Paper Chromatographic Determination of Tetracycline

M. K. YOUSSEF[A], EL. A. IBRAHIM, and I. A. ATTIA

Abstract □ A simple paper chromatographic method for separation and quantitative determination of tetracycline in pharmaceutical preparations was developed. The method is based on the complexation of the antibiotic with a mixture of urea and disodium edetate on a chromatographic paper at pH 7.4. Spots of tetracycline and its degradation products were located under UV light, and their respective R_f values were calculated. The method can be used to determine tetracycline phosphate, even in the presence of oleandomycin. The developed method proved satisfactory in monitoring the stability of the antibiotic base and its hydrochloride.

Keyphrases □ Tetracycline and tetracycline formulations—paper chromatographic analysis in presence of six degradation products □ Paper chromatography—analysis, tetracycline and tetracycline formulations □ Chromatography, paper—tetracycline and degradation products

Tetracycline antibiotics undergo rapid degradation through epimerization and/or oxidation (1–5). The ingestion of degraded tetracycline capsules was reported to cause a reversible Fanconi-type syndrome (6–10). Since the official microbiological methods suffer from many disadvantages, recent articles have been devoted to the development of simple, precise, and accurate methods for the determination of tetracycline in degraded pharmaceutical formulations.

Previous chromatographic separations of tetracycline involved paper saturated with McIlvaine's buffer at pH 3.5 (11, 12). However, epimerization of tetracycline on the chromatographic paper was reported under these conditions (13). The separation of epitetracycline from urine, using chromatographic paper[1] impregnated with edetic acid, was reported (14). Urea solution was successfully employed as an immobile phase for paper chromatographic determination of oxytetracycline (15). The formation of a slightly soluble tetracycline–urea complex also was reported (16).

Therefore, it was decided to use paper impregnated with a mixture of disodium edetate and urea for the quantitative separation and determination of tetracycline in the presence of its degradation products. This impregnating mixture resulted in better separation which led to more precise determination of tetracycline in the presence of its degradation products.

EXPERIMENTAL[2]

Separation of Tetracycline from Its Degradation Products—*Impregnating Solutions*—A 100-ml. quantity of urea solution (A) was prepared by dissolving 10 g. of urea in McIlvaine's buffer (pH 7.4) (17); 100 ml. of disodium edetate solution (B) was prepared by dissolving 5 g. of disodium edetate in McIlvaine's buffer (pH 7.4). A mixture of Solutions A and B (1:3) was used for impregnating a series of chromatographic papers[1]. The papers were then blotted between sheets of absorbent papers.

Solvents—A mixture of ethyl acetate–acetone (40:10), saturated with disodium edetate and urea, was used as a mobile phase for the papers impregnated with the mixture of Solutions A and B.

Method—A series of methanolic solutions of tetracycline base (1 mg./ml.) was prepared and stored in glass-stoppered bottles for 0, 1, 2, 3, 4, 5, 6, 7, 10, and 15 days at 26°. Other methanolic solutions of tetracycline hydrochloride (1 mg./ml.) were prepared and similarly stored for 0 and 10 days at 26°. Ten microliters from each fresh methanolic standard solution and sample solution were separately spotted on the impregnated chromatographic papers, prepared as previously described. The chromatograms were then allowed to dry partially in air; while still damp, they were transferred to the ascending chromatographic tank containing 100 ml. of the developing solvents. After the solvent front reached about 22 cm. above the starting line, the chromatograms were air dried. The separated spots were located under UV light after exposure to ammonia vapor for at least 15 sec. Mean R_f values of tetracycline and its degradation products were calculated.

Quantitative Analysis of Tetracycline Hydrochloride and Tetracycline Phosphate in Different Pharmaceutical Preparations—A starting line (10 cm.) was marked on a sheet of chromatographic paper (17.5 × 28 cm.), about 4 cm. from one end of the sheet and 2 cm. from the edges. The chromatogram was prepared as previously mentioned. Tetracycline hydrochloride solution [0.1 ml. (100 mcg.)] in methanol or a suitably diluted aliquot of another preparation was streaked on the line. The chromatogram was placed in the chromatographic tank as described previously. After the solvent front had moved about 22 cm., the chromatogram was air dried. A blank paper without antibiotic was similarly treated. The yellow fluorescent band of tetracycline was located under UV light after exposure to ammonia vapor. It was cut into 0.5-cm. squares, with equivalent areas taken from the blank paper. The bands of tetracycline and blank were separately eluted with 20 ml. of dilute hydrochloric acid (pH 1.8). The absorbance of tetracycline present in the eluate was determined spectrophotometrically at 355 nm. (17), within 1 hr., against the eluate of tetracycline standard using 2-cm. silica cells.

