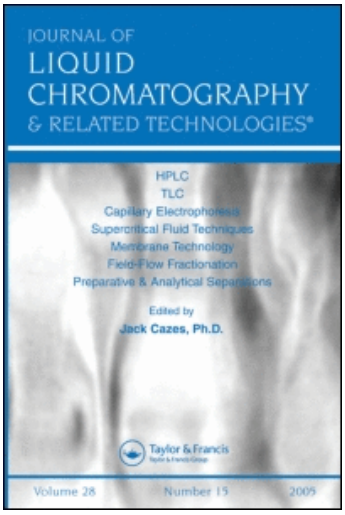

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Determination of Tetracycline and Related Compounds by High-Performance Liquid Chromatography

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DETERMINATION OF TETRACYCLINE AND RELATED COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An isocratic high-performance liquid chromatography method for the determination of tetracycline and its related compounds is described. The method uses a reverse phase (C_{18}) column, a modified acetonitrile/water mobile phase, and benzoic acid as the internal standard. Elution of all compounds of interest is complete within seven minutes. Results are presented for thirteen commercial capsule formulations and are compared with results by microbiological assay and thin-layer chromatographic methods.

INTRODUCTION

The most widely accepted method for the analysis of tetracycline (TC) and its formulations is the microbiological assay; it is the method of choice of both the British Pharmacopoeia 1980 (1) and the Code of Federal Regulations (2). The British Pharmacopoeia (BP) monograph specifies the large plate agar diffusion method, while that of the Code of Federal

Regulations (CFR) is the turbidimetric assay method. Non-chromatographic chemical methods generally encounter interference from one or more of the related compounds which may be present in TC. The more important of these compounds are 4-epi-tetracycline (ET), anhydrotetracycline (AT), 4-epianhydrotetra-cycline (EAT) and chlortetracycline (CT). (The latter may be found in TC which has been made by dehalogenation of CT.) Of these, EAT is the most significant, since it is nephrotoxic. The CFR specifies a spectrophotometric screening test for total anhydrotetracyclines in TC bulk substances and formulations; where the results exceed specified limits, determination of EAT by column chromatography is required. The BP determines all related compounds in bulk substances by thin-layer chromatography (TLC), but simply limits the concentration of the anhydrotetracyclines in capsules by a spectrophotometric test.

The microbiological assay and the chromatographic tests for related compounds are time consuming and their replacement by a single quantitative method for all compounds is desirable.

Tsuji and Robertson (3) introduced a gas-liquid chromatography method, but it necessitates a difficult derivatisation, and TC degradation may occur in the process.

In recent years a number of methods using high-performance liquid chromatography (HPLC) have been proposed; some of them employ ion exchangers, but the majority use reverse phase bonded materials. Complete resolution of TC and its related compounds has not been achieved in some of the proposed methods (4,5) while others do not present evidence for quantitative application to commercial formulations (6,7). While gradient elution systems are able to achieve good resolution in a short time, they may give rise to sloping baselines, e.g. the systems proposed by Mack and Ashworth (8). Tsuji and Robertson (9) developed a gradient elution system with a flat baseline, which they successfully applied to the analysis of pharma-ceutical preparations. A single step gradient system was employed by Muhammad and Bodnar (10) in a method for the related compounds in bulk substances and formulations.

The objective of this work was to develop an isocratic HPLC method suitable for the rapid determination of TC and its related compounds in bulk materials and in commercial capsule formulations.

MATERIALS

(a) Instrumentation

The instrument used was a Waters Associates liquid chromatograph, consisting of a 6000A pump, a U6K injector, a 440 dual channel detector, and an OmniScribe (Houston Instrument) dual pen recorder. A microBONDAPAK C_{18} (reverse phase) column, 3.9 mm X 30 cm (Waters Associates) was used for all separations.

(b) Solvents and Reagents

Acetonitrile (ACN) was supplied by Waters Associates; anhydrous methanol, diammonioum hydrogen phosphate and ortho-phosphoric acid were Analytical Reagent grade; ethanolamine and dimethylformamide (DMF) were Laboratory Reagent grade, and glass distilled water was freshly prepared before use. Benzoic acid (Merck, GR volumetric standard) was used as the internal standard.

The strong internal standard solution (SIS) was 0.5% W/v benzoic acid in 50% methanol, and the weak internal standard (WIS) was 0.05% benzoic acid in 50% methanol.

