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Development of a simple HPLC method for separation of doxycycline and its degradation products

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Abstract

A simple HPLC method for the separation of doxycycline and its degradation products 6-epidoxycycline and metacycline was developed. Numerous HPLC conditions were tested for the qualitative determination of doxycycline and its degradation products. The best result was achieved by using Phenomenex® Luna 5 μm C_8 250 × 4.6 mm column with a Phenomenex® C_8 4 × 10 mm I.D. guard column, and a mobile phase consisting of acetonitrile:water:perchloric acid ($HClO_4$) (26:74:0.25) adjusted to pH 2.5 with 5 M sodium hydroxide, a flow-rate of 1.0 ml/min and ultraviolet detection at 350 nm. Correlation coefficients for calibration curves within the detection range of 3–60 μl/ml were 0.9990 for doxycycline and 1.000 and 0.9994 for 6-epidoxycycline and metacycline, respectively (within the range 0.5–7 μl/ml). The resolution between metacycline and 6-epidoxycycline was 1.2 and between 6-epidoxycycline and doxycycline it was 1.9 which fulfils European Pharmacopoeia requirements. The within- and between-day precision was determined for both retention time and peak area. Preliminary results indicate that this method can also be applied for separating other tetracyclines such as minocycline, chlortetracycline, tetracycline and demeclocycline.

Keywords: Doxycycline; 6-Epidoxycycline; Metacycline; HPLC

1. Introduction

Over recent years there has been a growing use of doxycycline in clinical practice, which is not dependent on its antibacterial properties but on its inhibition effect on a group of enzymes referred to as matrix metalloproteinase's (MMP's) [1]. MMP's are enzymes which have a role in the immune response and in inflammatory conditions such as aphthous ulceration and gingivitis. As tetracycline drugs, (especially the semi-synthetic derivatives doxycycline and minocycline), distribute easily throughout most tissues of the mouth such as dentine, enamel of unerupted teeth and the gingivae they have been found to be effective in the treatment of various inflammatory diseases in the mouth. At least one pharmaceutical preparation

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has been developed to utilise this newly discovered activity of tetracycline drugs [2].

The determination of doxycycline and its degradation products by high performance liquid chromatography (HPLC) has been found to be rather difficult. Many of its degradation products are epimers, which are very similar structurally to doxycycline and to each other (Fig. 1a and b), consequently making separation problematical. Another HPLC separation problem appears when ODS columns are used, the retention time is long because of high hydrophobicity, but this problem can be bypassed by making the mobile phase sufficiently acidic to ionise the tetracycline which results in increased hydrophilicity and reduces the retention time.

Many HPLC methods have been used to determine tetracyclines in pharmaceutical formulations using for example silica gel [3,4], porous graphitic carbon [5], silica-based and polymer-based HPLC column stationary phases [6–15]. Most of these methods are time consuming and complex, some are very expensive and some even unable to separate tetracyclines from their degradation products.

This paper describes a simple and selective HPLC method for the separation of doxycycline and two of its degradation products, 6-epidoxycycline and metacycline, which fulfils the European Pharmacopoeia (Strasbourg, France) requirements for selectivity for those substances. The separation of these two degradation products has previously been fraught with difficulty. This proposed method is designed to be suitable for the quality assessment of these compounds in pharmaceutical products.

2. Experimental

2.1. Chemicals

Doxycycline was purchased from ICN® Biomedicals (Aurora, Ohio), metacycline and 6-epidoxycycline from European Pharmacopoeia. HPLC-grade acetonitrile and methanol were purchased from Rathburn (Walkerburn, Scotland) and perchloric acid pro analysis was obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

