Table V—Assay Results for Allantoin in Commercial Creams

Product	Sample Weight, g.	Weight Allantoin/g. Cream, mg.	Label Strength, %
A	1.1495	18.6	93.0
A	1.0523	18.4	92.0
B	1.0670	20.9	104.5
C	0.9141	20.2	101.0
D	3.2041	2.44	97.6
D	3.3492	2.47	98.8
E	1.2765	20.28	96.57

Chromatography and potentiometric titration of a mixture of allantoin and ammonium chloride showed that the ammonium ion is eluted with the allantoin and interferes with the quantification of allantoin in the titration. However, since no significant production of ammonia from allantoin in creams occurs, this causes no problem in the assay. Precipitation of ammonium ion from equimolar allantoin–ammonium-ion mixtures using sodium tetraphenyl borate was 96% effective in removing the ammonium-ion interference and resulted in 103% apparent recovery of allantoin. Smaller amounts of ammonium ion gave correspondingly smaller interferences.

REFERENCES

(1) G. Young and C. Conway, *J. Biol. Chem.*, **142,** 839(1942).

(2) S. A. Katz, R. T. Turse, and S. B. Mecca, *J. Soc. Cos. Chem.*, **15,** 303(1964).

(3) R. Crokaert, *Bull. Soc. Chim. Bio.*, **41,** 1001(1959).

(4) G. Siest, *Bull. Soc. Pharm. Nancy*, **64,** 55(1965).

(5) R. Zimmermann, *Naturwissenschaften*, **43,** 399(1956).

(6) G. D. Vogels, F. E. De Windt, and W. Bassie, *Recueil*, **88,** 940(1969).

(7) J. Wagner and E. Franzen, *Arch. Tierenahrung*, **9,** 11(1959).

(8) I. Bonadeo and G. Bottezzi, *Ital. Essenzy Profumi*, **50,** 78 (1968).

(9) H. S. Harned and B. B. Owen, "Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold, New York, N. Y., 1958, p. 683.

(10) A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **35,** 1358 (1941).

(11) "The Merck Index," 8th ed., Merck and Co., Rahway, N. J., 1968, p. 33.

(12) "Handbook of Chemistry and Physics," 45th ed., The Chemical Rubber Co., Cleveland, Ohio, 1964, p. D77.

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NMR Analysis of Mestranol Bulk Drug

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Abstract □ A double assay procedure for mestranol is described. The method is based upon measurement of the NMR spectrum of mestranol in pyridine, using diphenylacetic acid as an internal standard. The signals chosen are those from the methoxyl and ethinyl groups. Three commercial lots of the steroid were studied, and a TLC study of each lot is described. The impurities are tentatively identified.

Keyphrases □ Mestranol bulk drug—analysis □ TLC—separation □ Potentiometric titration—analysis □ NMR spectroscopy—analysis

The synthetic oral estrogen mestranol (17α-ethinyl-3-methoxyestra-1,3,5(10)-trien-17β-ol) is now in widespread use, chiefly as a component of oral contraceptive preparations. At present, the only official assay (1) for the raw material is a potentiometric titration. Other methods reported in the literature are colorimetric (2–4), UV (5–7), GLC (4, 6, 8, 9), TLC (7), and fluorometric (4, 10–12).

Mestranol lends itself very well to quantitative analysis by means of NMR spectroscopy, since the signals from the protons of both the ethinyl and methoxyl groups are single sharp peaks. With a suitable choice of solvent, these peaks appear in a region of the spectrum that is unaffected by signals from other protons.

The effect of solvents on the chemical shift of acetylenic protons is well documented (13). In particular, addition of pyridine to a dilute solution of a monosubstituted acetylene in carbon tetrachloride can result in deshielding of up to 1 p.p.m. of the acetylenic proton (14). Such significant deshielding is due to the fact that acetylenic compounds can form weak hydrogen bonds with molecules containing electronegative centers, such as acetone, acetonitrile, and pyridine (14–16).

Since the impurities present in mestranol bulk drug might interfere with either the signal from the ethinyl group or that from the methoxyl group, a study of the assayed samples was made by means of TLC.

