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# Gas chromatographic-mass spectrometric determination of tetracycline in biological fluids

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## ABSTRACT

A gas chromatographic-mass spectrometric (GC-MS) method is described for the determination of tetracycline (TC) residue in biological fluids. This method allows detection of residual TC in biological fluids by using single-ion monitoring (SIM), confirmation by a full scan electron impact (EI) mass spectrum is possible if residual level in a sample is ≥1 µg/mL. TC is extracted with chloroform from a sample and cleaned up by n-hexane washing followed by partition between chloroform and phosphate buffer solution. The cleaned up extract is acetylated in acetic anhydride-pyridine mixture (1: 2) at room temperature. The reaction mixture is injected into the GC-MS apparatus, and the detection has been conducted using SIM at m/z 149. The detection limit is 0.01µg/mL. This method could be adopted in the forensic laboratories as well as in the toxicological laboratories.



## KEYWORDS

Tetracycline;
Biological fluids;
Derivatization;
GC-MS;
SIM.

## INTRODUCTION

Antibiotics are now used extensively in food producing animals to maintain optimal health and to promote growth. The use of these drugs can leave drug residues in edible tissues. These drug residues may have direct toxic effects moreover; safety considerations may include allergic reactions of consumers and induction of resistant bacteria. Therefore, conditions of use must be established for animal drugs to assure human food safety as well as efficacy and safety to the animal species[1]. Tetracyclines (TCs) are commonly used for the prevention or treatment of disease in livestock production.

TC is an extremely important group of antibiotics having broad spectrum of activity against gram-positive and gram-negative bacteria, some large viruses, rickettsiae, spirochetes and mycoplasmas. The first TC was discovered in 1948 and it was second compound to be developed after chloramphenicol as a broad-based antibiotic. Chemically all of these TCs have an octahydronaphthacene ring skeleton, consisting of four fused rings (Figure 1). Besides natural TCs isolated from various strains of *Streptomyces*, many derivatives[2] *viz.* doxycycline and minocycline have been prepared by their chemical conversion.

TCs are amphoteric compounds soluble in polar and moderately polar organic solvents and have the ability to form strong complexes with multivalent cations; it is the latter feature that is primarily taken into consideration when developing extraction methodolo-

gies[3] for these agents. Oka and Patterson[4] extensively reviewed the literature upto 1995 on the methodologies utilized for the analysis of TCs. In addition to the review which was made by Shaikh and Moats[5], both treaties indicated that there is an enormous variety of extraction methodologies for TC analysis.

TC residues that exceed the maximum level may be of toxicological concern. Some individual may have an allergic reaction[6] to these compounds or resistance by some bacteria may be induced. Even when residue levels are below minimum levels that produce an allergic reaction, it is not known whether toxicity may result from continuous low-level intake. Adverse effects associated with high levels of antibiotic residues include allergic reactions to the residues, carginogenicity of the residues, and evolution of the microorganisms that are resistant to the antibiotics.

TCs may be extracted from tissues by using solutions of weak mineral acids, but once in aqueous solution, partition coefficients prevent extraction of TCs into volatile organic solvents for concentration. Because aqueous extracts of TCs as so dilute, there is no practical method for their identification. At the AOAC meeting, a method was presented[7] for the qualitative identification of TCs in tissues by using TLC.

There are a number of chemical methods available for measuring TCs in biological materials[8-10], but these neither are sensitive enough for residue work nor include the cleanup necessary for complex animal tissues. Conversely microbiological techniques[11] for measuring residues of TCs are reliable and sensitive but lack of specificity and can be time consuming. Methods used to determine TC residue levels include, but are not limited to, microbiological and chromatographic techniques[12] such as TLC and GC. However, microbiological methods lack specificity and TLC methods lack sensitivity.

Microbiological assays are most commonly used for the measurement of TCs in food, but they are time consuming, cannot identify certain TCs, and their precision appears to be variable. Therefore a precise chromatographic analysis method for the TCs has been required. Several liquid chromatographic (LC) methods were published[13-17] for the determination of TC residues in biological fluids. Most of these methods did not have the desired sensitivity. Various authors have published methods[18,19] for the determination of TCs in plasma and in urine but not in tissues.

