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Ion chromatographic analysis of tetracyclines using polymeric column and acidic eluent

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Abstract

High-performance ion chromatography (HPIC) is first successfully used to analyze tetracycline antibiotics (TCs) in this work. The TCs are well separated on a solvent compatible polymeric cation-exchange column within 12 min. Isocratic elution with acetonitrile-hydrochloride is very advantageous for routine analysis. HPIC may be seen as a specific variant of the more common high-performance liquid chromatography (HPLC) for water-soluble and polar pharmaceuticals with low hydrophobicity. The detection limits (signal-to-noise ratio=3:1) of oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), doxycycline (DC) are 10, 10, 20 and 20 $\mu g\ l^{-1}$, respectively. Samples are prepared by vortex mixing with an ethylenediaminetetraacetic acid disodium salt (Na_2EDTA)–McIlvaine buffer (pH 4.0) solution and the mixture filtrates through a molecular weight cut-off filter. The method has been successfully applied to monitor the OTC removal rate through every reactor in the process of OTC manufacturing wastewater treatment by bio-chemical technology. It is also applicable to determine the TCs residues in milk and milk powder with satisfying results.

Keywords: Antibiotics; Tetracyclines

1. Introduction

Tetracyclines(TCs) are broad-spectrum antibiotics with low price. They have been extensively used to control bacterial infections in both humans and animals over the past decades. Recent clinical studies of TCs have showed that they not only have an auxiliary treatment of tumor but also have some non-antibiosis, such as they can inhibit the activity of collagen enzyme and promote bone absorption, etc. [1,2]. Since oxytetracycline (OTC) and chlortetracycline (CTC) as feed additives were legally used in agriculture in the 1950s, they as well as tetracycline (TC) are still widely used in many countries for the prevention and treatment of disease and promotion of growth in current intense animal husbandry practices.

However, the failure to follow label directions for approved tetracycline antibiotics could result in unsafe TCs residues in food-producing animals, with potential adverse effects on human health. In China, maximum residue limits (MRLs) have been set for OTC, TC and CTC in a number of tissue types, including 0.1 mg/kg in milk, 0.25 mg/kg in edible tissue and 2 mg/kg in liver [3]. Safety evaluations of

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the TCs residues are often taken into account by regulatory agencies around the world. A critical part of such safety evaluations depend on the assay methods used to determine the quantities of residues present. So, new methods with high sensitivity, rapidity and specificity for the determination of the TCs residues must be developed accordingly.

The TCs drugs usually contain small amount of impurities such as 4-epimers, especially when they are out-of-date or stored under high temperature and humidity. The impurities possess almost no or very slight microbiological activity. 4-Epianhydrotetracycline (EATC) and anhydrotetracycline (ATC) have already proven toxic [4]. Chromatography of anhydrotetracycline is somewhat more difficult to achieve than that of the parent compounds because of the lower polarity of the former. Often a column might be found which appeared to chromatograph parent tetracyclines well but produced poor peak responses for anhydrotetracyclines. Therefore, there is also a need to determine the impurities in pharmaceutical formulations to control the TCs drug quality.

Oxytyetracycline manufacturing wastewater contains high concentrations of sulfate, ammonium and organics with inhibitory action to microorganism, which is difficult to biodegrade. In the process of biochemical technique for treatment of the OTC manufacturing wastewater, it is necessary to monitor the OTC removal rate of every reactor to know whether the inhibitory action to micro-organism has been reduced. However, the commonly used spectrophotometric method has been found to fail when determining the concentration of OTC in the OTC manufacturing wastewater owing to its low sensitivity and lack of selectivity. As a result, the analysis of tetracycline antibiotics is still an active area of research in recent years [5–9].