EXPERIMENTAL

Spectra were obtained at 60 Mc.p.s., using a Varian A-60A analytical NMR spectrometer. A sweep time of 50 sec. for a chart width of 500 c.p.s. was used for all integrals. A r.f. power of 0.25 mG. (nominal dial setting) gave the maximum integral amplitude (17) and was used for the integrations. Tetramethylsilane in chloroform was used as an external reference to measure chemical shifts.

Assay Procedure—*NMR*—Approximately 200 mg. of mestranol and 150 mg. of pure diphenylacetic acid (DPAA) were accurately weighed and dissolved in the minimum amount of pure pyridine (approximately 0.5 ml.). The NMR spectrum was obtained in the usual manner and integrated five times in each direction through the region of interest.

Potentiometric Titration—Approximately 200 mg. of mestranol,

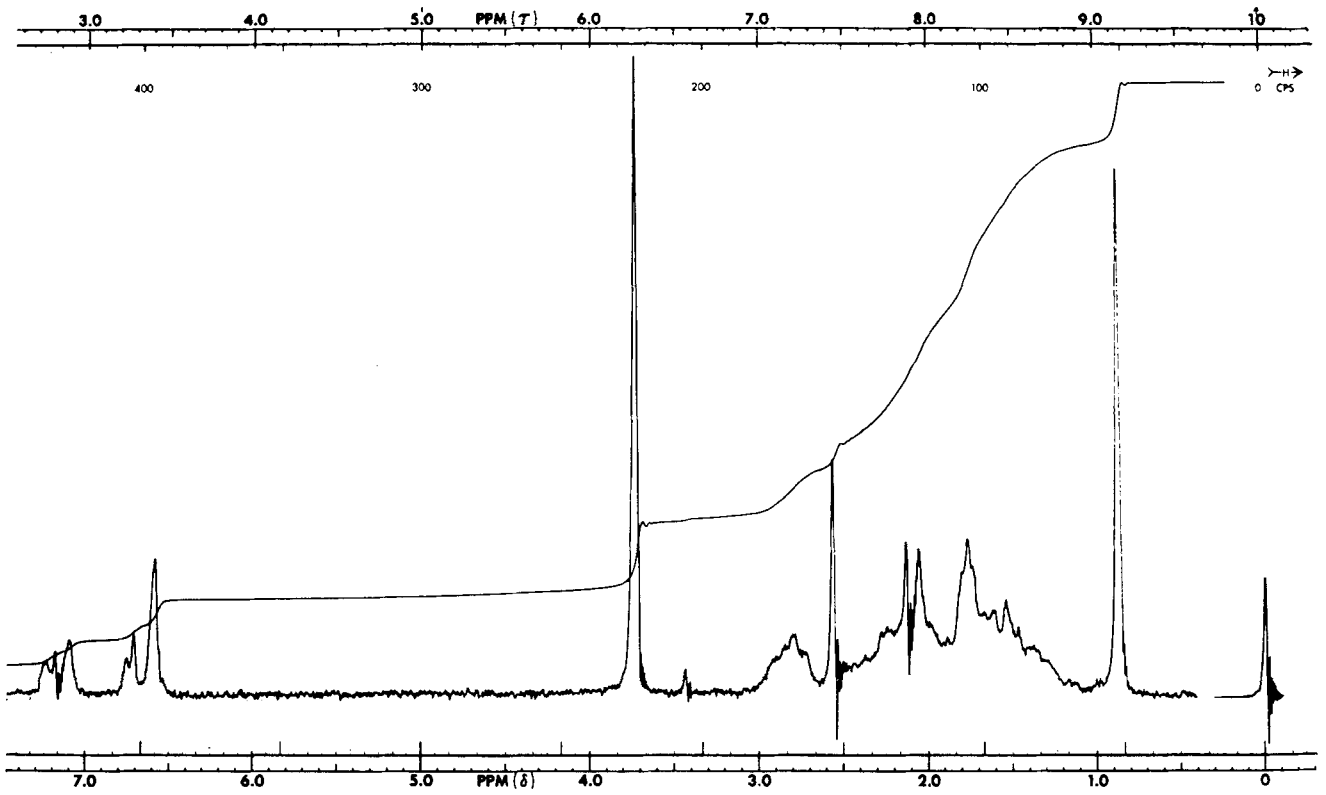

Figure 1—*NMR spectrum of mestranol in deuteriochloroform.*

accurately weighed, was dissolved in 40 ml. of tetrahydrofuran. To the solution was added 10 ml. of 5% silver nitrate solution, and the mixture was titrated with 0.1 *N* sodium hydroxide, the end-point being determined potentiometrically.