The focus of our research however has been on methods allowing the confirmation of TC in biological fluids. This paper reports the detection of TC by derivatization using GC-MS. This method could be applied in the determination of TC groups in forensic samples as well as in the environmental and toxicological samples.

## EXPERIMENTAL

### Apparatus

### GC column

Capillary column 5% phenyl methyl silicone, 30 mts width 0.25 mm I.d 0.25 film thickness.

### Gas chromatograph mass spectrometer

Perkin Elmer instrument with Autosystem XL GC, turbo mass MS, Auto sampler, 2µL injection, injector port 200, interface 200 degree, EI-mode 70 EV, Mass range 40-630 amu.

### Operating conditions

Injection port: 200 degrees, Oven isothermal: 250 degrees, carrier gas helium flow: 1mL/min; ionization voltage, 70eV.

### Reagents and chemicals

Standard reagents *viz.* methanol ($CH_3OH$), n-hexane (n-$C_6H_6$), chloroform ($CHCl_3$), sodium hydroxide (NaOH), sodium chloride (NaCl), sodium sulfate ($Na_2SO_4$) and disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) all used were AR grade.

Tetracycline hydrochloride was received from Hindustan Antibiotics Ltd., Bangalore, India as a free gift sample. Acetic anhydride and anhydrous pyridine were obtained from Merck (Darmstadt, Germany) and was pro analysis grade. De-ionized, doubly distilled water was used throughout.

TLC Plate Silica gel $F_{254}$ were obtained from Merck (Darmstadt, Germany)

### $Na_2HPO_4$ solution

1 gm of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) was taken in a beaker and then dissolved it in 100 mL of doubly distilled water.

**NaOH solution**

5 N sodium hydroxide (NaOH) solution was prepared using 20 gm of NaOH and dissolved in 100 mL of doubly distilled water.

**NaCl solution**

10 gm of Sodium chloride (NaCl) was taken in a beaker and dissolved it in 100 mL of doubly distilled water.

**Standard solutions**

**Stock solution**

Accurately weighed 100 mg of pure tetracycline hydrochloride dissolved in the methanol, the solution was made up in the 10 mL standard flask to make the concentration 10 mg/mL.

**Working solution**

Dilute standard solutions to 10, 5 and 1 µg/mL concentration with methanol from the above stock solution. These solutions have been stored in refrigerator with light protection.

**Extraction and cleanup**

Gastric cleavage samples 10 mL were taken in a 100 mL centrifuge tube. Then 20 mL of $CH_3OH$ were added into that, and then it has been homogenized for 10 minutes. The contents were rinsed twice well with 3 mL of $CH_3OH$ and the rinses were added into the centrifuge tube.

The centrifuge tube has been shaken very well for 10 minutes. The supernate solution was filtered through fluted paper into 100 mL separatory funnel. 20 mL of $CH_3OH$ was added into the residue, the centrifuge tube was shaken vigorously for 10 minutes.

The contents were centrifuged for 10 minutes at 2000 rpm after that the supernate solution was filtered through fluted paper. The filtrate was combined with first extract, which is in 100 mL separatory funnel.

Furtherly 20 mL of n-hexane were added into the combined $CH_3OH$ extract, which is in separatory funnel, it has been shaken vigorously for 5 minutes, and it has set aside to separate.

The lower $CH_3OH$ phase was drained into second 100 mL separatory funnel and the upper n-hexane phase was discarded and 2 to 3 mL of 1 N NaOH and 30 mL of $CHCl_3$ were added into the $CH_3OH$ phase and these were mixed by swirling.

In addition to this, 30 mL of 1% of $Na_2HPO_4$ solution were also added and it has been shaken vigorously for 2-3 minutes. It has set aside to separate for 15-20 minutes. The lower $CHCl_3$ phase was drained into third 100 mL separatory funnel.

