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SIMPLE METHOD FOR THE QUANTITATIVE ANALYSIS OF TETRACYCLINES BY DIRECT FLUORIMETRY AFTER THIN-LAYER CHROMATOGRAPHY ON CELLULOSE PLATES

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SUMMARY

A thin-layer chromatographic method is described in which tetracyclines are separated on a cellulose layer by development with aqueous solutions of certain salts (magnesium, calcium, barium and zinc chloride). The spots exhibit good fluorescence in UV light, allowing ready detection without the necessity for treatment with any reagents. This fluorescence can be used for the direct photometric determination of the tetracyclines with good accuracy.

INTRODUCTION

In the course of previous studies on the possibilities offered by different thin-layer chromatographic (TLC) methods for tetracycline antibiotics[1], we observed that on cellulose plates (both natural and microcrystalline cellulose) it was possible to achieve a differentiation of most tetracyclines by developing the plates with buffer solutions or aqueous solutions of certain salts. The method offered the substantial advantage of simplicity, without the need for careful and lengthy conditioning of the sorbent layers, unlike the methods that employ mixtures of organic solvents, where special impregnations of the layers are necessary.

In the same studies[1], we also observed that good fluorescence in UV light could be obtained with all of the tetracyclines by spraying the plates with a methanolic solution of salts of metals that have a known ability to form complexes with the tetracyclines (magnesium, calcium, aluminium and zinc chloride). The fluorescence so developed by the various tetracyclines in UV light was then employed in later studies[2] for the direct photometric determination of antibiotics after TLC.

We then observed that on developing cellulose TLC plates with aqueous solutions of certain salts that form fluorescent complexes with tetracyclines (magnesium, calcium, barium and zinc chloride), it was possible to obtain differentiations among these antibiotics comparable to those obtained after development with buffer solutions[1], with well shaped spots which, in UV light, developed a fluorescence that was sufficient both for ready detection on the plates and for a direct fluorimetric determination, without further treatment with any reagents.

TABLE I

SETTINGS OF THE VITATRON TLD 100 PHOTODENSITOMETER FOR SCANNING THE TETRACYCLINES AFTER DEVELOPMENT OF THE PLATES WITH SALT SOLUTIONS

Tetracycline	Developing solution											
	Magnesium chloride			Calcium chloride			Barium chloride			Zinc chloride		
	Level	Zero	Span	Level	Zero	Span	Level	Zero	Span	Level	Zero	Span
Tetracycline	e	7	8,3	f	6	8,5	e	7	9,3	e	7	9,9
Oxytetracycline	f	6	8	e	7	9	f	6	9,5	f	6	9,8
Chlorotetracycline	e	7	9	f	6	10				f	6	10
Demethylchlorotetracycline	e	7	9	e	7	8,7	f	6	9,5	f	6	9,8
Methacycline	f	6	10									
Doxycycline	f	6	9,6									
Minocycline	f	5	9,9	f	6	9,2	f	6	9,7	e	6	10
Anhydrotetracycline	f	6	10							f	6	10

EXPERIMENTAL

Samples

The following samples were used: tetracycline, 4-epitetracycline, anhydro-tetracycline, 4-epianhydrotetracycline, oxytetracycline, chlorotetracycline, demethyl-chlorotetracycline, methacycline, doxycycline and minocycline, all as hydrochlorides.

TLC plates

Plates were prepared using the Shandon Unoplan device with microcrystalline cellulose for TLC (Merck, Darmstadt, G.F.R., Cat. No. 2330). A 40-g amount of the sorbent suspended in 100 ml of water was used for five plates (20 × 20 cm) with a layer thickness of 0.25 mm. The plates were air-dried for at least 12 h and used directly for chromatography.

The following pre-coated plates were also used: cellulose pre-coated TLC plates without fluorescent indicator (Merck, Cat. No. 5716); CEL 300-10 MN pre-coated TLC plates and Polygram CEL 300 pre-coated plastic sheets, both with a 0.1-mm layer of cellulose MN 300 (Macherey-Nagel, Düren, G.F.R.); Polygram CEL 400 pre-coated plastic sheets with a 0.1-mm layer of microcrystalline cellulose (Macherey-Nagel); and Eastman Chromagram cellulose sheets (Cat. No. 6064) without fluorescent indicator (Eastman-Kodak, Rochester, N.Y., U.S.A.).