The mobile phase was prepared as follows:
In 760 mL water were dissolved 5.2 g diammonium hydrogen phosphate and 5.0 mL ethanolamine. To the solution were added 240 mL ACN and 60 mL DMF. Finally the pH was adjusted, using orthophosphoric acid, to 2.5. After filtration through a 0.5 µm pore size membrane filter (Millipore Corporation Type FH), the mobile phase was ultra-sonically degassed. The work involved in the development of this mobile phase is summarised below under METHODS.

(c) Tetracycline and Related Compounds Standards

In this work, the form of TC and its related compounds was the hydrochloride, unless otherwise specified.

The TC standard was a commercial sample of BP quality, supplied by Cyanamid Pty. Ltd. It was microbiologically standardised against the World Health Organisation's Second International Standard for Tetracycline (1970), giving a potency of 968 units/mg, "as is". An HPLC analysis by the method described here gave an ET content of 2.5%, an AT content of 0.2% and a negligible EAT content. The loss on drying (60°C, 5 mm pressure) was 0.7%. The microbiological and HPLC analyses were therefore consistent within experimental error, and the standard was assumed to be of 96.8% purity for this work. The ammonium salt of ET was prepared according to McCormick et al (11); AT was prepared by the method described by Schlecht and Frank (12), and EAT, by an adaptation of the latter method, from the ammonium salt of ET.

(d) Formulation Samples

All of the TC capsule formulations, for human use, on the Australian market, were sampled. There were thirteen different formulations (seven of which were buffered), representing nine manufacturers.

METHODS

(a) Mobile Phase Development

The evaluation and optimisation of the mobile phase were based on an analysis time-constrained concept. The retention time of the last eluting peak (AT) was adopted as a measure of analysis time. Optimisation of the system then became a matter of achieving the best resolution, within that time, for the least resolved pair, TC and ET.

The column suppliers recommend using ethanolamine in all mobile phases to reduce the adsorptive effect of active silanyl groups on the column; it was therefore routinely included at 0.5% v/v.

Preliminary work showed that acidified aqueous ACN was the most promising of the range tested, but that the system would

require modification in order to produce acceptable chromatograms. The column suppliers recommend working above pH 2 in order to avoid hydrolytic loss of the C_{18} phase. The pH range 2.5 to 3.3 was investigated by comparing the chromatograms given by different ACT/water mixtures acidified to pH 2.5, 2.9 and 3.3 with ortho-phosphoric acid. A system consisting of 24 ACN/76 water at pH 2.5 gave the best ET-TC peak separation; it was also effective in reducing the tailing of the AT peak which was observed at higher pH. Below pH 2.5, the ET-TC peak separation was further improved, but as continuous work at such acidities may have shortened the effective column life, it was decided to standardise all phases to pH 2.5.

Investigation of a number of solvents and modifiers showed that DMF and ammonium phosphate both markedly improved the chromatogram. The latter, when present at 1% w/v in the mobile phase (19ACN, 81 water, 6DMF) reduced the analysis time from 23 minutes (for mobile phase without the salt) to 9 minutes. However, the ET-TC peak separation was significantly better in the salt-free system, and it was necessary to compromise at an intermediate concentration. The salt, at 0.52 g diammonium phosphate per 100 mL ACN/water mixture, became a standard addition to all experimental phases.

It was found that additional marked improvement in the chromatogram could be affected by the addition of 5-10% DMF. This modifier was used by Knox and Jurand (13), who developed mobile phases based on simple mixtures of it with water. However, in the conditions used in this work, ACN could not be completely replaced by DMF to give satisfactory chromatograms. In particular, with DMF/water mixtures, AT and EAT were poorly resolved. When used at 5-10%, DMF brought about only small reductions in retention times, but had a striking effect in reducing tailing and in sharpening all peaks.

At this point, after evaluation of a number of chromatograms, the desired analysis time was fixed at seven minutes. Within that constraint, optimisation of the system with respect to ET-TC separation was undertaken by varying the ACN/water ratio and the DMF concentration.

