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Short Communication

Rapid determination of tetracycline antibiotics in serum by reversed-phase high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A rapid and accurate determination of tetracycline antibiotics in human serum by reversed-phase high-performance liquid chromatography with fluorescence detection has been developed, based on protein precipitation in serum. Various reagents for precipitation were investigated, and 24% trichloroacetic acid in methanolic solution gave the maximum recovery (at least 94.3%) and interference-free chromatograms of different three tetracyclines. At a concentration of 0.5 μg/ml, the precision (relative standard deviation) ranged from 1.12 to 1.94%. In the range 0.04–10.0 μg/ml for oxytetracycline and chlorotetracycline and 0.01–10.0 μg/ml for tetracycline, linear responses were observed. The detection limits of this method were 10–35 ng/ml for all three antibiotics. The proposed method was applied to the determination of serum concentrations in subjects receiving tetracycline antibiotics.

INTRODUCTION

Tetracycline antibiotics (TCs) are widely used as antimicrobial agents. Several determination methods for TCs in serum or plasma by high-performance liquid chromatography (HPLC) have been reported [1–7]. Because these methods mainly used spectrophotometric detection, they require tedious extraction and concentration steps by organic solvents or solid-phase extraction using a pre-column. These steps tend to result in unsystematic errors and are not suitable for automatic analysis. In contrast, Hermansson [8] has reported the rapid HPLC assay of TCs by only the precipitation of plasma as pretreatment. However, this method could not solve the problem of low recovery. More recently, we have described a highly sensitive method for TCs by reversed-phase HPLC with fluorimetric detection, and demonstrated a simple analysis for TCs in fish tissues without tedious sample treatment steps [9].

This paper deals with a rapid and accurate method for the determination of tetracycline (TC), oxytetracycline (OTC) and chlorotetracycline (CTC) in serum. The method is based on deproteinization of serum followed by sensitive HPLC analysis as previously described [9]. After pretreatment, conditions were optimized using control serum spiked with different TCs. The

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method was applied to the determination of serum concentrations in patients receiving TC antibiotics.

EXPERIMENTAL

Apparatus

The HPLC system consisted of an L-6200 delivery system (Hitachi, Tokyo, Japan), a Model 7125 loop injector (Rheodyne, Cotati, CA, USA), a prepacked Capcell C_{18} type SG-120 column (250 mm × 4.6 mm I.D., 5-μm; Shiseido, Tokyo, Japan) and an F-1200 spectrofluorimeter (Hitachi). The column temperature was held at 30 ± 0.2°C. The detector excitation and emission wavelengths were set at 390 and 512 nm, respectively. The flow-rate was 1.0 ml/min. The results were recorded on a D-2500 chromato-integrator (Hitachi).

Reagents

HPLC-grade methanol was purchased from Kanto Chemicals (Tokyo, Japan). Water was purified by distillation, followed by final clean-up through a Milli-Q Labo system (Nihon Millipore, Tokyo, Japan). TC and OTC hydrochloride were obtained from Wako (Osaka, Japan) and CTC hydrochloride from Sigma (St. Louis, MO, USA). Hyland Q-pack chemistry Control Serum I was purchased from Cooper Biomedical (Tokyo, Japan). All other reagents were of analytical-reagent grade. Standard solutions of each TC were prepared by diluting the stock solution (1 mg/ml of free compound in 0.1 *M* hydrochloric acid) to appropriate concentrations with 50 m*M* phosphate buffer (pH 7.4) or the control serum. The mobile phase consisted of methanol and 0.1 *M* sodium acetate buffer (pH 6.5) containing 35 m*M* calcium chloride and 25 m*M* disodium ethylenediaminetetraacetate (buffer A). The mixing ratios are indicated in the figure captions.

Sample procedures

An aliquot of serum (100 μl) was pipetted into a centrifuge tube. After the addition of 200 μl of 24% trichloroacetic acid (TCA) methanolic solution and 300 μl of buffer A, the tube was vortex-mixed for 1 min. Following centrifugation at 2000 *g* for 15 min, 50 μl of the resulting supernatant was injected into the HPLC column.

