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Analytical Methods

Analysis of residual oxytetracycline in fresh milk using polymer reversed-phase column

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Abstract

A simple and rapid reversed phase high performance liquid chromatograph (HPLC) method for analysis of oxytetracycline (OTC) was developed and applied in the determination of the antibiotic in fresh milk sample. Isocratic elution was performed with acetic acid:-water (pH 4.5):acetonitrile (4:68:28), using a polymer reversed-phase (PLRP) column and UV detection at 354 nm wavelength. The average recoveries of OTC spiked milk at 0.1, 0.2, 0.5 and 100 ng/mL were in excess of 92% with intraday and interday precision between 0.8% and 6.6% respectively. A good linearity was established between the range 100–1000 ng/mL with $r^2 = 0.9995$. The limit of detection and quantitation were 30 and 100 ng/mL respectively. The method demonstrated successful application for analysis of 100 milk samples. Two samples out of 70 from livestock keepers tested OTC positive while none of the 30 samples from milk centers tested positive.

Keywords: Oxytetracycline; Tetracyclines; Polymer reversed-phase column; Fresh milk; Deproteinization extraction

1. Introduction

Oxytetracycline (OTC) is one of the tetracyclines, a group of broad-spectrum antibiotics. It is produced by strains of *Streptomyces rimosus* and was introduced in 1950 (Jawetz, 1997). Chemically it is (4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (BP, 2005). See Fig. 1. Other antibiotics in this family are tetracycline, chlortetracycline, demeclocycline, methacycline, doxycycline, rolitetracycline and minocycline. Other related substances are 4-epioxytetracycline (EOTC) formed by epimerization of OTC at pH 2–6; 4-epitetracycline (ETC) formed by epimerization of tetracycline at pH 2–6; terrinolide (TL) and α- and β-apooxytetracycline (α-APO and β-APO) both are the result of acid on anhydrooxytetracycline (AOTC) and 2- acetyl-2-decarboxamidooxytetracycline (BP, 2005; Van Schepdael & Hoogmartens, 2002). Because of its antibacterial action, OTC is used widely not only in human but also in veterinaries as well as in the meat production industries, where it is used as feed additive or in drinking water to maintain optimal animal health for food production (Popadoyannis, Samanidou, & Kovatsi, 2000). It is extensively used in the following veterinary animals; cows, poultries, pigs, dogs and cats. For cows it is used to treat enteritis, pneumonia, endometritis, septicemia, mastitis, metritis and other secondary bacterial infections (Naoto, 1999; Veterinary Oxytetracycline, 2005).

Tetracyclines are excreted in milk; their concentrations in breast milk of lactating mothers are approximately 70% of maternal serum concentrations (Gideon & Martin, 1997). The same scenario happens in veterinaries and substantial levels of OTC have been detected in milk obtained from cattle on OTC therapy (Boatto, Pau, Palomba, Arenare, & Cerri, 1999; Long, Hsieh, Malbrough, Short, & Barker, 1990; Schenck & Callery, 1998). Consumption of milk with such OTC levels by humans predisposes them to serious health effects. Examples of such health risks include: induction of allergic reactions to some individuals (Rudzki & Rebandel, 1997); development of bacterial resistance (Rudzki & Rebandel, 1997), risk of teratogenicity when

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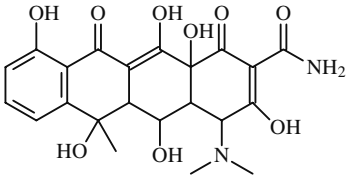

Fig. 1. Oxytetracycline structure.

OTC is administered in the first trimester of pregnancy (Stauffer, 1967); discoloration of primary and permanent teeth (Stauffer, 1967); possibility of hypoplasia in developing teeth when OTC is administered to infants, children below 12 years of age or mothers during the last two trimesters of pregnancy (Senyuva, Ozden, & Sarica, 2000).