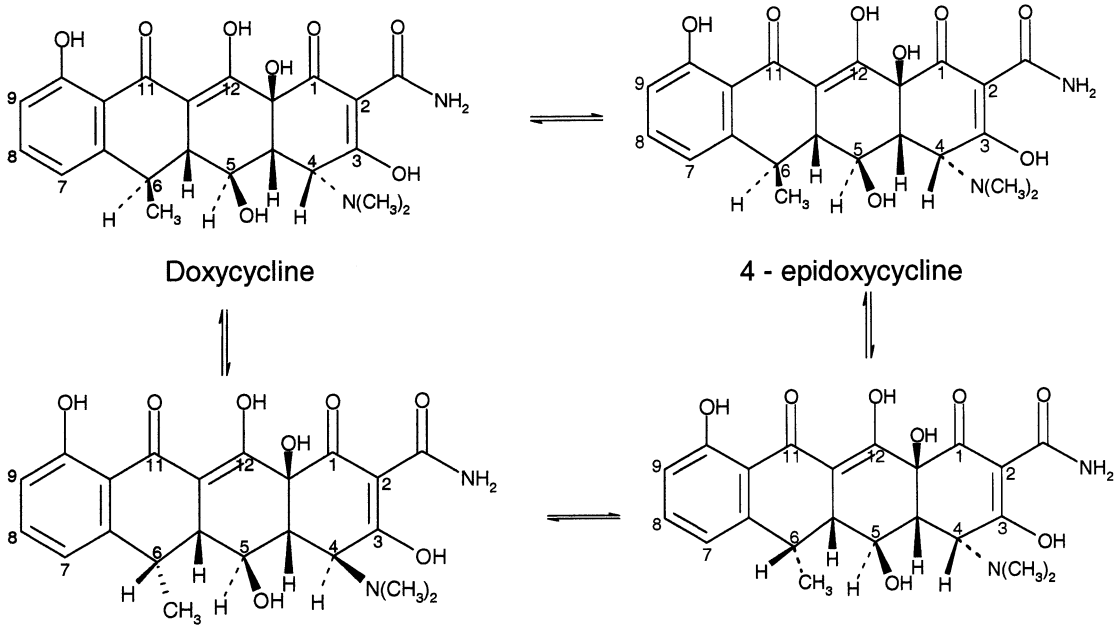
2.2. Chromatography

Separation was carried out with a gradient modular HPLC system equipped with a variable wavelength spectrophotometer. HPLC system Thermo Separation Products (TSP), Spectra series P200 pump UV 150 detector and TSP Chromjet Integrator (Spectra-Physics Analytical, Fremont, CA, USA) and Merck Hitachi Autosampler L7200 LaChrom (Hitachi ltd. Tokyo, Japan). The columns used in the development of the separation were Hypersil ODS C_{18} 100 × 4.6 mm, Cosmosil® C_{18} 150 × 4.6 mm, Cosmosil® C_{18} 250 × 4.6 mm, Phenomenex® Luna 5 μm C_8 150 × 4.6 mm and Phenomenex® Luna 5 μm C_8 250 × 4.6 mm (all purchased from Phenomenex, UK). The detector wavelength was set at 350 nm. A flow rate of 1.0 ml min^{-1} was used for the separation of doxycycline and its degradation products.

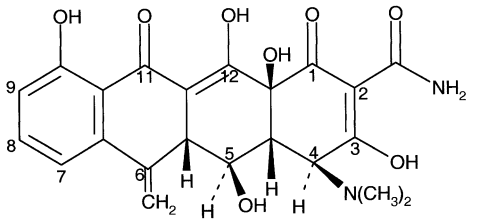
The mobile phase composition was acetonitrile: water: perchloric acid ($HClO_4$) (26:74:0.25) adjusted to pH 2.5 with 5 M sodium hydroxide. Samples and standards were dissolved in the mobile phase and 20 μl samples were injected into the HPLC system at room temperature.

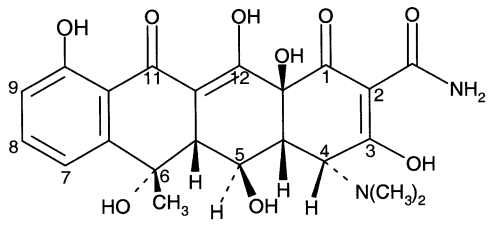
2.3. Preparation of solutions

Test solutions of doxycycline, metacycline and 6-epidoxycycline were prepared by dissolving them in the mobile phase in a concentration of 40 μg/ml for doxycycline and 7 μg/ml for metacycline and 6-epidoxycycline. Samples were measured the same day they were prepared. The calibration standards (3–40 μg/ml for doxycycline and oxytetracycline and 0.5–7 μg/ml for metacycline and 6-epidoxycycline) were prepared from stock solutions by dilution as follows; accurately weigh about 0.020 g of doxycycline HCl and 0.003 g of 6-epidoxycycline and metacycline each into a 200 ml volumetric flask. Dissolve in about 100 ml of the mobile phase and then make up to volume with the mobile phase. This is solution S which is then diluted accordingly; SI: 10 ml of S diluted to 25 ml, SII: 15 ml of SI diluted to 25 ml, SIII: 15 ml of SII



(a)





(b)

Fig. 1. (a) Doxycycline and its epimers. (b) Two common degradations products of doxycycline.

diluted to 25 ml, SIV: 10 ml of SIII diluted to 25 ml, SV: 5 ml of SIV diluted to 10 ml. Samples were filtered through a 0.45 μm nylon syringe filter (Millipore, Denmark) before they were injected into the HPLC system.

