Quantitation of Antibiotics Using High-Pressure Liquid Chromatography: Tetracycline

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A method for quantitative determination of tetracycline in serum, using high-pressure liquid chromatography, is reported. After extraction of the drug from serum, using methanol-trichloroacetic acid solution, tetracycline was separated by reverse-phase chromatography. Quantitation of tetracycline was based on a linear relationship between peak heights in the chromatograms and known concentrations of the drug in the original serum samples. Serum tetracycline concentrations as low as 0.3 μg/ml could be accurately measured. Serum samples obtained after a single intravenous injection of tetracycline to three human volunteers and six dogs were assayed by microbiological and chemical assays. Correlation coefficients of 0.95 and 0.97, respectively, were found. This chemical method is rapid (less than 30 min), accurate, sensitive, and reproducible, and it seems feasible for routine clinical use.

Routine determinations of antibiotic concentrations in serum or other body fluids are presently dependent primarily on microbiological assays (4, 5). The lack of uniform methodology, the frequent interference by the presence of another antibiotic in the sample, and the long time required to obtain the information desired limit the clinical usefulness of these techniques.

In an effort to alleviate many of these problems, the applicability of high-pressure liquid chromatography to measurement of antibiotic concentrations in biological fluids was investigated. For the initial studies reported here, tetracycline was selected because of its ease of detection due to a high absorption coefficient in the ultraviolet (UV)-visible spectrum and common clinical usage. Described here is a rapid, simple method for chemical determination of tetracycline in serum, involving disassociation of the drug from serum proteins and subsequent separation and quantitation by high-pressure liquid chromatography.

MATERIALS AND METHODS

Methanol. Absolute methanol (American Drug and Chemical Co., Culver City, Calif.) was redistilled prior to use in the chromatographic procedure.

Water. Water used throughout the procedure was deionized and freshly redistilled.

Trichloroacetic acid-methanol reagent. Trichloroacetic acid (Matheson, Coleman and Bell, Norwood, Ohio) was dissolved in absolute methanol to make a 20% solution (wt/vol).

EDTA solution. Ethylenediaminetetraacetic acid (EDTA) (Matheson, Coleman and Bell, Norwood, Ohio) was dissolved in distilled water to make a 0.005 M solution.

Chromatographic eluent. The mobile phase consisted of methanol-EDTA solution (3:7, vol/vol). This will be referred to in the text as methanol-EDTA. This eluent was filtered (Solvinert 0.5-μm filter, Millipore Corp., Bedford, Mass.) and deaerated under vacuum prior to use.

Liquid chromatography equipment. Chromatography was carried out on an ALC/GPC 200 liquid chromatograph (Water's Associates, Inc., Milford, Mass.) equipped with a model 6000 solvent delivery system and a model U6K Universal injector. The detector was a variable wavelength UV-visible spectrophotometer (SF 770; Schoeffel Instrument Corp., Westwood, N. J.). Absorbance of the eluent was monitored using an Omniscribe recorder (Houston Instrument, Houston, Tex.). The chromatography was carried out in a reverse-phase system, employing a MicroBondapak C_{18} column (30 cm by 4 mm, ID) (Water's Associates, Inc.).

In vitro serum samples. Tetracycline hydrochloride (USP reference standard), dissolved in 0.1 ml of distilled water, was added to 0.9 ml of dog serum. Serum blanks were prepared in the same manner, omitting tetracycline.

All samples were left at room temperature for 20 min. Control experiments with samples pre-

pared identically with human sera obtained from normal volunteers demonstrated that such samples did not behave differently from dog serum when carried through the extraction and chromatography procedures.

In vivo serum samples. Three young, healthy human volunteers received a single intravenous injection of 250 mg of tetracycline (Panmycin; Roerig, New York, N. Y.). Blood was drawn before and at various times up to 24 h after injection. Serum was separated by centrifugation and stored at −70 C until analyzed. Chemical and microbiological assays were performed in duplicate. During storage under the above conditions for 1 month, no change in antibiotic concentration was observed.

Tetracycline was also administered intravenously to six dogs. Three dogs received 3.5 mg/kg of body weight, and three were given 7.0 mg/kg of body weight in a single dose. Serial blood samples were obtained from 0 to 12 h after injection. Serum was separated and stored at −70 C until assayed in duplicate by microbiological and chemical assays.

