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Fluorometric Estimation of Oxytetracycline in Blood and Plasma

BRYAN SCALES▲ and DAVID A. ASSINDER

Abstract ☐ A sensitive spectrofluorometric method was developed for the estimation of oxytetracycline in whole blood or plasma. The method involves the use of protein precipitation, solvent extraction, and alkaline degradation procedures. Reproducible recoveries of oxytetracycline are obtained by adding β-mercaptopropionic acid at various stages of the extraction procedure. Whereas the alkaline decomposition product of oxytetracycline fluoresces at 337/410 nm., the reproducibility of the assay is improved by scanning the fluorescence spectrum from 300 to 500 nm. This enables any variation in background fluorescence of different samples to be estimated and accounted for when measuring low levels of oxytetracycline. The method will measure oxytetracycline to better than 0.1 mcg./ml. blood or plasma and is therefore suitable for monitoring the blood levels obtained after a therapeutic dose. The results obtained in man illustrate the variability of blood level profiles of oxytetracycline following its oral administration under closely controlled conditions.

Keyphrases ☐ Oxytetracycline—spectrophotofluorometric analysis in whole blood or plasma ☐ Plasma levels, oxytetracycline—after oral administration in man, spectrophotofluorometric analysis ☐ Spectrophotofluorometry—analysis, oxytetracycline in whole blood or plasma

A growing understanding of the importance of the blood levels of drugs and their relevance to the clinical evaluation makes it imperative that relatively simple and reliable methods are available for estimating this class of organic compounds in biological fluids. The present study started as an attempt to show biological equivalence, or a lack of it, in oxytetracycline hydrochloride[1] formulations and to correlate blood levels with disintegration times or dissolution times.

Initial problems occurred in the development of a satisfactory analytical method for oxytetracycline in serum, plasma, or whole blood. Although bioassay techniques have been recommended and widely used (1), an alternative method was preferred. Estimations by conventional polarography offered a rapid, although insensitive, method of analysis, and this could not be improved sufficiently even by the use of polarography (a.c.)(2). The more recent colorimetric assays for tetracyclines (3) also lack sensitivity. A variety of fluorescence methods have been reported in the literature; Hayes and DuBuy (4) utilized the conversion to the anhydroderivative on heating in acid, followed by a measurement of the fluorescence intensity at alkaline pH. Ibsen *et al.* (5) developed a method based on the increase in fluorescence of the oxytetracycline on chelation with magnesium, and Kahn (6) described its estimation in biological systems utilizing the extractability of fluorescent calcium–barbital complexes.

In this laboratory the reported fluorescence methods were largely unworkable and at best gave irreproducible results, probably due to photodecomposition of the oxytetracycline. During the investigations it was found that the ready decomposition which occurred during the various isolation stages could be most satisfactorily prevented by the inclusion of thiol reagents during the work-up procedures. This led to the development of a reliable, sensitive fluorometric method for the quantitation of oxytetracycline at the therapeutic levels encountered in whole blood or plasma.

EXPERIMENTAL[2]

Reagents—All hydrochloric acid solutions were prepared from concentrated hydrochloric acid[3] for Foodstuffs Analysis grade. This was free from the interfering materials often added to some commercially standardized solutions to prevent bacterial growth.

The following aqueous solutions were prepared in glass-distilled water: 0.1 *N* HCl containing β-mercaptopropionic acid (1.0 mg./ml.), trichloroacetic acid (30% w/v), 1.0 *N* NaOH, 0.02 *N* HCl, and

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[1] Oxytetracycline hydrochloride is the active drug substance in the pharmaceutical formulation Imperacin, marketed by I.C.I. Ltd., Pharmaceuticals Division.