With regard to the discoloration of teeth, the risk of this untoward effect is highest when OTC is given to neonates and babies prior to the first dentition. However pigmentation of the permanent dentition may develop if the drug is given between the ages of 2 months and 12 years, when the teeth are being calcified. An early characteristic of this defect is a yellow fluorescence of the dental pigment, which has an ultraviolet spectrum with an absorption peak at 270 nm (Senyuva et al., 2000). The deposition of the drug in the teeth and bones is probably due to its chelating property and the formation of a tetracycline calcium orthophosphate complex (Senyuva et al., 2000). As time progresses, the yellow fluorescence is replaced by a non-fluorescent brown color that may represent an oxidation product of the antibiotic, the formation of which is accelerated by light (Senyuva et al., 2000).

To prevent any harmful health effects to consumers, Food and Agricultural organization (FAO), World Health Organization (WHO), US Food and Drug Administration (FDA), European Union (EU) and Japan have established the maximum residual limit (MRL) of OTC in all circulating milk in their countries as 100 ng/mL Naoto, 1999; and the 'safe levels' set by the US Food and Drug Administration are 30 ng/mL for Oxytetracycline, 30 ng/mL for chlortetracycline and 80 ng/mL for tetracycline (Popadoyannis et al., 2000). Also the WHO recommends a maximum allowable level of 100 ng/mL for oxytetracycline (Boatto et al., 1999).

The widespread use of antibiotics in dairy cattle management may result in the presence of antibiotic residue in milk. While rapid screening tests are commonly used to detect presence of antibiotics in milk, more accurate chromatographic methods are required (Boatto et al., 1999) by Government Regulatory Authorities to identify and quantify the amount of antibiotic present. Recently developments in the chromatographic techniques for determinations of antibiotic residues in milk have been reviewed (Delgado & Remes, 1998; Long et al., 1990; Martindale, 2000; Naoto, 1999; Tanase et al., 1998). In order to monitor compliance to these limits several analytical methods have been developed and validated (Long et al., 1990; Naoto, 1999; Schenck & Callery, 1998; Senyuva et al., 2000; Tanase et al., 1998).

LC is the chromatographic approach most commonly used for the determination of tetracyclines in residues in bovine milk. The tetracyclines are amphoteric, forming crystalline salts with acids and bases.

Oxytetracycline like most tetracyclines has a strong tendency to bind irreversibly to the silanol groups in silica-based LC stationary phases resulting in peak tailing (Schenck & Callery, 1998).

This problem has been overcome by adding oxalic acid to the mobile phase (Long et al., 1990; Oka et al., 1994; Thomas, 1989) and use of RP-C8 end-capped (Naoto, 1999). Polymeric columns (PLRP) enjoy wide application in the analysis of amphoteric molecules like tetracycline, which could not give good separation on the classical reversed-phase columns.

The chemistry of polystyrene/divinylbenzene (PS/DVB) HPLC phases feature outstanding chemical and physical stability (Polymerlab, 2007). The media is inherently hydrophobic and reproducible, and does not require a bonded alkyl chain to confer hydrophobicity, unlike alkyl-functionalized silica reversed-phase materials.

Polymeric materials do not possess residual surface functionalities and do not, therefore, suffer from the typical silica problems of acidic silanol groups or other ionic species, which can interfere with the separation performance of the matrix and are difficult to remove entirely and reproducibly by endcapping. As a result, polymeric columns show excellent stability and reproducibility (BP, 2005; USP27, 2006).

The benefits of using polymeric HPLC columns for analysis of other tetracyclines are illustrated in the European and US Pharmacopeias (Phr. Eur., 2002; USP27, 2006).

Presently the application of these novel stationary phases for analysis of oxytetracycline has not been fully demonstrated. The present paper describes a simple procedure for PLRP–LC determination of residual OTC in milk with a simple and robust extraction based on deproteination and centrifugation and detection is performed by using an ordinary UV detector, which is more available and affordable to developing countries compared to the more expensive photo diode array detector.