Microbiological assay. Microbiological assay for tetracycline in both human and dog sera was performed according to a modification of a previously described method (4). The test strain organism used was *Bacillus cereus* ATCC 11778. An overnight culture of the organism was grown on solid agar, scraped from the plate, and inoculated into sterile physiological saline to a concentration equivalent to a McFarland 0.5 barium sulfate standard (2.8 × 10^6 bacteria/ml). The inoculum was prepared by pipetting 1.8 ml of bacterial suspension into 12 ml of remelted, cooled (45 to 50 C) test agar (antibiotic medium no. 2; Difco Laboratories, Detroit, Mich.). The organism suspension was thoroughly mixed, poured into petri plates (150 by 15 mm, diameter), and allowed to harden. Twenty-four wells with a diameter of 5.5 mm were punched out of each plate.

Standard solutions of tetracycline hydrochloride were prepared in dog (or human) sera at concentrations from 0.25 to 4.0 µg/ml. Wells were filled with 10 µl of standard antibiotic solution and serum samples. In some instances dilution of serum samples was necessary because of large zone sizes. Plates were incubated at 37 C, and zones of inhibition were read at 4, 8, 12, and 16 h. Distinct zone diameters were best visualized at 12 to 16 h. Zone size was plotted against known tetracycline concentration on semilog paper, and antibiotic concentration of serum samples was determined.

Procedure. (i) Extraction. A 1-ml amount of each sample was added dropwise to 1 ml of trichloroacetic acid-methanol reagent, stirring vigorously on a Vortex mixer, and left at ambient temperature for 10 min. The protein precipitate was sedimented by centrifugation for 10 min at 2,000 × *g*. The supernatants were withdrawn with a glass syringe and passed through 0.5-µm filters (Solvinert, Millipore Corp.).

(ii) Separation and detection. A 200-µl amount of the filtered supernatant was injected into the liquid chromatograph. Elution was performed by using methanol-EDTA as mobile phase at a flow

rate of 2.8 ml/min, requiring a pressure of about 4,000 lb/in². The eluent was monitored at 355 nm at an attenuation of 0.01 absorbance unit full scale. The recorder chart speed was 0.5 cm/min. Heights of the individual peaks were measured with compensation for base line drift (15).

(iii) Critical steps. Several steps in the procedure require special attention. (1) A new trichloroacetic acid-methanol solution must be prepared each day, since an older mixture seems to result in incomplete protein precipitation. (2) The serum sample to be assayed must be added dropwise to the acid-methanol solution, simultaneously stirring rapidly on a Vortex mixer, and then allowed to stand for 10 min. Otherwise, incomplete precipitation of proteins will occur at this step. (3) Before the liquid chromatography step, all samples and solvents should be filtered through a 0.5-µm filter to prevent particulate matter from obstructing filters in the solvent delivery system and at the column inlet. Deaeration of the solvents is important since air bubbles cause base line drift and excessive noise in the UV detector. (4) The injected volume should be constant and as small as possible, since better resolution and narrower peaks are obtained when small volumes are injected (15). To accurately determine tetracycline at a concentration of 0.3 µg/ml of serum, injection of 200 µl of filtrate is required. In the system described, this injection volume gives sufficiently distinct peaks. (5) The EDTA in the chromatographic eluent is of prime importance. With no EDTA present, tetracycline appears to bind to the packing material and is not eluted in a quantitative fashion. However, with addition of EDTA as part of the eluent mixture, tetracycline peaks are quantitative and reproducible.

RESULTS

Choice of detection wavelength. Solutions of tetracycline in the extraction reagent were found to have three absorption maxima in the UV spectrum, at 355, 270, 220 nm. It was noted that extracts of serum with no added drug contained materials with substantial absorption at wavelengths less than 310 nm. Some of this material coeluted with tetracycline in the chromatographic system used. When monitoring the eluent at 355 nm, however, none of this coeluting material was measurable, making this wavelength most suitable for detection.

