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Research Article

DEVELOPMENT AND COMPARISION OF AN HPTLC, HPLC AND LC-MS METHOD FOR DETERMINATIONOF TETRACYLINE ANTIBIOTICS

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ABSTRACT: Tetracycline (TC) antibiotics continue to play an important role in human and veterinary medicine and in animal nutrition. Comparison of three techniques, High Performance thin-layer chromatographic (HPTLC), HPLC and LC-MSMS for the determination of tetracycline has been carried out here. The purpose of this study was to determination of minimum responce tetracyclines and their metabolite by chromatographic techniqes. A rapid and easy-to-use method was developed on the liquid chromatography.

Keywords: HPTLC, HPLC, LC-MS, Tetracyclie atibiotcs

INTRODUCTION

The emergence of pathogenic bacteria resistant to many current antibiotics is a major public health concern and one of particular importance in clinical settings (nosocomial infections). Restocking the armamentarium of antibacterial agents, especially broad-spectrum antibiotics such as the tetracyclines, promises to be one of the most effective means to combat infectious disease, from hospital-acquired Gram-positive and Gram-negative pathogens to unforeseen and evolving microbial threats. To date, all commercial tetracycline antibiotics have been prepared by fermentation semi-synthesis, which is inherently limited (http://www.chem.harvard.edu).

The strategy for the discovery of new tetracyclines had not varied since the discovery of the first tetracycline (chlortetracycline) more than 60 years ago, which is to say semi-synthetic transformations of complex fermentation products. The human semi-synthetic evolution of the tetracyclines is marked by specific, impactful discoveries that led to the production of new antibiotics. The first enabling advance in tetracycline semisynthesis was achieved by Pfizer scientists: reductive removal of the C6-hydroxyl group of the natural products tetracycline and oxytetracycline (Stephens *et al,* 1958, McCormick *et al,* 1960,Wittenau *et al,1962*).The important and now generic antibiotics doxycycline and minocycline followed as a consequence, the latter arising from the additional discovery that electrophilic aromatic substitution at C7 becomes possible when the more stable 6-deoxytetracyclines are used as substrates (Spencer *et al,* 1963, Martell and Boothe, 1967, Church, *et al,*1971,Zambrano, 1969).Decades later, a team of Wyeth scientists synthesized 7,9-disubstituted tetracycline derivatives, leading to the discovery of the antibiotic tigecycline (Sum *et al,*1994, 1999).

Various methods have been used to analyze tetracyclines for impurities, such as microbiological assay, spectrophotometry, gas chromatography, high-performance liquid chromatography, and thin-layer chromatography. Microbiological analysis, the most sensitive technique for the residue analysis of tetracyclines in food products, requires a long period of incubation and lacks precision and specificity. Spectrophotometric methods are insensitive and interferences from other materials cannot always be excluded.

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Furthermore, because the microbiological assay and spectrophotometry techniques do not allow differentiation between the main component of tetracyclines and its impurities, they cannot be used for the purity control testing of tetracyclines pharmaceutical products. Gas chromatography methods require prior formation, under carefully controlled conditions, of the trimethylsilyl derivative.(Bobbitt and Ng, 1992, Ascalone,1978). For these reasons, the quantitative analysis of tetracyclines has been dominated by reversed-phase high performance liquid chromatography HPLC, however, HPLC requires that samples be injected sequentially (Chen and Lee,1994, Oka *et al,*1984, Iwaki *et al,*1992, Muritu *et al,*1994).

MATERIALS AND METHODS

Reagents and Solvents

Tetracycline standards were obtained from Sigma Aldrich. All of the standards were in powder or crystalline solid form. The samples were stored in a freezer (at 4 °C) inside a dark desiccator. Individual stock solutions were prepared with HPLC-grade Methanol. All stock solutions were stored at 4 °C in Glass bottles wrapped in aluminum foil. Fresh stock solutions were prepared each month. Working standards were made daily by diluting the stock solutions with methanol to the desired concentration. All of the solutions were protected from light during use. Other solvents and chemicals used for this study were either HPLC-grade or analytical-reagent grade. All organic solvents and ammonium hydroxide were obtained from Merck (EM Science, Gibbstown, NJ, USA). Solutions of saturated Na_2EDTA and oxalic acid (J. T. Baker) were prepared with distilled water.

