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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ASSAY OF OXYTETRACYCLINE

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SUMMARY

The British Pharmacopoeia monograph for oxytetracycline calcium describes an high-performance liquid chromatographic (HPLC) assay which requires packing of the column by the analyst. Presented in this report is an HPLC method for the assay of oxytetracycline which employs a commercially available reversed-phase column and a solvent system which gives improved separation of the antibiotic from common impurities. Results obtained using this method for both bulk and dosage forms of oxytetracycline are in accord with the results of the microbiological assays.

INTRODUCTION

Although the tetracycline group of antibiotics are subject to assay microbiologically, the British Pharmacopoeia monograph for oxytetracycline calcium includes an high-performance liquid chromatographic (HPLC) assay[1]. However, the column packing specified is a silica based strong cation exchanger which is not available commercially and must be prepared by the analyst. Since large differences in the retention time of tetracyclines using support material from different suppliers have been reported[2] and in order to avoid the inconvenience of column-packing and to reduce the possibilities of inter- and intra-laboratory variation which this causes, it seemed desirable to develop a method based on a commercially available packing. This report describes the application of an HPLC assay included in the United States Pharmacopeia for minocycline[3], to the assay of oxytetracycline and oxytetracycline-containing products.

EXPERIMENTAL

The HPLC system consisted of a Gilson 302 pump (Anachem, Luton, U.K.) and a Cecil 212 variable-wavelength detector (Cecil Instruments, Cambridge, U.K.) with computerised integration and data handling facilities (LDC 301, Laboratory Data Control, Stone, U.K.). All solvents were degassed with helium.

The British Pharmacopoeia method employed a 5-μm strong cation-exchange

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column (200 × 5 mm I.D.) with a mobile phase consisting of methanol (350 ml) and potassium dihydrogen phosphate (3.4 g) and sodium edetate (9.3 g) dissolved in 650 ml water, adjusted to pH 5.3. Sulphadimidine (0.3 g/l in methanol) was used as the internal standard. The flow-rate was 0.7 ml min^{-1} and the detection wavelength 263 nm.

For the present HPLC method, the system was fitted with a LiChrosorb RP-8 10-μm pre-injection guard column (30 × 4.5 mm I.D.). Samples (10 μl) were filtered through a 0.45-μm filter and applied through a loop injector (Rheodyne 7125) onto a Spherisorb S5 (5 μm) C_8 column (200 × 4.6 mm I.D.) (Phase Separations, Gwent, U.K.). The solvent system, 0.2 *M* ammonium oxalate–0.1 *M* Na_2EDTA–dimethylformamide (55:20:25) was pumped at a flow-rate of 1 ml min^{-1}. A detection wavelength of 280 nm was used. Paracetamol recrystallized from methanol (1.15 g/l methanol) was used as the internal standard.

Samples were prepared at concentrations of 50 mg/ml for qualitative assays and 0.25 mg/ml for quantitation of oxytetracycline in the sample. A higher concentration was used for quantitative tests so that impurities could be detected. Two dosage forms, capsules and tablets (oxytetracycline hydrochloride and oxytetracycline dihydrate respectively) and samples of bulk material (hydrochloride, dihydrate and calcium forms) were assayed using the above method.

Samples were prepared from oxytetracycline tablets as follows: ten tablets were ultrasonicated for 20 min in 0.1 *M* hydrochloric acid in methanol–water (15:85) (500 ml) until completely dispersed. A volume of 5 ml of the resulting suspension together with 5 ml methanol, 5 ml 0.1 *M* hydrochloric acid in methanol and 20 ml internal standard were made up to 100 ml with methanol–water (15:85). The capsules were assumed to contain 250 mg of oxytetracycline hydrochloride and samples were prepared in the same way as the tablets. The 2nd International Standard (NIBSC, Hampstead, London, U.K.) of oxytetracycline dihydrate was used as the assay standard and was made up in 10 ml 0.1 *M* hydrochloric acid in methanol, to which 20 ml internal standard were added followed by methanol–water (15:85) to make the volume up to 100 ml, giving a final concentration of 0.25 mg/ml. The bulk samples were made up in the same way as the assay standard.

Microbiological assays were carried out using *Bacillus pumilus* NCTC 8241, following the method recommended in the British Pharmacopoeia[1].

