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A rapid high performance liquid chromatographic (HPLC) assay for the determination of oxytetracycline in commercial pharmaceuticals

I.N. Papadoyannis *, V.F. Samanidou, L.A. Kovatsi

Department of Chemistry, Laboratory of Analytical Chemistry, Aristotle University of Thessaloniki, Thessaloniki GR-54006, Greece

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Abstract

In the present study, a simple, sensitive and rapid reversed-phase high performance liquid chromatographic (HPLC) method with ultraviolet detection for the analysis of oxytetracycline (OTC) is developed and applied to the determination of the antibiotic in commercial pharmaceutical preparations (powder, capsules, vaginal tablets and ointment). The isocratic elution is performed with methanol-0.01 M oxalic acid, pH 3.0 (30:70, v/v) at a flow rate of 0.95 ml/min, using a Silasorb C_8 analytical column, 250×4 mm, 10 μm. Codeine is used as internal standard. Absorbance is monitored at 250 nm where both analyte of interest and internal standard have significant absorption. Total analysis time was ~ 7 min. Data with respect to precision and accuracy and limits of detection are reported and discussed. The described method can be readily utilised for analysis of pharmaceutical products and pharmacokinetic studies as well. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Oxytetracycline; Pharmaceuticals; High performance liquid chromatography (HPLC)

1. Introduction

Oxytetracycline (OTC) is one of the tetracyclines, a group of wide spectrum antibiotics. Chemically, it is 4- (dimethylamino)-1, 4, 4a, 5, 5a, 6, 11, 12a-octahydro-3, 5, 6, 10, 12, 12a-hexahydroxy-6-methyl-1, 11-dioxo-2-naphthacenacarboxamide.

Because of its antibacterial action, oxytetracycline is used widely not only in medicine but also in veterinary medicine as well as in the meat production industry, where it is used as a feed additive or in drinking water to maintain optimal health for food-producing animals. Residues may remain in edible animal tissues and then affect human health and this is the reason why many researchers focus on the development of a rapid, accurate and sensitive method for the determination of this antibiotic. Several papers have been published concerning assay methods for oxytetra-

* Corresponding author. Tel.: +30-31-997793; fax +30-31-997719.

E-mail address: papadoya@chem.auth.gr (I.N. Papadoyannis).

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cycline. Among them, bioassay and fluorometry, which are commonly used, lack sensitivity and specificity, while chromatographic methods are generally preferred for their greater selectivity and sensitivity [1,2].

Many methods have been described for the determination of OTC in pharmaceutical preparations and various biological matrices. Official methods of microbiological approach are not only laborious, expensive and time consuming, but also poor in terms of sensitivity and specificity. A further problem of the bio-assay is the similar anti-microbial characteristics of degradation products (epi, anhydro, epianhydro) that may be present in varying amounts [3].

Among high-performance liquid chromatographic (HPLC) methods reported for the analysis of oxytetracycline, a large number concern reversed-phase bonded packing materials involving complicated aqueous-organic eluents, with pH in the range 1–2.5, where a number of bonded packing materials are unstable so that column life can be short. Furthermore, most published methods failed to introduce an internal standard which is most useful in quantitation [4–12].

Amperometric detection has also been suggested recently, however electrochemical detection is generally regarded as being more technically demanding, despite its higher degree of selectivity and sensitivity. Post-column derivatization and fluorescence detection require special attention and may be time consuming, when applied to pharmaceutical analysis [3,13].

In the present paper, a simple, accurate and rapid isocratic HPLC method for the determination of oxytetracycline, using codeine as internal standard, is developed. The method appears to be suitable for quality control in pharmaceutical industry, due to its sensitivity, simplicity, selectivity and lack of excipient interference.

2. Experimental

2.1. Chemicals and reagents

Oxytetracycline was purchased from Veterin Hellas.

Codeine, used as internal standard, was kindly provided by the Laboratory of Toxicology, Medicine School, Aristotle University of Thessaloniki.

HPLC-grade methanol was obtained from Riedel-de-Haen (Seelze, Germany).

Oxalic acid pro analysi was from Merck (Darmstadt, Germany). All other reagents used were of analytical grade. Bis de-ionised water was used throughout analyses. Pharmaceutical preparations with oxytetracycline as active ingredient were purchased as follows: ointment under the trade name Oxacycle, by Specifar, powder under the trade name Terramycin w/Polymyxin, vaginal tablets under the trade name Terramycin and capsules under the trade name Terramycin, by Pfizer Hellas.