Application of samples

Various amounts of each antibiotic (0.1–2 μg) in methanolic solution (0.1–0.5 mg/ml) were applied on the plates with a Hamilton microsyringe.

Solvent systems

Solutions 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 M in water were used for magnesium, calcium, barium, aluminium, and zinc chloride. Solutions 0.01, 0.02 and 0.04 M in water were used for uranium and thorium nitrate.

Detection of spots

After air-drying for 2–4 h, the spots could be detected by their fluorescence (yellow or green-yellow; blue for minocycline), developed by exposure to a UV lamp.

Quantitative analysis

The spots were quantified by means of a Vitatron TLD 100/Hg flying-spot densitometer, with a mercury lamp as a light source. Excitation of the fluorescence was achieved with a UVB filter (240–340 nm). The instrument settings were as follows: secondary filter (photomultiplier), U3; diaphragm, 0.25 nm; damping, 2; scanning speed, 1 cm/min; mode, + lin. The other settings of the densitometer and operating conditions were adapted in accordance with the developing solution used and with the appropriate tetracycline; the conditions found most suitable for the analysis are summarized in Table I.

RESULTS AND DISCUSSION

Separation of tetracyclines

Good differentiations among most of the tetracyclines (comparable to those obtained by development with citric acid–disodium phosphate buffer solutions at pH 3.6–5.6[1]) were obtained with aqueous solutions of magnesium, calcium, barium and zinc chloride.

The concentration of the solutions appeared to exert a weak influence on the separation of the tetracyclines, except for minocycline (see the curves in Fig. 1). The R_F values, however, in general showed a progressive increase up to a concentration of 0.15 M and then remained stable.
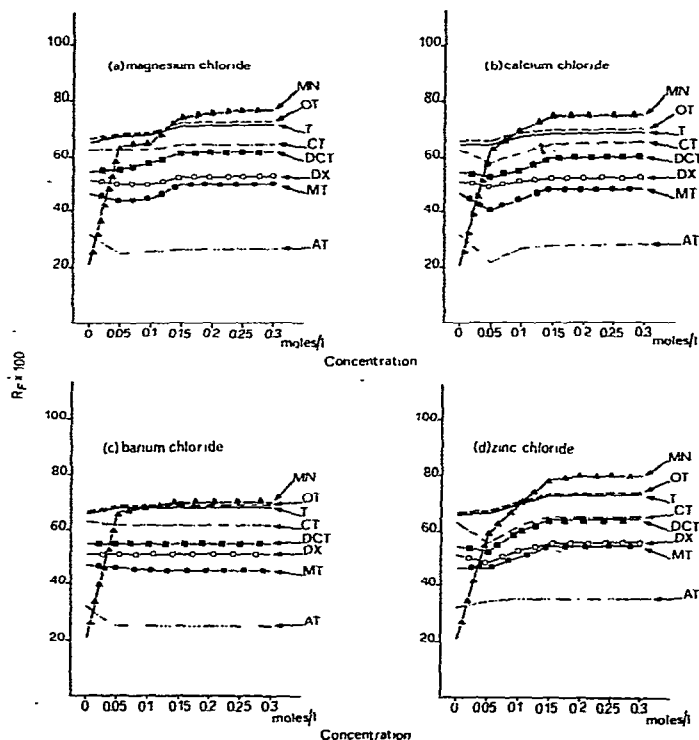

Fig. 1. Plots of $R_F \times 100$ values of tetracyclines on microcrystalline cellulose plates obtained by development with solutions of increasing concentration of: (a) magnesium chloride; (b) calcium chloride; (c) barium chloride; (d) zinc chloride. T = tetracycline; AT = anhydrotetracycline; OT = oxytetracycline; CT = chlorotetracycline; DCT = demethylchlorotetracycline; MT = methacycline; DX = doxycycline; MN = minocycline.

After air-drying, in UV light the spots exhibited good fluorescence at all concentrations of the development solutions. The spots were sharp and regular, with no trails or blurs.