HPTLC

Chromatography was performed on 20 cm × 10 cm aluminium pla-tes coated with 200-µm layers of silica gel $60F_{254}$ (E. Merck, Germany). Before use the plates were sprayed with 10% (w/v) aqueous disodium EDTA (ethylene diaminetetraacetic acid) solution, the pH of which had been adjusted to 9.0 with 10% (m/v) aqueous sodium hydroxide solution Samples were applied to the plates as bands 5 mm wide, 10 mm apart, by means of a 100-µL syringe. A constant 10 ul Volume used as a spot with the help of Nitrogen Flow for dry spot. Plates was activated, for at least 1 h at room temperature, and then in an oven at 110°C for 1 h, shortly before use. Linear ascending development by different solvent system were used to run Tetracycline's on plate like. Toluene : Ethylacetate (70:30), Acetonitrile : water (50:50), dichloromethane :methanol : water (59:35:6),methanol–acetonitrile–isopropanol (IPA)–water 5:4:0.5:0.5 (v/v) as mobile phase was per-formed in a 20 cm × 10 cm twin-trough glass chamber (Camag), with tightly fitting lid, previously saturated with mobile phase vapour for 30 min at room temperature (25 ± 2°C) and relative humidity 50 ± 5%. The development distance was 8 cm. After development the plates were dried in current of air from an air dryer. Best separation was achieved using mobile phase DCM: MeOH: H2O.After run plate was dried and observed in the presence of UV light at scanned at multi wavelength.
Densitometry scanning at 345 nm was then performed with a TLC Scanner in absorbance mode. The source of radiation was a deuterium lamp emitting a continuous UV spectrum in the range 190–400 nm. The slit dimensions were 5 mm × 0.45 mm and the scanning speed 20 mm s^{-1}.
Tetracycline's are showing very less mobility on Silica plate and we can detect up to 10 ppm level by HPTLC as shown in chromatogrm.(Fig-1).

HPLC

Instrumentation

The HPLC system (Agilent Technologies) equipped with a quaternary gradient pump and PDA Variable wavelength Detector, auto sampler, column oven, and software EZCHROME
For data analysis. The analytical column was a 5 µ (C18) column (4.6 × 150 mm, 5µm particle size) from Agilent Co. The optimized mobile phase for desorption andSeparation was a mixture of 0.01 M oxalic acid/acetonitrile/methanol (77:18:5, v/v/v), and the flow rate was kept 1.0 mL/min. The detection was performed at 280 nm with scanning range 340-360 nm. 25 ul samples injected, Chromatograph of Tetracycline's are given in (fig-2).

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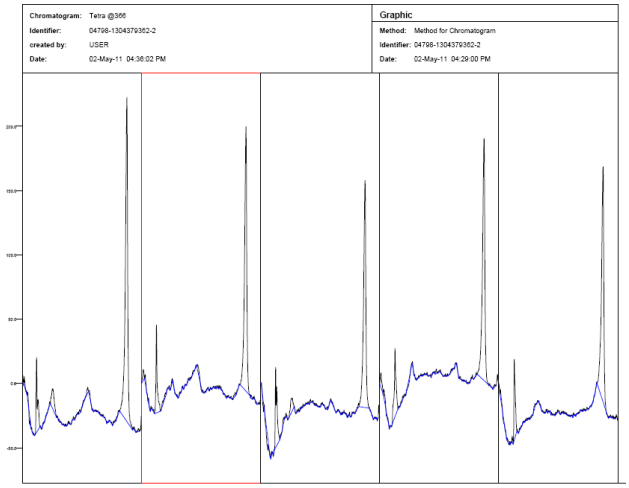
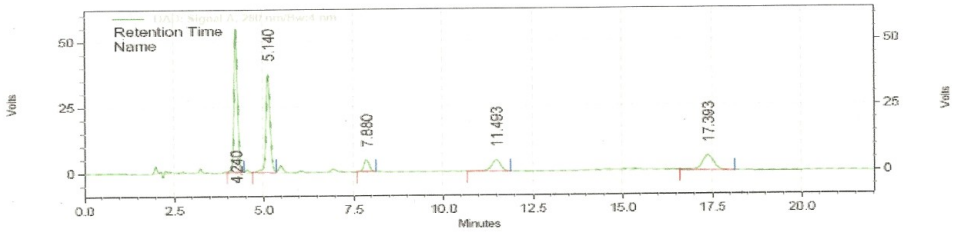


Figure-1 : Chromatograph of 10 ppm Tetracycline by HPTLC.