TLC was carried out on silica gel GF plates of 0.25-mm. thickness. The developing mixture was benzene–methanol (19:1), and spots were detected by spraying with concentrated sulfuric acid and heating for 20 min.

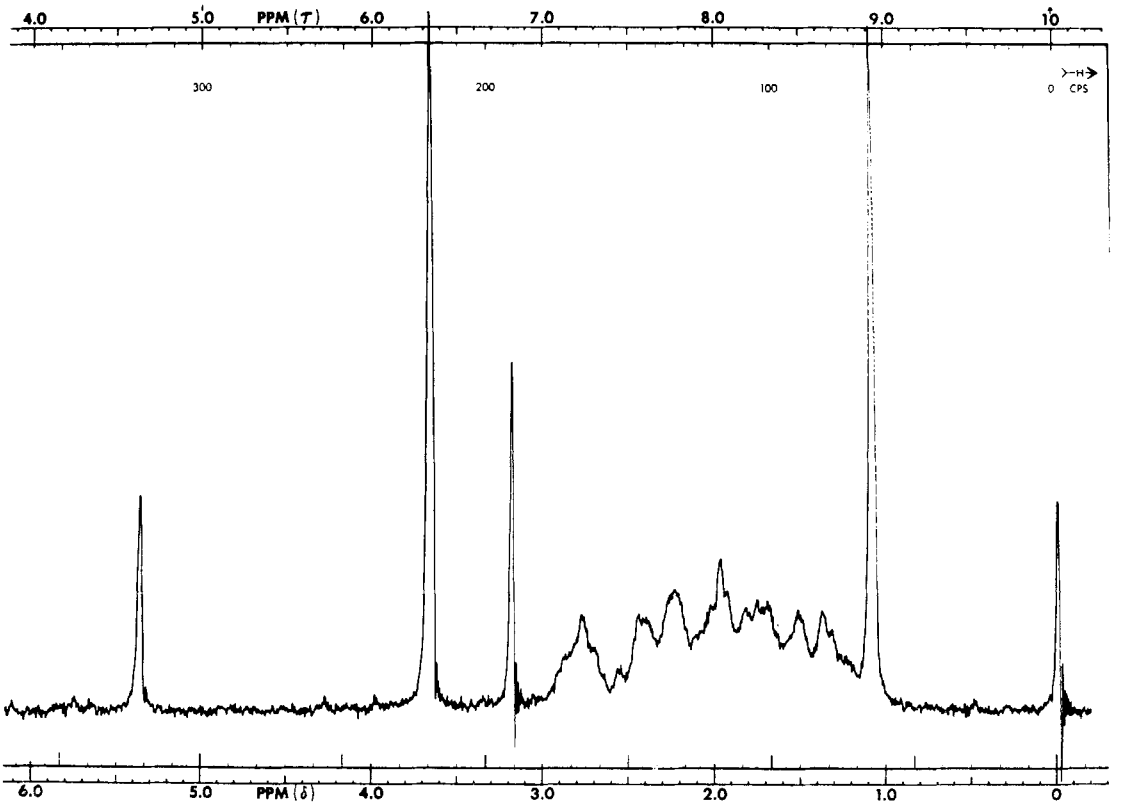

Figure 2—*Partial NMR spectrum of mestranol plus DPAA in pyridine.*

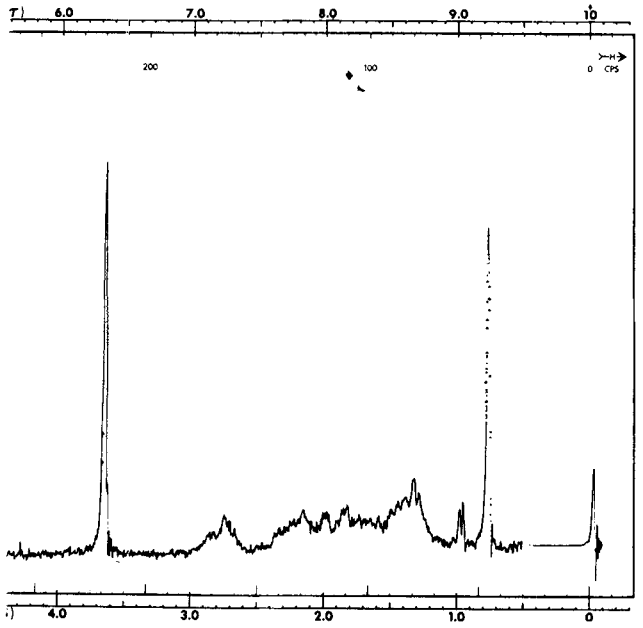

Figure 3—*Partial NMR spectrum of estrone-3-methyl ether in pyridine.*

Calculation—*NMR*—

$$\text{Wt. of mestranol (mg.)} = \frac{\text{E.W. mestranol}}{\text{E.W. DPAA}} \times \frac{\text{I mestranol}}{\text{I DPAA}} \times \text{wt. DPAA (mg.)} \quad \text{(Eq. 1)}$$

where E.W. is the molecular weight of the substance divided by the number of protons corresponding to the signal chosen for the assay, and I is the integral height.

Potentiometric Titration—

$$\text{Wt. of mestranol (mg.)} = 31.04 \times \text{number of ml. 0.1 } N \text{ sodium hydroxide} \quad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

NMR spectroscopy, based on the 60-Mc.p.s. spectrum, has been shown (18) to be of value in the analysis of meprobamate and chemically related substances. Information has been presented (19) demonstrating the use of NMR in the quantitative analysis of synthetic corticosteroids of the 1,4-dien-3-one type.