Good separations were also achieved with aqueous solutions of uranium nitrate (Fig. 2); the spots, although sharp and well shaped, were not fluorescent in
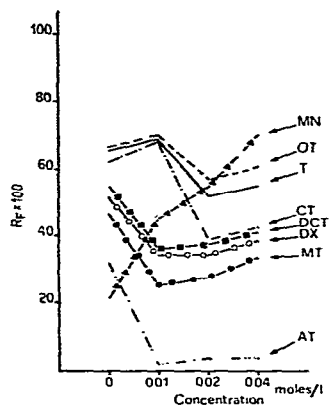
Fig. 2. Plots of $R_F \times 100$ values of tetracyclines on microcrystalline cellulose plates obtained by development with solutions of increasing concentrations of uranium nitrate. Abbreviations as in Fig. 1.

UV light and remained dark even after treatment with those reagents which usually develop a strong fluorescence with the same tetracyclines (ammonia vapour, amines, salts[1]).

The other solutions tested gave unsatisfactory results: the solutions of aluminium chloride always produced spots that were not well differentiated and with long tails, even if strongly fluorescent; the solutions of thorium nitrate showed similar results, but with non-fluorescent spots.

Substantial differences in the separation of the tetracyclines did not occur between the plates prepared in the usual manner in our laboratory and the pre-coated plates examined. According to the characteristics of the sorbent layer of each type of plate, the time for the development of the chromatograms varied (2 h for cellulose plates prepared in our laboratory; 2½ h for pre-coated cellulose plates (Merck) and for Polygram sheets; 2 h for pre-coated CEL 300 MN plates; only 45 min for the Eastman Chromagram sheets).

The different rates of development necessarily produced changes in the individual R_F values, but without influencing the mutual separation of the various tetracyclines, at equivalent concentrations of the solutions used for the development.

With pre-coated plates, more regular and sharper spots were obtained, without the interferences that the inevitable irregularities of the surface of the sorbent layer of the plates prepared in the usual manner in the laboratory can exert.

The various possible separations among the different tetracyclines can be enhanced by an appropriate choice of both the salt and its concentration, as shown by the curves in Figs. 1 and 2.

In any event, however, it was not possible to obtain a clear separation between the pairs tetracycline–epitetracycline and anhydrotetracycline–epianhydrotetracycline, owing to the small difference between their R_F values (see Table II).

The method, in addition to its simplicity, offers a good reproducibility of the results, independent of ambient and saturation conditions.

Direct fluorimetric analysis of tetracyclines

After development with the solutions of magnesium, calcium, barium and zinc

.TABLE II

$R_F \times 100$ VALUES OF TETRACYCLINES ON CELLULOSE PRE-COATED PLATES (MERCK, CAT. No. 5716) AFTER DEVELOPMENT WITH SALT SOLUTIONS

Tetracycline	Developing solution			
	Magnesium chloride, 0.25 M	Calcium chloride, 0.20 M	Barium chloride, 0.25 M	Zinc chloride, 0.15 M
Tetracycline	72	67	63	65
4-Epitetracycline	68	63	60	65
Anhydrotetracycline	29	25	24	22
4-Epianhydrotetracycline	25	16	17	18
Oxytetracycline	72	68	63	65
Chlorotetracycline	62	59	58	52
Demethylchlorotetracycline	64	57	54	55
Methacycline	54	47	44	50
Doxycycline	53	50	49	48
Minocycline	76	70	60	68

chloride, the tetracyclines exhibited fluorescences in UV light comparable to those developed after treatment with ammonia vapour or after spraying with methanolic solutions of certain amines[2] or solutions of the above salts[1,2].

We therefore studied the possibility of utilising this fluorescence for the direct photometric analysis of tetracyclines (as was possible after spraying with methanolic solutions of the same salts the plates, both cellulose and Kieselguhr, developed with various solvent systems[2]).

For our study, as pointed out in a preceding paper[2], pre-coated plates were used because, in order to allow a direct photometric analysis, they produce more regular spots and exert fewer interferences on the determinations than plates prepared in the usual manner owing to the inevitable irregularities of the surface layer. Of the different types of pre-coated plates available, we preferred Merck cellulose plates (Cat. No. 5616) because, by virtue of their denser sorbent layer, they gave spots that were more regular and sharper and consequently more strongly fluorescent, at equivalent surface areas.

First the stability of the fluorescence with time was checked. For this purpose, samples of methanolic solutions of each tetracycline were spotted on the plates and developed with a 0.2 M solution of the above four salts. After air-drying out of direct light, the fluorescence intensities of the spots were determined at suitable invervals between 4 and 30 h after the end of the development.