2. Experimental

2.1. Materials and reagents

Oxytetracycline 98% "as is" and tetracycline 99.5% reference standards were donated by Prof. Hoogmartens & Prof. Ann Van Schepdael of the Laboratory for Drug Analysis, KULeuven, Belgium. Acetonitrile from Sigma–Aldrich Laborchemikalien GmbH, Germany; acetic acid 100% (v/v) from Schuchardt, 85662 Hohenbrunn, Germany and trichloroacetic acid (TCA) 100% v/v bought from S.D. fine-CHEM Ltd., Mumbai. Double distilled water was prepared in our lab and sodium hydroxide was of analytical chemical grade. Fresh milk samples for

analysis were purchased from different centers selected by convenience.

2.2. Instrumentation and HPLC conditions

The chromatographic system was operated in an isocratic mode, consisting of commercial components: A Prostar 230 Varian pump, a Prostar 310 variable UV–Vis detector, operated at 354 nm and a sensitivity setting of 0.002 (AUFS), A Prostar 410 autosampler with a 9125 Rheodyne (CA, USA) injection valve (PA, USA) with a 20 mL loop (Kyoto, Japan). The PLRP-S 100A 8 mm analytical column was in- house packed in the Laboratory of Prof. Hoogmartens using polymer packing materials (PLRP-S 100A 8 mm) from Polymer Laboratories Inc., MA, USA. A centrifuge from Heltich Zentrifugen, Tubingen, Germany. The mobile phase consisted acetic acid–water (pH adjusted using 1 M NaOH solution to 4.5)–acetonitrile [4:68:28] at a flow rate of 0.8 mL/min.

2.3. Fresh milk samples

Fresh milk sample products were collected from shops, milk centers and farmers from all the three districts of Dar es Salaam city. Sampling procedures for the milk centers and farmers was done by convenience since there are no specific centers in some areas and the distribution of farmers is not normal. For the processed milk from milk industries, samples were collected basing on their sources and batch numbers. A total of 100 different samples were collected; 40 from shops (packed milk), 30 from milk centers and 30 from farmers.

2.4. Analytical procedures

2.4.1. Preparation of standard solutions

A stock standard solution of OTC was prepared by accurately weighing 10 mg of standard OTC powder, dissolved in 100 mL of 1% (v/v) acetic acid (in water) solution. Working standard solutions were prepared by diluting the stock solution with 1% acetic acid. These solutions were stored in a refrigerator. Naoto (1999) reported that the OTC solution is stable for up to one month upon storage in a refrigerator.

The mobile phase consisted of a solution of acetic acid–water (pH 4.5 adjusted with 1 M sodium hydroxide)–acetonitrile [4:68:28].

2.4.2. Extraction

Milk samples and standard spiked milk were subjected to a deprotenizing chemical procedure using TCA. A 2 mL quotient of milk sample was placed into a 10 mL test tube and shaken intensively with 3 mL of 20% (v/v) TCA for 1 min. The mixture was centrifuged for 15 min and 20 μL of the supernatant was injected into the HPLC system.

2.4.3. Extraction recovery

The recoveries of OTC were determined from blank milk samples spiked at 0.1, 0.2, 0.5 and 1.0 μg/mL. These spiked levels were prepared by adding 200 μL of four standard solutions of OTC (1.0, 2.0, 5.0 and 10.0 μg/mL) respectively to separate 2.0 mL portions of the milk samples, followed by thorough mixing. These fortified samples were allowed to stand at 4 °C for 16 h after OTC addition. Six samples of blank fresh milk were used for intraday precision determinations. For interday precision three injections were made for each sample concentration level for six days.