Three different concentrations of DMF (6, 8 and 10 volumes per 100 volumes ACN/water mixture) were examined. At each DMF concentration, the ACN/water ratio was varied and the ET-TC peak separations were measured according to the method proposed by Morgan and Deming (14). The results are shown graphically in Figs. 1, 2 and 3. It is evident that the analysis times increase,

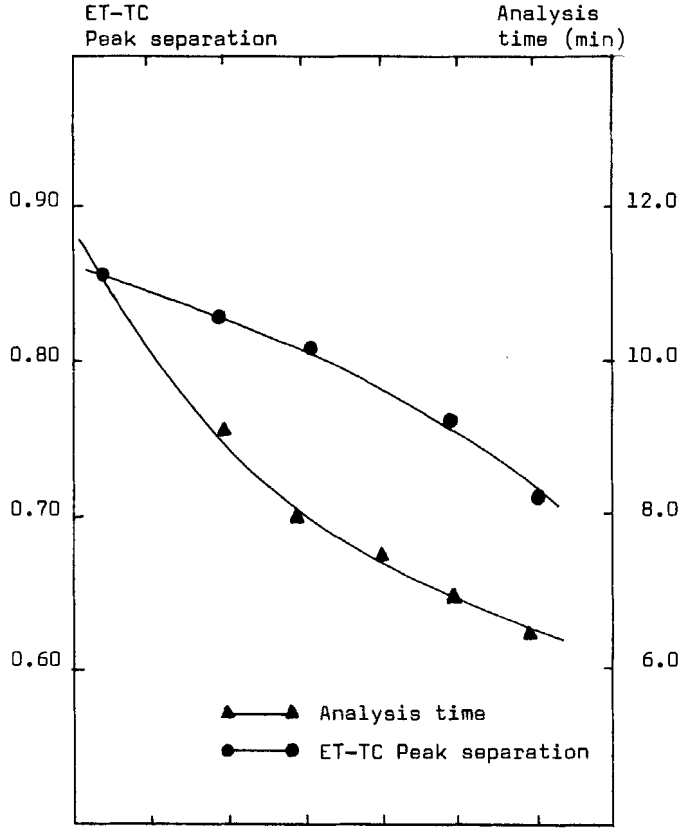

Figure 1

The dependence of ET-TC peak separation and analysis time on the ACN to water ratio of the mobile phase; DMF concentration constant at 6 volumes per 100 volumes ACN/water mixture.

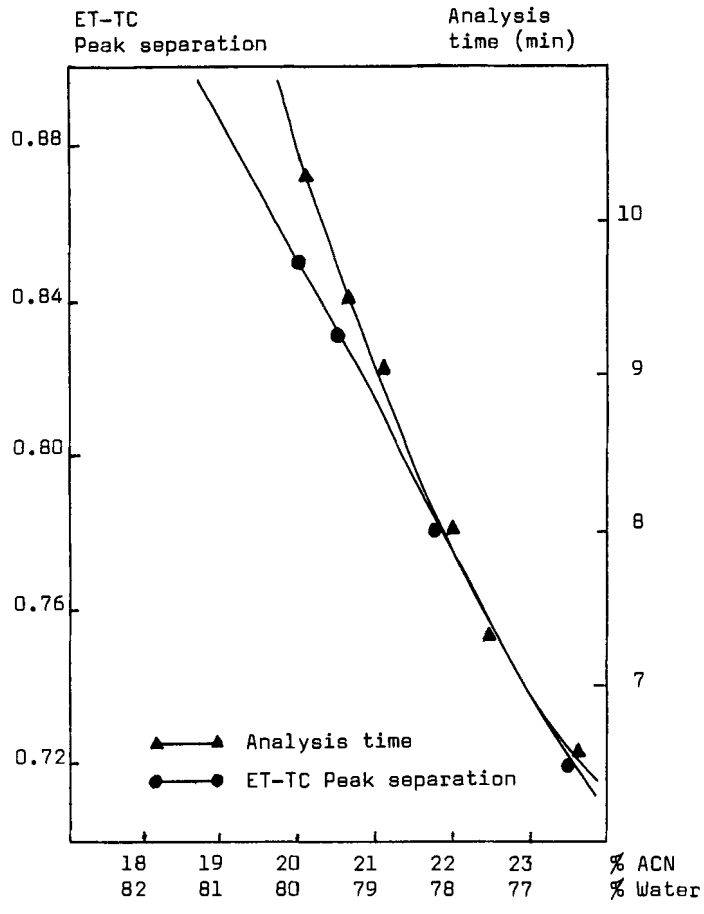

Figure 2

The dependence of ET-TC peak separation and analysis time on the ACN to water ratio of the mobile phase; DMF concentration constant at 8 volumes per 100 volumes ACN/water mixture.