The NMR spectrum of mestranol in deuteriochloroform (Fig. 1) possesses signals at 6.27 τ (3 protons, due to the 3-methoxy group) and at 7.44 τ (1 proton, due to 17α-ethinyl group). The signal from the methoxyl protons is in an isolated region of the spectrum and is thus available for use in quantitative analysis. The ethinyl proton signal is not suitable, however, since it is surrounded by a large number of other signals. On the other hand, in the NMR spectrum of mestranol in pyridine (Fig. 2), the methoxyl proton signal is very slightly shielded (6.33 τ), but the ethinyl proton signal is significantly deshielded (6.80 τ) and is isolated from all other signals, thus making it suitable for use in quantitative analysis. Therefore, using pyridine as the solvent, two simultaneous independent determinations can be carried out on the same molecule. The signal at 4.62 τ (Fig. 2) is due to the methine proton of the internal standard, DPAA.

Table I—Analysis of Mestranol Bulk Drug by NMR and Potentiometric Titration

	NMR				Potentiometric Titration	
	Percent Found, Based on Ethinyl Protons	Mean	Percent Found, Based on Methoxyl Protons	Mean	Percent Found	Mean
Lot A	99.0	98.9	101.8	101.2	99.6	99.4
	98.8		100.5		99.2	
Lot B	99.6	100.1	101.1	101.0	101.4	101.4
	100.4		101.0		100.6	
	100.3		100.9		102.2	
Lot C	99.9	99.6	100.1	100.3	98.6	98.4
	99.2		100.6		98.4	
	99.7		101.3		98.2	