3. Results and discussion

3.1. Optimisation of HPLC conditions

In the present work extracts obtained by previously described cleanup procedure (Fletouri, Botsoglou, Psomas, & Mantis, 1996; Moats & Kotula, 1993; Naoto, 1999) were analyzed on PLRP–LC to determine oxytetracycline content. These studies demonstrated that extraction/deproteinization was simple, rapid and gave essentially quantitative recoveries. The initial experiment using previously developed mobile phase (Naoto, 1999) did not give good separation. There was a co-elution of OTC peak with an endogenous peak as a result of peak tailing and fronting. The previous paper established that increasing the amount of acetonitrile in the mobile phase improves the selectivity and decreases retention time of OTC. The proportion of acetonitrile:water in the mobile phase was altered but only the retention time was changing, the selectivity between OTC and endogenous substance was not improved. In another trial the proportion of TCA in the extraction solvent was also changed with no significant gain in selectivity. When the pH of the aqueous component was increased from 2.48 to 4.5 with 1 M NaOH, baseline separation was obtained. Thus this study proposes the following optimal chromatographic conditions: PLRP-S 100 A 8 mm column, mobile phase consisting of acetic acid–water (pH adjusted using 1 M NaOH solution to 4.5)–acetonitrile [4:68:28] at a flow rate of 0.8 mL/min. and the analysis was done using chromatographic system operated in isocratic mode consisting of the commercial components. Typical chromatogram of standard OTC 100 ng/mL is shown in Fig. 2. Compared to existing method (Zhao, Zhang, & Gan, 2004) this method uses a simple UV–Vis detector that more readily available and convenient to use in many labs and particularly in developing countries.

3.2. Method validation

3.2.1. Recovery

Replicate injections (six) of spiked OTC milk at concentrations of 0.1, 0.2, 0.5 and 1.0 μg/mL enabled evaluation of precision. The peak areas and percentage recoveries were recorded at all the four levels. The intra and inter

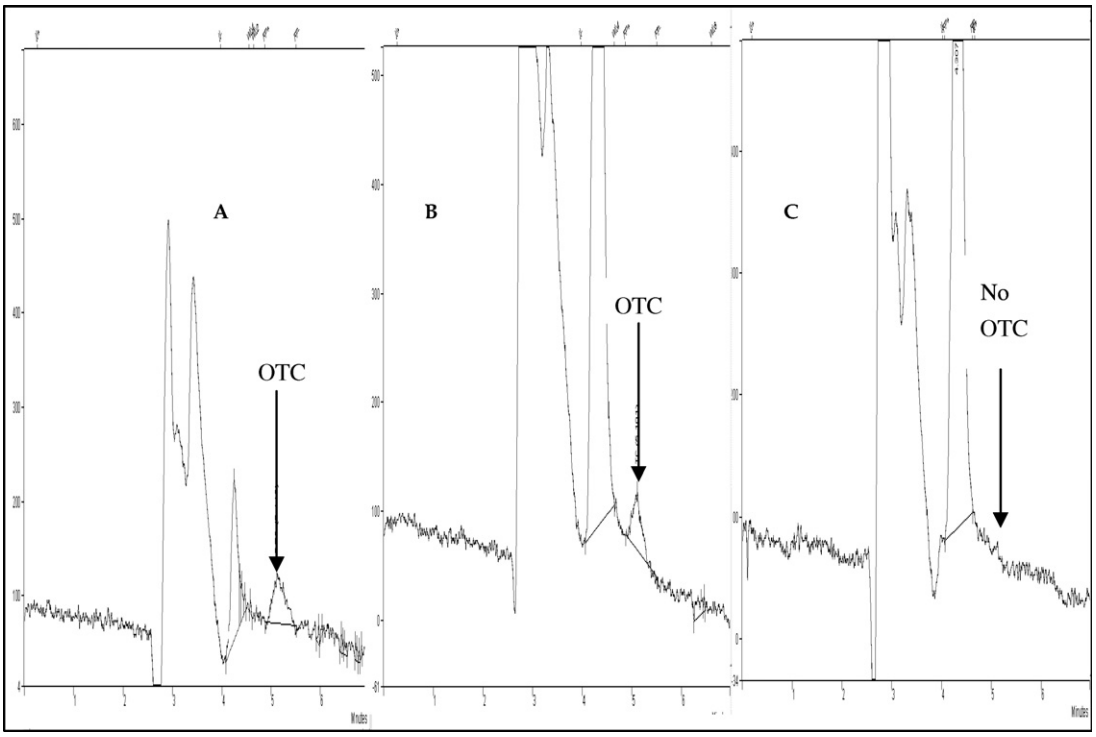

Fig. 2. HPLC chromatograms OTC retention time 5.0 min: (A) standard OTC (100 ng/ml), (B) typical chromatogram of fresh milk spiked with OTC at 100 ng/ml and (C) blank milk (UV detection at 354 nm).