Recovery. Control samples were prepared by adding tetracycline to distilled water instead of to serum. The efficiency of the extraction procedure was determined by comparison of absorption values of serum samples with those of controls (Table 1). Recovery of tetracycline from serum samples was consistently 95 to 105%. The absorption values of tetracycline in trichloroacetic acid-methanol-water solutions, not carried through the extraction procedure,

TABLE 1. Recovery of tetracycline from serum

Tetracycline hydro-chloride added to se-rum or distilled wa-ter (µg/ml)	Optical density at 355 nm[a]		Recovery[b] (%)
	Serum ex-tracts[c]	Water con-trols	
1	0.021	0.020	105
5	0.080	0.078	103
10	0.151	0.159	95

[a] Each absorption value is the mean of four separate determinations.

[b] Recovery $= \left(\dfrac{\text{serum value}}{\text{water control value}}\right) \times 100.$

[c] The absorption value of a serum blank has been subtracted.

were constant for at least 1 h and identical to the values presented in Table 1. Thus, no indication of alteration of tetracycline during the extraction procedure nor of loss during centrifugation or filtration was noted.

Separation of tetracycline by liquid chromatography. In the liquid chromatography system described above, the tetracycline peak had a retention time of 3.75 min. It was well separated from other detectable compounds in the trichloroacetic acid-methanol extracts (Fig. 1). Extracts of serum samples without added drug showed no interfering peaks when chromatographed (Fig. 2). There was a linear relationship between the tetracycline concentration in the samples and the peak heights recorded (Fig. 3). Samples of tetracycline in serum and in water gave identical slopes (Fig. 3 and 4), again showing a quantitative recovery of tetracycline in the extraction procedure. The linear relationship extended to at least 50 µg/ml, well above the concentrations shown in Fig. 3 and 4.

Sensitivity. The base line fluctuation of the detection system was approximately 3 mm. This allowed accurate determinations of tetracycline concentrations in serum to levels as low as 0.3 µg/ml (peak heights of about 8 mm).

Precision. The precision of the method was determined for two concentrations of tetracycline in serum within the therapeutic range, i.e., 2 and 7 µg/ml (11). Six samples of each concentration were extracted separately and determined on the same day. As shown in Table 2, this method had a satisfactory precision in the range of serum concentrations obtained with the doses of tetracycline used in clinical practice (11). At tetracycline concentrations of 2 and 7 µg/ml, the variation coefficients were 3.45 and 1.50%, respectively.

Specificity. No interfering peaks were found in serum extracts from 30 dogs and from five young, healthy human volunteers. Serum samples from nine patients not given tetracycline but treated with a variety of drugs, including other antibiotics (gentamicin, penicillin, cephalothin, ampicillin, amphotericin B, methicillin, and clindamycin), were assayed and showed no detectable peak at the retention time of tetracycline.

In vivo experiments. Figure 5 shows a typical time curve for the elimination of tetracycline in one of three human volunteers after a single dose of 250 mg of tetracycline intravenously. Values from chemical and microbiological analysis are plotted on the same graph. The insert in Fig. 5 represents the semilog plot of the data obtained with chemical assay. An initial phase of distribution of the drug after injection is followed by a linear phase of elimination, starting at 8 h. From this part of the
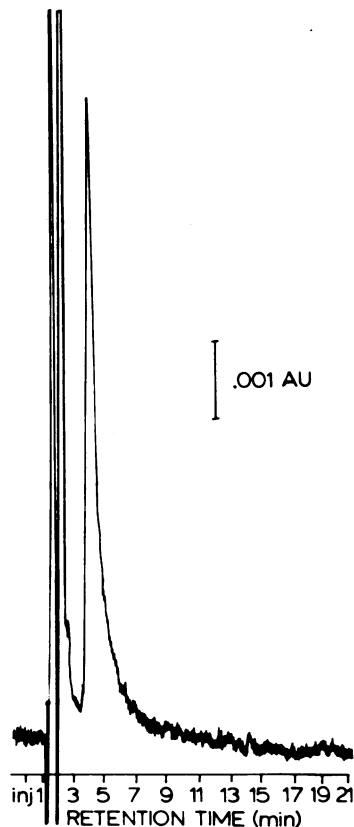