[2] All fluorometric measurements were carried out using either the Aminco-Bowman spectrophotofluorometer or the Hitachi MPF 2A or MPF 3 instruments. All glassware was cleaned preferably in Haemosol, rinsed in tap water, soaked overnight in 1 *N* HCl, and, after rinsing in distilled water, dried.
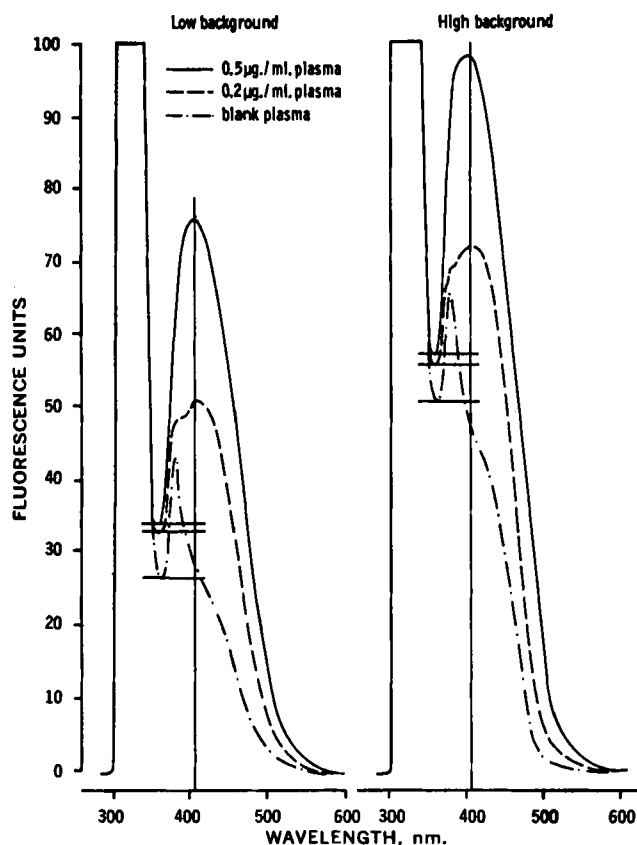
[3] British Drug Houses.

Figure 1—*Fluorescence spectra of low and high background extracts of oxytetracycline obtained from control and spiked whole blood.*

Figure 2—*Calibration curves obtained from the low and high background extracts illustrated in Fig. 1.*

a citrate–phosphate buffer prepared by diluting a mixture of 0.5 *M* citric acid (30.7 ml.) and 1.0 *M* disodium hydrogen phosphate (19.3 ml.) to 100 ml.

The organic solvents required were ether, freshly distilled ethyl acetate, and a solution of β-mercaptopropionic acid (50 mg./ml.) in methanol.

Standard Solutions—Oxytetracycline hydrochloride was dissolved at 1.00 mg./ml. in 0.1 *N* HCl containing β-mercaptopropionic acid (1 mg./ml.). This solution was then diluted 100- and 1000-fold, using the same solvent mixture, to give solutions of 10.0 and 1.00 mcg./ml., respectively.

Spectrofluorometric Determination of Oxytetracycline in Blood or Plasma—Into a 10-ml. glass-stoppered tube were placed 1.0 ml. whole blood or plasma, 1.0 ml. water, 1.0 ml. 0.1 *N* HCl containing β-mercaptopropionic acid (1.0 mg./ml.), and 1.0 ml. trichloroacetic acid (30% w/v). The mixture was thoroughly shaken for 5 min. and centrifuged for 10 min. at 2000 r.p.m. (1500×*g*), and a 3.0-ml. aliquot of the supernate was placed in a 50-ml. tube. The trichloroacetic acid was extracted by shaking with 2 × 10-ml. portions of ether; after the last traces of ether had been aspirated, the pH of the aqueous phase was adjusted to 4.0 by the addition of 0.10 ml. 1.0 *N* NaOH and 0.60 ml. citrate–phosphate buffer. This buffered aqueous phase was extracted with 15.0 ml. ethyl acetate. After centrifugation, a 14.0-ml. aliquot was transferred to a tube containing 0.10 ml. of a methanol solution of β-mercaptopropionic acid (50 mg./ml.) and evaporated to dryness at 60° in a stream of dry nitrogen.

The residue obtained was dissolved in 3.0 ml. 0.02 *N* HCl, and the solution was made alkaline by the addition of 0.5 ml. 1.0 *N* NaOH prior to heating in a boiling water bath for 15 min. to develop the maximum fluorescence. The fluorescence intensity of the cooled solution was determined over the 300–500-nm. range while exciting at the peak activation wavelength of 337 nm. (Fig. 1).

Calibration Curves—Two types of calibration curves were constructed to aid in the development of the method and to check the recovery during routine analysis.