Sample Name: Tetra_Mix_02.5ppm
Data File: D:\EZChrom Elite\Enterprise\Data\Tetra\20110528\Tetra_Mix_02.5ppm.dat
Method: D:\EZChrom Elite\Enterprise\Projects\Default\Method\Tetracycline.met
Acquired: 5/29/2011 1:08:43 AM Printed: 5/29/2011 2:58:27 PM
Inj Vol: 25 Vial No. 21



DAD: Signal A, 280 nm/Bw:4 nm Results

Pk #	Name	RT	Area	Area %	Height	Height Percent
1		4.240	772914	39.81	115265	51.522
2		5.140	621208	32.00	78502	35.090
3		7.880	100926	5.20	9148	4.089
4		11.493	159446	8.21	8843	3.953
5		17.393	286792	14.77	11960	5.346
Totals			1941286	100.00	223718	100.000

Figure-2 : Chromatograph of Tetracycline by HPLC

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LC-MSMS

Agilent 6460 series

- Column: Agilent C18, 4.6×150mm, 5 um;
- Flow rate : 0.4 mL/min
- Temperature : 30^{0}C

MS Source settings

- Source: ESI
- Ion polarity: Positive
- Drying Gas flow rate: 10 L/min
- Drying Gas temp. 350^{0}C
- Nebulizer: 45psi
- Vcap.: 4000V

Table-1 : Chromatography conditions

S.No.	Compound	Molecular Mass	Transitions		Fragmentor	Collision Energy
1	Chlorotetracycline	479	Quantifier: Qualifier:	444 462	125 125	22 15
2	Oxytertracycline	461	Quantifier: Qualifier:	426 443	125 125	20 10
3	Doxycycline	445	Quantifier: Qualifier:	428 154	125 125	15 30
4	Tetracycline	445	Quantifier: Qualifier:	410 427	125 125	20 15
5	4-Epitetracycline	445	Quantifier: Qualifier:	427 410	125 125	10 20

Source Parameters

Gas temp.	350°C
Gas Flow	10L/min
Nebulizer	45 psi
Shaeth Gas temp.	350°C
Shaeth Gas Flow	10L/min