day recoveries with their corresponding rsd % are summarized in Table 1. Acceptable results were obtained and the average recoveries were greater than 92% with % rsd ranging between 0.8 and 6.6% in all the levels tested.

3.2.2. Linearity of calibration curve

The standard curve was plotted as the peak area vs. concentration with five levels. The equation of standard curves was: $y = 3936.3x - 74.475$, $r^2 = 0.9993$, (range 100.0–1000.0 ng/mL), standard error of slope $S_{y,x\ \text{slope}} = 52$, of intercept $S_{y,x\ \text{intercept}} = 27$, and ($n = 3$) number of standards, where y is the OTC peak area and x is the concentration of OTC in n/mL and $S_{y,x}$ = standard error.

3.2.3. Accuracy

A six level calibration curve was prepared by spiking milk with standard OTC. Separate three control standards at 0.2, 0.3 and 0.5 μg/mL levels were prepared and analysed. The accuracy data are summarized in Table 2.

Table 1
Recoveries of oxytetracycline from milk at four concentration levels

Spike concentration (μg/ml)	% Recovery for intra day[a]	% Recovery for inter day[b]
0.1	94.0 (5.4)	94.9 (4.5)
0.2	94.2 (2.0)	93.1 (6.6)
0.5	97.2 (1.9)	95.8 (5.4)
1.0	92.8 (0.8)	95.1 (6.2)

Data are averages, [a]($n = 6$); [b]($n = 18$); in % RSD in parentheses.

3.2.4. Specificity

The specificity was tested to evaluate the ability of this method to accurately and precisely determine OTC in the presence of the potential interfering compounds like the other tetracycline analogs with their relative retention times in the parenthesis; tetracycline (1.2), chlortetracycline (1.4), doxycycline (2.1), minocycline(2.8) and the interference of the sample matrix and reagents used were evaluated by performing a blank determination in which it was found that no interference, that is no peak was eluted with the retention time of OTC. The chromatograms are depicted in Fig. 2.

3.2.5. Limit of detection (LOD) and quantitation (LOQ)

The limit of detection is considered to be the quantity yielding a detector response approximately equal to thrice the background noise. Thus, the minimum detectable quantity was found to be 40 ng/mL. The limit of quantitation is the lowest amount that can be analyzed within acceptable precision and accuracy, was found to be 100 ng/mL RSD 5.4%, $n = 6$ at signal to noise ratio of 10.

3.3. Monitoring residues in marketed milk

A total of 100 samples were analyzed, only two samples from the farmers that had OTC (<100 ng/mL), which was below LOQ. All samples from local milk centers and milk industries (packed) tested negative for OTC. Samples from milk centers; stores and farmers gave negative results because of dilution as a result of mixing milk from OTC

Table 2
Accuracy of oxytetracycline from milk at three levels

Spiked concentration (μg/ml)	Accuracy (%)	RSD (%)	Replicates (*n*)
0.2	82.9	2.5	6
0.3	94.8	1.3	6
0.5	97.4	2.1	6

treated cows and that from untreated cows. A study spanning throughout the year will rule out any possible seasonal variation of disease pattern and hence the extensive use of oxytetracycline.

4. Conclusions

The present method, based on polymer reversed-phase allowed good separation of OTC without requiring ion-pairing reagents. It is simple, accurate, and sensitive with good robustness. The extraction procedure described is simple and cost effective suiting application in resource-constrained laboratories in developing countries.

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