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RAPID DETERMINATION OF TETRACYCLINE AND LUMECYCLINE IN HUMAN PLASMA AND URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and accurate method for the determination of tetracycline in human plasma and urine is presented. Determination of tetracycline in plasma is based on precipitation of plasma proteins with trifluoroacetic acid, followed by injection of the centrifuged plasma sample onto a μBondapak C_{18} column. Acetonitrile in phosphate buffer pH 2.2 is used as mobile phase. Only tetracycline, and no trace of lumecycline can be detected in plasma and urine after administration of lumecycline, indicating that lumecycline is completely degraded to tetracycline, lysine and formaldehyde in the gastrointestinal tract prior to absorption.

Determination of tetracycline in urine was performed by injection of urine diluted with phosphoric acid onto a μBondapak Phenyl column. The precision of determination of tetracycline in plasma, expressed as the relative standard deviation, was < 3% at tetracycline concentrations of 0.05 and 3.7 μg/ml. Urine determinations were made with a precision of < 1.5% at tetracycline concentrations of 0.5 and 6.7 μg/ml.

INTRODUCTION

Different techniques such as microbiological [1, 2] and fluorimetric methods [3—5] have been applied for the analysis of tetracyclines. However, these methods suffer from a lack of selectivity. By use of a chromatographic method, like high-performance liquid chromatography (HPLC), this problem can be overcome. During the last few years numerous papers have been published on the chromatography of the tetracyclines and their potential impurities [6—12].

A few papers, which deal with the determination of tetracycline in biological material using HPLC, have also been published [13—16]. The determination of tetracycline in plasma is based on extraction of tetracycline as a complex with calcium [13—15] or as an ion pair [16] into an organic solvent, followed by

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extraction into an acidic water phase prior to its chromatographic isolation. Determination of tetracycline in urine has been performed by injection of a urine extract [13—15], or by injection of diluted urine onto the column [16]. To obtain a sufficiently high detection selectivity for tetracycline in plasma and urine from cattle, sheep and swine, Sharma and co-workers [13, 14] monitored the eluate at 355 nm. Detection of tetracycline in this range (357 nm) was later applied for the analysis of tetracycline in human plasma and urine [16].

The present paper describes a rapid method for the determination of tetracycline and lumecycline in human plasma and urine. The time-consuming extractions of tetracycline from plasma are replaced by a rapid isolation step. The method is based on precipitation of the plasma proteins with trifluoroacetic acid, followed by injection of the centrifuged plasma sample onto the column. The eluate is monitored at 357 nm. The aim of this study was to design a rapid and accurate method suitable for use in a comparative study of the bioavailability of tetracycline and lumecycline in man.

EXPERIMENTAL

Apparatus

The pump was an Altex Model 100 solvent delivery system and the injector a Waters Model U6K. A Spectra-Physics Model 770 UV detector with variable wavelength was used. The detector was operated at 357 nm. Peak areas were calculated using a Hewlett-Packard 3370 B integrator. pH was measured with an Orion Research Model 701 A digital pH-meter, with an Ingold Type 401 combined electrode.

Chemicals

Tetracycline hydrochloride was kindly supplied by ACO Läkemedel AB (Solna, Sweden) and lumecycline was obtained from Carlo Erba (Milan, Italy). Capsules containing tetracycline hydrochloride or lumecycline corresponding to 300 mg of tetracycline as base were prepared in a Swedish pharmacy. The solid phases μBondapak C_{18} (10 μm) and μBondapak Phenyl (10 μm) were obtained from Waters Assoc. (Milford, MA, U.S.A.). The acetonitrile was of grade S quality and purchased from Rathburn Chemicals (Walkerburn, Great Britain). Trifluoroacetic acid (TFA) für die Spectroskopie was obtained from E. Merck (Darmstadt, G.F.R.). All other chemicals used were of analytical grade and used without further purification.

Column preparation

The columns 100 × 3.2 mm (for plasma samples) or 150 × 3.2 mm (for urine samples) were made of 316 stainless steel with a polished inner surface, equipped with modified Swagelok connections and Altex stainless-steel frits (2 μm).

The columns were packed by a modification of the balanced density slurry technique described previously [17]. The support was suspended in chloroform (10 ml chloroform per solid phase) and poured into the packing column which was filled by hexane. Acetone was used as driving liquid in the Haskel pump which was operated at 6.2 MPa. After packing, the columns were washed by

pumping 100 ml of acetonitrile through the columns followed by 100 ml of acetonitrile—water (1:1).