Methods used to determine TCs residue levels include, but are not limited to, microbiological and chromatographic techniques such as thin-layer, gas, and liquid chromatography. Microbiological methods lack specificity and thin-layer chromatographic methods lack sensitivity. The most often employed reversed-phase high-performance liquid chromatography (RP-HPLC) encountered a serious problem, namely the TCs have a tendency to bind irreversibly to the silanol groups on silica-based materials (C_8, C_{18}) which result in peak tailing and low column efficiency. This has been overcome by adding oxalic acid (pH 2.0) to the mobile phase [10–13] to suppress the dissociation of the TCs molecules and by using polystyrene-divinylbenzene (PS-DVB) LC columns such as the Polymer Labs. PLRP-S. However, the reversed-phase silica-based materials are unstable when the pH is 2.5 or less, at which the highest column efficiencies are obtained [14]. Thus, the columns have to be flushed with a neutral solvent (e.g. water–acetonitrile, 50:50) for 1 h at the end of each working day in order to significantly prolong column life [8,9], which accordingly increases the consumption of organic solvent. Poly (styrene-divinylbenzene) (PS-DVB) packing materials are known to be very stable in extreme pH conditions (pH1–13). They are generally utilized to separate the common impurities and degradation products from the parent TCs drugs [15–18]. Capillary electrophoresis (CE) has ever been applied to determine the impurities in tetracycline hydrochloride [19]. It is rarely used in the analysis of TCs drug residues at low parts-per-billion level mainly because of the higher limit of detection caused by small volume of sample introduced ($<$100 nl). However, by developing a proper sample pretreatment method, CE can also simultaneously determine CTC, DC, OTC and TC residues in bovine milk, serum and urine [20].

In addition, several ion-exchange column chromatographic assays were also used for the analysis of TCs and were summarized by Knox et al. [14] in 1979. It was found that reversed-phase bonded materials (ca. 5 μm) gave better separations with plate heights ranging from 0.1 to 4 mm, and cation-exchangers were better than anion-exchangers. The ion-exchangers were pellicular materials of fairly large particle size (ca. 40 μm), which result in the low separation efficiency with typical plate height (H) values$>$5 mm. From then on, reversed-phase bonded materials became dominant in the analysis of TCs drugs and ion-exchangers were rarely used due to their low efficiency. The British Pharmacopoeia monograph for oxytetracycline calcium includes an HPLC assay [21], but the column packing specified is silica based strong cation-exchanger, which is not available commercially and must be prepared by the analyst.

Since ion chromatography (IC) was first introduced by Small in 1975, it proved a powerful

technique for the analysis of inorganic cations and anions in aqueous systems. To realize the full potential of the IC ion-exchange resins for liquid chromatography, they lack only solvent compatibility. However, with the development of the IC technique goes on, ion-exchange resins for IC has overcome the shortcomings of the conventional ion-exchange materials and progressed to organic ion problem solving. Some new IC columns, which are solvent amenable ion-exchange packing materials with high column efficiency, have been developed and are commercially available. Thus, the IC analysis of the amphoteric TCs is possible. To the best of our knowledge, no ion chromatographic separation of the TCs has been reported hitherto.

The aim of this paper is to present a novel HPIC method for the simultaneous analysis of OTC, TC, CTC and DC. The four TCs can be assayed in a single isocratic elution, which is advantageous to practical application. To validate the newly established method, it has been successfully applied to monitor the OTC removal rate through every reactor in the process of OTC manufacturing wastewater treatment by bio-chemical technology and the acquired results can be used to evaluate the function of the reactors. It is also applicable to determine the tetracycline residues in milk and milk powder samples.

2. Experiments

2.1. Instrumentation

Chromatographic analysis were performed on a metal-free Dionex-4000i ion chromatography (Dionex, Sunnyvale, CA, USA), which included one advanced gradient pump (AGP), one eluent degas module (EDM) and a VDM-2 variable wavelength absorbance detector at 350 nm. A Dionex OmniPac PCX-100 analytical column (250×4 mm I.D.) was used. The column packing consists of a highly crosslinked microporous ethylvinylbenzene/divinylbenzene polymeric core covered with a pellicular layer of cation-exchange latex. The retention times represent the average values from at least three injections with a relative standard deviation of less than 5%. A 513 μl injection loop was used. The flow-rate of eluent was 1.0 ml/min. All measurements were made at room temperature (16±2°C) and all samples were filtered through a 0.45-μm and 30 000 Da filters in turn prior to injection. In all cases, injection of the sample was done at least in triplicate.

Molecular weight cut-off filters at 30 000 Da was kindly gifted by the Membrane Division of the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences (Beijing, China). Centrifuge (the Tenth Plant of Shanghai Operation Instrument, Shanghai, China). Vortex mixer

The spectrophotometric experiments were carried out by a Shimadzu UV-120-02 spectrophotometer (Kyoto, Japan) with 1-cm quartz cell.

