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Rapid liquid chromatographic method for simultaneous determination of tetracyclines antibiotics and 6-Epi-doxycycline in pharmaceutical products using porous graphitic carbon column

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Abstract

A rapid and selective high performance liquid chromatographic (HPLC) method has been developed for the separation and determination of five commercially used tetracyclines. The chromatography was performed on a porous graphitic carbon (PGC) column, using 0.05 M potassium phosphate buffer (pH 2.0) — acetonitrile (40 + 60) as the mobile phase and ultraviolet detection at 268 nm. The method permits the simultaneous determination of oxytetracycline, metacycline, chlortetracycline and doxycycline as well as the separation of one of their common impurities (6-Epi-doxycycline) in bulk powder and pharmaceutical preparations with detection limits of 0.5–2 μg ml^{-1} and recoveries of 98.9–100.5%. Correlation coefficients for calibration curves in the range of 5–50 μg ml^{-1} were greater than 0.999 for all tetracyclines. The within- and between-day precision was determined for both retention times and peak area. It is suggested that the proposed HPLC-PGC method should be used for routine quality control and dosage form assay of tetracyclines in pharmaceutical preparations. The chromatographic behaviour of the five tetracyclines was examined under variable mobile phase compositions, the results revealed that elution order and selectivity were dependent on the buffer agent used. Comparison between retentions obtained with PGC and with silica-based stationary phase (ODS), showed similar variations of the capacity factors with the mobile phase composition, but with a different elution order. © 2000 Published by Elsevier Science B.V.

Keywords: Porous graphitic carbon column; Tetracyclines; 6-Epidoxycycline; Isocratic elution

1. Introduction

The tetracyclines are referred to as broad-spectrum antibiotics, due to their ability to fight many different types bacteria (Gram-positive and Gram-negative bacteria), being used in human

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and veterinary medicines as well as feed additives. Many tetracyclines are commercially available, such as oxytetracycline, methacycline, chlortetracycline and doxycycline that are permitted for human administration. Under abnormal conditions (heat, pH, humidity), tetracyclines undergo reversible epimerisation at position C-4 and C-6 to form a mixture of degradation products. These degradation products or contaminants of tetracyclines have very low antibiotic activity [1], in addition some of them have shown certain toxicity [2]. Therefore, it is very important that their contents can be controlled simply and precisely.

Many high performance liquid chromatographic (HPLC) methods have been described for the determination of common tetracyclines antibiotics using silica gel [3,4], silica-based and polymer-based stationary phases [5–15]. One of the main reasons is the increasing interest in finding robust systems for evaluation of different pharmaceuticals and their contaminants. These methods are time consuming in addition the alkyl bonded silica based stationary phases suffer from a number of drawbacks, including poor stability at extremes of pH and a variety of unwanted interactions due to the surface heterogeneity [16]. Furthermore, the use of polymeric columns has been mostly in conjunction with elevated column temperature, and required the use of viscous organic modifier, and alkaline mobile phases containing additives such as tetrabutylammonium ion pairing agent or EDTA [17,18].

However, these separations and reproducibility levels have also not been satisfactory due to the instability of packing materials and the complicated separation systems; in addition, the highly stable and selective porous graphitic carbon (PGC) is preferable for the separations and determination of these compounds. PGC was introduced as an alternative for HPLC [19,20]. This material has several advantages, most notably its physical and chemical stability as well as superior selectivity towards diasteriomers and geometric isomers [21]. PGC has successfully been used in the separation of drugs and pharmaceuticals [22–27] and also is most applicable to the separation of small ionisable molecules which are not retained on octadecylsiloxane (ODS) columns [28].

This paper describes a rapid and selective HPLC method for simultaneous separation and determination of tetracycline antibiotics and one of their common impurities 6-Epi-doxycycline (6-EpD) using PGC column. This proposed method designed to be suitable for the quality assessment of these drugs in pharmaceutical products. The effect of the mobile phase composition in the retention and elution order of tetracyclines on PGC was further investigated.