Suppose, if two phases do not readily separate centrifuge 5 minutes at 2000 rpm. We have to add additionally 30 mL of $CHCl_3$ into the aqueous phase and it should be extracted again. The $CHCl_3$ extract was combined into third 100 mL separatory funnel and it was washed with 30 mL of NaCl solution.

The $CHCl_3$ phase has been transferred into 100 mL round bottom flask and solvent has been evaporated at 40 - 45° C.

**Derivatization procedure**

In an auto sampler vial 1 mL of working solution containing concentration of 1µg/mL was pipetout. It was evaporated to dryness and the dry residue was dissolved in 500 µL of anhydrous pyridine in addition to the pyridine solution 250 µL of acetic anhydride were added and the vial was closed tightly. The residue was dissolved by swirling and allowed to stand at room temperature overnight. This sample solution has been reserved in refrigerator. This is ready for injection into GC-MS apparatus.

**Evaluation of repeatability, reproducibility and linearity**

The repeatability of the derivatization procedure and the chromatographic analysis was determined by five replicate injections of the standards prepared as described above. The reproducibility of the method was determined by 5 replicate injections performed within 3 days.

Linearity of the detector response was evaluated for total tetracycline hydrochloride by plotting the total peak area *versus* total concentration and performing linear least squares regression analysis.

## RESULTS AND DISCUSSION

TC, chemically it contains an octahydronaphthacene ring skeleton, consisting of four fused rings. Structure of the TC and its fragmentation pathways are described in figure 1. Its molecular weight is relatively large, and it

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has polar functional groups such as hydroxyls and carbonyls. The derivatization reactions generally used for GC determination are silylation, acetylation, and ether formation. In this study we have chose to utilize the acetyl derivative, because it is easily prepared and rather stable, and the increase in molecular weight by derivatization is not so large. TC was acetylated in a mixture of acetic anhydride-pyridine at room temperature. The reaction mixture was checked by TLC analysis to ensure that no TC remained.

The reaction rate of acetylation at an elevated temperature is faster than that at room temperature. However, reaction at room temperature was employed because an ordinary round-bottom flask with ground-glass joint, such as was used for evaporation of solvent, may be used and the manipulations are simple and easy. The amount of acetylated product decreased about half after a week reservation in a refrigerator. Our experiences indicated that the GC-MS determination must be performed within 2-3 days after derivatization.