Data collection and the operation of all components in the system were controlled by Dionex AI-450 chromatographic software interfaced via an ACI-2 advanced computer interface to a 80486 based computer.

2.2. Reagents

All reagents were analytical grade unless specified otherwise. All solutions were prepared with de-ionized water throughout. Standards of oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), doxycycline (DC), 4-epitetracycline (ETC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC) hydrochloride salts were bought from the Institute of Chinese Pharmaceutical and Biological Product Inspection (Beijing, China). Hydrochloric acid (HCl, G.R), ethylenediaminetetraacetic acid (EDTA) disodium salt, citric acid, sodium phosphate dibasic dodecahydrate and acetonitrile were all from Peking Chemical Works, Peking, China.

The stock solutions were prepared by dissolving the TC standards in hydrochloric acid (0.04 mol l^{-1} for OTC and DC, 0.01 mol l^{-1} for TC, ETC, ATC and EATC, 0.1 mol l^{-1} for CTC) to give concentrations of 1 mg ml^{-1} for OTC and TC; 0.1 mg ml^{-1} for CTC, DC, ETC, EATC and ATC. Working standard solutions were prepared daily by diluting the stock solutions to the desired concentration with 0.01 mol l^{-1} HCl. All solutions were stored at <4°C in plastic bottles wrapped in aluminum foil. The standard solution could be kept for at least 1 week.

To make 0.1 mol l^{-1} EDTA-McIlvaine buffer, 27.6 g sodium phosphate dibasic dodecahydrate, 12.9 g citric acid monohydrate and 37.2 g ethylenediaminetetraacetic acid (EDTA) disodium salt were dissolved, respectively, then mixed in 1000 ml flask and diluted to volume.

2.3. Eluent

0.2 mol l^{-1} hydrochloric acid–27.9% acetonitrile. The eluent was degassed for only 15 s prior to use. Excessive degas can result in the loss of acetonitrile. As the Dionex ion chromatographic equipment is made of polyether ketone (PEEK), it is not damaged by the HCl eluent. In this study, the peak area was used for quantification.

2.4. Sample preparation

(1) The OTC manufacturing wastewater collected from oxytetracycline production plant of North China Pharmaceutical Corporation (Shijia Zhuang city, HeBei Province, China) was filtered through a 0.45 μm and a 30 000 Da filter membrane in turn. The filtrates were clear and colorless and could be directly injected into the IC system after dilution.

(2) Milk and milk powder were purchased from local market. A 10 ml milk sample and 20 ml 0.1 mol l^{-1} EDTA-McIlvaine buffer (pH 4) were placed into a 50-ml flask and dilute to volume with deionized water. Then the mixture was stirred for 20 min on a Vortex mixer. Centrifuged at 3500 rev./min for ca. 15 min. The supernatant was filtered through a 0.45 μm and a 30 000 Da filter membrane in turn. If the clear, colorless filtrates will not be analyzed at once, they can be stored at −20°C and protected from light for 3 days. Tetracyclines are not stable in pH 4 McIlvaine buffer, but no losses occur under these conditions for 3 days [8].

3. Results and discussion

3.1. Choice of separation system

The correct selection of a separation system is the first thing to be considered. Selection of column types in combination with solvents is depedent upon the type of the analyte ions. As a general rule, hydrophilic ions are better separated on hydrophilic column packings, and hydrophobic analytes are better on hydrophobic packings [22]. Tetracyclines are derived from a system of four six-membered rings arranged linearly with characteristic double bonds (see Fig. 1). They are amphoteric compounds with high polarity and an isoelectric point between 4 and 6. They are soluble in aqueous and polar (or moderately polar) organic solvents, and have several functional groups resulting in strong complexing properties. Three prototropic dissociations have been observed for the TCs, with $pK_1=3.3$, $pK_2=7.5$, $pK_3=9.4$. In strongly acidic pH (for example 1–2.5), the TCs molecules exist in the fully protonated form as a singly charged cation [14] which is favorable for the successful separation of the TCs by cation-exchange ion chromatography. Thus a Dionex OmniPac PCX-100 analytical column, which is advantageous for low hydrophobic analytes is employed. Unlike the conventional ion-exchangers with pellicular materials of fairly large particle size (ca. 40 μm) and relatively low separation efficiency (typical plate height (H) values >5 mm), this column is latex-coated pellicular cation-exchanger. The latex is on the order of 60–100 nm in diameter, fully sulfonated. The substrate is 8-μm diameter particles made from ethylvinylbenzene (EVB) cross-linked with >50% divinylbenzene (DVB). The OmniPac PCX-100 is coated with a medium hydrophobicity stationary phase and is a solvent compatible cation-exchange column. It remains stable between pH 0 and pH 14