Plasma and urine samples

Blood samples were collected in 10-ml heparinized Venoject tubes according to a protocol approved by our Ethical Committee. Plasma was separated immediately after collection and frozen at −20°C until analyzed. Urine samples were stored at −20°C until analyzed.

Standard curves

The standard samples were prepared by spiking drug-free plasma and urine with tetracycline. Tetracycline HCl was dissolved in 0.1 *M* phosphoric acid.

Determination of tetracycline in biological material

Plasma. To 0.5 ml of plasma 65 μl of TFA were added. The tube was agitated in a Whirl mixer for 0.5 min and centrifuged for 5 min at 5400 *g*. Two hundred and fifty microlitres were injected onto the μBondapak C_{18} column. The column was eluted with phosphate buffer pH 2.2 (μ = 0.1) containing 16% (v/v) acetonitrile. The flow-rate was 0.5 ml/min.

Urine. The urine was diluted with 0.1 *M* phosphoric acid (1:1) and 25 μl (or more if needed) were injected onto the μBondapak Phenyl column. The mobile phase and the flow-rate were the same as above.

RESULTS AND DISCUSSION

Chromatographic retention, selectivity and peak symmetry

The retention of tetracycline and its most commonly found impurity, epitetracycline, is easily regulated by varying the acetonitrile concentration

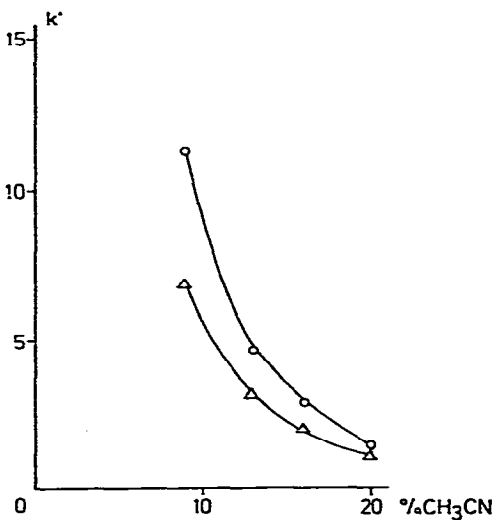

Fig. 1. Regulation of the capacity factor (*k'*) with acetonitrile. Mobile phase: sodium phosphate buffer, pH 2.2, containing acetonitrile. Solid phase: μBondapak C_{18} (10 μm). Column: 100 × 3.2 mm. Samples: tetracycline (○), epitetracycline (△).

in the mobile phase as demonstrated in Fig. 1. The separation factor (α) decreases with increasing acetonitrile concentration. Baseline separation between tetracycline and epitetracycline was obtained on a 100 × 3.2 mm μBondapak C_{18} column within 7 min using a mobile phase of 16% acetonitrile in phosphate buffer pH 2.2. The separation factor was 1.42.

Wide variations in the chromatographic properties of tetracyclines have been observed using solid phases obtained from different manufacturers, despite the fact that the nominal alkyl chain lengths attached were identical [12]. By use of μBondapak C_{18} or μBondapak Phenyl as solid phase almost symmetrical peaks were obtained (asymmetry factor 1.2). This is a prerequisite for high sensitivity for the method. Tetracyclines have been isolated from plasma and urine using LiChrosorb RP-2 as solid phase and a mobile phase similar to the above [16]; however, there was a significant tailing of the tetracycline peak in this separation system. LiChrosorb RP-8 has also been used for chromatography of tetracyclines. It was, however, necessary to add tertiary amines to the mobile phase [10] or to pretreat the solid phase [18] in order to obtain good chromatographic conditions for tetracyclines using this solid phase. Sharma and Bevill [14] reported that new μBondapak C_{18} columns must be conditioned before use, in order to obtain reproducible results in chromatography of tetracyclines. These data disagree with the present findings and there is no obvious explanation for the discrepancy. However, a slightly higher asymmetry factor of the tetracycline peak was observed during the first injections on a new column.

Stability of tetracycline and lumecycline

Due to the sensitivity to epimerization, dehydration and oxidation of tetracyclines [19—21] the stability of tetracycline at different stages of the method was investigated. The stability of tetracycline in plasma stored at room temperature was investigated by repeated injection of a plasma sample containing 2.1 μg/ml tetracycline. No significant degradation was observed after 6 h.