2.2. Apparatus

The chromatographic system operating in isocratic mode, consisted of the commercial components: a Shimadzu (Kyoto, Japan) LC-9A pump, an SSI500 variable UV/VIS detector (SSI, State College, PA, USA), operating at 250 nm and a sensitivity setting of 0.002 (AUFS), a 9125 Rheodyne (CA, USA) injection valve, with a 20-μl loop and an HP 3396 II integrator (Hewlett-Packard, Avondale, PA). The analytical column, a Silasorb RP-8, 250×4 mm ID, 10 μm, was purchased from Rigas Labs (Thessaloniki, Greece).

A glass vacuum-filtration apparatus obtained from Alltech Associates, was employed for the filtration of the buffer solution, using 0.2-μm membrane filters (Schleicher and Schuell, Dassel, Germany). Degassing of solvents was achieved by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany) prior to use.

UV spectra for selecting the working wavelength of detection were taken using a Varian DMS 100 S UV/VIS double-beam spectrophotometer.

2.3. Chromatographic conditions

The RP-8 Silasorb column was used at ambient temperature 22°C. The mobile phase consisting of CH_3OH-0.01 M oxalic acid, pH 3.0 adjusted with

dilute HCl (30:70 v/v) was delivered isocratically, at a flow rate of 0.95 ml/min and this combination was selected among others investigated, as leading to optimal resolution between oxytetracycline and codeine, optimal peak shape, as well as for convenience regarding total time of analysis. The pH of the mobile phase was selected as 3.0, to minimise the formation of isomeric analogues, which occurs rapidly in alkaline medium [14]. The use of CH_3CN was also examined leading to higher retention times and thus increasing the total cost of analysis.

Prior to use, mobile phase was filtered through 0.2-μm membrane filters and degassed by sonication in an ultrasonic bath.

A wide variety of compounds such as xanthine derivatives, tropane alkaloids, anthracene derivatives and other tetracyclines, were assayed as regards their application as internal standard. Codeine was selected taking into consideration its elution properties, as well as spectra characteristics.

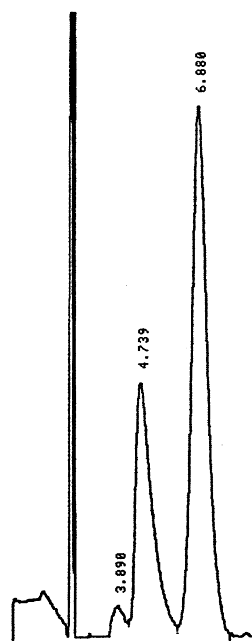

Fig. 1. High-performance liquid chromatogram of OTC 3 ng/μl (6.880 min) in the presence of codeine (internal standard) 3.85 ng/μl (4.739 min).

2.4. System suitability

The analytical column was equilibrated with the eluting solvent system used. After an acceptable stable baseline was achieved, the standards and then the samples were analysed. The resolution factor R_s was calculated between the peak of oxytetracycline and that of codeine and was found to be 3.02, signifying complete separation. The respective chromatogram of standard solution is given in Fig. 1.

The small peak appearing at 3.89 min may be due to degradation product, more likely the epimer at C-4 position, but it could not be identified due to the lack of standard compound. No such peak was noticed in real sample chromatograms.

2.5. Preparation of standard solutions — calibration curve

Stock standard solution of 100 ng/μl oxytetracycline was prepared in methanol and stored at −18°C. Stock standard solution of 77 ng/μl codeine was prepared in methanol and stored at 4°C. A total of ten working standard solutions in the range 0.5–20 ng/μl were prepared by appropriate dilution with methanol. All solutions containing oxytetracycline were protected from direct sun and artificial light throughout the analyses, because of the instability of OTC to light. These solutions were stable for 1 month, when stored at −18°C.

2.6. Sample pretreatment for analysis of OTC in pharmaceutical preparations

The developed method was applied to the analysis of oxytetracycline in commercially available pharmaceuticals in four dosage forms: capsules, vaginal tablets, ointment and powder. Each formulation was treated differently according to the following conditions.