Standard Calibration Curves for Extracted Oxytetracycline—Aliquots of the standard solutions of oxytetracycline (10.0 and 1.00
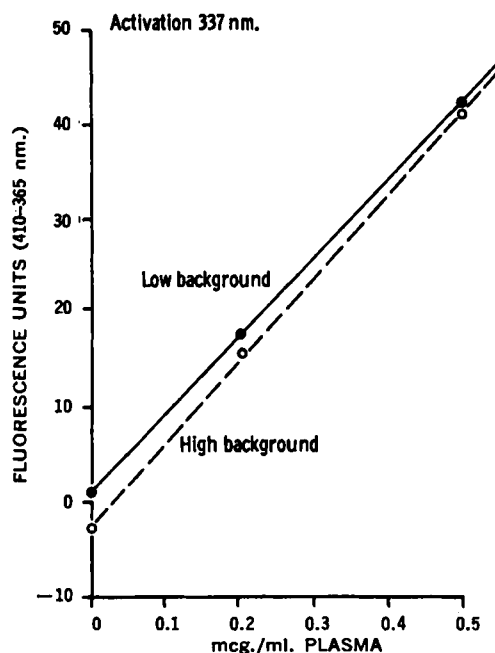
mcg./ml.) were added to the mixture of 1.0 ml. biological fluid and 1.0 ml. water. The volume was increased to 3.0 ml. where necessary by the addition of 0.1 *N* HCl containing β-mercaptopropionic acid; 1.0 ml. trichloroacetic acid (30% w/v) was then added and the samples were taken through the full extraction and fluorescence development procedure already described. Such a standard curve was prepared with every batch of samples to be analyzed.

Calibration Curves for Oxytetracycline Added to Extracts of Blood or Plasma—Samples of whole blood or plasma that were free from oxytetracycline were taken through the full extraction procedure. After evaporation of the ethyl acetate extracts to dryness, standard solutions of oxytetracycline (0.0–10.0 mcg.) were added to the residue. Then 3.0 ml. 0.02 *N* HCl was added to dissolve the residue, the solution was made alkaline, and the fluorescence was developed as already described.

Preparation of Oxytetracycline Solution for Oral Administration—Solutions of oxytetracycline sufficient for a single oral dose were prepared by dissolving a mixture of oxytetracycline hydrochloride (500 mg.) and ascorbic acid (2.30 g.) in 20.0 ml. distilled water in screw-capped vials. The proportion of these two components was similar to that used in intravenous formulations[4]. The solution was administered within 15 min. of preparation.

Administration and Blood Sampling—Normally healthy male volunteers were fasted for at least 10 hr. prior to, and 3 hr. after, the dose of oxytetracycline (500 mg.), which was taken at about 9:00 a.m. Any formulation remaining in the vials was washed out and swallowed with at least 60 ml. water.

Venous blood (8.0 ml.) was taken and oxalated at the times shown in the figures, usually at 0, 1, 2, 3, 5, 7, and 24 hr. When plasma samples were required, the blood was centrifuged and the plasma separated, usually within 30 min. of being obtained.

One week elapsed between subsequent doses to each volunteer, ensuring that plasma levels had fallen well below the limits of detection. All samples were assayed in duplicate, the second assay being carried out 1–5 days after the first assay.

RESULTS AND DISCUSSION

Development of Method—Once it had been established that the fluorescence intensity of the alkaline oxytetracycline solution was sufficient to permit the detection of oxytetracycline at levels of 0.01 mcg./ml. of final solution, it became a relatively simple task to develop the extraction technique and eliminate or control unreliable
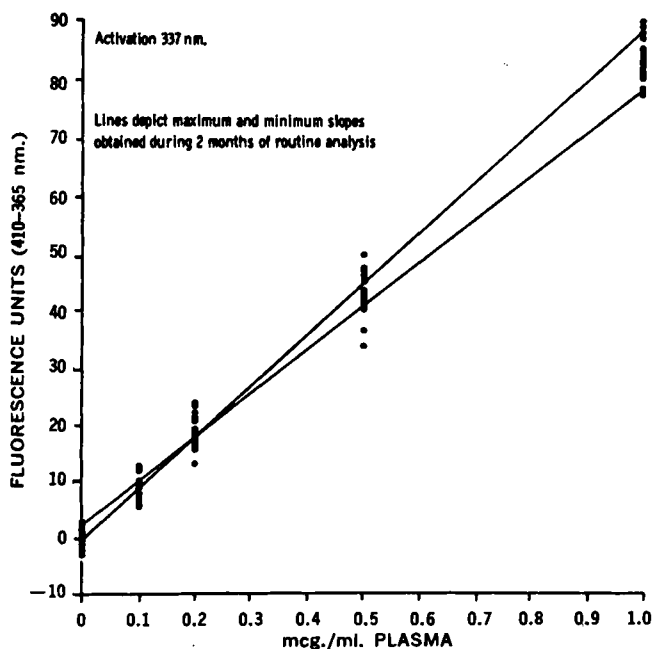
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[4] Pfizer.