Before injection of the plasma samples onto the column the plasma

TABLE I

STABILITY OF TETRACYCLINE IN PLASMA AFTER PRECIPITATION OF PROTEINS WITH TRIFLUOROACETIC ACID AND PERCHLORIC ACID

Trifluoroacetic acid		Perchloric acid	
Storage time (min)	Area (μV·sec)	Storage time (min)	Area (μV·sec)
0	9723	0	9100
30	9578	30	8864
37	9401	60	8605
60	9365	67	8452
68	9278	120	8039
120	8998	129	8168
126	8840		

proteins were precipitated by addition of an acid. Two different acids were tested, perchloric acid (60 μl/ml of plasma) and TFA (130 μl/ml of plasma). The stability of tetracycline in plasma after precipitation of proteins was investigated chromatographically and the results are demonstrated in Table I. There was a 12% decrease of the tetracycline concentration in plasma samples precipitated with perchloric acid after storage for 120 min at room temperature. Tetracycline was slightly more stable in plasma precipitated with TFA, and the tetracycline concentration decreased 8% under the same conditions. TFA was therefore preferred for the bioanalytical method. To avoid problems with degradation of tetracycline the plasma samples were injected onto the column directly after precipitation and centrifugation.

In a recent publication lumecycline was shown to be very sensitive to degradation in water solutions of pH 2.2 and 7.5 [10]. Under these conditions lumecycline was degraded very quickly to tetracycline, lysine and formaldehyde. Healthy volunteers were administered a lumecycline capsule containing lumecycline corresponding to 300 mg of tetracycline. Only tetracycline and no trace of lumecycline could be detected in their plasma 0.5, 1 and 3 h after administration of the capsule using the method described under Experimental for plasma analysis, with the exception that the column was eluted with 9% acetonitrile in phosphate buffer pH 2.2. This suggests that lumecycline is completely degraded to tetracycline in the gastrointestinal tract prior to absorption. Blank plasma spiked with lumecycline was also analysed to ensure that lumecycline can be detected in original plasma samples after treatment with TFA. Two peaks were obtained in the chromatogram, corresponding to lumecycline and tetracycline, respectively. From the above results it is obvious that lumecycline is completely degraded by the time it reaches the systemic circulation and can be analysed as tetracycline.

The stability of tetracycline in urine (pH 6.0) at room temperature was also determined by repeated injection of a urine sample containing 6.7 μg/ml tetracycline. A 5.3% decrease in the tetracycline concentration was observed after 22 h.

Handling of plasma samples before chromatography

Tetracyclines have previously been determined in plasma using extraction of tetracycline into an organic phase as a complex with calcium [13,14] or as an ion pair [16], followed by extraction into an acidic water phase and chromatographic isolation. The extraction procedures are very time-consuming, and help to decrease the precision of the determinations. Therefore precipitation of the plasma proteins is preferable in the isolation of tetracycline prior to the chromatographic step. The plasma proteins were precipitated in three different ways, using either acetonitrile, perchloric acid or TFA.

To precipitate the plasma proteins in 1.0 ml of plasma, 1.0 ml of acetonitrile, 60 μl of perchloric acid or 130 μl of TFA were required. Chromatography of an acetonitrile-precipitated plasma sample containing tetracycline resulted in elution of tetracycline in the front, using the separation system described under Experimental. This is the result of the high acetonitrile con-

centration of the plasma sample (50%, v/v) compared with that in the mobile phase (16%, v/v), and the pH of the sample (7—7.5) under which conditions the tetracycline exists in zwitterionic form [7]. Better results were obtained by chromatography of plasma samples precipitated with perchloric acid or TFA. This is probably caused by the lack of acetonitrile in the injected sample and the low pH of the acid-precipitated sample, where tetracycline exists as a singly charged cation. No significant differences in the chromatographic properties of tetracycline were observed by injection of a plasma sample precipitated with perchloric acid or TFA. However, TFA was preferred because of the slightly higher stability of tetracycline in TFA than in perchloric acid solutions (see above).

Recovery and precision

The recovery of tetracycline from plasma was studied at two different concentrations, 0.12 and 3.50 μg/ml. Drug-free plasma and phosphate buffer pH 7.5 ($\mu = 0.1$) were spiked with tetracycline and analysed according to the method described under Experimental. The recovery of tetracycline was calculated by comparing the data from the two series and the results are summarized in Table II.

The precision of the determination of tetracycline in plasma was determined by analysing original plasma samples containing three different concentrations of tetracycline, 0.06, 0.73 and 3.70 μg/ml. The precision of analysis of tetracycline in urine containing 0.5 and 6.6 μg/ml was determined using the described procedure. The relative standard deviations were calculated and the results are summarized in Table III.

