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Fluorometric Determination of Tetracyclines in Small Blood and Tissue Samples

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Abstract □ A reported fluorometric method to determine tetracyclines in biological material was modified to determine low levels of the antibiotics when using only small amounts of starting material. The method is applicable to tissue distribution studies in small experimental animals and also can be used for serial determinations in blood samples of these animals and for biopsy material. The tetracyclines are extracted in the presence of ethylenediaminetetraacetic acid, and the phosphate precipitation steps are omitted. This omission makes the procedure considerably less time consuming. The recoveries of the tetracyclines, oxytetracycline and doxycycline, are ~80–100%. The values obtained by this method were consistent with those from microbiological assays, and its reliability was shown further in a comparison with the fate of radioactive tetracycline.

Keyphrases □ Tetracyclines—fluorometric determination in small blood and tissue samples □ Oxytetracycline—fluorometric determination in small blood and tissue samples □ Doxycycline—fluorometric determination in small blood and tissue samples □ Fluorometry—analysis, tetracyclines in small blood and tissue samples

Several methods are available to determine the amount of tetracycline in biological material. Microbiological assays often are preferred even though their precision is low and data can be obtained only after prolonged incubation (1).

Spectrophotometric methods (2) are insensitive, and interference from other material cannot always be excluded. High-pressure liquid chromatography (HPLC) (3) and polarographic analysis (4) of tetracyclines also have been described, but they require special equipment. Fluorometric analysis is relatively simple and specific for the tetracyclines (5, 6).

BACKGROUND

Studies have been made of the inhibition of mitochondrial protein synthesis by tetracyclines and the consequences of this inhibition on the functional activity of cells, tissues, and whole animals (7–10). Because of the many other possible effects of tetracyclines, which occur mainly at drug concentrations much higher than those at which mitochondrial protein synthesis is impaired, careful control of tetracycline levels and amounts is necessary in such studies.

For these reasons, the fluorometric method described by Poiger and Schlatter (6) was selected for measurement of tetracycline concentrations in tissues and serum samples of rats after prolonged drug administration. This method is based on buffer extraction of tetracyclines from blood and tissue samples. After deproteinization and elimination of phosphates, the tetracyclines are extracted into an organic solvent as ion-pairs with calcium and trichloroacetate ions. Fluorescence of the tetracyclines is induced by the addition of a base and magnesium ions. The method gives reproducible results, although the recoveries are low—on the order of 35–40% for tetracycline and chlortetracycline. Only for oxytetracycline was 60% recovery from serum recorded (6).

A major disadvantage of the method is the large quantity of material required (3 ml of serum or 2 g of tissue). Therefore, the amounts of tetracyclines in blood and tissue samples of experimental animals such as mice and rats are difficult to determine with this method. By critical evaluation of the various steps of the method, it was possible to modify it in a way that allows the detection of small amounts of the tetracyclines in as little as 0.2 ml of serum or 50 mg (wet weight) of tissue.

EXPERIMENTAL

Chemicals and Antibiotics—All chemicals were analytical grade. Oxytetracycline[1] (as hydrochloride), doxycycline[2] (as hydrochloride), and [7-^{3}H-(N)]tetracycline[3] (0.94 Ci/mmole) were used as obtained.

Experimental Animals and Preparation of Homogenates—In all recovery studies, after the addition of tetracyclines (oxytetracycline, doxycycline, or tetracycline), rat tissue homogenates or serum samples were incubated for 20 min at 37° to obtain maximal binding. Tissue homogenates were prepared in saline using a glass–polytef homogenizer.

Extraction of Tetracyclines—Two milliliters of 0.1 *M* glycine–hydrochloric acid (pH 2) was added per milliliter of a 20% homogenate or dilutions thereof or per milliliter of serum with a known tetracycline content. Proteins were precipitated by the addition of 2 ml of 1 *M* trichloroacetic acid. The mixture was shaken vigorously and then centrifuged. Four milliliters of the supernate was mixed with 1.2 ml of a solution containing sodium acetate (2 *M*) and lead nitrate (0.06 *M*) and allowed to stand for 45 min at room temperature before centrifugation. Then 0.85 ml of saturated potassium iodide solution was added to 5 ml of the supernate, and the mixture was left for 15 min. After centrifugation, 5 ml of the supernate was mixed with 50 μl of 1 *M* calcium chloride and saturated with sodium chloride.