This procedure was used to determine the tetracycline content of capsules, injections, suspensions, pediatric drops, and ointments. Previously published methods (15) for preparing samples of oxytetracycline before application to the chromatographic papers were successfully used in the case of the tetracycline preparations. The method was also used to determine tetracycline phosphate in pharmaceutical preparations containing oleandomycin. The calculated potencies of the investigated tetracycline dosage forms are compiled in Table I.

The method was utilized to monitor the stability of tetracycline hydrochloride and tetracycline base solutions in methanol during 6 days of storage at 26°.

RESULTS AND DISCUSSION

Epimerization of tetracycline is known to proceed at a higher rate at pH 2–6 (1). Accordingly, epimerization is expected to take place on paper previously saturated with McIlvaine's buffer, pH 3.5, and this is actually the case (13). Therefore, the pH of the impregnating solution was raised to 7.4 in order to avoid such epimerization.

Separate use of either urea or disodium edetate solution in McIlvaine's buffer (pH 7.4) as an immobile phase proved to be unsatisfactory for the complete separation of tetracycline from its degradation products. The impregnation of the chromatographic papers with urea solution resulted in a marked lowering of the R_f values with the formation of intermingled zones of tetracycline and

[1] Whatman No. 1.

[2] The following apparatus and reagents were used: (*a*) spectrophotometer, Carl Zeiss Jena; (*b*) UV lamp, HPW-125 W, type 57202 E/7, Philips; (*c*) ethyl acetate, acetone, methanol, disodium edetate, urea, and hydrochloric acid, all analytical grade; (*d*) tetracycline hydrochloride, tetracycline base, and troleandomycin USP or NF grade; and (*e*) tetracycline hydrochloride, international standard, provided by the World Health Organization.

Table I—Stated and Calculated Potency Values of Tetracycline in Different Dosage Forms

Dosage Form[a]	Stated Potency	Calculated Potency[b]	Standard Deviation
Tetracycline Hydrochloride Preparations			
Capsule	250 mg./capsule	252.8 mg./capsule	±4.20
Suspension	125 mg./5 ml.	93.5 mg./5 ml.	±1.15
Ointment	30 mg./g.	30.5 mg./g.	±0.99
Tetracycline Phosphate Preparations			
Suspension	125 mg./5 ml.	121.7 mg./5 ml.	±3.04
Injections:			
No. 1 (reconstituted with the provided vehicle)	100 mg./2 ml.	81.4 mg./2 ml.	±2.60
No. 2 (reconstituted with methanol)	100 mg./2 ml.	94.5 mg./2 ml.	±1.60
Pediatric drops	100 mg./ml.	97.7 mg./ml.	±1.80
Preparations Containing Tetracycline Hydrochloride and Oleandomycin			
Synthetic mixture	91 mg./136 mg.	90.8 mg./136 mg.	±2.40
Capsule	167 mg./capsule	169.3 mg./capsule	±0.94
Suspension	83.3 mg./5 ml.	84.2 mg./5 ml.	±0.95
Pediatric drops	66.7 mg./ml.	68.4 mg./ml.	±3.20
Injection	66.7 mg./2 ml.	65.2 mg./2 ml.	±1.40

[a] Commercial samples. [b] Average of four determinations.

epitetracycline; both adhered to the starting line. On the other hand, the use of a disodium edetate solution as an immobile phase produced diffuse spots with higher R_f values. The impregnation of the chromatographic papers with the mixture of Solutions A and B (1:3) gave the best results for the quantitative separation of tetracycline from its degradation products.