Changes in fluorescence intensity were found indistinctly for all of the tetracyclines within the first 12 h after the development of the plates, as shown by the curves in Fig. 3. The changes then decreased up to the next 20 h, when the fluorescence for most tetracyclines became almost constant or changed sufficiently slowly to be negligible within the time required for scanning the plate. Attempts to stabilise the fluorescence intensity by drying the plates in an air-oven failed owing to the alteration of the tetracyclines in the presence of the salts. In order to ensure consistent results for the purpose of quantitative analysis, determinations were therefore always made between 20 and 30 h after the development of the plates, when the fluorescence of the tetracyclines was more stable.
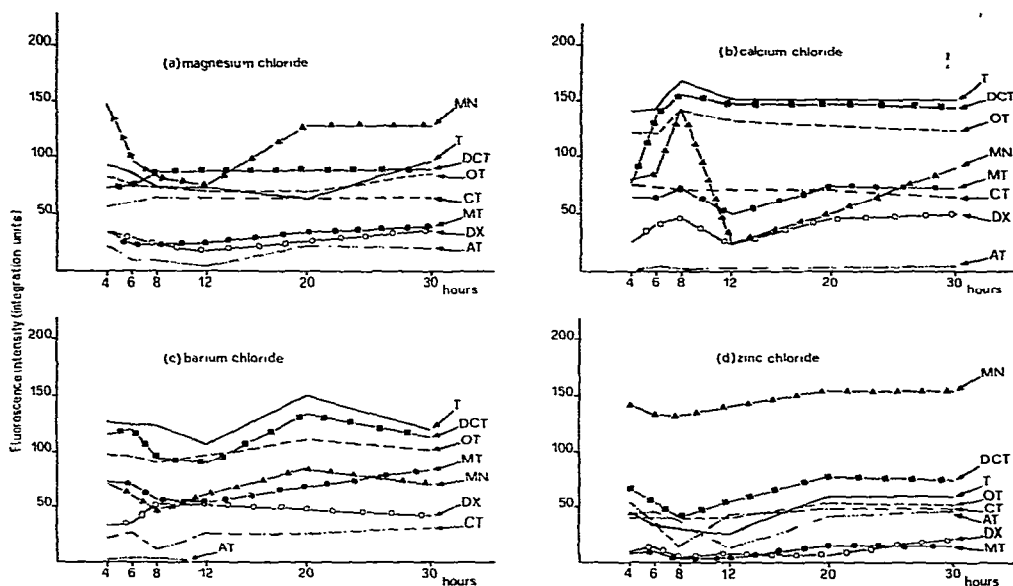
Fig. 3. Variation with time of the fluorescence intensity exhibited by 0.8 μg of the various tetracyclines after development with 0.2 M solutions of the four salts on cellulose pre-coated plates (Merck, Cat. No. 5716): (a) magnesium chloride; (b) calcium chloride; (c) barium chloride; (d) zinc chloride Abbreviations as in Fig. 1.

Finally, in order to check the influence of the salt concentration on the fluorescence intensity of the antibiotics, a photometric determination of the fluorescence was made on samples of each tetracycline developed with solutions of the four salts of different concentrations. As shown by the curves in Fig. 4, the fluorescence intensity of most tetracyclines generally changed as the concentration of the salt solutions changes, then remained constant from a certain concentration, depending on the salt. Only with magnesium chloride did the concentration exert little influence on the fluorescence, with the exception of minocycline and anhydrotetracycline (see Fig. 4a).

The concentrations of the four salts that gave the highest fluorescence intensity with all of the tetracyclines examined were: magnesium chloride 0.25 M, calcium chloride 0.20 M, barium chloride 0.25 M and zinc chloride 0.15 M. Even under such conditions, differences in fluorescence intensity were found among the tetracyclines. The fluorescence intensities exhibited by equal amounts of the various tetracyclines are given in Table III (calculated on the basis of the integration numbers obtained on the Vitatron UR 402 recorder and related to the fluorescence of the tetracycline).