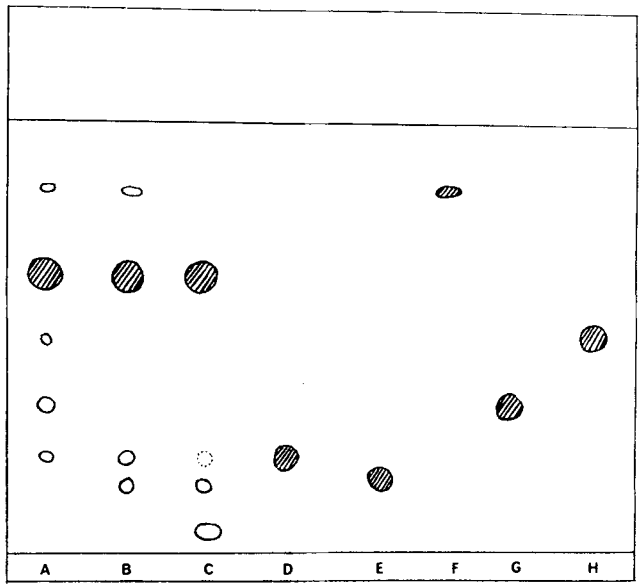

Figure 4—*Schematic TLC chromatogram of three commercial lots of mestranol and likely impurities. Key: A–C = samples, D = 17β-ethinylestradiol, E = estradiol, F = estrone-3-methyl ether, G = estrone, and H = estradiol-3-methyl ether. The adsorbent is silica gel GF, 0.25 mm.; the solvent is benzene–methanol (19:1).*

Mestranol is synthesized from estrone *via* estrone-3-methyl ether (20); both of these are, therefore, likely to occur as impurities. Other possible foreign related steroids in the final raw material are estradiol, estradiol-3-methyl ether, and 17α-ethinylestradiol. Of these, two (estrone-3-methyl ether and estradiol-3-methyl ether) interfere with the signal from the methoxyl protons of mestranol, and one (17α-ethinylestradiol) interferes with the signal from the ethinyl proton. Figure 3 shows a partial NMR spectrum of estrone-3-methyl ether in pyridine, with the signal from the methoxyl protons, at 6.3 τ, being in exactly the same position as the corresponding signal in mestranol (Fig. 2).

Figure 4 shows a schematic representation of the results of a TLC study of the three commercial lots investigated, together with the most likely impurities. Lot A contained significantly more methoxyl than ethinyl impurities, together with some estrone, which would not be detected by either the NMR or the BP potentiometric method (1). Lot B contained rather more methoxyl than ethinyl impurities, together with some estradiol, which again would not be detected. Lot C contained a trace of 17α-ethinylestradiol but no methoxyl impurities; in addition, there was a very faint but diffuse spot, close to the origin, which was not identified.

Results obtained from the analysis of the samples are shown in Table I. The potentiometric titration (1) is essentially an estimation of ethinyl protons, since the alkyne is treated with silver nitrate, liberating nitric acid which is determined by titration with standard alkali. Therefore, the results obtained from the NMR ethinyl proton signal should agree with those obtained by titration. The results from Lot A are in good agreement; the results from Lots B and C differ by 1.3 and 1.2%, respectively. The NMR method gives an average deviation of ±0.6% (19); the accuracy of the titration method was not determined, but if it is no worse than the NMR procedure, the results obtained from the ethinyl protons by the two techniques are not significantly different.

Of immediate interest in the NMR method is a comparison of the pairs of results. For pure mestranol, the results should be identical (within the experimental limits ±0.6%). In the case of Lots B and C, the results are in good agreement. The results obtained from Lot

A, however, show a difference of 2.3%, the methoxyl signal giving the higher answer. This is probably because this sample contained both estrone-3-methyl ether and estradiol-3-methyl ether as impurities in amounts which appeared, from TLC, to be significantly greater than the amount of 17α-ethinylestradiol present. Therefore, the result would be expected to be biased by an increase in the methoxyl signal.

The NMR procedure as described offers an attractive alternative assay for mestranol bulk drug. Simultaneous independent answers, from two different and isolated functional groups in the same molecule, act as a built-in check for the procedure.

REFERENCES

(1) "British Pharmacopoeia," General Medical Council, London, England, 1968, p. 595.

(2) A. P. Shroff and R. E. Huetteman, *J. Pharm. Sci.*, **56,** 654 (1967).

(3) D. C. Tsilifonis and L. Chafetz, *ibid.*, **56,** 625(1967).

(4) R. J. Templeton, W. A. Arnett, and I. M. Jakovljevic, *ibid.*, **57,** 1168(1968).

(5) R. A. Bartow, *J. Pharm. Pharmacol.*, **19,** 41(1967).

(6) A. P. Shroff and J. Grodsky, *J. Pharm. Sci.*, **56,** 460(1967).