Figures 1 and 2 show the decomposition of tetracycline hydrochloride and tetracycline base in methanol. The acid decomposition products of tetracycline were obtained by heating tetracycline solution in 2 *N* HCl on a steam bath for 10 min., and they were then detected by spotting on the chromatogram after the appropriate dilution with methanol. Figure 2 shows that the acid-catalyzed decomposition of tetracycline proceeds mainly through the formation of anhydro derivatives of the antibiotic and its epimer. When the antibiotic was eluted from the chromatogram using ammonium chloride solution (10% w/v), it showed a shift of λ_{max} toward the lower wavelength, thus confirming the complex formation of the antibiotic on the chromatogram. The ammonium chloride solution was chosen as the eluent because tetracycline was reported to be stable in it (13). However, the use of the ammonium chloride solution gave low recoveries of the antibiotic. Dilute hydrochloric acid (pH 1.8) was found to be satisfactory as an eluting medium. The

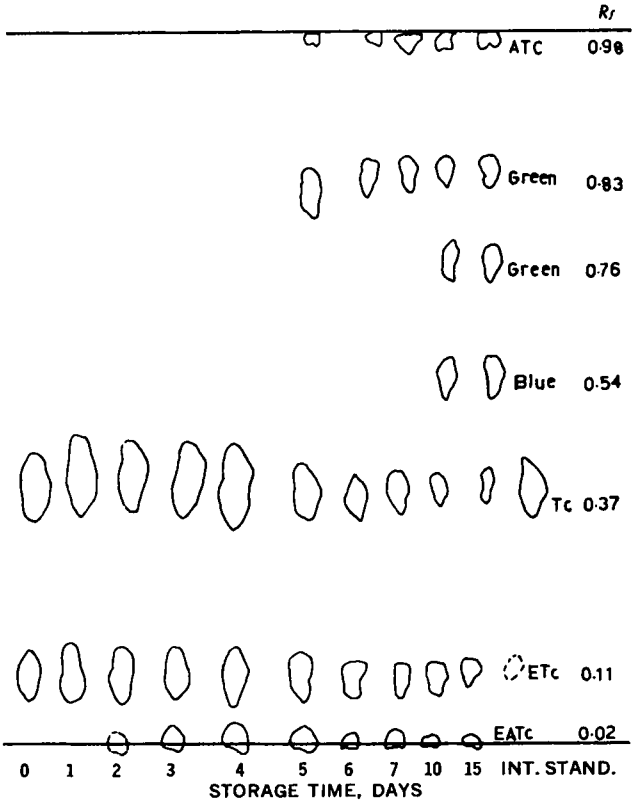

Figure 1—*Schematic chromatogram of the degradation products of tetracycline base in methanol. Key: TC, tetracycline; ETC, epitetracycline; ATC, anhydrotetracycline; and EATC, epianhydrotetracycline.*

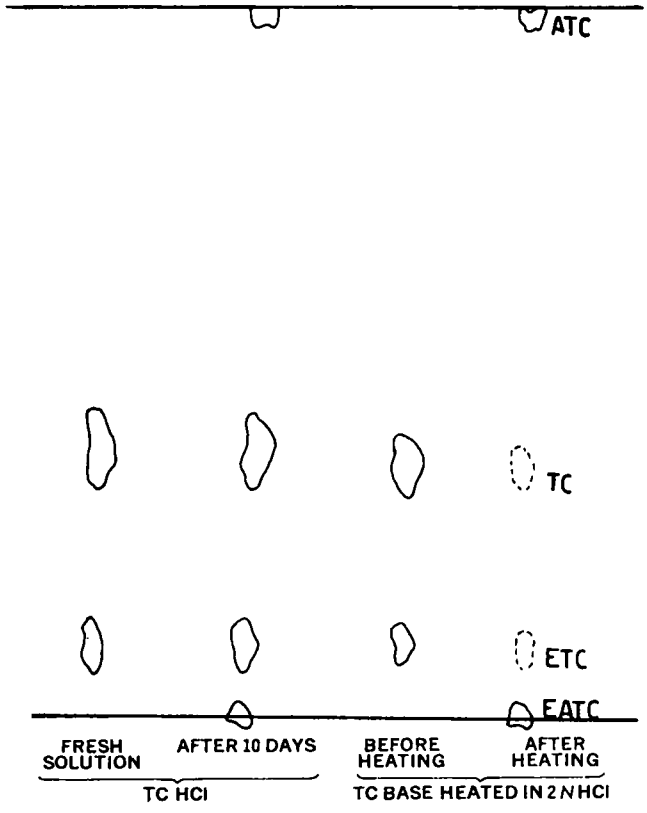

Figure 2—*Schematic chromatogram of the degradation products of tetracycline hydrochloride in methanol and the catalytic decomposition products of tetracycline base in hydrochloric acid. Key: TC, tetracycline; ETC, epitetracycline; ATC, anhydrotetracycline; and EATC, epianhydrotetracycline.*