The pH was adjusted to 9.5 with concentrated ammonia. After addition of 3 ml of ethyl acetate, the tetracycline was extracted by shaking the mixture for 1.5 min. Deviations from this general procedure will be indicated.

Fluorescence Measurements—Fluorescence of tetracyclines extracted into the organic layer was induced by adding 0.5 ml of a solution containing 200 mg of barbital sodium and 600 mg of magnesium acetate in 100 ml of methanol to 3 ml of the ethyl acetate extract just prior to the measurement of fluorescence. When small amounts of tissue or serum were used, 1 ml of water, treated as for a sample containing tetracycline, was a suitable blank. In other cases, a sample of tissue or serum without tetracycline was used as a blank because of the background fluorescence.

After reading the fluorescence of a sample, a known amount of tetracycline was added to the cell to calculate the recovery of tetracycline from the sample.

Fluorescence measurements were carried out on a fluorescence spectrophotometer[4] equipped with a xenon arc lamp. The spectral bandwidth was 10 nm for both excitation and emission. The instrument settings were 400 nm for excitation and 505 nm for emission.

[1] Vendarcin, Gist-Brocades, Delft, The Netherlands.
[2] Pfizer Inc., Brussels, Belgium.
[3] New England Nuclear, Dreiech, West Germany.
[4] Perkin-Elmer MPF-43, Charles Goffin, Maastricht-de Bilt, The Netherlands.

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Table I—Recovery of [^{3}H]Tetracycline Measured after Every Step of the Fluorometric Determination Procedure where 10 μg of [^{3}H]Tetracycline Was Added/5 ml of Homogenate (20% w/v)

Step in Procedure	Recovery, % 0.25 ml of Homogenate	10 ml of Homogenate
After trichloroacetic acid precipitation	71	32
After lead phosphate precipitation	100	87
After lead iodide precipitation	91	93
After ethyl acetate extraction	43	81
Counting of radioactivity (net recovery)	28	25
Fluorometric measurement (net recovery)	22	30

Microbiological Assay—The tetracycline content of the tetracycline solutions was determined microbiologically by measuring the growth inhibition of *Bacillus cereus* D220 (11).

Radioactivity Measurements—Tritiated tetracycline was used in some experiments. Radioactivity was counted in a liquid scintillation counter using various scintillators on xylene basis[5].

RESULTS AND DISCUSSION

Recovery Studies with Labeled Tetracycline—To gain insight into the causes of the low recoveries found with the method of Poiger and Schlatter (6), the recovery of tritiated tetracycline after each step of the procedure was investigated. The method was applied to small (0.25-ml) and large (10-ml) amounts of a 20% (w/v) liver homogenate containing 10 μg of [^{3}H]tetracycline/g (wet weight) of tissue.

A considerable amount of tetracycline was coprecipitated with the proteins, reducing the recovery in the supernate, especially if large amounts of homogenate were used (Table I). An important loss also was observed in the extraction of tetracycline into ethyl acetate; the reduced recovery in this step seemed to be inversely proportional to the amount of tissue present. Table I also shows that the values for the recovery based on the radioactivity and fluorescence measurements agree rather well. This finding indicates that the tetracycline is not decomposed during the procedure.