and the ET-TC separations improve, with increasing water content. The relationship between analysis time and peak separation for the three DMF concentrations is shown in Fig. 4. For the desired analysis time of seven minutes, a mobile phase containing 6 volumes DMF per 100 volumes ACN/water mixture provides better peak separa-

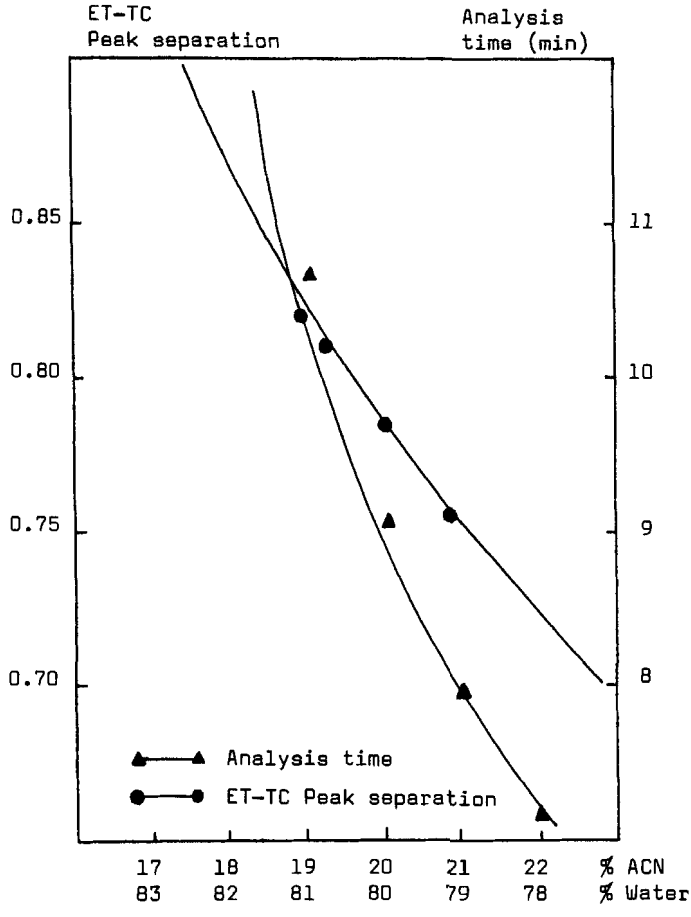

Figure 3.

The dependence of ET-TC peak separation and analysis time on the ACN to water ratio of the mobile phase; DMF concentration constant at 10 volumes per 100 volumes ACN/water mixture.

tion than the higher DMF concentrations. Reference to Fig. 1 indicates that the ACN to water ratio should be approximately 24 to 76 to achieve the required analysis time.

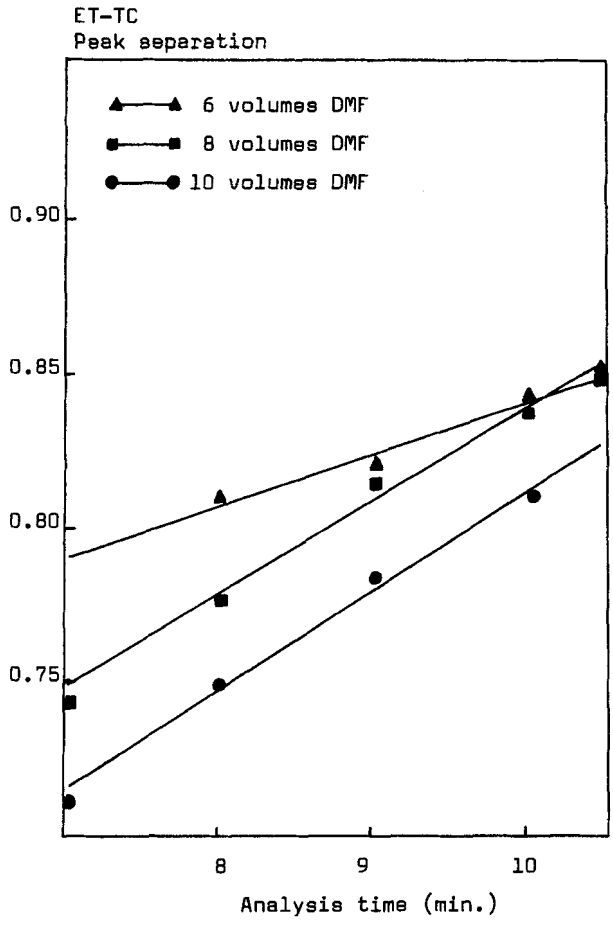

Figure 4.