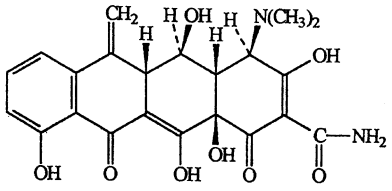
2. Experimental

2.1. Chromatography (instrumentation and conditions)

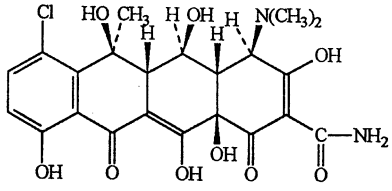
The analytical separation was carried out with a gradient modular HPLC system equipped with an UV 166 variable wavelength spectrophotometer (Beckman Instruments Inc., USA). The detector wavelength was set at 268 nm as required by different analytes. A flow rate of 1 ml min^{-1} was used for the separation of tetracyclines. The column was carbon column (100 × 4.6 mm i.d., 7 μm particle size) packed with Hypercarb porous graphitic carbon (Shandon, Runcorn, UK) was used for retention measurements. The mobile phase composed of two components, acetonitrile and buffer solutions with different ratios. The buffer solutions tested were potassium phosphate, trichloroacetic acid, acetic acid, sodium acetate and ammonium acetate with a concentration of 50 mM and a pH of 2.0 otherwise stated.

2.2. Reagents

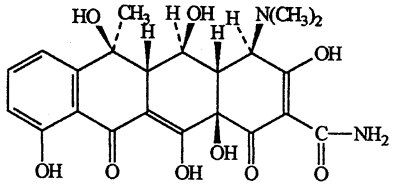
HPLC-grade acetonitrile was obtained from Prolabo (Paris, France). LC-grade water was prepared by purifying demineralised water in a Milli-Q filtration system (Millipore, Bedford, MA, USA). Reference standards for oxytetracycline-HCl (OTC), methacycline-HCl (MC), chlortetracycline-HCl (CTC) and doxycycline-HCl (DC) were kindly supplied by laboratory Ibn Al-Baytar (Tunis, Tunisia). The degradation product was obtained from Janssen Chimica (Beerse, Belgium). Dosage forms were obtained from local pharma-



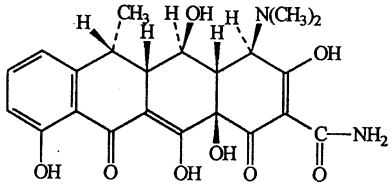
Méthacycline



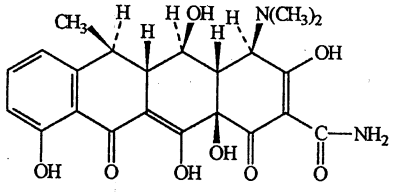
Chlortetracycline



Oxytetracycline



6-épidoxycycline



Doxycycline

Fig. 1. Structures of tetracycline compounds studied.

cies; Doxamycine Tablets '100 mg DC', Monocycline Tablets '200 mg DC', Vibramycine Tablets '200 mg DC', Oxytetracycline Tablets '250 mg OTC', Metacycline Tablets '200 mg MC' and Oxytetracycline ointment '3% OTC'. All other chemicals were of analytical grade and were obtained from Prolabo. The tetracycline compounds studied are shown in Fig. 1.

2.3. Preparation of solutions

Stock solutions containing 1 mg ml^{-1} of tetracyclines were prepared in the mobile phase and stored at 4°C. The working standards (5–50 μg ml^{-1}) were prepared from the stock solutions by dilution with the appropriate volume of the mobile phase. Drug tablets were prepared by crushing 20 tablets and an accurately weighed portion of the mixed powder equivalent to the antibiotic content of one tablet was transferred to 100 ml volumetric flask and dissolved by sonication. The sample was filtered and diluted to make a final concentration in the range of 10–20 μg ml^{-1}. Drug ointments were prepared by dissolving in cyclohexane and the drug was extracted from the organic phase with the buffer solution of the mobile phase. All tetracycline compounds were protected from light.