RESULTS AND DISCUSSION

Spherisorb S5 C_8 was used throughout this work as it was found to be superior in performance to μBondapak C_{18} 5 μm, which was selected as a representative C_{18} packing. Better separation of oxytetracycline from its common contaminants was achieved under the conditions described, compared to that achieved with the British Pharmacopoeia method (Fig. 1). Improved peak symmetry and reduced base width of the oxytetracycline peak are also features of the present method, as is the improved resolution of the impurities. Authentic samples were used to identify contaminants. A linear relationship was obtained between both peak height and oxytetracycline concentration and peak area and oxytetracycline concentration up to 4 mg/ml. The repeatability of the system was determined for peak areas of six replicate injections and gave a standard deviation of 2.4%; reproducibility measured over six days for

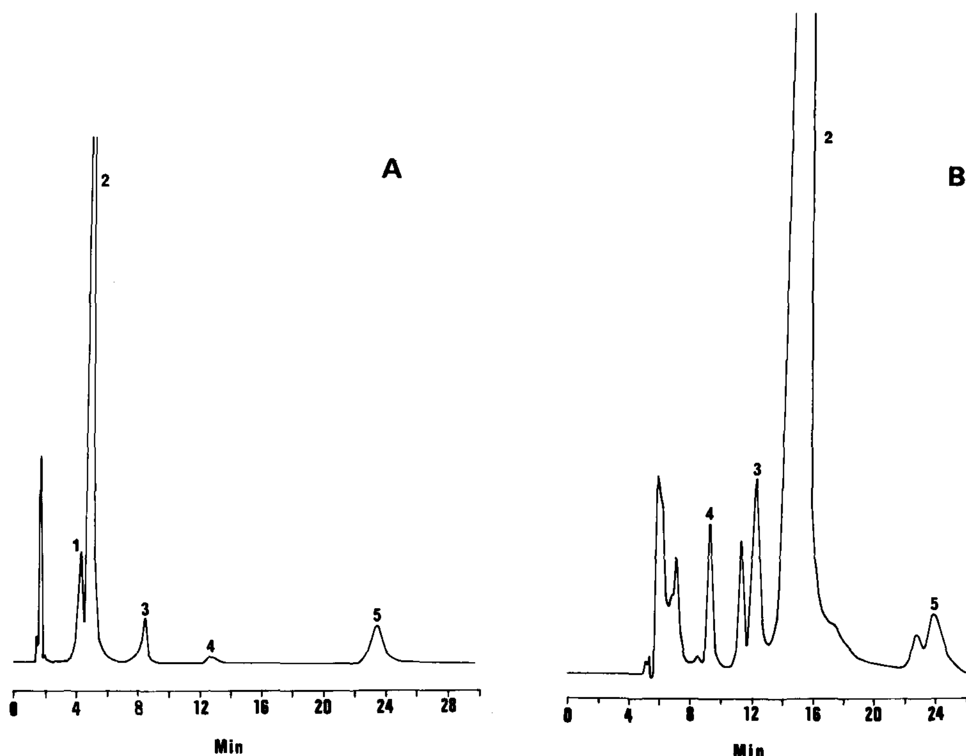

Fig. 1. HPLC separation of oxytetracycline and common contaminants. (A) by the present method, (B) by the British Pharmacopoeia HPLC method. Peaks: 1 = 4-epi-oxytetracycline, 2 = oxytetracycline, 3 = α-apo-oxytetracycline, 4 = β-apo-oxytetracycline; 5 = anhydro-oxytetracycline.

six independent assays gave a standard deviation of 1.52%. Repeatability and reproducibility of the British Pharmacopoeia method determined in the same way, gave standard deviations of 5.3% and 5.2% respectively, however, column efficiency may have been reduced by the age of the packing material.

Nine commercial samples of oxytetracycline tablets (dihydrate form) were prepared and assayed as described above; the results were compared with those obtained using the British Pharmacopoeia HPLC method and a microbiological assay (Table I). Results calculated from both peak heights and peak areas in the present method, with the exception of samples 3 (height) and 6 and 8 (area), all fall within the fiducial limits. In contrast none of the results calculated from peak heights or areas obtained using the British Pharmacopoeia HPLC method, except samples 5 (height) and 7 and 9 (height and area), fall within the fiducial limits defined by the microbiological assay. Three samples of capsules (hydrochloride form) were also assayed and the results compared with those of the microbiological assay (Table II).

Three different batches of bulk oxytetracycline in the hydrochloride, dihydrate and calcium forms were also assayed and the results compared with those from the biological assay (Table III). The HPLC assay results were calculated from the ratio of peak height or peak area of the standard and test samples, × 880, which is the potency (I.U./mg) of the 2nd International Standard.

TABLE I

COMPARISON OF OXYTETRACYCLINE CONTENT OF NINE DIFFERENT SAMPLES OF TABLETS (250 mg) USING THE BRITISH PHARMACOPOEIA HPLC METHOD, THE PRESENT HPLC METHOD AND MICROBIOLOGICAL ASSAY (mg/TABLET)

Sample	*British Pharmacopoeia HPLC method*		*Present HPLC method*		*Biological assay mean (fiducial limits)*
	Peak area	*Peak height*	*Peak area*	*Peak height*	
1	–	–	258.3	254.7	251.3 (242.6–260.3)
2	–	–	267.6	264.9	256.5 (246.0–267.4)
3	244.3	245.7	256.8	251.4	261.0 (254.6–267.4)
4	257.4	256.7	274.2	274.2	270.3 (263.5–277.1)
5	281.2	270.7	280.2	281.3	275.6 (269.4–282.0)
6	255.1	251.9	276.9	279.7	274.4 (270.1–278.8)
7	261.3	263.6	256.0	258.2	264.7 (255.7–274.1)
8	272.0	262.3	262.7	–	256.2 (250.8–261.8)
9	240.1	242.7	255.7	251.8	247.8 (240.0–255.7)