Figure 3—*Calibration curves obtained for oxytetracycline extracted from spiked whole blood and plasma. Points are those accumulated over a 2-month period. The lines show the extremes in slopes obtained during this time.*

stages. This was done most expediently by examining the latest stages first. Thus, it was found that the simple evaporation of solutions of oxytetracycline in ethyl acetate (1 mcg. in 15 ml.) was sufficient to destroy all oxytetracycline. This effect was presumably due in part to photodecomposition since, when the evaporation was carried out in darkness, a 20–45% recovery was usually obtained. The addition of antioxidants such as β-mercaptoethanol and β-mercaptopropionic acid resulted in a 98–100% recovery of oxytetracycline from the solvent. Ascorbic acid could not be used because of the production of fluorescent impurities during treatment of the residue with alkali.

The use of hydrochloric acid free from metallic antibacterials was essential, since their presence led to a large and variable reduction (5–30-fold) in fluorescence of the oxytetracycline.

The trichloroacetic acid precipitation of protein and the removal of excess trichloroacetic acid by ether extraction prior to pH adjustment are advisable, since these procedures helped to reduce the background fluorescence to acceptable levels. The extraction of excess trichloroacetic acid also simplifies the problem of pH adjustment to 4.0 prior to the extraction of oxytetracycline into the ethyl acetate.

The addition of higher concentrations of hydrochloric acid to the whole blood or plasma prior to protein precipitation did not increase the recovery of oxytetracycline; and since the recovery was virtually the same from whole blood, plasma, and water, it is presumed that the standardized conditions were adequate to prevent any absorption and losses due to protein binding.

The assay was also carried out on a number of occasions using 4.0 ml. whole blood. The stages up to that of pH 4.0 adjustment were scaled up proportionately; the volume of ethyl acetate used for the oxytetracycline extraction was doubled, but subsequent stages were unchanged. As expected, the background contamination was slightly increased, but the method could still be used to detect 0.1 mcg. oxytetracycline in the 4.0-ml. sample. This largescale method was not used because it was more cumbersome and time consuming, and the sensitivity of the assay based on a 1-ml. sample was perfectly adequate.

Stability of Oxytetracycline in Whole Blood and Plasma—The stability was checked by spiking samples of the fluids, as in the preparation of calibration curves, and storing at 0–4° or at room temperature for up to 3 weeks prior to assaying. There was no distinction between calibration curves prepared from freshly spiked and from stored samples. The stability of oxytetracycline in whole blood or plasma was also independent of the presence of

Table I—Comparison of Plasma Level Profiles after Oral Administration of 500 mg. Oxytetracycline Hydrochloride in Solution to Four Subjects (see Fig. 4)

Subject	Weight, kg.	Dose Sequence	Maximum Plasma Level, mcg./ml.	Area under Curve (0–7 hr.), hr. × mcg./ml.
M.B.C.	58.0	1	2.95	17.4
		2	2.71	14.4
		3	2.46	14.8
	Mean ± SD		2.71 ± 0.24	15.53 ± 1.63
D.A.A.	65.8	1	1.22	5.8
		2	2.46	8.0
		3	2.00	11.6
	Mean ± SD		1.89 ± 0.63	8.47 ± 2.93
A.J.W.	72.5	1	2.10	12.4
		2	3.40	15.2
		3	2.06	10.8
	Mean ± SD		2.52 ± 0.76	12.80 ± 2.23
D.M.F.	86.6	1	1.58	9.0
		2	1.46	7.1
		3	1.31	7.3
	Mean ± SD		1.45 ± 0.14	7.80 ± 1.04
Overall mean			2.14	11.15

β-mercaptopropionic acid, although it was invariably added during routine assays since it was present in the standard aqueous solutions used for spiking samples.