The relationship between ET-TC peak separation, DMF concentration in the mobile phase and the analysis time.

(b) Analysis of Capsule Samples by HPLC

A weighed quantity of the mixed contents of 20 capsules equivalent to about 250 mg TC was shaken with 60 mL anhydrous methanol for 20 minutes. For capsules containing TC base,

sufficient 1M hydrochloric acid should be added to the suspension to convert the base to hydrochloride, as solutions of the base are relatively unstable. Some samples required brief untrasonication in order to complete the solution of the tetracycline. After making the volume to 100 mL, the solution was filtered to remove insoluble excipients (Filtrate A).

For the determination of tetracycline, filtrate A was diluted 1 to 5 with anhydrous methanol, and the diluted solution mixed 1 to 1 with SIS just before injection.

For the determination of related compounds, filtrate A was mixed 1 to 1 with WIS just before injection.

The standard solution contained in 100 mL anhydrous methanol: 50 mg TC, 20 mg ET (ammonium salt), 10 mg CT, 2.5 mg AT and 2.5 mg EAT. For the determination of tetracycline, the standard solution was mixed 1 to 1 with SIS, and for the determination of related compounds, it was mixed 1 to 1 with WIS. As with the sample solutions, the mixing with the internal standards was carried out just prior to injection, since there was a slow increase in the ET content (about 10% in 2 hours) of the mixed solutions. Three successive injections of each mixed solution were made (standard and sample), in order to determine the mean ratio of TC (or related compound) to internal standard, before discarding the solution. The unmixed standard solution in methanol was stable for at least six hours and therefore one preparation was used for a number of samples.

(c) Analysis of Bulk Substances by HPLC

Solutions of TC bulk substances for HPLC were prepared to contain 500 mg TC in 100 mL anhydrous methanol. In the case of TC base, sufficient 1M hydrochloric acid was added to the solution to effect conversion to the hydrochloride. For determination of TC, the solution was diluted 1 to 10 with methanol before mixing 1 to 1 with SIS. For determination of related compounds, the solution was mixed 1 to 1 with WIS. The standard solution and its dilutions were those specified above for the analysis of capsules.

(d) Chromatographic Conditions

The chromatographic conditions were as follows:

Temperature	:	Ambient (20^{o} - 22^{o}C)
Flow rate	:	2.5 mL/min
Chart speed	:	1 cm/min
Detector	:	280 nm 0.5 AUFS for tetracycline 0.1 AUFS for related compounds
Injection volume	:	10 µL

Quantitation was effected via peak heights, these being measured directly from the recorder chart graduations.

(e) Microbiological Assays of Capsules

Microbiological assays for TC were carried out on the capsules using the large plate agar diffusion system, in which the standard and unknown preparations were contrasted at two corresponding dose levels (3.0 and 0.75 units/mL).

The TC standard and the capsule contents were dissolved in 0.01 M hydrochloric acid, and diluted to the assay working doses with phosphate buffer pH 5.8.

A seed layer of Medium C, pH 6.6 (BP A 67) was inoculated with a spore suspension of Bacillus cereus ATCC 11778 and the antibiotic solutions applied to the surface of the medium by the use of Schleicher and Schuell 740-E, 12.7 mm diameter paper discs.

The statistical validity of the test results, the potency of the unknown and the fiducial limits of error of the potency estimate were calculated from the 64 responses (zones of inhibition) obtained from each plate assay.

(f) TLC Examination of Capsules

The capsule samples were examined for the presence of related compounds by TLC on kieselguhr, using the method of Murray (15). The sample solutions examined were those prepared for the HPLC

analyses (Filtrate A), and the standard solutions contained 2.5 mg AT, 2.5 mg EAT, 10 mg CT and 20 mg ET (ammonium salt), each separately dissolved in 100 mL anhydrous methanol. These standard solutions correspond to proposed limits of 1% for AT and EAT, 4% for CT and 8% for ET. The intensities of the spots from the samples were compared with those from the standards, and were arbitrarily rated as follows:

0 - no spot detected
1 - faint compared with standard
2 - moderate compared with standard
3 - intense, but less than standard
4 - more intense than standard

(g) Precision and Statistical Evaluation

In order to establish the precision of the HPLC assay method for TC, ten determinations were carried out on Sample 1 over a period of three days.