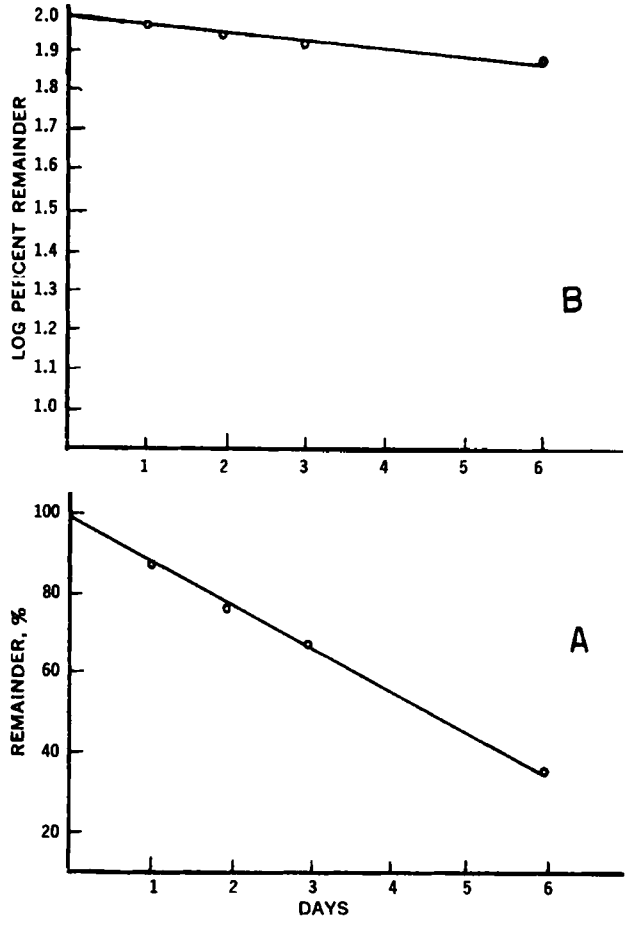

Figure 3—*Stability of tetracycline base (A) and tetracycline hydrochloride (B) solutions in methanol at 26°.*

absence of degradation spots on rechromatographing the acid eluate of tetracycline indicated that the antibiotic was not decomposed during the development and that it was stable in this solution for at least 1 hr. after elution. The absorbance of the antibiotic acid eluate at 355 nm. after 1 hr. was not changed. These results were parallel to those obtained by McCormick *et al.* (3).

The maximum load of the paper was found to be 10 mcg./spot. This necessitates the use of a horizontal line technique for applying at least 100 mcg. of the antibiotic to the chromatogram, providing a final concentration of about 5 mcg./ml. in the eluate.

Tetracycline hydrochloride, international standard, was used to study the precision and accuracy of this method. The zones representing tetracycline and 4-epitetracycline in the standard sample were combined and eluted with dilute hydrochloric acid (pH 1.8). The absorbance of the eluate at 355 nm. was compared with that of total tetracycline taken to find out the recovery of the antibiotic. Five samples gave an average recovery of 99.2 ± 1.7%.

Table I shows stated and calculated potencies of various dosage forms of tetracycline such as capsules, suspensions, injections, pediatric drops, and ointments. The data indicate that the calculated potencies of tetracycline hydrochloride capsules and ointments were close to the stated potencies. The calculated potency of tetracycline hydrochloride suspension was found to be much less than the stated potency. On observing the chromatogram of this preparation, it was found that the zone representing 4-epitetracycline was much larger than that observed in the other preparations. In addition, anhydrotetracycline was clearly detected on the chromatogram. From Table I, it is obvious that the presence of oleandomycin did not interfere with the chromatographic determination of tetracycline hydrochloride in the tested pharmaceutical preparations. The data reveal that the method is satisfactory for the determination of the antibiotic content of tetracycline phosphate in suspensions, injections, and pediatric drops. Paper chromatographic determination of tetracycline phosphate injection, after reconstitution with the provided aqueous vehicle, indicated that its potency was much less than the stated value. On the other hand, the direct measurement of the same injection, after reconstitution with methanol and immediate application on the chromatogram, gave higher results (Table I). This unexpected result might be attributed to the interference of certain additives present in the injection formulation. This suggestion is supported by the fact that the additives present in the injection are not completely dissolved in methanol. Consequently, the interference effect of these substances was greatly eliminated by the direct use of methanol as a reconstituting solvent prior to the application on the chromatogram. On the other hand, the administration of this type of tetracycline phosphate injection should be carried out promptly to avoid the degradation of the antibiotic in the reconstituting aqueous vehicle.