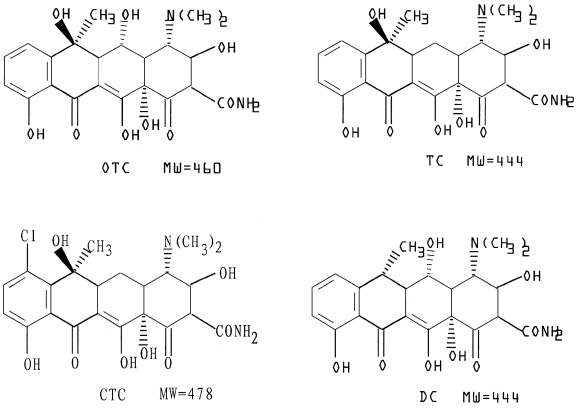

Fig. 1. Structures of tetracyclines.

and compatible with eluents containing 1–100% organic solvents, which facilitates eluent selection [23]. In this study, acetonitrile was preferred to methanol because of its lower column backpressure it creates. In addition, column cleanup and sample solubility may all be enhanced through addition of organic solvents to the eluent.

In the IC separation of organic cations it has long been known, or at least suspected, that the mechanism involved more than simple ion-exchange [24]. Hoffman [25,26] has shown that two mechanisms occur in such cases: ion-exchange and hydrophobic interaction between the sample cations and the resin matrix. There is no exception of the TCs. Because the TCs have similar structures and the same charges, the retention behavior was mainly controlled by hydrophobic interaction. The elution sequence of OTC, TC, CTC and DC was mainly in agreement with their relative degrees of lipophilicity. The *P* values (partition coefficient in octanol–water at pH 2.1) were used to depict the lipophilicity of the TCs. They were 0.0035, 0.014, 0.15 and 0.52 for OTC, TC, CTC and DC respectively [27]. For DC was the strongest lipophilic analyte of the above four TCs ($P=0.52$), it was the last to elute and the acetonitrile concentration had a significant effect on its retention time as illustrated in Fig. 2. Acetonitrile, a neutral molecule, can compete with TCs for adsorption on a lipophilic ion-exchange site. In fact, of course, only an ion can occupy an ion-exchange site. In reality, the effect of added solvent is to improve the solvation of the environment surrounding the ion-exchange site. This improved solvation, lessens the tendency of hydrophobic analytes to interact with the polymer matrix of the ion-exchange phase by a secondary hydrophobic mechanism. Thus, with the increase in acetonitrile concentration, the retention times of OTC, TC, CTC and DC were reduced as shown in Fig. 2 when 0.2 mol l^{-1} HCl remains unchanged in the eluent. Taking the separation and detection into account, the concentration of acetonitrile was set at 27.9% (V/V).

HCl as an easily available acid is normally the first choice as the acidic component of PCX-100 eluent. It is used to fully protonated the TCs and additional amounts of it in the eluent will help to elute the analytes. Fig. 3 illustrates the effects of HCl concentration on retention times of the TCs when 27.9% (V/V) CH_3CN remains unchanged. The retention times of the TCs also decrease with the increase in HCl concentration. However, when the HCl concentration increases to a certain degree, it will be immiscible with acetonitrile. So, 0.2 mol l^{-1} is selected as the HCl concentration for safety.