3. Results and discussion

3.1. Chromatography

By appropriate choice of mobile phase conditions, the baseline separation of the five tetracyclines was achieved using PGC column (Fig. 2). The analysis time of the five tetracyclines on PGC was very short with a maximum retention time of 11.2 min. These separation conditions are much better than the separation obtained by reversed phase ODS columns [7], by the United States Pharmacopoeia XXIII [29] or by the British Pharmacopoeia 1993 [30] official methods. Improved peak symmetry and reduced peak widths are also features the present method along with the improved resolution of the tetracycline from their impurities (see Table 2). This method can be used

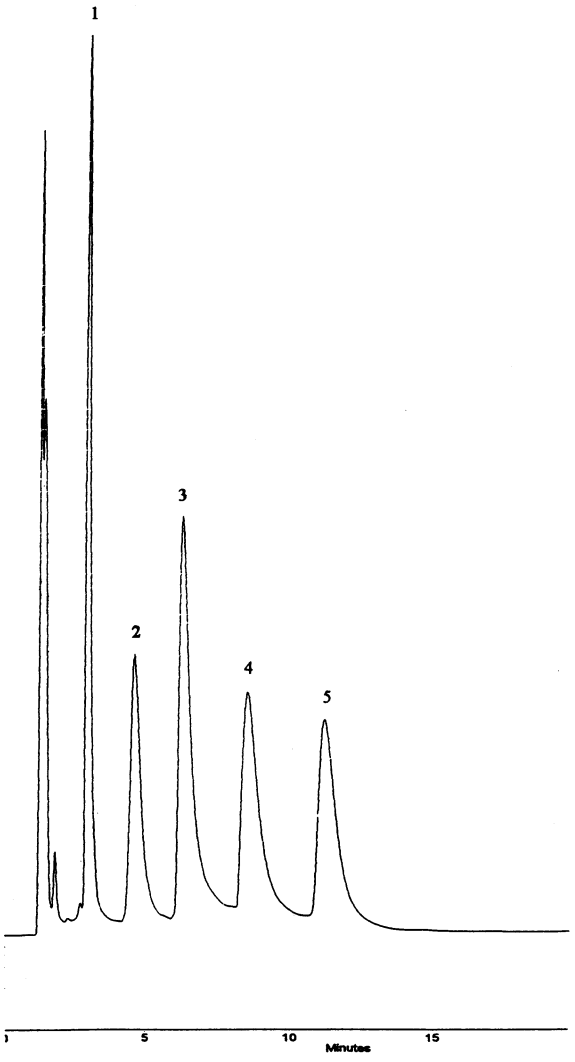


Fig. 2. The separation of various tetracycline compounds on a porous graphitic carbon column. Column: 100 × 4.6 mm i.d. packed 7 μm porous graphitic carbon. Eluent: potassium dihydrogenophosphate buffer (50 mM, pH 2.0) containing 40% (v/v) acetonitrile. Flow rate 1 ml min^{-1} and injection volume 20 μl. Peak1: OTC, Peak2: 6-EpD, Peak3: MC, Peak4: CTC, Peak5: DC.

for controlling tetracyclines contaminates within the limits that specified in the current official USP and BP methods.

The main advantage of PGC method is that the results (replicates of samples and standards) can be obtained rapidly in less than 1 h. Furthermore, this column showed fast equilibrium with the changes in the mobile phase composition and a high stability during the analysis.

3.1.1. Comparison of mobile phase effects

The effect of potassium phosphate, trichloroacetic acid (TCA), acetic acid, sodium acetate and ammonium acetate buffers (pH 2.0) on the retention of tetracyclines on PGC were studied, the results are summarised in Table 1.

Initially, a phosphate buffer (50 mM) containing different ratios of acetonitrile (35–60% v/v) as the mobile phase was tested for the chromatography of OTC, 6-EpD, MC, CTC, DC. The higher the ratio of the organic modifier in the mobile phase, the lower the retention time of the tetracycline compounds. The retention time of doxycycline decreases from 22.1 min at 35% acetonitrile to 4.2 min at 50% acetonitrile, but this ratio (50%) was not effective for the separation of all tetracycline compounds. Therefore, 40% of acetonitrile was selected as the ratio of the organic modifier for the baseline separation of the five tetracyclines (Fig. 2).

Using the same organic phase ratio, the retention order of tetracyclines with phosphate and trichloroacetic acid buffers (50 mM) were similar, OTC, 6-EpD, MC, CTC, DC. These compounds were retained to a higher extent with the TCA buffer (Fig. 3). The chromatographic parameters of tetracyclines on PGC using the appropriate phosphate and TCA conditions were shown in Table 2. The resolution of these compounds on PGC was better achieved with phosphate buffer (R_s 2.1–2.7) than with TCA buffer (1.1–2.4). These results are within the validated standard values for antibiotics determination which require; a resolution between the target antibiotic and the contaminant (e.g. 6-EpD) peaks not less than 1.5, the column efficiency is not less than 1000 theoretical plates and the tailing factor is not more than 2.0.