	Positive	Negative
Capillary	3500V	3500V
Nozzle Voltage	500V	500V

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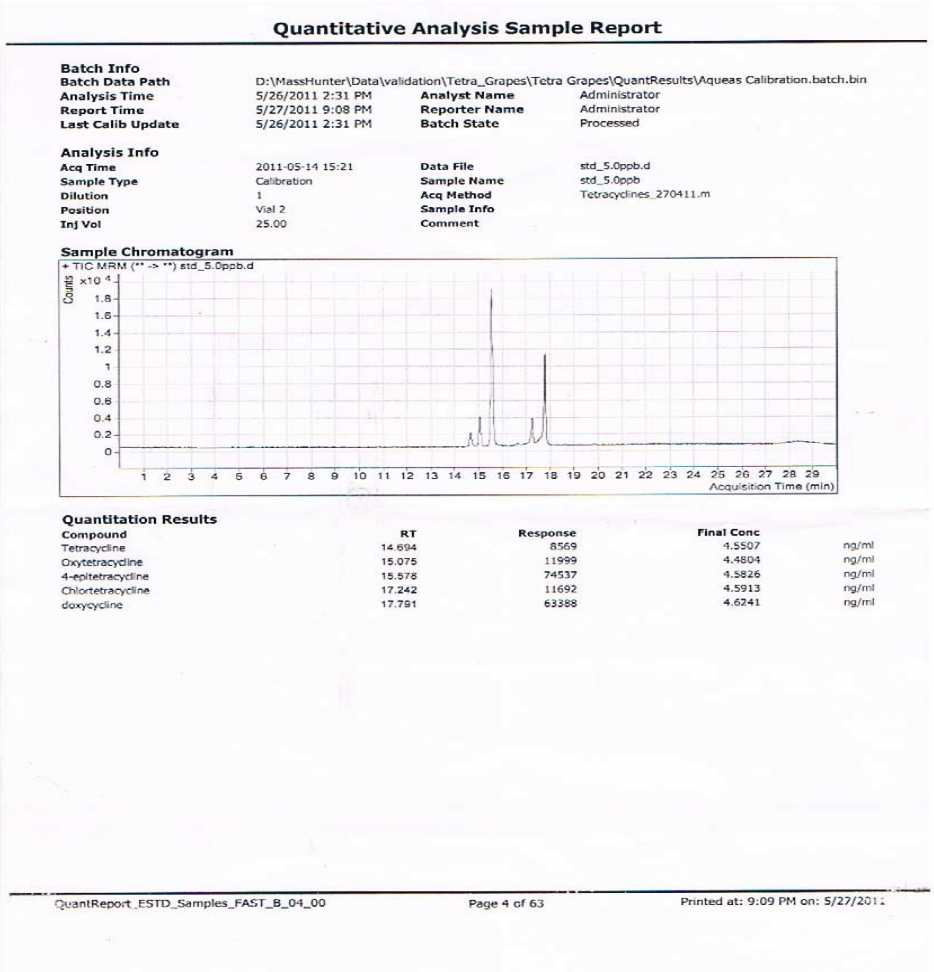
Mobile Phase

A	1%HCOOH in H_2O		
B	1%HCOOH in Acetonitrile:Methanol (50:50)		
Gradient			
Time	A%	B%	Flow
0	85	15	0.4
5	85	15	0.4
10	60	40	0.4
15	10	90	0.4
22	10	90	0.4
25	85	15	0.4
30	85	15	0.4
Column	Agilent ZORBAX Eclipse XDB (2.6X150mmX5µm)		



Quantitative Analysis Sample Report

Batch Info
Batch Data Path: D:\MassHunter\Data\validation\Tetra_Grapes\Tetra Grapes\QuantResults\Aqueas Calibration.batch.bin
Analysis Time: 5/26/2011 2:31 PM
Report Time: 5/27/2011 9:08 PM
Last Calib Update: 5/26/2011 2:31 PM
Analyst Name: Administrator
Reporter Name: Administrator
Batch State: Processed

Analysis Info
Acq Time: 2011-05-14 15:21
Sample Type: Calibration
Dilution: 1
Position: Vial 2
Inj Vol: 25.00
Data File: std_5.0ppb.d
Sample Name: std_5.0ppb
Acq Method: Tetracyclines_270411.m
Sample Info:
Comment:

Sample Chromatogram

Quantitation Results

Compound	RT	Response	Final Conc	
Tetracycline	14.694	8569	4.5507	ng/ml
Oxytetracycline	15.075	11999	4.4804	ng/ml
4-epitetracycline	15.578	74537	4.5826	ng/ml
Chlortetracycline	17.242	11692	4.5913	ng/ml
doxycycline	17.791	63388	4.6241	ng/ml

QuantReport_ESTD_Samples_FAST_B_04_00 Page 4 of 63 Printed at: 9:09 PM on: 5/27/2011

Figure-3 : Chromatograph of Tetracycline by LC-MS

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CONCLUSION

By this study it is clear that Tetracycline's can be detected by any of these techniques. HPTLC is technique is simple, less expensive, easily available technique and is useful for higher concentration like 10 ppm and 100 ppm level. This technique is very useful for pharmaceuticals industry for qualitative purpose. HPLC is also very good technique but instrument is costly, needed experienced operator and maintenance is high but we can detect quantitatively up to 1 ppm of individual tetracycline. This technique is very useful for food and pharmaceuticals industry for qualitative and quantitative analysis.LC MSMS is latest technique and we can detect easily up to 1.0 ppb level by this technique qualitative and also quantitative. Machine is very costly and maintenance is high and needed special training for operate. This technique is very useful for food, pharmaceutical and clinical, industry also for research Institutes.

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