3. Results and discussion

3.1. Chromatography

Of the columns tested it was only by using Phenomenex® Luna 5 μm C_8 250 × 4.6 mm that good separation of metacycline and 6-epidoxycycline was achieved, from each other as well as from doxycycline. The retention time of the compounds with this method is approximately the same as with the official method of the European Pharmacopoeia (Ph.Eur 4th ed.) [16], about 30 min and Ph.Eur requirements of resolution and peak symmetry are fulfilled with this method (Fig. 2 and Table 1). The main advantage of this method compared with the Ph.Eur method is that it is simpler to carry out with regard to the preparation of samples and the conditions used and thus is less time consuming. There is also no need for column heating equipment and it is less costly with the Phenomenex® Luna 5 μm C_8 costing half as much as conventional styrene-divinylbenzene copolymer (SDVB) columns.

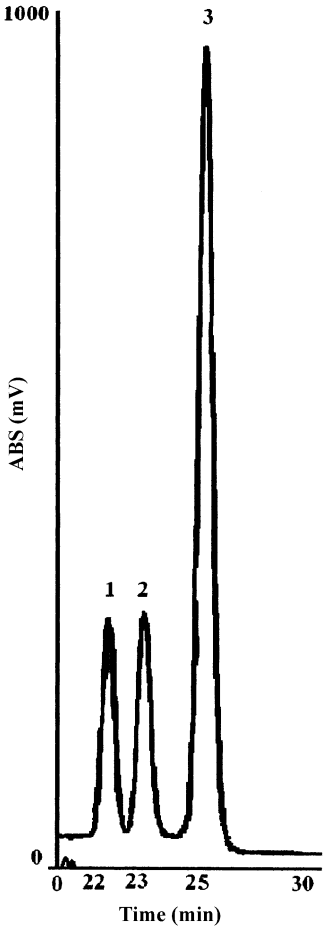


Fig. 2. Example of the chromatogram, peak 1, metacycline; peak 2, 6-epoxycycline; peak 3, doxycycline.

Table 1
Retention times and resolutions

Substance	Retention time (min)	Resolution
Metacycline	22.22	
6-epidoxycycline	23.30	1.2[a]
Doxycycline	25.16	1.9[b]

[a] Between metacycline and 6-epidoxycycline.
[b] Between 6-epidoxycycline and doxycycline.

Preliminary studies of the separation capabilities of the described method for other tetracyclines show good separation of all the investigated compounds (Fig. 3), indicating that this method could be applied for the analysis of different tetracyclines.

3.2. Mobile phase

3.2.1. Mobile phase composition

Mobile phase compositions consisting of perchloric acid buffer, water and acetonitrile in different ratios (30–50%) were tested. The higher the ratio of the buffer in the mobile phase, gave higher retention time of the tetracyclines and better resolution but also broadened the peaks. After testing various compositions, 26% of acetonitrile was selected as the ratio of the organic modifier for the baseline separation of doxycycline

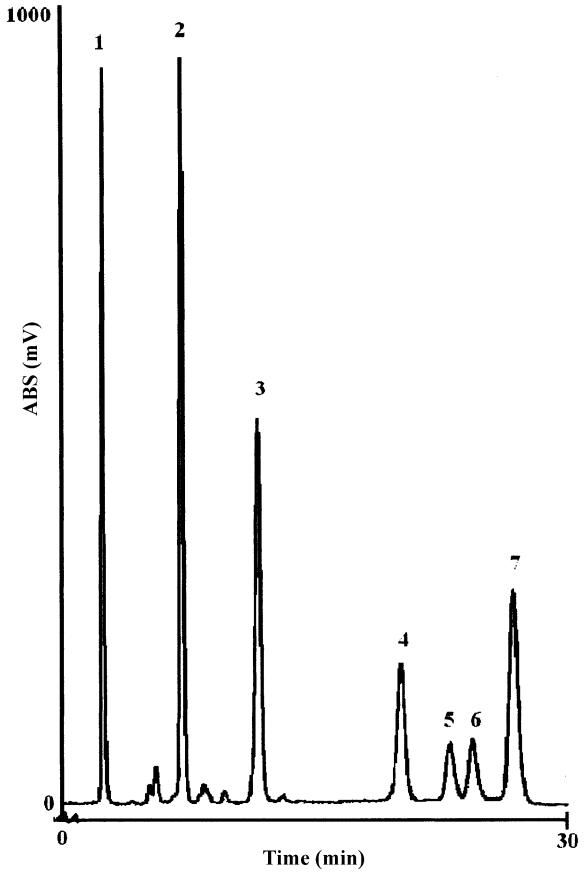


Fig. 3. Example of chromatogram, total run time is about. 30 min. Peak 1, tetracycline; peak 2, demeclocycline; peak 3, chlortetracycline; peak 4, minocycline; peak 5, metacycline; peak 6, 6-epidoxycycline; peak 7, doxycycline.

and its degradation products as it gave the best resolution.