The elution order of tetracyclines with acetic acid, sodium acetate and ammonium acetate buffers (50 mM) were similar, 6-EpD, MC, OTC, DC, CTC. However, this elution order was different to that obtained with phosphate and TCA buffers. With these buffers, OTC showed a higher retention than 6-Ep and MC and CTC was re-

tained longer than DC. The higher retention times for the tetracyclines were obtained with ammonium acetate followed by sodium acetate and acetic acid. These results indicated that acetic acid has a higher interaction with the PGC stationary phase than the other two buffers. Among the many mobile phases tested, phosphate buffer proved to be the eluent of choice for the PGC-tetracycline assay validation. A baseline resolution (R_s between 2.1 and 2.7) was achieved between

Table 1
Effects of buffer components on the retention factors (k') of tetracycline compounds on a porous graphitic carbon column[a]

Tetracycline compounds	Potassium phosphate	TCA	Sodium acetate	Ammonium acetate	Acetic acid
OTC	0.46	3.38	7.46	7.23	1.77
6-EpD	0.75	6.11	2.69	3.0	0.73
MC	1.13	8.92	5.77	5.54	1.15
CTC	1.62	12.61	13.62	13.23	3.92
DC	2.23	17.07	10.38	10.0	2.61

[a] Mobile phase 50-50 acetonitrile/buffer. Column size: 100 × 4.6 mm i.d., 7 μm, concentration of buffer 50 mM.

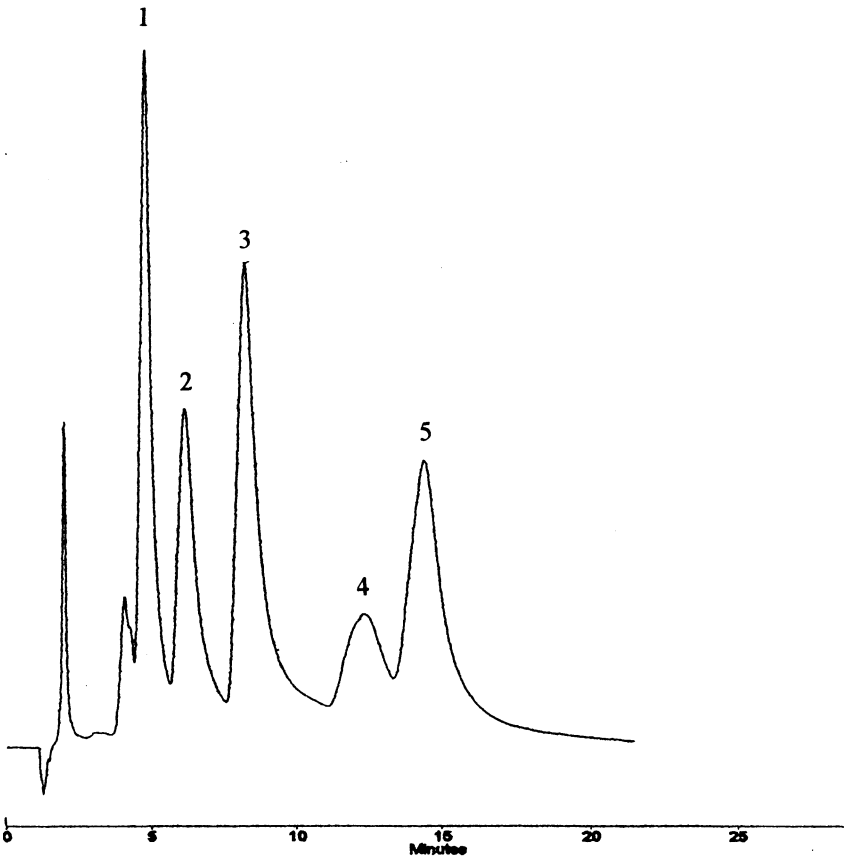


Fig. 3. The separation of various tetracycline compounds on a porous graphitic carbon column. Column: 100 × 4.6 mm i.d. packed 7 μm porous graphitic carbon. Eluent: trichloroacetic acid (50 mM, pH 2.0) containing 60% (v/v) acetonitrile. Flow rate 1 ml min^{-1} and injection volume 20 μl. Peak1: OTC, peak2: 6-EpD, Peak3: MC, Peak4: CTC, Peak5: DC.