After development with 0.25 M magnesium chloride solution, the fluorescence of the antibiotics was sufficient for the quantitative determination of the tetracyclines down to 0.1 μg for tetracycline, oxytetracycline, chlorotetracycline, demethylchlorotetracycline and minocycline. For methacycline, doxycycline and anhydrotetracycline, the smallest amount that could be determined was 0.5 μg.

After development with 0.2 M calcium chloride solution, tetracycline, oxytetracycline, chlorotetracycline, demethylchlorotetracycline and minocycline could be determined down to 0.25 μg; methacycline, doxycycline and anhydrotetracycline,
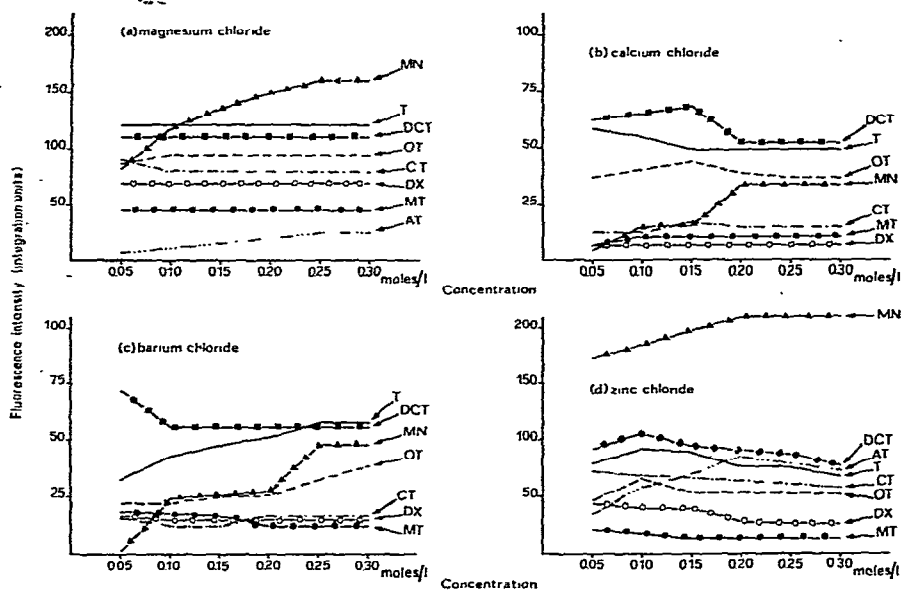
Fig. 4. Plots of fluorescence intensity of the various tetracyclines against concentrations of the salt solutions used for development of the plates (cellulose pre-coated plates, Merck, Cat. No. 5716): (a) magnesium chloride; (b) calcium chloride; (c) barium chloride; (d) zinc chloride. Abbreviations as in Fig. 1.

TABLE III

RELATIVE FLUORESCENCE ON CELLULOSE PRE-COATED PLATES (MERCK, CAT. No. 5716) OF TETRACYCLINES AFTER DEVELOPMENT WITH SALT SOLUTIONS
Fluorescence of tetracycline = 100.

Tetracycline	Developing solution			
	Magnesium chloride, 0.25 M	Calcium chloride, 0.20 M	Barium chloride, 0.25 M	Zinc chloride, 0.15 M
Tetracycline	100	100	100	100
Oxytetracycline	74	86	54	64
Chlorotetracycline	90	20	24	57
Demethylchlorotetracycline	102	111	93	104
Methacycline	42	18	23	12
Doxycycline	58	14	19	45
Minocycline	134	77	84	210
Anhydrotetracycline	20	>1	>1	74

however, exhibited fluorescence with intensities that were insufficient for the correct determination of the amounts applied on the plates.

With 0.25 M barium chloride solution, fluorescence intensities sufficient for the determination of tetracycline, oxytetracycline, demethylchlorotetracycline and minocycline down to 0.5 μg were developed; the fluorescence intensities of chlorotetracycline, methacycline, doxycycline and anhydrotetracycline, although strong enough for detection on the plates, were insufficient for quantitative determinations.

Finally, after development with 0.15 M zinc chloride solution, the fluorescence intensities were strong enough for the determination of tetracycline, oxytetracycline, demethylchlorotetracycline and minocycline down to 0.25 μg and chlorotetracycline and anhydrotetracycline down to 0.5 μg; methacycline and doxycycline gave fluorescence intensities that were insufficient for quantitative analysis.

In any case, the largest amount of each antibiotic that could be determined, with sharp and regular spots with the plates used, was not over 2 μg.

