bio-Gel P-2 column with 0.1 *M* acetic acid as eluent. (a) Mixture of minocycline (MN) and oxytetracycline (OT); (b) mixture of rolitetracycline (RT) and tetracycline (T).

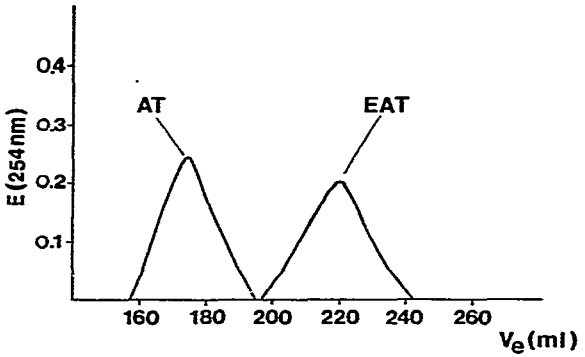

Fig. 5. Elution diagram of a mixture of anhydrotetracycline (AT) and 4-*epi*-anhydrotetracycline (EAT) on a Bio-Gel P-2 column with 0.1 *M* sodium chloride in 0.1 *M* acetic acid as eluent.

CONCLUSION

The results obtained suggest some possible practical applications of the method. One application may be the analytical separation of certain tetracyclines. Thus, by eluting the column with 0.1 *M* acetic acid, minocycline, which has the lowest V_e value (see Figs. 1 and 2), can be separated from the other tetracyclines. Its separation from rolitetracycline, which showed the same V_e value with the above eluent, might be achieved by elution with 0.1 *M* sodium chloride solution (see Table II). Similar possibilities exist for the separation of doxycycline and methacycline.

A more interesting aspect of the method concerns rolitetracycline, which so far could not be separated from tetracycline or from its carboxamide derivatives by the various thin-layer chromatographic methods[4]. By chromatography on both dextran and polyacrylamide gel with 0.1 *M* acetic acid as the eluent, rolitetracycline could be separated from both tetracycline and from its carboxamide derivatives with therapeutic uses, such as pipacycline, lymecycline and guamecycline, which have always shown higher V_e values (see Figs. 1 and 2). Tests carried out on samples of proprietory medicines containing rolitetracycline, in comparison with a standard sample, showed the clear possibility of this analytical application. The same test carried out on Bio-Gel P-2 and P-4 columns with an old sample of rolitetracycline showed the possibility of effecting a clear detection and a good isolation of some its degradation products: after the peak of the unchanged rolitetracycline there appeared a peak corresponding to tetracycline and 4-*epi*-tetracycline, followed, at a considerable distance, by two distinct peaks of anhydrotetracycline and 4-*epi*-anhydrotetracycline.

On elution of Bio-Gel P-2 and P-4 columns with 0.1 *M* acetic acid, it was possible to detect impurities in tetracycline, such as anhydrotetracycline and 4-*epi*-anhydrotetracycline, the latter being important on account of the risk of nephrotoxicity.

The bands of the separated tetracyclines might be subjected to a quantitative micro-determination, *e.g.*, by spectrophotometry or fluorimetry. Finally, the two gels tested might be useful for the purification of certain tetracycline antibiotics.

REFERENCES

1 M. H. J. Zuidweg, J. G. Oostendorp and C. J. K. Bos, *J. Chromatogr.*, 42 (1969) 552.
2 A. Albert and C. W. Rees, *Nature (London)*, 177 (1956) 433.
3 D. Kodrnja and A. Gertner, *Acta Pharm. Jugosl.*, 25 (1975) 111.
4 E. Ragazzi and G. Veronese, *Farmaco, Ed. Prat.*, 29 (1974) 27.