Table 2
Chromatographic parameters of tetracyclines on PGC column using the optimum separation conditions: acetonitrile–phosphate buffer (pH 2.0) 40–60% v/v; and acetonitrile–trichloroacetic acid buffer (pH 2.0) 60–40% v/v[a]

Tetracycline compounds	KH_2PO_4				TCA			
	k'	A_s	N	R_s	k'	A_s	N	R_s
OTC	1.27	1.2	3220	2.7	2.77	1.3	1720	1.5
6-EpD	2.62	1.4	2710	2.1	3.62	1.5	1496	1.8
MC	3.85	1.6	2550	2.3	5.46	1.7	1280	2.4
CTC	5.54	1.6	2040	2.45	8.85	1.3	1020	1.1
DC	7.48	1.5	2965		10.38	1.2	1180	

[a] Column size: 100 × 4.6 mm i.d., 7 μm, concentration of buffer 50 mM.

the peaks of tetracyclines in a shorter retention time than that obtained with TCA. The PGC column efficiency is more than 1000 (2040–3220) theoretical plates and the tailing factor between 1.2 and 1.6.

3.1.2. Mobile phase pH

The high stability of the PGC material over a wide range of pH values makes it feasible to use the mobile pH to provide an effective means of varying selectivity and optimising separation. The pH of the mobile phase was chosen as 2.0 to minimise the formation of isomeric analogues, which occurs rapidly in alkaline medium [13]. Furthermore, at the alkaline pH, these compounds were most likely to be suppressed and their hydrophobocity increases and as a consequence these solutes will interact strongly with the hydrophobic surface of PGC leading to excessive retention. However, at acidic pH these compounds were in the ionised forms, therefore their hydrophobocity is decreased and so their retention. The effects of separating tetracyclines at high and low pH have been demonstrated by the separation of OTC antibiotic. At pH 7.5, OTC is eluted as a broad peak with long retention time ($\cong$ 12 min) while under acidic mobile phase conditions (pH 2.0), OTC was eluted with a satisfactory peak shape and retention time ($\cong$ 3 min). The range of pH stability of PGC columns (1–14) will thus allow a greater flexibility in optimising the separation of closely related compounds compared to the more limited pH range available on conventional bonded silica columns.

3.1.3. Comparison of retention effects on PGC and ODS columns

The retention of tetracycline compounds on PGC have been compared with ODS columns (μ-Bondapak and PLRP-S, 250 × 4.6 mm i.d.). The retention required for the separation of the tetracycline compounds by ODS columns was greater than 30 min when using isocratic elution of 84/16 phosphate buffer pH 2.5/acetonitrile [7] and greater than 22 min when using a gradient elution of oxalic acid — acetonitrile-methanol [6]. Furthermore, and under these chromatographic conditions the elution order of these compounds on ODS was; OTC, CTC, 6-EpD, MC and DC, where the elution order on PGC using a higher ratio of acetonitrile (40%) was; OTC, 6-EpD, MC, CTC and DC.

The difference in tetracyclines elution order between PGC and ODS suggest that the molecular interactions determining solute retention are different for the two materials. This difference is believed to be associated with the sensitivity of PGC toward the number of contact points or total contact area of the solutes with the surface of PGC and/or electronic interactions, which proved that the retention mechanism was different from that observed on silica based stationary phases [31,32]. These results indicates that PGC stationary phase has effectively proved to be unique, showing selectivity towards the separation of compounds difficult or impossible to achieve on silica, silica based or polymer based stationary phases. In addition to its high selectivity towards pharmaceuticals, PGC showed a faster equilibra-

tion time (< 5 min) when changing the mobile phase composition than ODS columns.

3.2. Validation of the analytical method

To validate our PGC-HPLC method, a series of tests were performed on the proposed method using the optimised chromatographic conditions chosen (Fig. 2).