Within the above-mentioned limits, good proportionalities were obtained between the fluorescence intensities and the amounts of each antibiotic applied (Fig. 5).
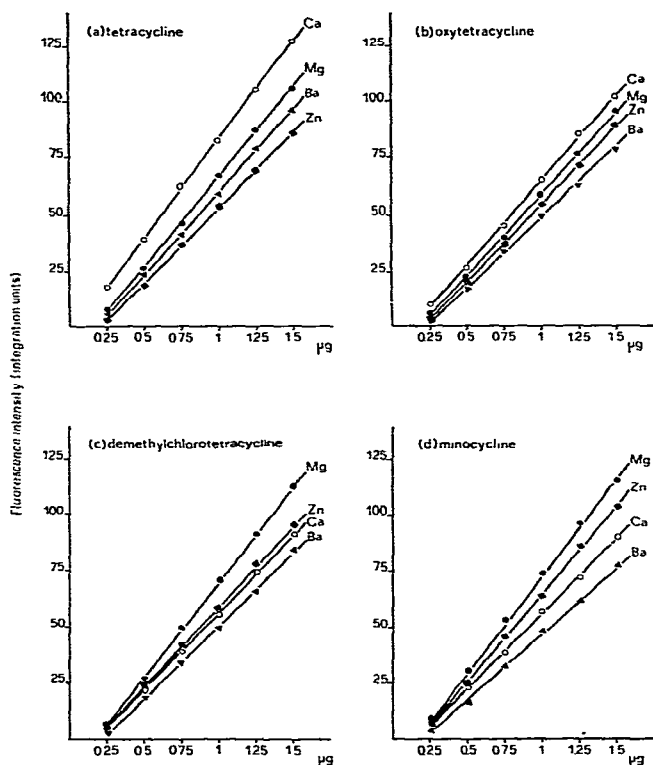

Fig. 5. Curves of fluorescence intensity against amount of antibiotic in the determination of tetracyclines by direct photometry after development of the plates with salt solutions: Mg = 0.25 M magnesium chloride; Ca = 0.20 M calcium chloride; Ba = 0.25 M barium chloride; Zn = 0.15 M zinc chloride.

Accuracy of the methods

The relative standard deviations in the determinations of 0.5–1 μg of each substance (summarized in Table IV) indicate a satisfactory reproducibility of the methods. Some differences in the values obtained with the same amounts of antibiotic from plate to plate could occur as a consequence of differences in the thickness and smoothness of the sorbent layers; hence, in comparing values obtained on different plates, it is advisable to carry out a preliminary selection of the plates.

TABLE IV

RELATIVE STANDARD DEVIATIONS (%) IN THE DIRECT FLUORIMETRIC DETER-
MINATION OF TETRACYCLINES ON PRE-COATED CELLULOSE PLATES (MERCK,
CAT. No. 5716)

Tetracycline	Developing solution			
	Magnesium chloride, 0.25 M	Calcium chloride, 0.20 M	Barium chloride, 0.25 M	Zinc chloride, 0.15 M
Tetracycline	± 1.49	± 2.54	± 1.34	± 2.15
Oxytetracycline	± 2.83	± 2.22	± 1.63	± 2.35
Chlorotetracycline	± 4.45	± 1.68		± 2.54
Demethylchlorotetracycline	± 2.01	± 2.52	± 2.23	± 3.46
Methacycline	± 3.49			
Doxycycline	± 3.21			
Minocycline	± 3.76	± 2.61	± 3.90	± 2.36
Anhydrotetracycline	± 2.21			± 7.19

CONCLUSIONS

By using a solution of magnesium, calcium, barium or zinc chloride for the
development of cellulose TLC plates, it is possible to differentiate tetracyclines; the
spots exhibit good fluorescence in UV light, without the necessity for treatment with
any reagents for their detection. This fluorescence can be used for the determination
of the tetracyclines by direct photometry of the spots obtained; while magnesium
chloride is suitable for the determination of all of the tetracyclines examined, the other
three salts produce fluorescences that are useful for the direct photometric determina-
tion only of some of the tetracyclines.

REFERENCES

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2 E. Ragazzi and G. Veronese, *Farmaco (Pavia), Ed. Prat.*, 29 (1974) 372.