A statistical comparison of the two assay methods was made, using the paired t-test and by simple regression.

RESULTS & DISCUSSION

A chromatogram of a mixture of TC, all the related compounds, and the internal standard is shown in Fig. 5.

Calibration studies showed that the detector responses were linear for all five compounds of interest, up to the following concentrations, in mg/mL:

TC 0.65, ET 0.60, AT 0.038, EAT 0.038, CT 0.15

These concentrations represent 130% of the expected (label strength) for TC in capsules, 300% of the proposed upper limit for ET, and 150% of the proposed upper limits for AT, EAT and CT in

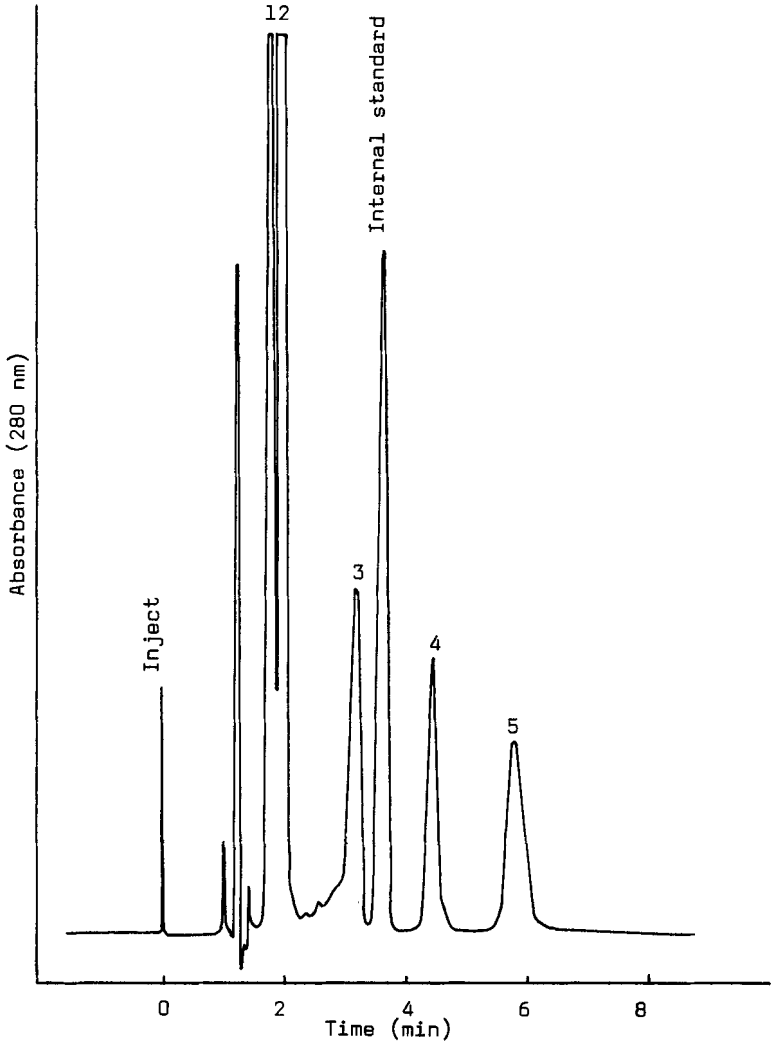

Figure 5

HPLC separation of tetracycline (peak 2) and its related compounds (4-epitetracycline, peak 1; chlortetracycline, peak 3; 4-epianhydrotetracycline, peak 4; anhydrotetracycline, peak 5).

Chromatographic conditions are described in the text

capsules. The linearity of response indicates that the method is valid for the determination of all compounds in a formulation, providing there is no interference from excipients. Confirmatory evidence for the validity of the method as applied to capsules is afforded by the results of the microbiological assay and the TLC examinations. These are presented, together with the HPLC results, in Table 1.

The chromatograms of samples 11 and 12 included peaks which were initially interpreted as being from CT, and the peak heights indicated contents of 1.00% and 1.50% respectively in the samples. However, no spots for CT were detectable in the TLC, and a closer examination of the HPLC chromatogram showed that the retention time of the compound in question was 0.1 minute longer than that for CT. The identity of the compound was not established.