TABLE II

RECOVERY OF TETRACYCLINE FROM PLASMA

Tetracycline conc. (μg/ml)	Recovery* (%)
0.12	68.7 ± 2.3
3.50	72.8 ± 1.1

*Recovery ± relative standard deviation ($n = 5$).

TABLE III

PRECISION OF PLASMA AND URINE DETERMINATIONS

Sample	Tetracycline conc. (μg/ml)	Relative S.D.* (%)
Plasma	0.05	2.66
Plasma	0.73	1.31
Plasma	3.70	2.69
Urine	0.50	1.40
Urine	6.67	1.30

*Calculated for $n = 5$.

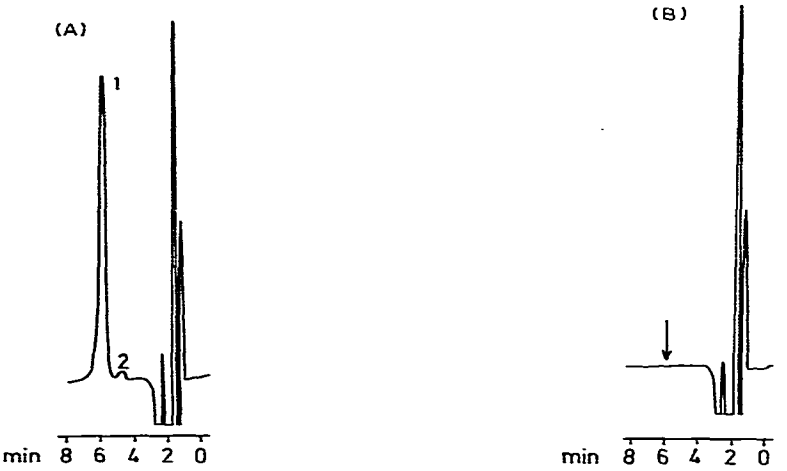

Fig. 2. (A) Chromatogram of a plasma sample containing 2.45 μg/ml of tetracycline (1) and epitetracycline (2). (B) Blank plasma chromatogram. Mobile phase: 16% acetonitrile in phosphate buffer, pH 2.2 (μ = 0.1). Column and solid phase as in Fig. 1.

Determination of tetracycline and lumecycline in plasma and urine

The plasma concentration of tetracycline and lumecycline (measured as tetracycline, see above) was determined as described under Experimental. Fig. 2A demonstrates the isolation of tetracycline from human plasma 4 h after administration of a capsule containing lumecycline corresponding to 300 mg of tetracycline. A blank plasma chromatogram is demonstrated in Fig. 2B. Using the described conditions, no interfering peaks were present in the chromatograms. This is largely the result of the high detection selectivity achieved at 357 nm [13, 16].

The quantitation of tetracycline was performed using external standards prepared as described under Experimental. Standard curves were prepared by plotting the peak areas against the sample concentration. The curves were linear in the studied concentration range, 0.1—4 μg/ml. Correlation coefficients were in all cases better than 0.9990.

With repeated injection of plasma samples precipitated as described under Experimental, there was a small tendency for pressure to increase, although no cavity was formed on the top of the column. This problem was overcome by changing the stainless steel frit on the column top after injection of about 125 plasma samples. The pressure increase is probably caused by accumulation of precipitated proteins on the frit.

The retention time of tetracycline and the resolution between tetracycline and epitetracycline decreases after repeated injections of plasma samples. This effect can be due to changes in the solid phase, due to the strongly acidic plasma samples injected, or to coating of the solid phase with components from the plasma samples. The column was repacked twice during the analysis of 750 plasma samples.

Figs. 3A and B demonstrate the plasma concentrations of tetracycline in two subjects who received a single oral dose of tetracycline (300 mg) and lumecycline (corresponding to 300 mg of tetracycline), respectively. The half-