Blood samples of 5 ml collected from patients

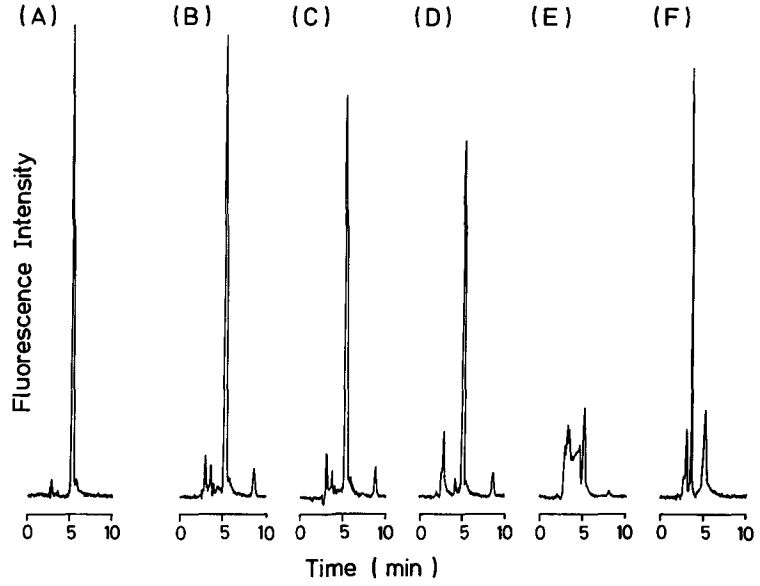

Fig. 1. Chromatograms of OTC standard (A) and spiked OTC in serum deproteinized by various reagents, 24% TCA–methanol (B), 24% TCA–water (C), methanol (D), acetonitrile (E) and acetone (F). A two-fold volume of the reagent was added to the sample. Mobile phase, methanol–buffer A (30:70, v/v); flow-rate, 1.0 ml/min; sample concentration, 1.0 μg/ml.

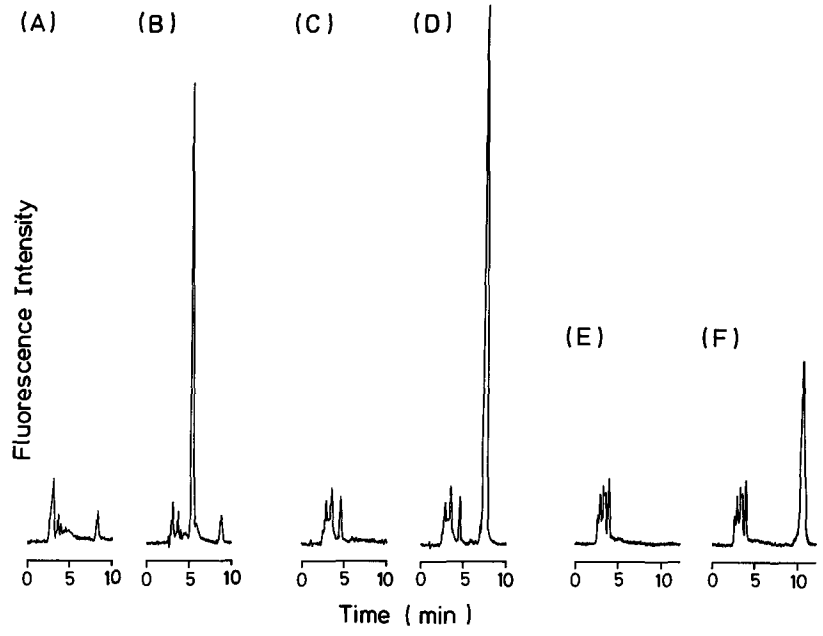

Fig. 2. Chromatograms of serum blank (A, C and E), spiked OTC (B), TC (D) and CTC (F) in serum. Mobile phase, methanol–buffer A; ratio: 30:70 (A and B); 60:40 (C and D); 55:45 (E and F). Flow-rate, 1.0 ml/min. Sample concentration, 1.0 μg/ml.

were allowed to stand for 30 min at room temperature and centrifuged at 3000 *g* for 15 min to give serum. This was treated as described above.