In order to further improve the cation-exchange selectivity, sodium chloride, potassium chloride, sodium perchlorate and ammonium acetate were ever added to the eluent, It was found that they all reduced the retention times of the TCs, but ammonium acetate as a highly hydrated salt was the best. It can enhance salting-out effects and change the selectivity of the column stationary phase. It could

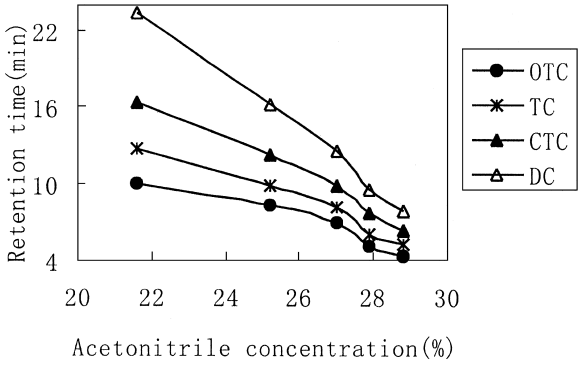

Fig. 2. Effects of the acetonitrile concentration on retention times of OTC, TC, CTC and DC. 0.2 mol l^{-1} HCl remains unchanged. Eluent flow-rate: 1 ml/min; detection wavelength: 350 nm; injection volume: 513 μl.

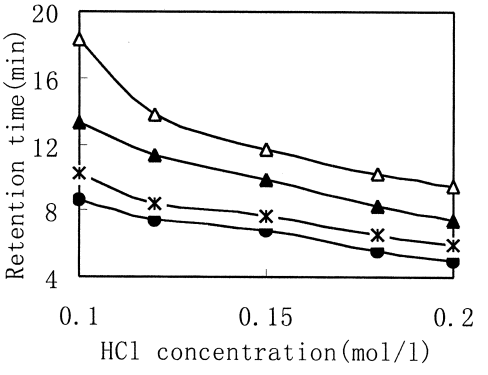

Fig. 3. Effects of the HCl concentration on retention times of OTC, TC, CTC and DC. 27.9% CH_3CN remains unchanged. The other conditions are the same as in Fig. 2.

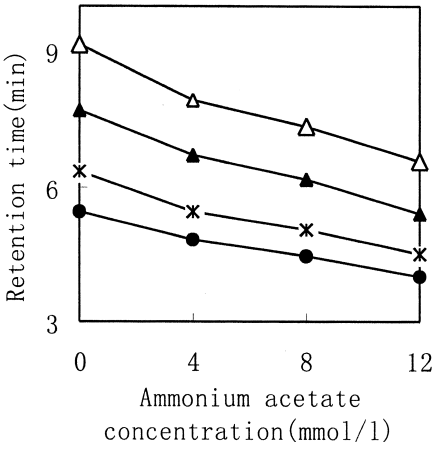

Fig. 4. Effects of the NH_4AC concentration on retention times of OTC, TC, CTC and DC. 0.2 mol l^{-1} HCl and 27.9% CH_3CN remain unchanged. The other conditions are the same as in Fig. 2.

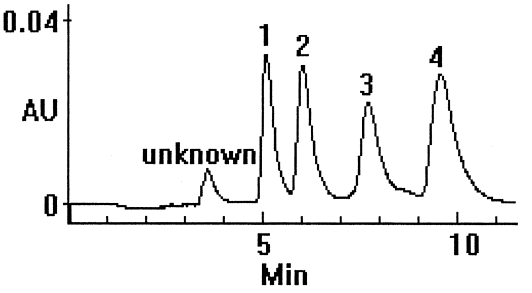

Fig. 5. Chromatogram of TCs in a synthetic standard solution. Column: OmniPac PCX-100 analytical column (250×4 mm I.D.). Eluent: 0.2 mol l^{-1} HCl+27.9% (V/V) CH_3CN; peaks: 1, OTC (1 mg l^{-1}); 2, TC (1 mg l^{-1}); 3, CTC (2 mg l^{-1}); 4, DC (2 mg l^{-1}), respectively. Injection volume: 513 μl. Detection wavelength: 350 nm.

reduce the retention times of the TCs especially CTC and DC. However, OTC and TC could not be baseline separated with the increase of ammonium acetate as Fig. 4 shows when 0.2 mol l^{-1} HCl and 27.9% CH_3CN remain unchanged. There was no obvious advantage of adding ammonium acetate to the eluent. So, to minimize reagent consumption, ammonium acetate was not used. In the end, 0.2 mol l^{-1} HCl and 27.9% CH_3CN were chosen as the eluent for the analysis of the above four TCs. Because the present used IC eluent is more strongly acidic, EDTA is no longer needed to prevent TCs from chelating with the metallic impurities in the eluent.