FIG. 1. High-pressure liquid chromatogram of an extract of serum containing tetracycline. The tetracycline peak has a retention time of 3.75 min. MicroBondapak C_{18} column (30 cm by 4 mm, ID). Mobile phase: methanol-EDTA (3:7, vol/vol). Flow rate: 2.8 ml/min. Detector: UV at 355 nm.
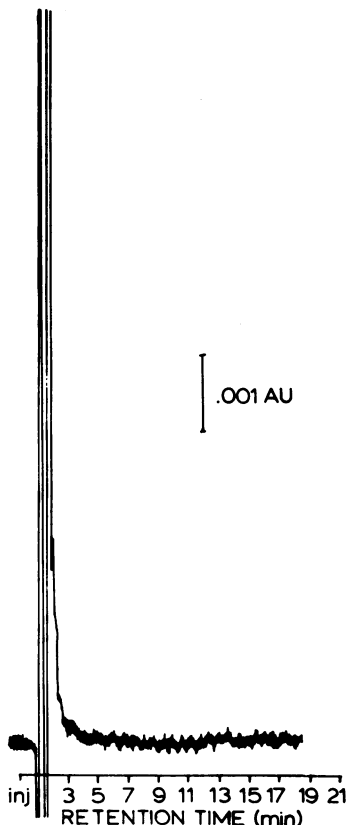
FIG. 2. *High-pressure liquid chromatogram of and extract of serum with no added tetracycline. Same chromatographic conditions as described in the legend to Fig. 1.*
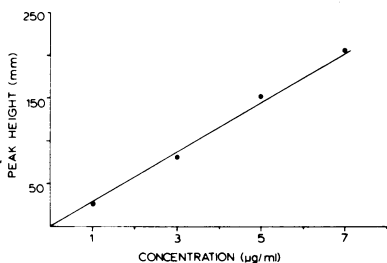
TABLE 2. *Precision of tetracycline determination*[a]

Tetracycline hydrochloride added to serum (μg/ml)	Mean[b] (μg/ml)	Variation coefficient (%)
2	2.00 ± 0.07 (1.90–2.05)	3.47
7	7.00 ± 0.11 (6.86–7.13)	1.50

[a] Values represent six separate observations at each concentration.
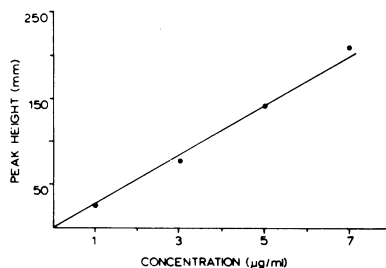[b] Values represent mean ± standard deviation. Range is shown in parentheses.

FIG. 4. *Chromatographic peak heights for controls as a function of original concentrations of tetracycline in water samples.*

FIG. 3. *Chromatographic peak heights for serum extracts as a function of original concentrations of tetracycline in serum samples.*
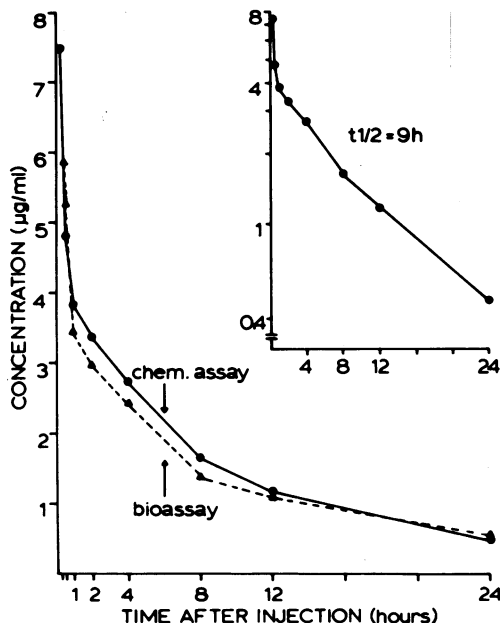

FIG. 5. *Serum levels of tetracycline in a human (male, 60 kg) after a single injection of 250 mg intravenously. Symbols: ●, chemical assay; ▲, microbiological assay. Insert shows semilog plot of data obtained by chemical assay. Abscissa and ordinate are the same as in main graph. t1/2, half-life of tetracycline in serum.*

curve, a half-life of 9 h for the antibiotic in serum can be calculated, well in agreement with earlier data obtained by microbiological assay (10). Similar values (8.5 and 8.3 h) were found for the two other volunteers. The tetracycline concentrations measured 15 min after injection of 3.5 mg/kg of body weight to three dogs were 5.6, 5.8, and 7.4 μg/ml; with the

dose of 7.0 mg/kg of body weight, values of
12.1, 12.6, and 12.2 µg/ml were recorded. The
shapes of the elimination curves after the two
doses were similar (Fig. 6).