TABLE II

COMPARISON OF PRESENT HPLC METHOD WITH MICROBIOLOGICAL METHOD (mg/CAPSULE) FOR THE ASSAY OF OXYTETRACYCLINE HYDROCHLORIDE IN CAPSULES (250 mg)

Sample	*Present HPLC method*		*Biological assay mean (fiducial limits)*
	Peak area	*Peak height*	
1	260.6	255.6	249.4 (242.4–256.7)
2	262.4	256.8	246.5 (241.7–251.4)
3	254.1	246.4	250.6 (246.7–254.5)

The results presented in both Tables II and III compare favourably with those obtained using a microbiological assay and there seems to be no significant advantage in using peak area in preference to peak height for calculating potencies.

Comparison of capacity ratios (British Pharmacopoeia method: 4.8; present method: 2.2) and theoretical plate values (British Pharmacopoeia method: 6332 m^{-1}; present method: 29 687 m^{-1}) indicates that the reversed-phase column gives superior resolution to the ion-exchange column employed in the British Pharmacopoeia method for assay of calcium oxytetracycline (Fig. 2). This suggests that the present method may have greater potential for resolving impurities than the current official method.

It has been reported that the use of organic ammonium compounds decreases retention times of tetracyclines[2] and that the presence of oxalic acid improves separation[4]. In the present method the use of ammonium oxalate in the solvent confers both these advantages on the system. Also, the use of EDTA in the solvent system gave results which support previous findings[4,5] that tailing of peaks is reduced in the presence of EDTA by preventing possible chelation of tetracycline with metal ions and subsequent adsorption to the column.

TABLE III

COMPARISON OF OXYTETRACYCLINE CONTENT OF BULK MATERIAL IN HYDROCHLORIDE, DIHYDRATE AND CALCIUM FORMS USING THE PRESENT HPLC METHOD AND BIOLOGICAL ASSAY

Values in I.U./mg; based on the ratio of standard: test height or area ×880 (unit/mg 2nd International Standard).

Sample	*Present HPLC method*		*Biological assay mean (fiducial limits)*
	Peak area	*Peak height*	
Hydrochloride			
1	854.2	861.3	869.1 (855.4–882.7)
2	904.6	913.4	904.5 (882.7–927.3)
3	857.7	871.7	895.4 (873.0–918.2)
Dihydrate			
1	879.6	882.3	878.3 (867.8–889.0)
2	878.1	900.4	895.3 (877.7–913.2)
3	892.0	910.0	887.3 (870.9–902.7)
Calcium			
1	682.4	693.1	691.5 (667.6–716.3)
2	681.8	696.0	694.6 (678.3–711.2)
3	770.1	765.9	754.1 (743.6–764.6)

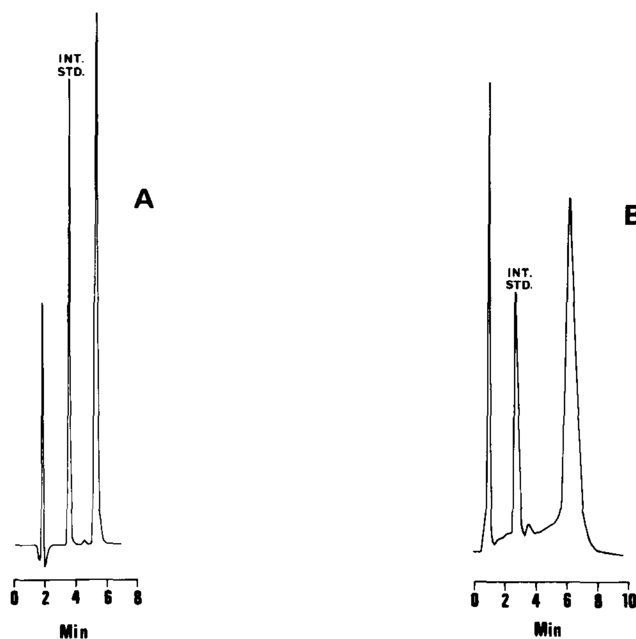

Fig. 2. HPLC chromatogram of oxytetracycline using (A) the present HPLC method and, (B) the British Pharmacopoeia HPLC method. INT. STD. = Internal standard.

Optimisation of solvent conditions, the use of a commercially available column which will reduce variation between assays, and the ready availability of paracetamol as an internal standard are advantages which may make the method reported here a suitable alternative to the biological assay for both bulk and dosage forms of oxytetracycline.

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