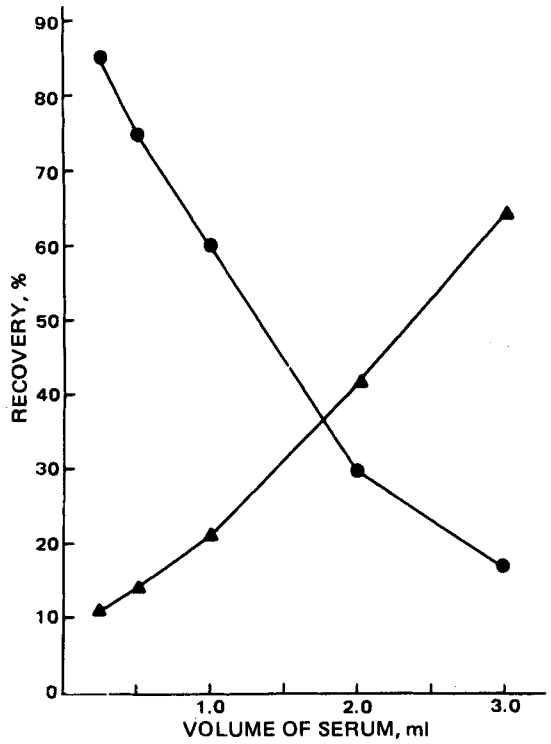

Figure 1—*Percentage recovery of 1 μg of oxytetracycline added to various amounts of human serum. Key: ●, recovery in the presence of 75 mM ethylenediaminetetraacetic acid during glycine treatment of the samples; and ▲, recovery without the addition of ethylenediaminetetraacetic acid.*

[5] Lipoluma and Hydroluma, J. T. Baker Chemicals, B. V. Deventer, The Netherlands.

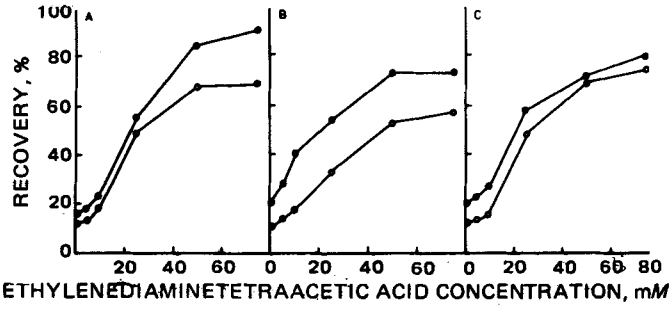

Figure 2—*Recovery of 0.1 μg of oxytetracycline (●) or 0.1 μg of doxycycline (▲) added to 0.2 ml of human serum (A), 0.25 ml of rat liver homogenate (20% w/v) (B), and 0.25 ml of rat kidney homogenate (20% w/v) (C). Recovery was measured in the presence of varying amounts of ethylenediaminetetraacetic acid.*

Modification of the Poiger and Schlatter Method—Various mechanisms such as chelation have been proposed for the binding of tetracyclines, especially to proteins (12). Addition of ethylenediaminetetraacetic acid during the first extraction may influence these processes and improve the efficiency of the extraction as well. However, the presence of ethylenediaminetetraacetic acid also influences other steps in the procedure. Because of its chelating properties, addition of ethylenediaminetetraacetic acid results in the removal of lead ions. Precipitation of lead phosphate or lead iodide thus is counteracted if enough ethylenediaminetetraacetic acid is added. The presence of lead ions as well as of phosphates inhibits the extraction of tetracyclines into ethyl acetate. Especially in samples containing only a little phosphate, excess lead may not be removed completely after the addition of potassium iodide. This condition may lead to a lower recovery. Therefore, the effects of ethylenediaminetetraacetic acid on the recovery of tetracyclines from serum and tissues were investigated under various conditions.

Figure 1 shows this effect for 1 μg of oxytetracycline added to various amounts of human serum. Ethylenediaminetetraacetic acid was added to the homogenate to give a final concentration of 75 m*M* together with the glycine. To measure the recovery of 1 μg of oxytetracycline added to 2 ml of serum, only 3 ml of the supernate after trichloroacetic acid precipitation was used. Before the addition of ethyl acetate, the calcium chloride concentration was raised to 150 m*M* to compensate for the high ethylenediaminetetraacetic acid content of the samples. Excess calcium chloride was used not only in the ethylenediaminetetraacetic acid-treated samples but also in the untreated ones. Recoveries varied with the amount of serum used (Table I). Ethylenediaminetetraacetic acid clearly improved the recovery, especially at the lower serum concentrations. Small amounts of serum and various organ homogenates incubated with doxycycline or oxytetracycline showed similar high recoveries if ethylenediaminetetraacetic acid was present during the glycine treatment (Fig. 2).