Correlation with microbiological assay. The
serum samples obtained after injection of
tetracycline into the three human volunteers
were assayed by the microbiological as well as
the chemical method. The correlation coef-
ficient, calculated from these 24 samples, was
0.95 (Fig. 7). In the 42 samples obtained af-
ter administration of two different doses of the

antibiotic to six dogs, an equally good cor-
relation between the microbiological and
chemical assays was found (correlation coef-
ficient, 0.97) (Fig. 8).

DISCUSSION

The common use of antibiotics and the po-
tential toxicity of many of these agents make it
imperative that rapid and accurate determina-
tions of antibiotic concentration in serum and
other body fluids be readily available. Espe-
cially in patients with hepatic and/or renal
dysfunction, knowledge of antibiotic levels in
the circulation becomes critical in order to
administer appropriate doses, assess thera-
peutic efficacy, and prevent adverse side ef-
fects.

Most clinical laboratories utilize microbio-
logical assays as the principal method for de-
termining antibiotic concentration in body
fluids. However, there is a lack of uniform
methodology (for instance, different media and
test organisms are used for a given anti-
biotic), which can be applied to most clinically
important antibiotics. In addition, another
antibiotic concurrently present in a biological
sample often interferes with the microbio-
logical method. Furthermore, these procedures
are time consuming, with the desired informa-
tion not being available to the clinician for
as long as 48 h.

More recently, other procedures have been
developed for some antibiotics, for instance,
radioimmunoassay for gentamicin (13), radio-
enzymatic assay for gentamicin, tobramycin,
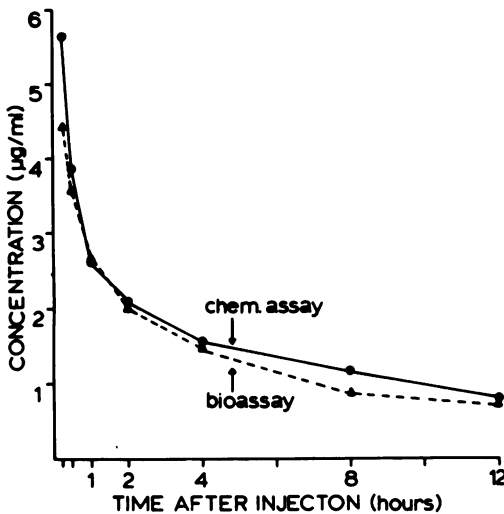BB-K8, and sisomicin (6, 16), and fluorometric

FIG. 6. Serum levels of tetracycline in a dog (30 kg) after a single injection of 3.5 mg/kg of body weight intravenously. Symbols: ●, chemical assay; ▲, microbiological assay.
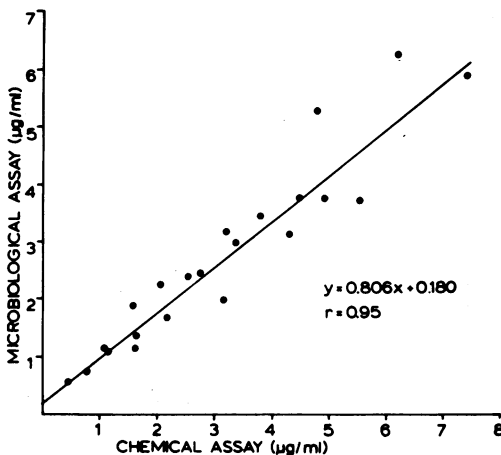
FIG. 7. Linear regression of tetracycline con-
centrations in human serum samples determined by
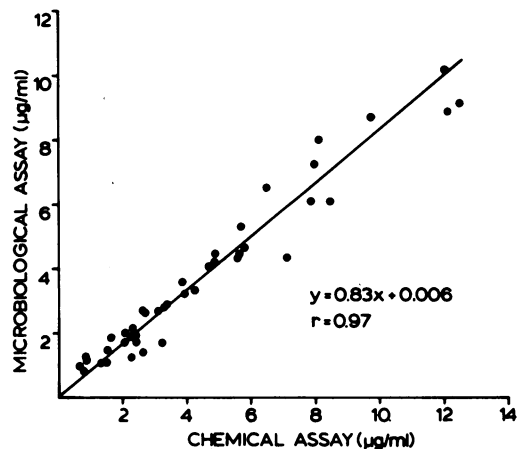chemical and microbiological assay. r = correlation
coefficient.