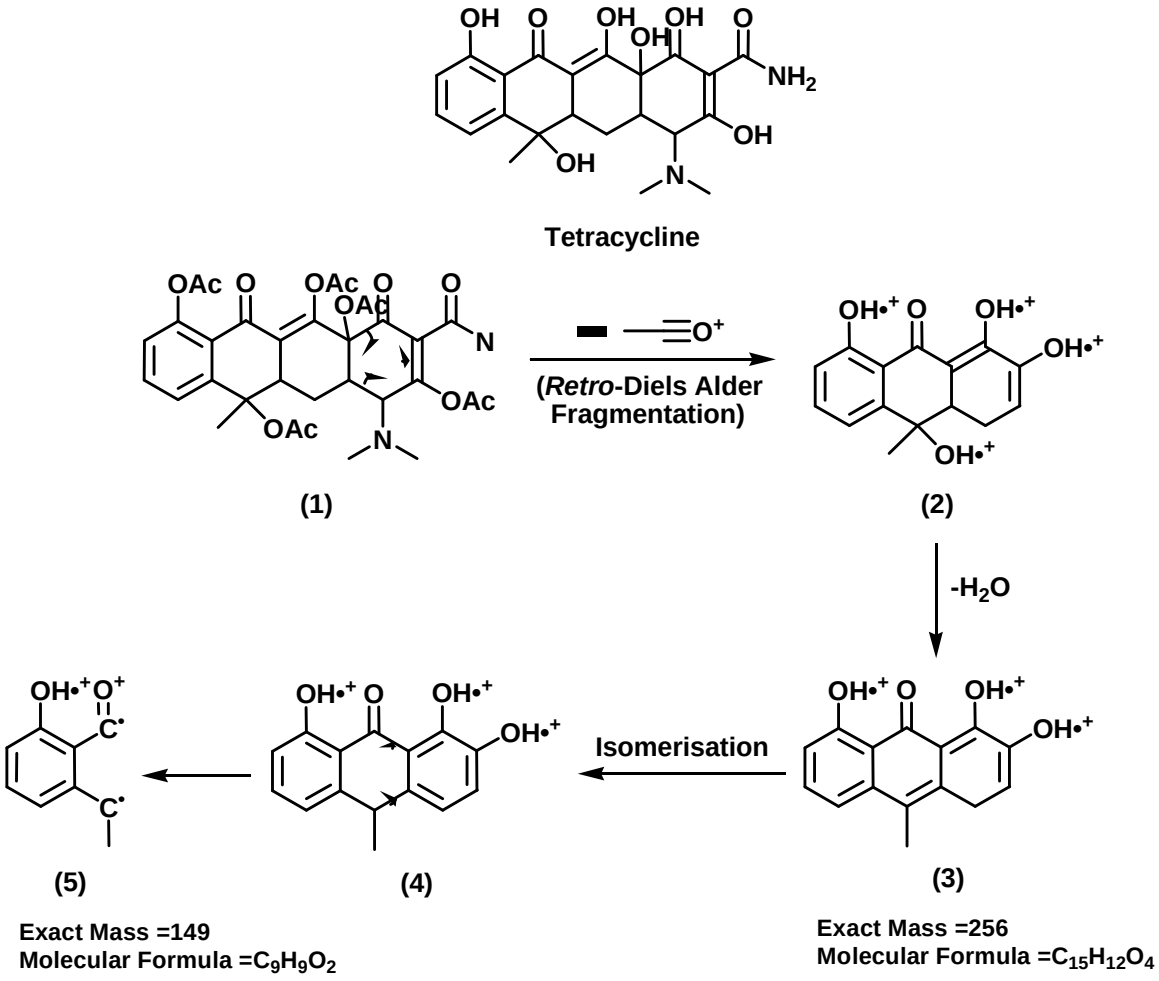


**Figure 1 : Suggested fragmentation pathway of tetracycline**

The derivatized product was injected into the GC-MS apparatus, and a total ion chromatogram (TIC) and a full scan mass spectrum were obtained. Figure 3 shows the mass spectrum, in which the base peak of m/z 149 is very prominent and m/z 85, 133, 191, 223 and 249 are observed but relatively week. The m/z 149 ion is considered to be the radical cation (Figure 1). Sphon[20] suggested that simultaneous monitoring of 3 ions is usually regarded as a minimum for identification by selected ion monitoring of GC-MS spectra. In this work, we attempted to detect tetracycline acetate using selected ion monitoring at m/z 149,133 and 223. However, the sensitivity of detection is much lower (<1/10) than that using single-ion monitoring at m/z 149 only.

The detection limit was determined by analysing samples at various concentrations with this method, and

it was found that 0.01 μg/mL of residual TC in a sample can be detected using SIM. Figure 2 shows the TIC at equal sensitivity of extracts from blank samples. The peak of tetracycline acetate can be clearly identified, thus the detection limit was determined to be at least 0.01 μg/mL in sample. We determined the lowest concentration of TC, which enables a full scan mass spectrum to be obtained by analyzing samples at various concentrations. Figure 3 shows the full scan mass spectrum of the peak indicated at retention time 1.33. However, many background ions appeared on the full scan mass spectrum of this peak, and confirmation was not possible. Thus it is possible to confirm tetracycline by a full scan mass spectrum if residual concentration in a sample is $\geq$ 1 μg/mL.

Although an internal standard is not used in this method, the separation of the GC column and the selectivity of SIM detection, considered together, offer the reliable screening of residual TC in biological fluids and a confirmation of the presence of TC in samples of high residue level is possible. This method could be applied in the determination of TC group of antibiotics in forensic samples as well as in the toxicological and in environmental samples.