Further evidence for the stability of oxytetracycline in the biological samples was obtained when carrying out replicate assays of unknown samples. Identical results were obtained when the replicate assays were carried out over a 2-week period.

Fluorescence Spectrum of Oxytetracycline and Its Quantitative Interpretation—The fluorescence spectra of extracts of whole blood containing 0.00, 0.20, and 0.50 mcg. oxytetracycline/ml. are shown in Fig. 1. For the majority of samples, the background fluorescence due to the Raman spectrum and to tissue impurities is low. In this case, the calibration curves obtained by plotting total fluorescence intensity at 410 nm. *versus* oxytetracycline concentration are quite acceptable, although there is a positive fluorescence blank value equivalent to about 0.25 mcg. oxytetracycline/ml. blood. Occasionally, the background scatter is increased due to poor sample preparation or to aging of the biological sample. In these circumstances, the use of such samples would normally be unacceptable in the analytical procedure. The value of scanning the full fluorescence spectrum rather than reading the fluorescence at a selected wavelength lies in the fact that this enables the background contribution of each sample to be estimated from the scan. On measuring the background fluorescence of blank samples, the fluorescence intensity of the valley at 365 nm. is equivalent to that at 410 nm.

The equivalence of fluorescence intensity of blank samples at the valley and 410 nm. is independent of the instrument and of the bandpass used in the excitation and fluorescence monochromators. For these reasons, a more accurate method of estimating fluorescence intensity due to oxytetracycline in any sample is to measure the peak height at 410 nm. above the valley at 365 nm. An example of one of the worst cases of a high background due to poor sample preparation is shown in Fig. 1, and even in this case the background is only equivalent to −0.025 mcg. oxytetracycline/ml. blood when calculated by this difference method. In general, calibration curves prepared by this method (Fig. 2) have a blank value which falls within the range of 0.00 ± 0.02 mcg. oxytetracycline/ml. biological sample.