FIG. 8. Linear regression of tetracycline con-
centrations in dog serum samples determined by
chemical and microbiological assay. r = correlation
coefficient.

methods for tetracyclines (8, 9, 12). These procedures suffer the major disadvantage of restricted applicability to only a few antibiotics and are not adaptable to a wide variety of chemotherapeutic agents. This has limited the general acceptance by most clinical laboratories in utilizing these assay systems. For example, fluorometric methods available for determination of tetracycline levels in biological samples are generally rapid and sensitive. However, since no separation step is employed, a complicated extraction system is necessary. The specificity of these methods relies on the specificity of the detection system, and there is a possibility that interfering substances present in serum could alter the measured antibiotic values.

The method described in this report involves rapid and simple extraction of antibiotic from serum samples. The introduction of a subsequent chromatographic separation allows the use of a simple detection principle (UV absorption) without impairing the specificity of the assay.

This general approach, due to the versatility of high-pressure liquid chromatography, appears to be applicable to the determination of a variety of substances in biological fluids. This technique has been applied to the quantitation of several drugs in biological fluids (for example, see references 7, 17), including quantitation of serum and urine levels of cephalothin (2, 3), cephoxitin (2), and flucytosine (1). This form of chromatography is rapid (separation is achieved in minutes), covers a wide molecular weight range (100 to 10,000), and does not necessarily require derivatization of the compounds under analysis. A wide variety of columns and solvent systems can be used, thus permitting separation of virtually any compound that exhibits solubility in aqueous or organic solvents (15). Detection of various substances, particularly antibiotics, is feasible because many of these agents possess properties of absorption or fluorescence in the UV-visible spectrum. Antibiotics that do not exhibit these characteristics, for instance, the aminoglycosides, may be detected by changes in refractive index or, if this method is not sensitive enough to allow measurement of therapeutic levels of the drugs in biological fluids, chromophores may be attached to facilitate detection by UV-visible absorption or fluorescence.

By using this general approach, a method for quantitation of tetracycline in serum was developed. This drug, like most antibiotics, exhibits significant binding to serum proteins.

When added to serum in vitro, 50% of tetracycline is bound to these proteins (14). Therefore, determination of the total concentration of tetracycline requires quantitative extraction of the drug from serum proteins. By treating the serum samples with 20% trichloroacetic acid in methanol, a virtually protein-free extract was obtained containing 100% of the added antibiotic. Several other extraction procedures were attempted but resulted in either incomplete protein precipitation or incomplete extraction of tetracycline.

Introduction of the chromatographic separation step greatly enhanced the specificity of the assay. Direct spectrophotometric determination of tetracycline in the filtered serum extracts was made impossible by even slight hemolysis in the serum samples, since heme, produced from hemoglobin during the extraction, has a substantial absorption at 355 nm. This problem was eliminated by the introduction of the liquid chromatography, in which products of hemolysis did not produce interfering peaks.

Correlation between tetracycline concentrations in serum, as determined by the described chemical analysis and by microbiological assay, was extremely satisfactory. The two methods are comparable with regard to absolute values obtained and show a correlation coefficient of ≥ 0.95. However, the bioassay was unable to detect tetracycline at concentrations below 0.6 $\mu g/ml$, whereas the chemical assay was sensitive enough to allow accurate determinations of the drug to levels as low as 0.3 $\mu g/ml$ of serum. Moreover, the high precision and reproducibility of the latter method was demonstrated by variation coefficients of less than 3.5% in the therapeutic concentration range. The method also appears to be very specific: no interference was found in sera from dogs, normal human volunteers, or patients treated with a variety of other drugs. Finally, the chemical analysis is easily performed in a short period of time. A single determination of tetracycline from the time the serum sample is received takes approximately 25 min, and four samples can be determined in 1 h. Therefore, in comparison to a microbiological assay, the technique developed in this investigation is more rapid, sensitive, specific, and accurate.

Extraction of tetracycline from serum, followed by high-pressure liquid chromatographic separation, is a useful technique for quantitative measurement of circulating tetracycline levels, with definite advantages over existing methods, and seems feasible for routine clini-

cal use. The possible application of this methodology to the quantitation of other antibiotics in serum and other biological fluids is presently under investigation.

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