Table II—Microbiological and Chromatographic Estimations of Tetracycline Hydrochloride in Capsules and Suspensions

Dosage Form	Stated Potency	Calculated Potency: Microbiological Method	Calculated Potency: Chromatographic Method
Capsule	250 mg./capsule	278.8 mg./capsule	252.8 mg./capsule
Suspension	125 mg./5 ml.	97.4 mg./5 ml.	93.5 mg./5 ml.

The official microbiological method (18) was used to determine the antibiotic content of tetracycline hydrochloride capsules and suspensions. The cup-plate method was employed, and *Bacillus cereus* (var. mycoides, ATCC 9643) was the test organism used (19). The results obtained (Table II) are comparable to those obtained by the developed chromatographic procedure within 10%.

Figure 3 shows the stability of tetracycline base and hydrochloride in methanol. In the case of tetracycline base, the antibiotic decomposed through a zero-order reaction rate (K = 11.04/day), while the antibiotic decomposition of tetracycline hydrochloride followed the first-order rate (K = 0.052/day). Tetracycline hydrochloride was distinguished by its higher stability compared to the tetracycline base.

So far, the quantitative separation of tetracycline from its degradation products by chromatography has been more effectively carried out by TLC and column procedures (20, 21). The reported degradation products, which appeared as three spots on the chromatogram, were characterized as epianhydrotetracycline, epitetracycline, and anhydrotetracycline. However, previously attempted methods for the quantitative separation using paper chromatography failed to separate all of the degradation products of the antibiotic (11–14). By using the developed method, the quantitative separation of tetracycline from six degradation products was realized; three degradation products were characterized. The running time was rather short (3 hr.). Several precautions were taken to guard against degradation of tetracycline on the chromatogram, [*i.e.*, raising the pH to 7.4 to eliminate the possibility of epimerization (1) and using urea for impregnating the chromatogram and saturating the running solvents] and they contributed to the formation of a more stable complex (16). Furthermore, urea helped in the separation of the degradation products and led to the formation of well-defined spots.

In conclusion, the developed method proved adequate for the control determinations of tetracycline in different pharmaceutical preparations. It is also satisfactory as a stability-indicating assay for the selective determination of tetracycline in the presence of its decomposition and reaction products. The accuracy of the determination of tetracycline is superior to the commonly employed microbiological procedure, which has an uncertainty of about 15%, an order of magnitude less precise than the presented chromatographic method.

REFERENCES

(1) A. P. Doerschuk, B. A. Bitler, and J. R. D. McCormick, *J. Amer. Chem. Soc.*, **77**, 4687(1955).

(2) J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Origoni, W. H. Muller, R. Winterbottom, and A. P. Doerschuk, *ibid.*, **78**, 3547(1956).

(3) *Ibid.*, **79**, 2849(1957).

(4) C. R. Stephens, L. H. Conover, P. N. Gordon, F. C. Pennington, R. C. Wagner, K. J. Brunings, and F. J. Pilgrim, *J. Amer.*