Figure 3 shows the result of treatment with ethylenediaminetetraacetic acid at increasing concentrations, measured immediately after trichloroacetic acid precipitation, for different samples containing 0.1 μg of [^{3}H]tetracycline. The presence of ethylenediaminetetraacetic acid im-

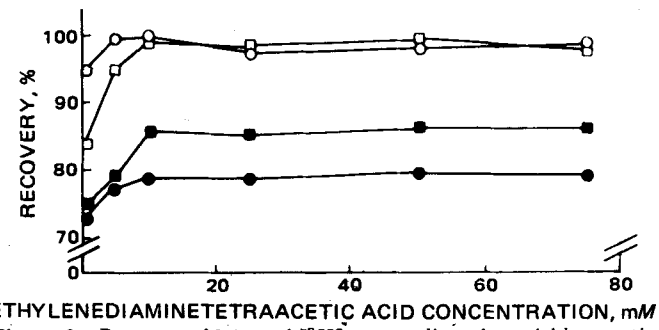

Figure 3—*Recovery of 0.1 μg of [^{3}H]tetracycline after trichloroacetic acid precipitation added to 0.1 ml of human serum (○), 1.0 ml of human serum (●), 0.25 ml of rat liver homogenate (20% w/v) (□), and 0.5 ml of rat liver homogenate (20% w/v) (■).*

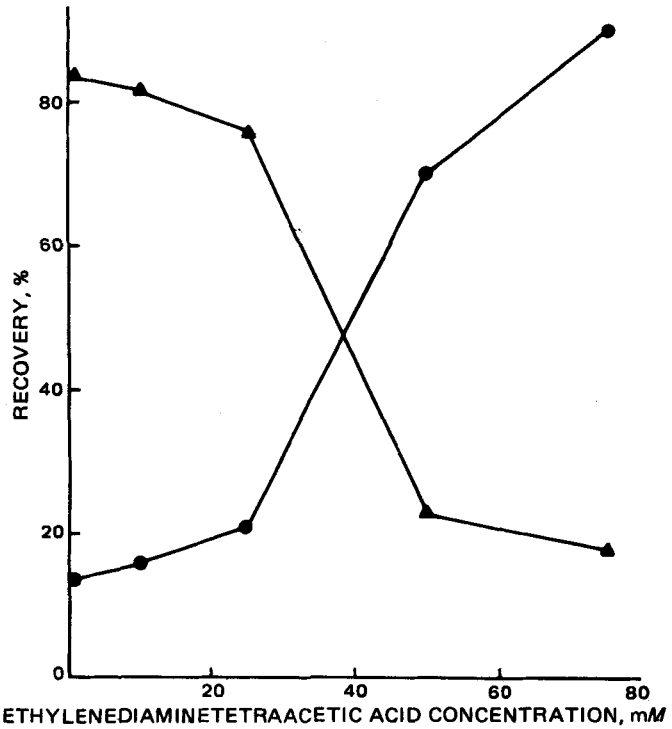

Figure 4—*Recovery of 1 μg of doxycycline/ml of distilled water or phosphate buffer at different ethylenediaminetetraacetic acid concentrations. Key: ●, distilled water; and ▲, 75 mM potassium phosphate buffer (pH 7.1).*