2.6.1. Powder

According to the label, the powder contained 3% w/w oxytetracycline. A total of 0.5 g of the powder were dissolved in 100 ml of methanol. The

Table 1
Intra-calibration analysis (within-day run) of oxytetracycline ($n = 8$) in methanolic solutions

Added OTC (ng)	Found ± S.D. (ng)	RSD (%)
19.6	22.8 ± 0.7	3.1
58.8	54.6 ± 4.2	7.7
78.4	78.9 ± 8.7	11. 0
117.6	122.2 ± 7.0	5.7

concentration of this solution was 150 ng/μl. From this stock solution three working solutions were prepared by dilution to obtain concentrations of 1.5–3.0 and 4.5 ng/μl.

2.6.2. Vaginal tablets

A total of six tablets containing 100 mg of oxytetracycline, as stated on the label of commercial product, were weighed giving an average tablet weight of 1.4215 g. After fine powdering of the tablets with a porcelain mortar, an accurately weighed portion of the pooled sample equivalent to the antibiotic content of one tablet, was incubated at 37°C for 5 min and then was quantitatively transferred to a 100-ml volumetric flask and diluted to volume with methanol. The concentration of this solution was 1000 ng/μl. From this stock solution three working solutions of 1, 2 and 3 ng/μl were prepared by dilution.

2.6.3. Ointment

The commercial product contained 50 mg of oxytetracycline per g of ointment. By means of sonication 2 g of ointment were dissolved in 50 ml of methanol. The supernatant was transferred into a 100-ml volumetric flask and diluted to volume with methanol. The concentration of this solution was 1000 ng/μl. From this stock solution, three working solutions of 1, 2 and 3 ng/μl were prepared by dilution.

2.6.4. Capsules

A total of six capsules, containing 250 mg of oxytetracycline as stated on the label, were weighed and the average capsule weight was found to be 0.4290 g. After fine powdering of the capsule contents in a porcelain mortar, an accurately weighed portion of the pooled sample equivalent to the antibiotic content of one capsule, was quantitatively transferred to a 100-ml volumetric flask and diluted to volume with methanol. The concentration of this solution was 2500 ng/μl. After subsequent dilutions, three working solutions of 1, 2 and 3 ng/μl were prepared.

All working solutions originated from pharmaceutical formulations contained the internal standard codeine at a concentration of 3.85 ng/μl. Aliquots of 20 μl were injected onto the HPLC analytical column.

3. Results and discussion

3.1. Performance characteristics of the proposed method

Optimised chromatographic conditions were set and the following analytical characteristics were evaluated:

- Precision and accuracy
- Calibration data
- Linearity of calibration curve
- Limits of detection and quantitation
- Selectivity
- Real sample analysis

3.2. Precision and accuracy

In order to verify the repeatability, replicate injections of standard solutions at low, medium and high concentration levels were made and peak areas were measured in comparison to the peak area of the internal standard. Statistical evaluation revealed relative standard deviations at different values for eight injections. Results of intra-day assay are presented in Table 1.

Intermediate precision was examined during routine operation of the system over a period of 8 consecutive days. Results are illustrated in Table 2.

3.3. Calibration data

Calibration curve was constructed in the presence of internal standard, at ten concentrations of oxytetracycline, covering the range from 0.5 to 20.0 ng/μl. Aliquots of 20 μl of these solutions were injected into the HPLC system and peak area ratios of oxytetracycline to those of codeine were recorded and plotted versus oxytetracycline concentration. All determinations were repeated eight times and results were treated statistically. Calibration of the method was performed by injection of standards covering the entire working range. Least squares linear regression analysis gave a correlation coefficient $r = 0.9994$ and the following calibration curve:

$$Y = (1.03449 \pm 0.01836) + (0.00710 \pm 9 \times 10^{-5})X$$

where Y is peak area ratio of OTC to codeine and X is amount of OTC in ng.