Under the optimized experimental conditions, the chromatogram of a synthetic standard solution is shown in Fig. 5 where the concentrations of OTC, TC, CTC and DC are 1, 1, 2 and 2 mg l^{-1}, respectively, in which the four TC peaks are well separated within 12 min. The theoretical plate height, *H*, is obtained from the chromatogram by:

$$H = (L/16)(w_t/t_R)^2 \quad (1)$$

where L is the column length; w_t is the peak width at the baseline (in seconds); and t_R is the retention time of a solute (in seconds). The calculated H for OTC, TC, CTC and DC were 0.2, 0.3, 0.2 and 0.2 mm, respectively. Compared with the conventional ion-exchangers (typical plate height (H) values >5 mm), the efficiency of OmniPac PCX-100 analytical column was obviously increased at an order of magnitude and can be compared favorably with that of reversed-phase bonded materials.

3.2. Choice of detection wavelength

In general, the absorbance detection is the first choice in HPLC analysis of the TCs owing to its simplicity and reliability. The TCs have two maximum absorption peaks in the range 200–400 nm. The maximum absorption of TCs usually varied with different pH, for example, the increase of pH will results in a bathochromic shift. The above four tetracyclines were usually detected in visible wavelengths ranging from 350 to 380 nm by various reported HPLC methods. In this study, it was found that the absorptivities of TCs in ultraviolet wavelength range are usually higher than those in visible wavelength range. However, background absorbance from the eluent increases rapidly below 270 nm and the matrices in real samples may also have absorption which may interfere with the determination of the above four TCs. Under the present optimized IC separation conditions, the OTC, TC and DC exhibit absorption maxima at 350 nm except CTC at 370 nm. In the end, 350 nm was selected as the optimize

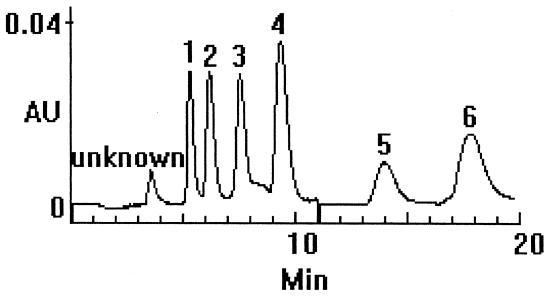

Fig. 6. Chromatogram of parent TCs and their impurities. The other conditions are the same as in Fig. 5. Peaks: 1, OTC (1 mg l^{-1}); 2, TC (1 mg l^{-1}); 3, CTC (2 mg l^{-1}); 4, DC (2 mg l^{-1}); 5, EATC (2 mg l^{-1}); 6, ATC (2 mg l^{-1}), respectively. EATC and ATC are detected at 425 nm. The four parent TCs are detected at 350 nm.

detection wavelength. A relatively clean chromatogram could be obtained, which accordingly reduces signal-to-noise ratios.

3.3. Interference study

The interference mainly comes from the impurities of the TCs. For, a small amount of ETC, EATC and ATC are the common degradation products of the TC drug especially when the TC drug was improperly stored. A small amount of impurities such as demeclocycline (DMCTC) may also exist in CTC drugs. Under the optimized IC separation conditions, only ETC co-eluted with TC and DMCTC co-eluted with CTC. Aanhydro-tetracyclines such as EATC and ATC were strongly retained and eluted after DC. They usually have maximum absorption ranging from 420 to 445 nm, which was obviously different from their apparent TC drugs. So they were detected at 425 nm by a wavelength-switching technique. The elution times were 14.10 and 17.74 min, respectively as shown in Fig. 6. However, The TCs residues in real samples usually contain OTC, TC and CTC at tens- of nanograms per gram or milliliter levels. Few ETC and DMCTC have been found in edible animal tissues and milk. So, the newly established IC method is not suitable for purity control of TC and CTC drugs. It can be applied to monitor the residues of OTC, TC and DC in food-producing animals and bovine milk. It was also applied to monitor the OTC removal rate through every reactor in the process of OTC manufacturing wastewater treatment by biochemical technology, and the acquired results can be used to evaluate the function of reactors.