RESULTS AND DISCUSSION

In the conventional HPLC methods, prolonged pre-treatment results from the low detection sensitivity, involving extraction and concentration using organic solvents or pre-column solid-phase extraction. The method previously reported by us [9] indicated that TCs in serum could be determined without tedious pre-treatment with a sensitivity at least equal to that of the conventional methods. In the present work, pretreatment methods of serum suitable for HPLC were investigated. Fig. 1 shows chromatograms of OTC spiked in serum deproteinized by various reagents: 24% TCA–methanol solution, 24% aqueous TCA, methanol, acetonitrile and acetone. Deproteinization by 24% TCA–methanol solution gave interference-free chromatograms and maximum recoveries for all three TCs (Fig. 1 and Table I). For hours after the centrifugation, the 24% TCA–methanol solution continued to precipitate proteins in the serum samples. The precipitate interfered with the injection of the sample into the HPLC column. In order to resolve this problem, not only deproteinization reagent but also buffer A were added to the serum,

TABLE I

RECOVERY OF THE TETRACYCLINES FROM SERUM BY THE USE OF VARIOUS DEPROTEINIZATION REAGENTS

Sample concentration, 1 μg/ml; reagent: serum ratio, 2:1.

Reagent	Recovery (%)		
	OTC	TC	CTC
24% TCA–methanol	97.8	99.2	100.8
24% TCA–water	90.0	79.7	63.2
Methanol	75.3	64.9	62.8
Acetonitrile	89.1	77.7	75.2
Acetone	90.2	89.5	82.0

and then the mixture was centrifuged. All three TCs could be detected without interference from endogenous materials (Fig. 2).

Linear responses were observed in the range 0.04–10.0 μg/ml for OTC and CTC, and 0.01–10.0 μg/ml for TC. The equations of the regression lines for calibration graphs were $y = 1.645x + 0.036$ for OTC, $y = 2.935x - 0.067$ for TC, and $y = 1.747x - 0.055$ for CTC (y = peak area $\cdot 10^{-5}$, x = μg/ml). The correlation coefficients for the three TCs were greater than 0.999. The detection limits of TC, OTC and CTC were 30, 10 and 35 ng/ml, respectively. They were of the same order of magnitude as those obtained by the conventional methods. Several analyses were performed to determine the recovery of TCs from serum and the reproducibility of the method. The recovery of OTC spiked at levels of 0.5, 2.0 and 5.0 μg/ml was 100.1–102.9%, for TC 99.5–100.3% and for CTC 94.3–101.2%. The recoveries were almost constant at various concentrations. Table II gives the relative standard deviations of the peak areas for the three TCs for intra- and inter-assay determinations. The results were comparable with those obtained by the conventional methods.

The use of an autosampler in HPLC analysis requires stability of the target material. Under the conditions reported here, all three TCs were stable for at least 24 h in the resulting sample mixture at room temperature.

Fig. 3 shows the serum concentrations of TCs in two patients who received a single oral dose of Terramycin capsule (Pfizer, containing 250 mg of OTC) or 10 ml of Achromycin V syrup (Lederle, containing 250 mg of TC). The half-lives of OTC and TC, calculated from the curves, were found to be *ca.* 9 and 10 h, respectively. These values were in fair agreement with previous data [10,11]. The results show that the present method is suitable for drug level monitoring of TCs.

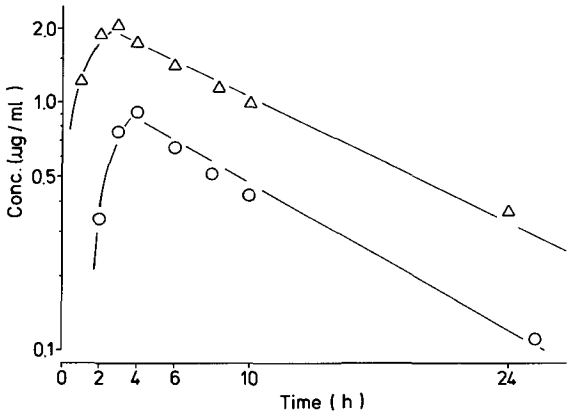

Fig. 3. Drug concentrations in the serum of two subjects receiving 250 mg of TC or OTC: (△) TC; (○) OTC.

CONCLUSION

These results prove the excellent simplicity and accuracy of the present method, and suggest that it may serve for the determination of TCs in biological fluids for pharmacokinetic studies. In addition, if a more sensitive detection is required, this may be easily achieved by adding concentration steps as described in the conventional method.

TABLE II

REPRODUCIBILITIES OF THE TETRACYCLINS SPIKED IN SERUM

Compound	Relative standard deviation (%)			
	Intra-assay ($n = 8$)			Inter-assay ($n = 5$)
	0.5 μg/ml	2.0 μg/ml	5.0 μg/ml	1.0 μg/ml
OTC	1.12	1.30	1.46	2.00
TC	1.52	1.03	0.77	2.31
CTC	1.94	1.11	1.51	1.66

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