3.2.2. Mobile phase pH

Three factors were considered when the pH of the mobile phase was chosen. Firstly doxycycline has to be in its ionised form in order to decrease the retention time, hence the mobile phase pH has to be lower than the pK_a of doxycycline which is 3.4. Secondly the lifetime of the column stationary phase is reduced at low pH, especially at pH lower than 2.0 and thirdly the formation of isomeric analogues decreases as the pH values is lowered. In view of these considerations a pH value of 2.5 was chosen.

3.3. Validation of the analytical method

To validate the HPLC method, a series of tests were made using the most promising conditions.

3.3.1. Linearity

A calibration curve was made for each of the three compounds. The concentrations examined were between 3 and 60 μg/ml for doxycycline and 0.5–7 μg/ml for 6-epidoxycycline and metacycline. The correlation coefficients (R^2) were 0.9990 for doxycycline, 1.0000 for 6-epidoxycycline and 0.9994 for metacycline.

3.3.2. Selectivity

The selectivity of the method for the determination of doxycycline and its degradation product was studied by mixing exact weights of the tetracyclines into the mobile phase. As shown in Fig. 2 and Table 1, there was adequate resolution of all these compounds, especially between doxycycline and its two degradation product.

3.3.3. Precision and accuracy

The within-day precision (expressed as the relative standard deviation (R.S.D.)) for area under the curve (AUC) and retention times was determined for all three substances by repeated analysis (n = 7). Average within-day R.S.D. values obtained for retention times were 1.16% and for areas under curve 1.44%. The average R.S.D. values for between-day precision obtained for AUC were 1.01% (Table 2).

4. Conclusion

A method was developed for the separation of doxycycline and its degradation products; 6-epidoxycycline and metacycline; which is reliable, inexpensive and simple in procedure in comparison with the European Pharmacopoeia method. The results indicate that the described method can be used for qualitative analysis of those three

Table 2
Precision

Compound	R.S.D. (%)	AUC R.S.D. (%)	Between-day precision R.S.D. for AUC (%)
Doxycycline	1.16	1.12	1.07
6-epidoxycycline	1.15	1.63	0.64
Metacycline	1.16	1.58	1.31

compounds. Further work will determine the quantitative limits of the method. The simplicity of the method reported here makes it a suitable alternative to official methods used for the quality control of doxycycline.

This study also indicates that this method can be used for both qualitative and quantitative analysis of most tetracycline compounds but, in order to establish that, further research will be needed.

Acknowledgements

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References

[1] L.M. Golub, H.M. Lee, G. Lehrer, A. Nemiroff, T.F. McNamara, R. Kaplan, N.S. Ramamurty, J. Periodontol. Res. 18 (1983) 516.
[2] L.R. Johnson, N.H. Stoller, Compendium Continuing Educ. Dentistry 20 (1999) 19.
[3] W. Naidong, K. Verresen, R. Busson, E. Roets, J. Hoogmarteins, J. Chromatogr. 586 (1991) 67.
[4] R. Böcker, J. Chromatogr. 586 (1983) 255.
[5] L. Monser, F. Darghouth, J. Pharm. Biomed. Anal. 23 (2000) 353.
[6] S. Croubels, H. Vermeersch, P. De Backer, M.D.F. Santos, J.P. Remon, C. Van Peteghem, J. Chromatogr. B 708 (1998) 145.
[7] A.G. Kazemifard, D.E. Moore, J. Pharm. Biomed. Anal. 16 (1997) 689.
[8] M.D. Santos, H. Vermeersch, J.P. Remon, M. Schelkens, P. De Backer, R. Ducatelle, F. Haesebrouck, J. Chromatogr. B 682 (1996) 301.
[9] D.S. Vienneau, C.G. Kindberg, J. Pharm. Biomed. Anal. 16 (1997) 111.
[10] M.C. Carson, M.A. Ngoh, S.W. Hadley, J. Chromatogr. B 712 (1998) 113.
[11] J.F.M. Nouws, G. Loeffen, J. Schouten, H. Van Egmound, H. Keukens, H. Stegeman, J. Dairy Sci. 81 (1998) 2341.
[12] P.D. Bryan, J.T. Stewart, J. Pharm. Biomed. Anal. 12 (1994) 675.
[13] P.D. Bryan, J.T. Stewart, J. Pharm. Biomed. Anal. 11 (1993) 971.
[14] W. Naidong, K. Verresen, E. Roets, J. Hoogmartens, J. Chromatogr. 596 (1991) 61.
[15] J. Hoogmartens, N.H. Khan, H. Vanderhaeghe, A.L. van der Leeden, M. Oosterbaan, G.L. Veld-Tulp, W. Plugge, C. van der Vlies, D. Mialann, R. Melamed, J.H.M.B. Miller, J. Pharm. Biomed. Anal. 7 (1989) 607.
[16] European Pharmacopoeia, fourth ed., Strasbourg, France, 2001, p. 1088.