Sources of Variability in Analysis—The day-to-day variation in overall extraction efficiency of oxytetracycline (42 ± 2%) and in instrument sensitivity was quite small. The individual points from extracted calibration curves obtained from whole blood and plasma over 2 months[5] are accumulated in Fig. 3. Also illustrated are the
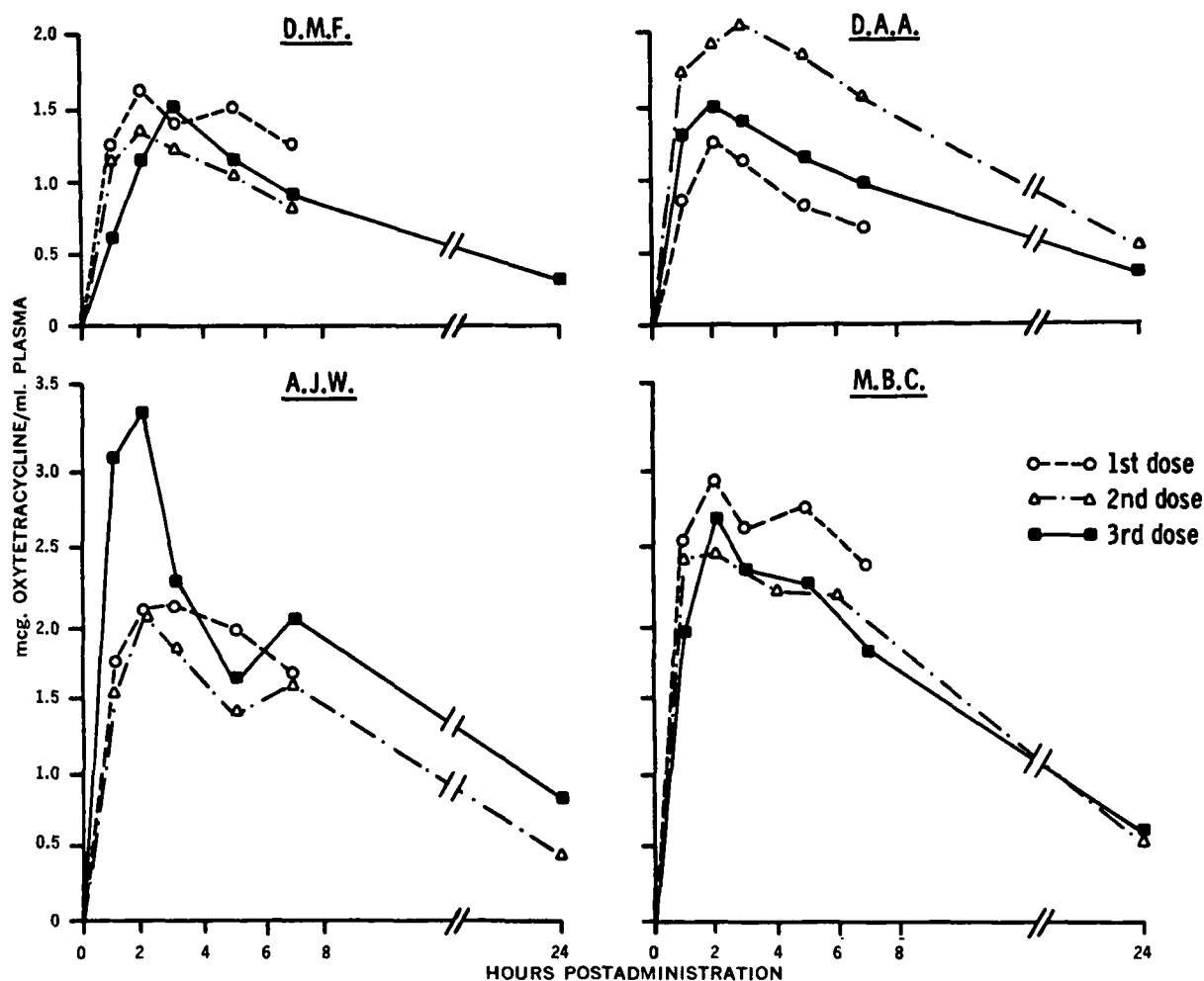

Figure 4—*Plasma level curves of oxytetracycline in four volunteers following the oral administration of a solution of 500 mg. oxytetracycline hydrochloride.*

extremes of slopes encountered during this period of routine assays. It was not necessary to adjust instrument sensitivity during the analytical runs, although this could have been done by use of fluorescence standards. The only instrument checks carried out during routine use were that the activation wavelength was correctly set at the optimum of about 337 nm. and that the activation and fluorescence slits were each set for a bandpass of 8 nm.

The slight fluctuations in slope (Fig. 3) encountered over a 2-month period were usually caused by the necessary periodic renewal of extraction solvents and reagents. For this reason it was preferred to calculate the concentration of oxytetracycline in the biological samples by direct application of the calibration curve from extracted spiked blood samples, rather than to rely solely on calculations based on a 42% extraction efficiency and a nonextracted standard curve.

Table II—Analysis of Variance of Data in Table I

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio
Maximum Plasma Level				
Between subjects	3	3.01	1.00	3.80[a]
Within subjects	8	2.11	0.26	—
Area under Curve				
Between subjects	3	121.08	40.36	9.34[b]
Within subjects	8	34.55	4.32	—

[a] Marginally significant ($p < 0.1$). [b] Highly significant ($p < 0.01$).

Plasma Levels after Oral Dosing—The solution was administered to four fasted male volunteers on three separate occasions at weekly intervals. The results of the plasma level analyses are given for each volunteer in Fig. 4. All samples were assayed in duplicate, and agreement was better than ±4% for levels above 1.2 mcg./ml. and better than ±8% for levels in the 0.2–1.2-mcg./ml. range. The drug appears to be fairly rapidly absorbed, with peak plasma levels being achieved between 2 and 3 hr. after dosing, and by 24 hr. the level had fallen to about 23 ± 2% of the peak value in all subjects. The absence of measured plasma levels in the 7–24-hr. period permits only a rough estimate of the plasma half-life, which in these volunteers probably falls in the 8–10-hr. range; this compares favorably with the literature value of 9.2 hr. (7).