3.4. Analytical data

Under optimized experimental conditions, all four TCs showed good linearities between the concentrations and peak area responses. The detection limits, defined as the signals three times the noise levels, were also calculated. The use of a large loop could lower the detection limits and did not have any detrimental effect on peak symmetry. However, the absorbances of impurities in real samples would increase accordingly. Therefore, 513 μl loop was used. The precisions were evaluated by performing seven replicate analysis of a standard solution where the concentrations of OTC, TC, CTC and DC were 1, 1, 2 and 2 mg/l. The relative standard deviations (RSD) were 1.4, 1.8, 3.0 and 2.1% for OTC, TC, CTC and DC respectively. All the results are listed in Table 1.

Table 1
Linearities and detection limits for tetracyclines

Analyte	Concentration range (mg/l)	Regression[a] equation	Correlation coefficient ($n=5$)	Detection limit (μg/l)
OTC	0.05–2.0	$A=(5.38c+0.085)\cdot 10^4$	0.9996	10
TC	0.05–2.0	$A=(6.35c-0.052)\cdot 10^4$	0.9998	10
CTC	0.10–4.0	$A=(4.44c-0.026)\cdot 10^4$	0.9991	20
DC	0.10–4.0	$A=(6.85c-0.032)\cdot 10^4$	0.9995	20

[a] A, peak area response (arbitray unit); c, concentration of analytes (mg/l).

3.5. Real samples

For most HPLC methods, TCs residues must be extracted firstly from the biomatrices and concentrated prior to chromatographic analysis. The most commonly utilized technique for the extration and cleanup of TCs from biomatrices method is solid-phase extraction (SPE) or matrix-phase dispersion. A troublesome feature of SPE for TCs is poor reproducibility. The residues must be eluted and concentrated by evaporation, and Onji et al. found that considerable losses of TCs could occur during evaporation of eluates [8].

To avoid this problem, a promising technique based on use of molecular weight cutoff filters to analyze the TCs residues in milk was developed [8,11]. This technique could avoid losses inherent in lengthy, conventional cleanup procedures. Precision and sample throughput was enhanced. In addition, no organic solvents were used in this sample pretreatment procedure, and thus the toxicity was lowered. So, it was applied in our present study for the analysis of TCs residues in milk and the OTC removal rate through every reactor in the process of oxytetracycline manufacturing wastewater treatment by bio-chemical technology. Under the present IC separation condition, any evident change in the separation performance of the analytical column was not observed after more than one hundred analysis of real sample analysis.

Five samples of OTC manufacturing wastewater which collected from aerator tank ($1^{\#}$), aerobic column ($2^{\#}$), denitrification column ($3^{\#}$), acidulated column ($4^{\#}$) and raw wastewater ($5^{\#}$), respectively, were first filtered through a 0.45 μm and then a 30 000 Da filter membrane. The filtrates were clear and colorless and could be directly injected into the IC system after dilution. The results are shown in Table 2. The chromatogram of sample $1^{\#}$ was shown in Fig. 7. Spike studies were performed by sample $1^{\#}$. Three concentration levels of OTC were added (0.2, 0.4 and 0.6 mg/l). The average recoveries ($n=3$) ranged from 93.55 to 97.52%. It must be pointed out that this method essentially measured the fraction of free state of OTC in wastewater and not the total concentration of the OTC, which included stable complexes with metals. However, the free state of the OTC more closely represented the OTC that was actually available to species and gave important information on inhibitory action to organism in the process of denitrification. If the total concentration was required, an ethylenediaminetetraacetic acid disodium salt (Na_2EDTA)-McIlvaine buffer (pH 4.0) solution must be added to release the OTC from the OTC–metal complex. Because the stability constants of metal-EDTA are usually larger than those of metal–OTC such as $\lg K_{Fe^{3+}-EDTA} = 25.1$ and $\lg K_{Fe^{3+}-OTC} = 22.5$ [28].