(7) E. P. Schulz, *ibid.*, **54,** 144(1965).

(8) J. T. France and B. S. Knox, *J. Gas Chromatogr.*, **4,** 173 (1966).

(9) A. R. Umbreit and J. V. Wisniewski, *Facts Methods*, **5,** 9(1964).

(10) R. Hüttenrauch and I. Keiner, *Pharmazie*, **20,** 242(1965).

(11) J. P. Comer, P. E. Hartsaw, and C. E. Stevenson, *J. Pharm. Sci.*, **57,** 147(1968).

(12) L. J. Cali and A. J. Khoury, *Automat. Anal. Chem., Technicon Symp.*, **1,** 196(1966).

(13) J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," vol. 2, Pergamon Press, Oxford, England, 1966, p. 745.

(14) M. M. Kreevoy, M. B. Charman, and D. R. Vinard, *J. Amer. Chem. Soc.*, **83,** 1978(1961).

(15) E. B. Whipple, J. H. Goldstein, L. Mandell, G. S. Reddy, and G. R. McClure, *ibid.*, **81,** 1321(1959).

(16) R. E. Richards and J. V. Matton, *Trans. Faraday Soc.*, **57,** 28(1961).

(17) J. L. Jungnickel and J. W. Forbes, *Anal. Chem.*, **35,** 939 (1965).

(18) J. W. Turczan and T. C. Kram, *J. Pharm. Sci.*, **56,** 1643 (1967).

(19) H. W. Avdovich and B. A. Lodge, APhA, Montreal meeting, 1969.

(20) F. B. Colton, L. N. Nysted, B. Riegel, and A. L. Raymond, *J. Amer. Chem. Soc.*, **79,** 1123(1957).

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Fluorometric Determination of Reserpine and Related Compounds by Reaction with Vanadium Pentoxide

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Abstract □ A rapid and fairly specific fluorometric procedure has been developed for the routine quantitative determination of reserpine and its derivatives alone and in tablet formulations. The method is based on the formation of fluorescence induced by the oxidation of reserpine with a reagent containing vanadium pentoxide in phosphoric acid. The oxidation product exhibits a greenish-yellow fluorescence, with the maximum around 500 mμ in an acidic alcoholic solution. The dependence of the intensity of fluorescence upon the nature of the solvent, reagent concentration, and other parameters is discussed. The fluorogen developed follows Beer's law over a very wide range, from 0.004 to 2 mcg./ml. of sample solution. The advantages and disadvantages of the proposed method are discussed, and the applicability in different formulations is demonstrated.

Keyphrases □ Reserpine and derivatives in tablets—determination □ Vanadium pentoxide–reserpine reaction—fluorescence □ Phosphoric acid effect—reserpine–vanadium pentoxide fluorogen □ Fluorometry—analysis

Since the isolation of reserpine[1] from the various Rauwolfia roots was completed, its double therapeutic effect as an antihypertensive and a tranquilizer has been recognized. When the usefulness of the reserpine as an effective human medicine was realized, Szalkowski and Mader (1) developed a quantitative method for its determination. This method underwent many modifications (2, 3) which, however, did not alter the methodology of the original procedure significantly.

In recent years, photometric methods (4) have been introduced for the quantitative determination of reserpine. These methods are based on reactions with suitable reagents resulting in the formation of chromophores or fluorogens, which can be measured by colorimetric or fluorometric techniques. Colorimetric measurements (5) are applied mostly for pharmaceutical formulations where the sensitivity of the determination is not critical. Fluorescence determinations are used for reserpine in feeds and biological materials (6), where extremely sensitive and selective methods are required. A direct UV method (7) is frequently used for the determination of reserpine and has the advantage of speed of assay where the concentration of reserpine is high enough for UV absorption. Column chromatographic methods (8) are highly specific and fairly sensitive, but they are time consuming. TLC methods (9) are often used for the separation of the active alkaloids, but these methods are primarily qualitative rather than quantitative.

The development of a specific method for the determination of reserpine is difficult because of its structural similarity to other active alkaloids isolated from the Rauwolfia root. The greenish-yellow color produced

[1] Serpasil, Ciba Pharmaceutical Co.