Table 2
Inter-day precision of oxytetracycline analysis in the presence of codeine ($n = 8$) in methanolic solutions

Added OTC (ng)	Found ± S.D. (ng)	RSD (%)
19.6	22.8 ± 2.3	10.1
58.8	57.3 ± 3.7	6.4
78.4	77.0 ± 3.3	4.3
117.6	127.2 ± 4.4	3.4

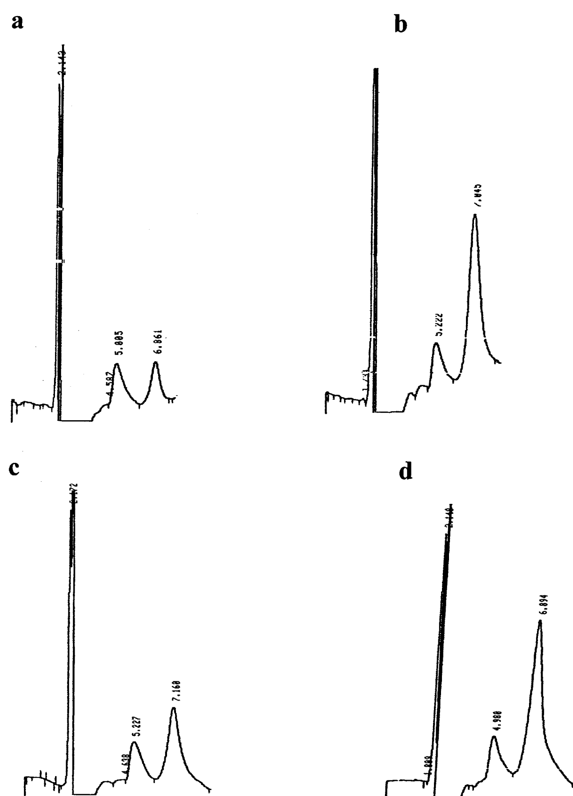

Fig. 2. High-performance liquid chromatogram of OTC in pharmaceutical preparations: (a) 1 ng/μl in vaginal tablets; (b) 2 ng/μl in capsules; (c) 2 ng/μl in ointment; (d) 3 ng/μl in powder. Internal standard concentration 3.85 ng/μl.

3.4. Linearity of calibration curve-limits of detection and quantitation

The linearity of calibration curve was observed up to 20.0 ng/μl. The limit of detection was assessed in the presence of the internal standard and considered to be the quantity yielding a detector response approximately equal to twice the size of background noise. Thus the minimum detectable quantity, expressed in ng injected onto the column, was found to be 2.0 ng. The limit of quantitation considered to be the lowest amount that can be analysed with sufficient accuracy and reproducibility was found to be 10.0 ng.

3.5. Selectivity

The selectivity of the developed RP-HPLC method for the determination of oxytetracycline in pharmaceutical preparations was investigated at the retention times of the analyte and the internal standard. Typical chromatograms obtained from commercial pharmaceuticals are illustrated in Fig. 2 (a–d). These chromatograms indicate that no peaks from excipients were noticed.

3.6. Real sample analysis

Experimental results of oxytetracycline analysis in pharmaceuticals, with reference to the amount stated in the labels of the commercial products, are presented in Table 3. These results demon-

Table 3
Experimental results for the analysis of oxytetracycline in pharmaceuticals by RP-HPLC with codeine as internal standard

Sample	Labelled amount (mg)	Found (mg)	RSD (%)	Analysed quantity (ng)	Found (ng)
Powder	30/g of powder	31 ± 3/g of powder	9.7	30	24
				60	65
				90	112
Vaginal tablets	100/tablet	97 ± 8/tablet	8.2	20	20
				40	36
				60	70
Ointment	50/g of ointment	45 ± 6/g of ointment	13.3	20	15
				40	36
				60	65
Capsules	250/capsule	257 ± 23/capsule	8.9	20	15
				40	46
				60	71

strate that the content of active compounds as obtained by this method, corresponds to the label.

4. Conclusion

There are not many literature references concerning the determination of oxytetracycline in pharmaceuticals. The determination methods that are proposed are time consuming, introduce complicated eluting systems and most of all fail to use an internal standard for quantitation. The HPLC analysis method of oxytetracycline described in the present study is characterised by sufficient accuracy, precision and reproducibility, as well as sensitivity and selectivity. Codeine proved to be a very suitable internal standard for this purpose. At the working pH conditions, no epimerisation products at C-4 were noticed in the chromatograms of commercial pharmaceuticals.

The developed method offers a short analysis time of oxytetracycline, which is a prerequisite in routine analysis of pharmaceutical preparations. Thus the method is suitable for the screening of formulated samples.

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