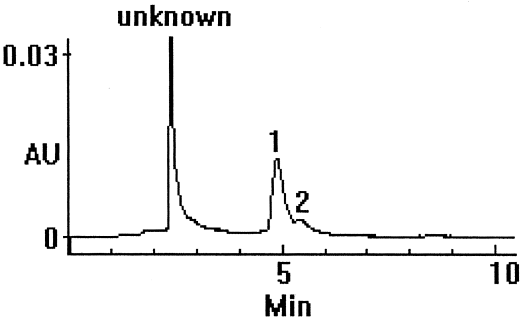

Fig. 7. Chromatogram of the $1^{\#}$ wastewater sample. Sample solution was diluted 1:50 (V/V) with 0.01 mol l^{-1}HCl prior to injection. Chromatographic conditions are the same as in Fig. 5. Peaks: 1=OTC (0.32 mg/l), 2=TC.

Table 2
Analysis of OTC manufacturing wastewater

Sample	$1^{\#}$	$2^{\#}$	$3^{\#}$	$4^{\#}$	$5^{\#}$
IC value (mg/l)	15.78	19.88	20.06	19.90	43.45
$1^{\#}$ Spiked (mg/l)	0.2		0.4	0.6	
Recovery (%)	95.55		93.55	97.52	
RSD (%)	3.8		3.2	2.2	

No tetracycline residues were detected in both bovine milk and milk powder samples. Spike studies were performed by milk. Three concentration levels of each analyte were added and the recovery results were showed in Table 3. The chromatograms of milk and fortified milk were shown in Fig. 8a and b, respectively.

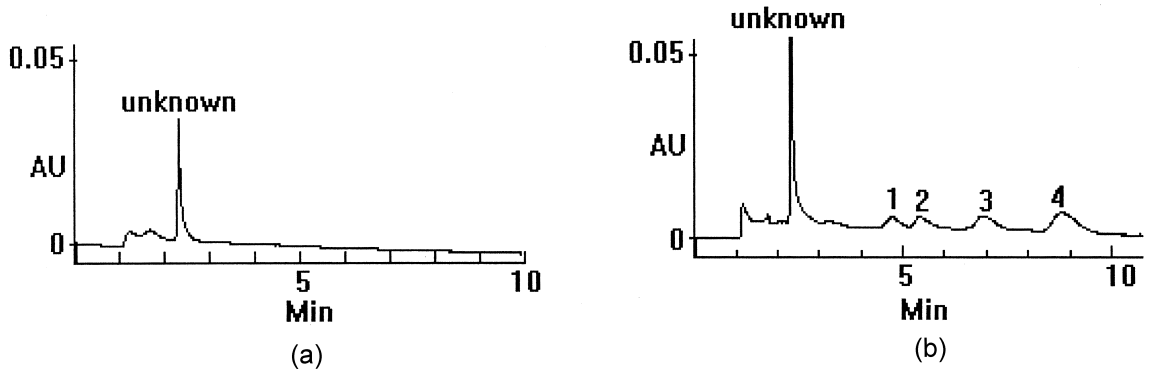

Fig. 8. Chromatograms of milk (a) and fortified milk (b). Sample solution was diluted 1:5(V/V) with 0.01 mol l^{-1} HCl prior to injection. The other conditions are the same as in Fig. 5.

Table 3
Recovery of tetracyclines from fortified milk sample

Tetracycline	Spiked (mg/l)	Mean recovery (%)	RSD (%)
OTC	0.1	75.71	8.2
	0.2	83.09	4.2
	0.4	88.15	5.3
TC	0.1	92.85	5.4
	0.2	92.31	7.4
	0.4	81.16	3.7
CTC	0.5	77.89	7.9
	1.0	79.59	5.8
	1.5	87.74	3.9
DC	0.5	72.19	6.2
	1.0	84.38	6.8
	1.5	87.63	7.6

4. Conclusions

A HPIC method for the simultaneous determination of the four TCs in a single isocratic elution was developed. The OmniPac PCX-100 column shows a very good selectivity towards OTC, TC, CTC and DC. The applicability was verified by the determination of the four TCs residues in milk and milkpowder. It was also applicable to monitor the OTC removal rate through every reactor in the process of OTC manufacturing wastewater treatment by bio-chemical technology, and the acquired results can be used to evaluate the function of reactors. The proposed method can give reliable and reproducible results with simple ultra-filtration in sample pretreament procedure. In addition, HPIC may be seen as a specific variant of the more common HPLC method for water-soluble; polar pharmaceuticals with low hydrophobicity.

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