The results of the ten HPLC assays on capsule Sample 1 ranged from 97.3% to 98.8% label strength, with a mean of 98.18% and a coefficient of variation of 0.48%.

In the statistical comparison of the HPLC and the microbiological assay data, the paired t-test gave a value for t of 0.508. A linear regression line, constrained to pass through the origin, when fitted to the data, resulted in the equation $Y = 1.0033\ x$, where x is the potency estimated by the microbiological assay and y is the HPLC assay. These results suggest that there is no significant difference in the two methods.

Comparison of the TLC and HPLC results for related compounds indicates excellent agreement in the visual appraisal of TLC spots and the quantitation of peak heights in HPLC. The latter procedure is clearly superior with respect to speed and precision, but TLC may be valuable in certain circumstances for identification purposes, since observations may be made on both R_f value and the colour of the fluorescent spot; in HPLC only the retention time is useable for identification. It is suggested that HPLC is the method of choice for determining

TABLE 1

HPLC Analyses, Microbiological Assays and TLC Examination of 13 TC Capsule Samples

Sample	H.P.L.C. Analysis, percent					Microbiological Assay Percent	T.L.C. Examination Spot Intensity Relative to Standard			
	TC[b]	AT[c]	EAT[c]	CT[c]	ET[c]		AT	EAT	CT	ET
1	98.6	0.40	0.05	ND	4.52	96	2	1	0	3
2	95.8	0.54	0.15	ND	6.90	96	2	1	0	3
3[a]	106.1	0.33	0.06	ND	6.29	103	2	1	0	3
4	102.8	0.46	0.08	ND	4.97	101	2	1	0	3
5	105.3	0.40	0.07	ND	3.62	102	2	1	0	3
6[a]	102.7	0.45	0.11	ND	6.11	101	1	1	0	3
7[a]	101.8	0.32	0.06	ND	5.42	102	2	1	0	3
8[a]	96.2	0.38	0.06	ND	5.34	98	2	1	0	3
9[a]	100.2	0.70	0.23	ND	4.55	101	3	1	0	3
10	95.2	0.98	0.25	ND	6.00	96	3	1	0	3
11[a]	96.2	1.03	0.23	ND	8.42	96	3	1	0	4
12[a]	88.8	1.52	0.34	ND	9.46	92	4	2	0	4
13	98.9	2.25	1.42	0.53	4.63	101	4	3	2	2

a Buffered Sample
b Mean of 3 determinations, expressed as % of label
c Expressed as % of assayed TC
d For ratings, see text
ND Not detected

related compounds, and that the TLC examination should be used for confirmation of identity where doubt exists.

The proposed method determines TC and the related compounds by means of two chromatograms obtained at different detector sensitivities on one channel at 280 nm. Where a dual channel detector is used, it is possible to simultaneously determine TC and the related compounds, by employing different sensitivities for the channels, both set to 280 nm. Under such conditions it would be valid to use the internal standard peak heights on the first channel (TC) for the quantitation of the related compounds on the second channel. For the quantitation of the related compounds it is desirable for the sample and standard solutions to be of similar composition. Thus an appropriate standard solution for the simultaneous determination of TC and related compounds in a bulk substance would contain 50 mg TC, 0.25 mg AT, 0.25 mg EAT, 1.00 mg CT and 2.00 mg ET (ammonium salt) in 100 mL anhydrous methanol. These quantities of the related compounds are only 10% of those used in the standard solution recommended for the two chromatogram system. The TC standard would contribute significant amounts of the related compounds to the standard solution; these amounts should therefore be determined with suitable precision so that accurate determination of the related compounds is assured. Where samples are subject only to a limit test for the related compounds, the single chromatogram system could be used for rapid screening purposes.

With regard to the reverse phase column life, there was no detectable drop in plate count in the column used for much of this work, after more than 400 injections. The column was dedicated to tetracycline work, and was well washed with water and then 70% methanol after each day's use. These factors, together with the restriction of the mobile phase to pH 2.5, may be responsible for the excellent stability of the column.

It is planned to investigate the application of this method to other TC formulations (ointments, oral suspensions, injections), to tetracycline - nystatin formulations and to other tetracycline antibiotics and their formulations.

ACKNOWLEDGEMENTS

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