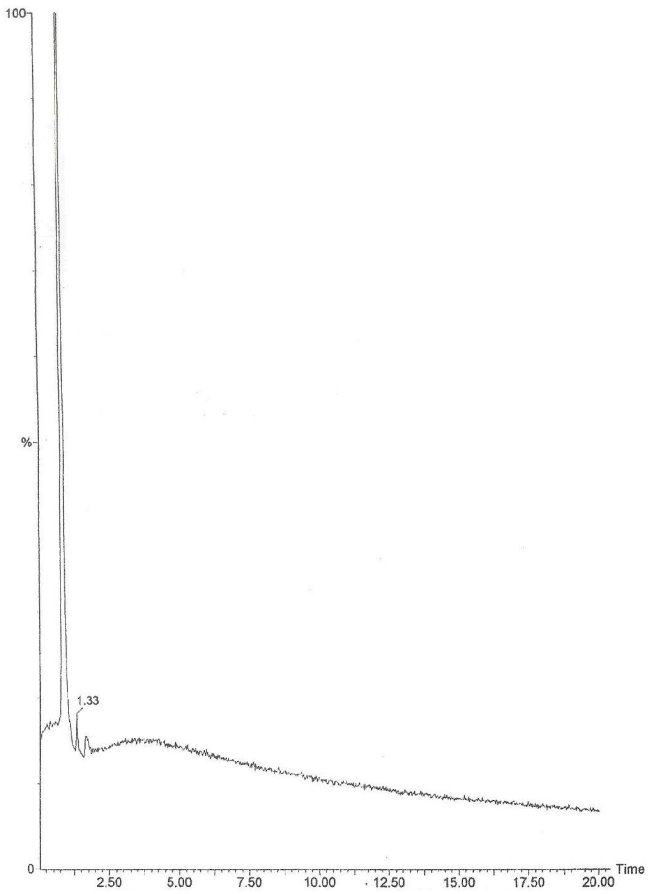


**Figure 2 : Total ion chromatogram (70 eV) of tetracycline**

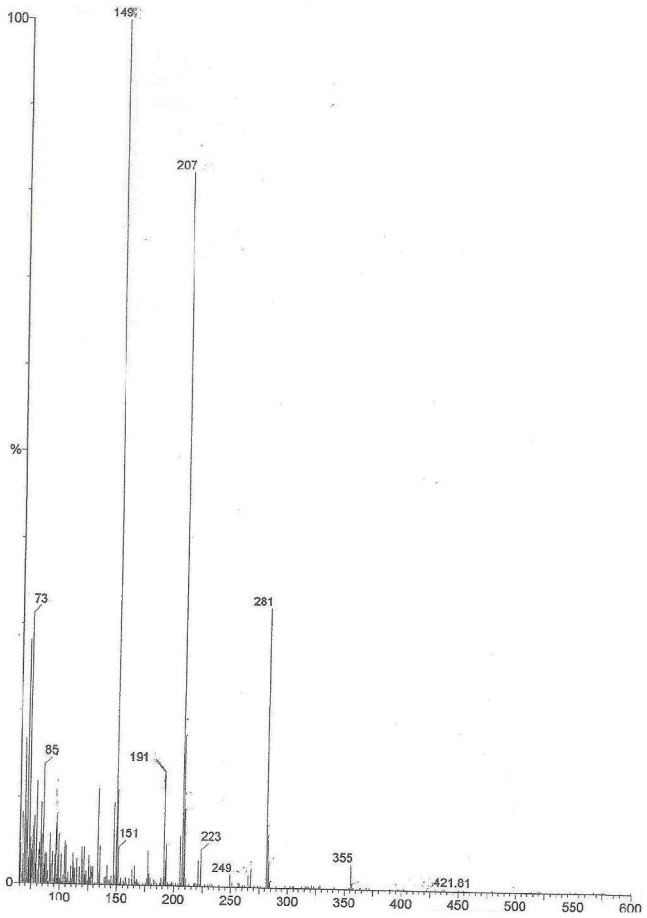


**Figure 3 : Electron impact (El) mass spectrum of the peak indicated at retention time 1.33 min.**

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