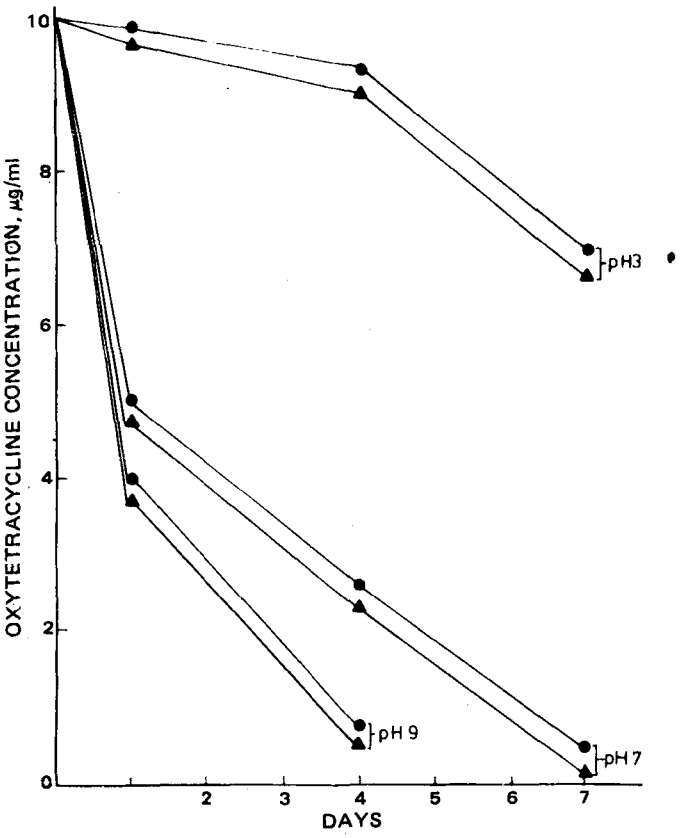

Figure 5—*Stability of solutions of different pH values containing 10 μg of oxytetracycline/ml. Key: ●, fluorometric assay; and ▲, microbiological assay.*

proved the recovery in this step in both serum and tissue but only to a small extent. The increase of ~10% was already reached at 10 m*M* ethylenediaminetetraacetic acid. Figure 3 also illustrates that the recovery was lower if more biological material was used.

Figure 4 shows the effect of ethylenediaminetetraacetic acid on the recovery of doxycycline in the presence and absence of 75 m*M* phosphate. Recovery was lowered from 80 to ~20% by the addition of ethylenediaminetetraacetic acid in the presence of phosphate but was raised from 15 to 90% in the absence of phosphate. It can be concluded that in the presence of phosphate and the absence of ethylenediaminetetraacetic acid, a large proportion of the lead ions are removed as lead phosphate, resulting in little lead iodide precipitation and negligible interference of phosphate or lead during the ethyl acetate extraction. In the presence of ethylenediaminetetraacetic acid and phosphate, the phosphate cannot be removed because of chelation of the lead ions. However, removal of phosphate is necessary because it strongly inhibits the extraction of tetracyclines into ethyl acetate (6).

On the other hand, small amounts of tissue or serum contain little phosphate. The excess lead ions that are not precipitated as lead phosphate now inhibit the extraction of tetracyclines into ethyl acetate. This implies that potassium iodide does not completely remove the lead ions. Addition of ethylenediaminetetraacetic acid seems to be more successful, resulting in much better recovery. Table II confirms these conclusions: the presence of ethylenediaminetetraacetic acid does improve the recovery from small amounts of liver homogenate but has no net effect on the recovery from large amounts.

In summary, it is obvious that the use of small samples of biological material, which contain little phosphate, to determine the tetracycline content of a particular sample offers several advantages, especially if the phosphate-precipitating steps are omitted. The procedure is less time consuming because of the omission of the phosphate-removing steps. Recoveries are between 80 and 100%. The high recoveries are the result of the low amount of trichloroacetic acid-precipitated material, which reduces the quantity of coprecipitated tetracycline, and of the avoidance of any loss caused by the unnecessary phosphate-precipitating steps.

The reliability of the modified method is indicated by the comparison of recoveries obtained by fluorometric and radioactivity counting measurements (Tables I and II). The recoveries were identical. A comparison of the amount of oxytetracycline measured fluorometrically and microbiologically is shown in Fig. 5.