3.2.1. Selectivity

The selectivity of the method for determination of tetracyclines (especially doxycycline) in the presence of degradation product was studied by spiking the tetracycline samples with 6-epidoxycycline. As shown in Fig. 2, there was adequate resolution of all these compounds, especially between DC and its degradation product 6-EpD. No interference of 6-EpD was detected for doxycycline or for other tetracyclines. The purity of doxycycline peak was verified using a diode array detector (Beckman Model 168). Furthermore, the selectivity of this method was assessed by analysis of standard solutions containing freshly prepared chlortetracycline and chlortetracycline that had been degraded in two different ways: after storage at room temperature for 1 week and after storage at room temperature for 4 weeks. Fig. 4 shows the presence of decomposition peaks along with undecomposed CTC. The areas of decomposition peaks (unknowns) increase as a function of time, in the mean time the peak area of undecomposed CTC decreases. So the proposed method is capable for resolving tetracyclines from the degradation products that could be formed during manufacture or storage. Further investigations are needed for the identification of the unknown CTC decomposition peaks.

3.2.2. Linearity

The calibration curves were constructed separately for each drug using the above mentioned conditions. The concentrations examined were between 5 and 50 μg ml^{-1} for OTC, MC, CTC and

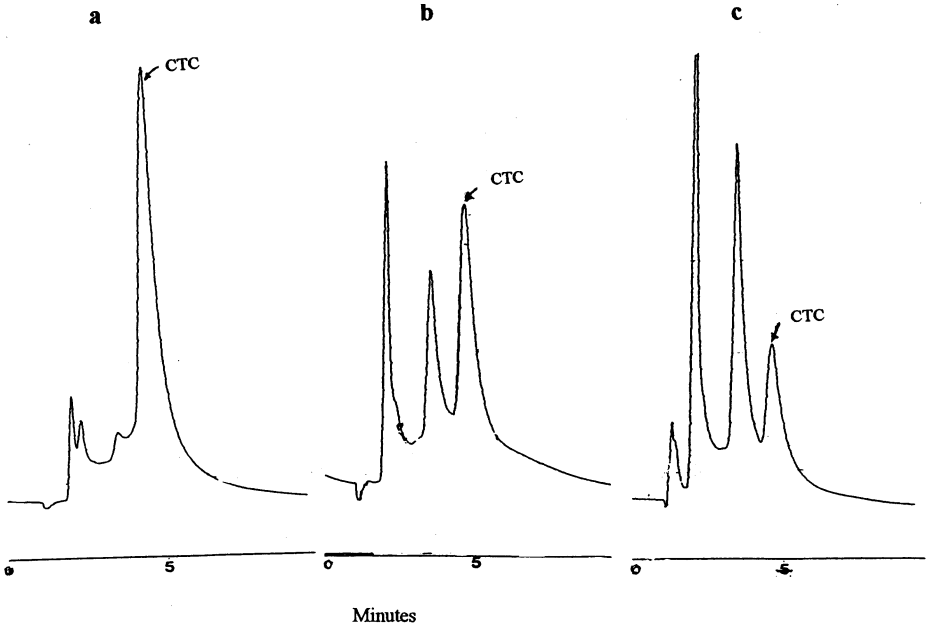


Fig. 4. A typical chromatogram from the analysis of chlortetracycline bulk drug substance in aqueous solutions: (a) immediate analysis after preparation of the solution; (b) after storage of the solution at ambient temperature for 1 week; (c) after storage of the solution at ambient temperature for 4 weeks. Column: 100×4.6 mm i.d. packed 7 μm porous graphitic carbon. Eluent: potassium dihydrogenophosphate buffer (50 mM, pH 2.0) containing 45% (v/v) acetonitrile. Flow rate 1 ml min^{-1} and injection volume 20 μl.

Table 3
Calibration curve parameters for the analysis of tetracyclines on PGC, obtained by linear regression analysis of peak area versus concentration in μg ml^{-1}

Tetracycline compounds	Slope	Intercept	R^2
OTC	27173	32040	0.9993
6-EpD	18268	1397	0.9990
MC	19921	7271	0.9993
CTC	17153	6546.3	0.9997
DC	8848	6883	0.9999

DC and 6-EpD. The correlation coefficients (R^2) of tetracyclines calibration curves (peak area versus concentration) were between 0.9993 and 0.9999. The equations of these curves ($y = mx + b$) were then used to calculate the unknown concentrations in the samples. The least squares linear regression analysis data are shown in Table 3.