The most disconcerting observation in this brief study was that although attempts were made to obtain reproducible plasma level profiles by: (a) administering the oxytetracycline in a form free from complications of variable disintegration and dissolution rates, and (b) minimizing disturbances in absorption due to variations in the contents of the GI tract, the results showed that this had not been achieved.

Any assessment of the reproducibility of plasma level profiles in one individual should include *inter alia* a comparison of the times to attain the peak level, the actual level achieved at the peak, and the area under the curve (hr. × mcg./ml.) over a chosen period of time. The peak plasma levels that occurred reproducibly at from 2 to 3 hr. after dosing and the area under the curves for the 0–7-hr. period are listed in Table I. Although the number of subjects was small and the within-subject variability was occasionally high, the results of the analysis of variance for these two parameters (Table II) show that there are significant differences between individuals with respect to their plasma level profiles.

The variability encountered in the plasma level profiles of any one individual was not an artifact due to changes in protein binding since, unlike microbiological assay techniques, the present fluorometric method measures total oxytetracycline. Neither was the variation between subjects caused by weight differences in the subjects, since none of the analyses was improved by taking the weight of the subjects into account. It is possible that plasma level profiles could be made more reproducible by stabilizing physiological conditions, e.g., transit times (8). However, such changes could only be of use in bioavailability studies and not in general clinical practice; even then, attempts at such stabilization could be beneficial to one formulation and detrimental to another.

In the present studies the mean peak plasma levels of oxytetracycline (2.14 mcg./ml.) attained after oral administration of a solution of 500 mg. of the hydrochloride are only 40% higher than the serum levels of the reference oxytetracycline (1.55 mcg./ml.) used in the study of Blair et al. (9), in which the dose of 250 mg. of the hydrochloride was administered in a capsule. This difference could be unreal, caused by the relatively few subjects used in the present study, but it could equally be the result of different physiological conditions of the GI tract.

The present results illustrate the difficulty in obtaining reproducible plasma level profiles of oxytetracycline, even under fairly carefully controlled conditions. The variability could be much greater after the oral administration of more complicated formulations than that used here. One way around the problem is to study plasma levels achieved after multiple doses, as in normal clinical practice, and the results of such studies will be the basis of further reports.

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Specificity of Antibodies Produced by Linear Antigenic Polypeptides of a Known Primary Structure: Synthesis and Use of Poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine Methyl Ester

BRIAN J. JOHNSON▲ and CHARLES CHENG

Abstract □ The specificity of antibodies formed in rabbits in response to the antigenic challenge of poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I) was studied with respect to the role of the glutamyl residue. For this purpose the polypeptide poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine methyl ester (II) was prepared. When using this material, it was found by cross-reaction and absorption studies that the antibodies to the antigen I possess a high affinity for the γ-carboxyl groups of the glutamyl residue.

Keyphrases □ Polypeptides, linear—synthesis, immunochemical properties □ Antibodies, polypeptide produced—specificity □ Poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)glycine methyl ester—synthesis, used to study antibody specificity

It has previously been reported that the antibodies produced by rabbits against the polypeptide poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I) (1-3) are most probably dependent upon the conformation of the antigen (4-7). These antibodies also have been shown to possess a specificity for the phenolic hydroxyl groups and the aromatic moiety of the tyrosyl

residue (8). However, no such specificity has been shown for the alanyl residue (9). The next phase of this work has been to study the specificity of these antibodies pertaining to the role of the glutamyl residue. This paper reports the characterization of the specificity of antiserum produced by rabbits against the antigen I as studied by cross-reactions and absorption studies. For this purpose the following polymer was synthesized and used:poly(L-tyrosyl-γ-glycyl-L-glutamyl-L-alanylglycyl)-glycine methyl ester (II).

DISCUSSION[1]

The polypeptide II was prepared from the polymerizing unit, L-tyrosyl-γ-(tert-butyl-glycyl)-L-glutamyl-L-alanylglycine pentachlorophenyl ester hydrochloride (IX), which was synthesized as outlined in Scheme I and as detailed in the Experimental section. The polymerization of IX was performed by the procedure that has been shown to produce linear high molecular weight polypeptides (1, 2, 4-8). After removal of the protecting tert-butyl groups, the

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[1] All amino acids are of the L-variety.