Solutions of oxytetracycline in 0.9% NaCl at different pH values were protected against light and kept at 20° for several days. The amount of oxytetracycline was determined fluorometrically and microbiologically on Days 1, 4, and 7 after preparation of the solutions.

Oxytetracycline values obtained fluorometrically are slightly elevated compared to those of the microbiological measurements, but the decrease in the oxytetracycline content is comparable. The stability of oxytetracycline depends on the pH of the solution (Fig. 5). Tetracycline solutions should be prepared fresh daily, unless it is possible to use an acid solution. For example, in tissue distribution studies, solutions of tetracyclines at pH 2 seemed to be suitable (and stable) in continuous intravenous infusions for up to 4 days. The comparable results obtained by the microbiological, fluorometric, and radioactivity assays suggest that the modified fluorometric determination gives fully reliable results in this type of study.

Böcker and Estler (13, 14) recently described a sensitive HPLC method for the determination of tetracyclines. The method presented in this paper has approximately the same sensitivity. It also is fast, and many samples can be handled at the same time. The HPLC method is more specific in that it discriminates among the various tetracyclines and their breakdown products. However, for most purposes such specificity is not required since a mixture of tetracyclines seldom is used. In the case of treatment with rolitetracycline, tetracycline can be formed. However,

Table II—Recovery of [^{3}H]Tetracycline Measured in the Presence of Ethylenediaminetetraacetic Acid after Every Step of the Fluorometric Determination Procedure where 10 μg of [^{3}H]Tetracycline Was Added/5 ml of Homogenate (20% w/v)

Step in Procedure	Recovery, % 0.25 ml of Homogenate	Recovery, % 10 ml of Homogenate
After trichloroacetic acid precipitation	94	41
After lead phosphate precipitation	100	100
After lead iodide precipitation	92	91
After ethyl acetate extraction	85	38
Counting of radioactivity (net recovery)	77	17
Fluorometric measurement (net recovery)	75	13

the antibacterial action of both antibiotics is similar. Moreover, the breakdown products comprise only a small (nondetectable) fraction of the tetracyclines after *in vivo* administration (14). In many cases, the fluorometric method, as presented here, may become the method of choice.

REFERENCES

(1) "Methods of Analysis of the Association of Official Analytical Chemists," 12th ed., Association of Official Analytical Chemists, Washington, D.C., 1975.

(2) P. P. Ascione, J. B. Zagar, and G. P. Chrekian, *J. Pharm. Sci.*, **56**, 1396 (1967).

(3) A. P. de Leenheer and H. J. C. F. Nelis, *ibid.*, **68**, 999 (1979).

(4) L. G. Chatten, R. E. Moskalyk, R. A. Locock, and K.-S. Huang, *ibid.*, **65**, 1315 (1976).

(5) K. W. Kohn, *Anal. Chem.*, **33**, 362 (1961).

(6) H. Poiger and C. Schlatter, *Analyst*, **101**, 802 (1976).

(7) H. de Vries and A. M. Kroon, *Biochim. Biophys. Acta*, **204**, 531 (1970).

(8) W. P. Gijzel, M. Strating, and A. M. Kroon, *Cell Differ.*, **1**, 191 (1972).

(9) W. P. Gijzel and A. M. Kroon, *J. Mol. Med.*, **3**, 157 (1978).

(10) J. A. Leezenberg, H. Wesseling, and A. M. Kroon, *Eur. J. Clin. Pharmacol.*, **16**, 237 (1979).

(11) J. Hoette and A. P. Struyk, *J. Lab. Clin. Med.*, **51**, 638 (1959).

(12) H. Zia and J. C. Price, *J. Pharm. Sci.*, **65**, 226 (1976).

(13) R. Böcker and C. J. Estler, *Arzneim.-Forsch.*, **29**, 1690 (1979).

(14) *Ibid.*, **29**, 1693 (1979).