Chem. Soc., **78**, 1515(1956).
(5) D. A. Hussar, P. J. Niebergall, E. T. Sugita, and J. T. Doluisio, *J. Pharm. Pharmacol.*, **20**, 539(1968).
(6) J. M. Gross, *Ann. Intern. Med.*, **58**, 523(1963).
(7) G. W. Frimpter, *J. Amer. Med. Ass.*, **184**, 111(1963).
(8) L. I. Ehrlich and H. S. Stein, *Pediatrics*, **31**, 339(1963).
(9) *Ibid.*, **31**, 698(1963).
(10) S. M. Rosenthal, *Pediatrics*, **31**, 697(1963).
(11) G. B. Selzer and W. W. Wright, *J. Antibiot. Chemother.*, **7**, 292(1957).
(12) G. Coppi, *Farmaco, Ed. Pract.*, **15**, 407(1960).
(13) E. Addison and R. G. Clark, *J. Pharm. Pharmacol.*, **15**, 268 (1963).
(14) R. G. Kelly and D. E. Buyske, *J. Antibiot. Chemother.*, **10**, 604(1960).
(15) A. Sina, M. K. Youssef, A. A. Kassem, and I. A. Attia, *J. Pharm. Sci.*, **60**, 1544(1971).
(16) L. L. Smith, *J. Org. Chem.*, **23**, 221(1958).
(17) "Documenta Geigy, Scientific Tables," 6th ed., J. R. Geigy S. A., Basle, Switzerland, pp. 314, 644.
(18) "The British Pharmacopoeia," The Pharmaceutical Press, London, England, 1968, p. 1001.
(19) F. Kavanagh, "Analytical Microbiology," Academic, New York, N. Y., 1963.
(20) A. A. Fernandez, V. T. Noceda, and E. S. Carreras, *J. Pharm. Sci.*, **58**, 443(1969).
(21) W. W. Fike and N. W. Brake, *ibid.*, **61**, 615(1972).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 11, 1972, from the *Faculty of Pharmacy, Pharmaceutics Department, Assiut University, Assiut, Egypt.*

Accepted for publication June 7, 1973.

The authors are grateful to Dr. A. Sina, Chairman of El-Nasr Pharmaceutical Chemicals Co., Egypt, for his helpful discussion.

▲ To whom inquiries should be directed.

Analysis of Adrenocortical Steroids in Pharmaceutical Preparations by High-Pressure Liquid–Liquid Chromatography

MICHAEL C. OLSON

Abstract □ The adrenocortical steroids hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate were separated by liquid–liquid chromatography. A commercially prepared reverse-phase cyano ethyl silicone column was used in conjunction with a UV precision photometer. Linearity studies were carried out using the internal standard technique and peak height measurements. Responses of each steroid were linear over the working range when *equal* injection volumes were used. The results of a study of the controlled decomposition of hydrocortisone in basic solution indicated a good separation of drug from the degradation products. Samples of adrenocortical steroids in various dosage forms, primarily creams and ointments, were analyzed by the proposed procedure, yielding a single steroid assay value and an identification and quantitation of each foreign steroid. The sample preparation was a simple dissolution of the steroid in alcohol plus the addition of an internal standard prior to injection.

Keyphrases □ Adrenocortical steroids in pharmaceutical preparations—analyzed by high-pressure liquid–liquid chromatography □ Steroids (hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate)—analyzed by high-pressure liquid–liquid chromatography □ High-pressure liquid–liquid chromatography—analysis of adrenocortical steroids □ Chromatography, high-pressure liquid–liquid—analysis of adrenocortical steroids

Liquid–liquid chromatography is actively being investigated for drug analysis (1–9). Studies of various groups of steroids have demonstrated that both qualitative detection (10–16) and quantitative measurements (17–19) may be performed. Except for these particular works (17–19), no other reports appear to have been published dealing with the quantitative analysis of adrenocortical steroids.

The official compendial approaches (20, 21) to the assay of four adrenocortical steroids—hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate—employ well-established methods. The steroids are either extracted with alcohol for total steroid content or separated by TLC to isolate a single steroid, after which the actual measurement is carried out by blue tetrazolium colorimetry. When tablets are assayed, the foreign steroid content is determined as the difference between the results of the single steroid and the total steroid assays. The problems with the blue tetrazolium color reaction—*viz.*, its nonspecificity for individual steroids and the many compounds that interfere with it, have been thoroughly documented (22–26).

Other analytical methods for steroid analysis are available (27–31). These include spectrophotometry and colorimetry, approaches that possess the same problems in varying degrees as does the blue tetrazolium color reaction, and GLC where the problem is the labile nature of the corticosteroids. If steroid derivatives are formed to ensure thermal stability, complete and specific derivatization must be achieved.

This work describes a study of a liquid–liquid chromatographic method for the analysis of hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate and the basic aqueous decomposition of hydrocortisone. The linearity of the detector response to the concentration of the steroid was confirmed for each steroid and actual commercial products; creams, ointments, lotions, and suppositories were analyzed for the presence, identification, and quantity of foreign steroids and for the main steroid component in terms of the single steroid assay value. This method was fast, accurate, and specific for each individual steroid studied.