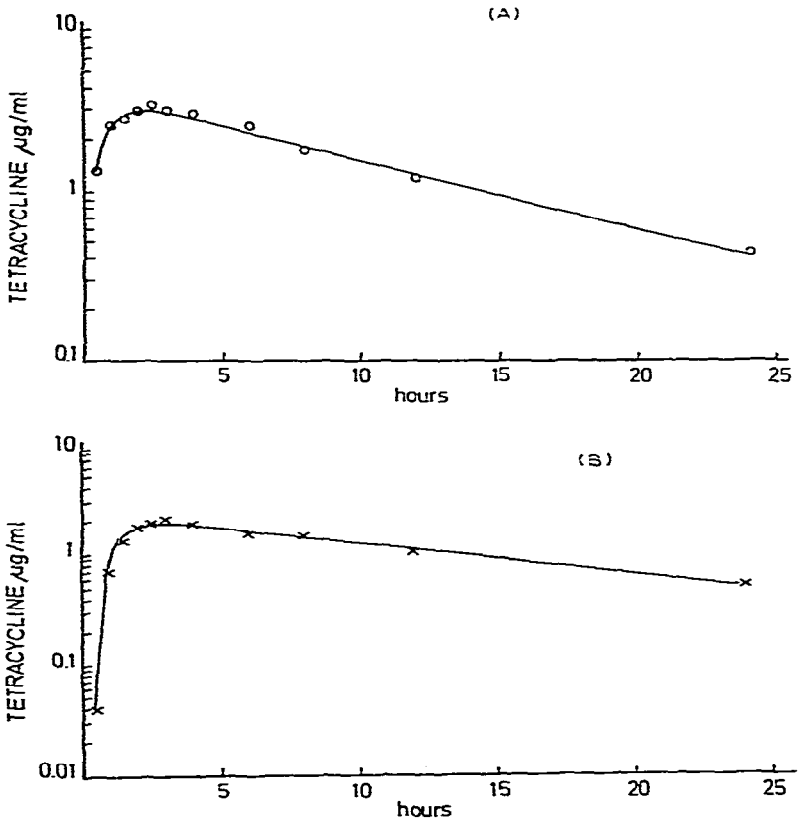

Fig. 3. Plasma concentrations in two subjects receiving 300 mg of tetracycline (A), and lumecycline (B) corresponding to 300 mg of tetracycline. (○) Tetracycline, (×) lumecycline (determined as tetracycline).

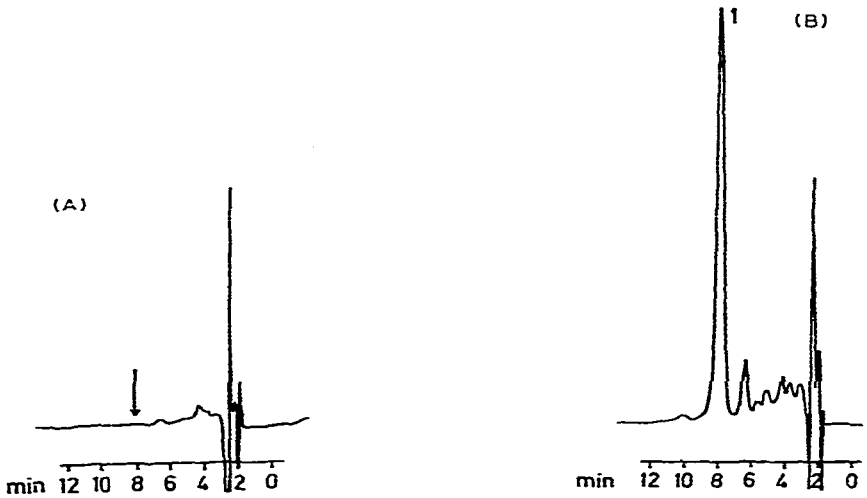

Fig. 4. Isolation of tetracycline from human urine. (A) Blank urine chromatogram. (B) Chromatogram of urine containing 6.6 µg/ml tetracycline. Solid phase: µBondapak Phenyl. Column: 150 × 3.2 mm. Mobile phase: as in Fig. 2.

lives of tetracycline were calculated from the curves and were found to be 7.4 and 10.5 h for tetracycline and lumecycline, respectively.

Urine samples containing tetracycline were analyzed as described under Experimental. Fig. 4A and B demonstrate a blank urine chromatogram and a chromatogram of urine from a subject 16 h after receiving an oral dose of lumecycline (corresponding to 300 mg of tetracycline). μBondapak Phenyl was preferred as solid phase for the urine analysis of tetracycline, because of the more selective isolation of tetracycline from endogenous compounds obtained on this solid phase compared with μBondapak C_{18}. No deterioration of the column has been observed after injection of urine onto the column. Quantitation of tetracycline in urine was performed as described for plasma samples (see above).

The described methods are now in use in a study for comparison of the bioavailability of tetracycline and lumecycline.

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