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Sepiolite, a Potential Excipient for Drugs Subject to Oxidative Degradation

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Received May 5, 1980, from the *Departments of *Agronomy and ‡Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN 47907.* Accepted for publication July 29, 1980.

Abstract □ Sepiolite, a member of the fibrous mineral group of clays, is relatively free of surface ferric iron and does not accelerate significantly the oxidative degradation of hydrocortisone. The compatibility of sepiolite with drugs that can undergo oxidative degradation is in sharp contrast to the catalytic effect of attapulgite, another fibrous mineral, which contains a significant amount of surface ferric iron and which therefore accelerates oxidative degradation. Sepiolite adsorbs hydrocortisone by a weak adsorption mechanism, which was shown by IR spectroscopy to be chiefly due to hydrogen bonding. However, accelerated oxidative degradation of the adsorbed hydrocortisone does not occur. Maximum adsorption occurs at pH 7–8.5. Desorption occurs readily by washing with water. Sepiolite has similar rheological properties to attapulgite. The results of this study suggest that sepiolite may be useful as a pharmaceutical excipient for drugs that undergo oxidative degradation.

Keyphrases □ Sepiolite—potential as excipient for drugs subject to oxidative degradation □ Excipients—potential of sepiolite as excipient for drugs subject to oxidative degradation □ Clays—sepiolite, potential as excipient for drugs subject to oxidative degradation □ Adsorption—sepiolite, potential excipient evaluated for use with drugs subject to oxidative degradation

A recent study (1) demonstrated that hydrocortisone is adsorbed weakly by attapulgite and subsequently undergoes oxidative degradation, catalyzed by both adsorbed iron oxides and hydroxides as well as by structural ferric iron at the clay surface. Other clays such as montmorillonite (2–4) and hectorite (5, 6) promote the oxidation of organic compounds. Surface-adsorbed contaminants or structural ferric iron at the clay surface have been suggested as being responsible for the oxidation of organic materials by these clays.

BACKGROUND

Attapulgite, which frequently is termed palygorskite in clay mineralogy literature, is a member of the fibrous mineral group of clays. The fibrous minerals are similar to the smectite group of clays, which includes montmorillonite, hectorite, and saponite, since they are 2:1 type minerals consisting of a layer of magnesium octahedra sandwiched between two silica tetrahedral sheets. However, their properties are significantly different from the smectite group since crystal growth is limited to the C-dimension, resulting in ribbons of the 2:1 layer attached at their longitudinal edges. A cross section of the fiber gives a checkerboard arrangement of ribbons and voids with no possibility of expansion. In addition, the fibrous minerals have little or no true cation-exchange capacity. However, because of the very thin nature of the ribbons, the external surface area is moderately high. Fibrous minerals also are very porous due to the channels between the ribbons. However, little adsorption occurs within the pores since the dimensions of the pores can accommodate only small molecules such as water, ammonia, and lower alcohols (7).

Sepiolite also belongs to the fibrous mineral group of clays and has an ideal formula, external surface area, internal surface, and channel dimensions that are very similar to attapulgite (Table I). However, there is a striking difference in the ferric iron content of natural samples. Sepiolite samples contain much less ferric iron than is found in attapulgite samples. Since the surface ferric iron is responsible for catalyzing the oxidative degradation, it was decided to investigate the effect of sepiolite on the oxidative degradation of hydrocortisone. In addition, since sepiolite has not been mentioned as a pharmaceutical excipient or as a GI adsorbent, the rheological properties of sepiolite suspensions were compared to attapulgite suspensions to determine if sepiolite could be considered for such use.

EXPERIMENTAL

Materials—All chemicals were official or reagent grade. The vallecas sepiolite studied was obtained from a deposit near Madrid, Spain, and was used as received. X-ray diffractograms indicated that sepiolite was the major mineral present, although a small amount of calcite also was found. The total iron content was determined by hydrofluoric acid dissolution (8).

Hydrocortisone Assay—A high-pressure liquid chromatographic (HPLC) method that was recommended for the analysis of hydrocortisone tablets (9) was modified slightly for this study. The liquid chro-