3.2.3. Precision

The within- and between-day precision (expressed as the relative standard deviation (RSD)) of the proposed method was determined for both retention times and peak areas by repeated analysis ($n = 6$) of each tetracycline standard solution (Table 4). The within-day RSD values obtained for retention times were less than 1.0%, and for peak areas were between 1.55 and 2.0%. The between-day RSD values obtained for retention time were 0.7–1.2%, and for peak areas 1.7–2.6%.

3.2.4. Analytical recovery, reproducibility and limit of detection

The PGC-HPLC recovery assessment was performed by analysing real drug samples (tablets and ointments) spiked with known amounts (10 μg ml^{-1}) of drug standards. The recovery of OTC from drug ointment was 98.8% and the recoveries of other tetracyclines from tablets were 99.8–101%. The detection limit was measured by injection of 50 μl of standard solutions and the values of tetracycline compounds were 0.5–2.0 μg ml^{-1} (signal-to-noise ratio of 3:1). The reproducibility of the retention times and peak areas are summarised in Table 4.

3.2.5. Analysis of tetracycline formulations

The newly developed PGC-HPLC method was applied to the analysis of commercially available tetracycline formulations from different manufacturers. The formulations collected were treated as mentioned in Section 2.3, filtered through a filter of 0.45 μm before injection. The assay was repeated six times for each type of preparation. The quantitative results of these assays are summarised in Table 5. The results demonstrated that the content of active compounds correspond to each formulation label.

4. Conclusion

The proposed HPLC-PGC method showed the successful baseline separation of the five tetracy-

Table 4
Reproducibility, recovery and limit of detection of chromatographic analysis of tetracyclines using isocratic elution and a PGC column

Tetracycline compounds	Retention time[a] mean ± S.D. (min)	RSD (%)	Peak area[b] RSD (%)	Recovery[c] (%)	Limit of detection (μg ml^{-1})
OTC	2.95 ± 0.02	0.67	1.55	98.8	0.5
6-EpD	4.71 ± 0.04	0.85	1.79	99.1	1.2
MC	6.32 ± 0.04	0.64	2.0	99.8	1.2
CTC	8.51 ± 0.05	0.67	1.8	101.0	2.0
DC	11.20 ± 0.09	0.80	1.95	100.2	2.0

[a] Chromatographic conditions as in Fig. 2
[b] A total of 20 μl injection of 20 μg ml^{-1} solutions of tetracyclines ($n = 6$)
[c] A total of 20 μl injection of tetracycline samples spiked with 10 μg ml^{-1} standard tetracycline solutions ($n = 6$).

Table 5
Results of the analysis of different forms of commercialised drugs using PGC-HPLC method

Commercialised formulations		Label amount	% Found ± S.D. ($n = 6$)
Monocycline	DC	200 mg	98.8 ± 1.4
Doxamycine	DC	100 mg	97.9 ± 2.0
Vibramycine	DC	200 mg	100.5 ± 1.7
Mynocine	DC	100 mg	99.3 ± 1.2
Metacycline	MC	200 mg	98.8 ± 2.0
Oxytetracycline	OTC	250 mg	98.5 ± 1.6
Oxytetracycline ointment	OTC	3%	98.2 ± 1.9

clines in less than 12 min using isocratic elution. The best separation was achieved using 50 mM potassium phosphate buffer (pH 2.0) with 40% acetonitrile. These separation conditions are much better than that obtained by the USP XXIII, or by the BP 1993 official methods. This method is precise, reproducible and specific for individual tetracyclines and can be used for qualitative and quantitative analysis of tetracyclines in pharmaceutical products and for controlling there contaminates within the limits specified in the current official methods. The simplification of the working condition of the method reported here make it a suitable alternative to other officially methods used for quality controlling of tetracycline antibiotics. Furthermore, PGC column showed a rapid equilibrium and a high stability during the analysis. The chromatographic behaviour of tetracyclines on PGC is different from that obtained with other reversed phase sorbents.

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