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NMR Analysis of Synthetic Corticosteroids of the 1,4-Dien-3-one Type

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Abstract □ A procedure for the analysis of synthetic corticosteroids of the 1,4-dien-3-one type is described. The method is based upon NMR spectroscopy. Spectra are determined in dimethyl sulfoxide containing an internal reference substance, fumaric acid. Both bulk drugs and formulations can be assayed using this method, and comparison is made with results obtained from official assays on the steroids and their formulations. The average deviation obtained in the NMR method was 0.6%. A procedure for water-soluble 1,4-dien-3-ones is also described. This method uses triethylamine hydrochloride as an internal standard.

Keyphrases □ Corticosteroids, 1,4-dien-3-one type—analysis □ NMR spectroscopy—analysis □ Fumaric acid—internal standard

Synthetic corticosteroids of the 1,4-dien-3-one type are at present assayed (1, 2) by colorimetric methods based on the reduction of certain tetrazolium derivatives by the α-ketol side chain at C-17. Such methods do not distinguish between 1,4-dien-3-ones and related corticosteroids of the 4-en-3-one type, which may be present as impurities and which frequently possess different corticoid activity to that desired in the 1,4-dien-3-one. NMR spectroscopy affords a method of distinguishing easily between the two groups of steroids. 1,4-Dien-3-ones possess three vinylic protons, of which the chemical shift usually differs from that of the single vinylic proton of 4-en-3-ones. It is, therefore, theoretically possible to detect the two groups of compounds in the presence of each other and, hence, to develop a much more specific assay procedure.

Present methods used in the determination of prednisolone sodium phosphate (PSP) (1, 2) are also relatively nonspecific. The BP method (1) for the bulk drug relies upon dissolution in water and measurement of the extinction of the solution at 247 mμ; for

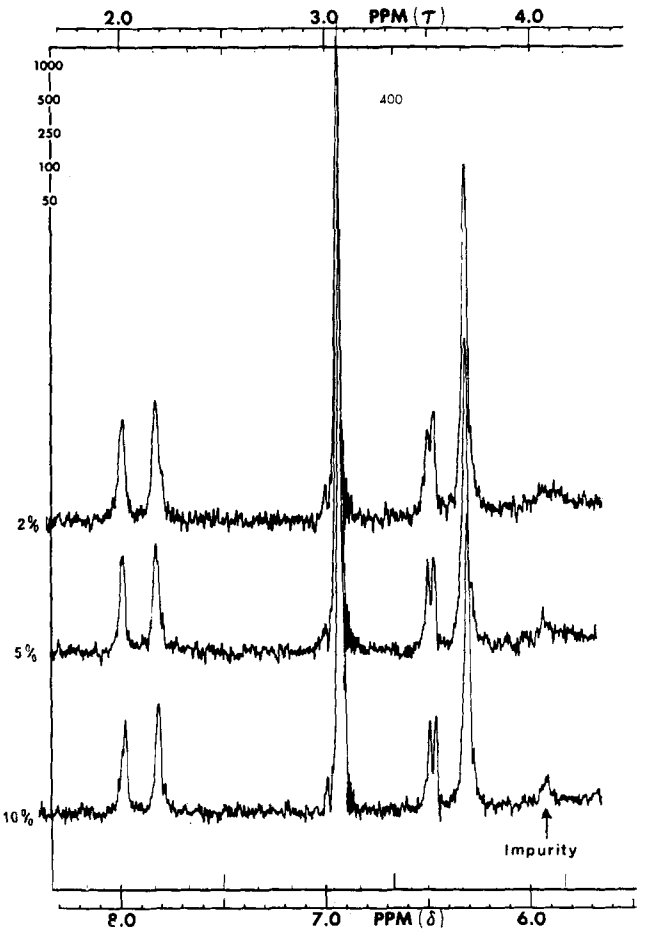

Figure 1—*Partial NMR spectrum of steroidal 1,4-dien-3-one in dimethyl sulfoxide containing fumaric acid and 2, 5, or 10% of added steroidal 4-en-3-one.*

Table I—Analysis of Bulk Water-Insoluble Steroids by NMR

Steroid	Sample No.	Amount Weighed, mg.	Amount Found, mg.	%	Deviation from Mean %
Betamethasone	1	77.9	78.5	100.8	0.5
	2	106.8	107.7	100.8	0.5
Dexamethasone	1	108.0	107.0	99.1	1.2
	2	100.1	100.1	100.0	0.3
Fluocinolone acetonide	1	104.5	105.2	100.7	0.4
Prednisolone	1	82.3	83.3	101.2	0.8
	2	107.3	107.9	100.6	0.3
	3	133.3	134.0	100.5	0.2
	4	140.9	140.0	99.4	0.9
	5	85.9	87.3	101.6	1.3
	6	76.0	77.0	101.3	1.0
	7	75.7	76.3	100.8	0.5
Prednisolone acetate	1	69.4	69.8	100.6	0.3
	2	101.8	102.4	100.6	0.3
	3	95.2	95.3	100.1	0.2
	4	93.2	92.9	99.7	0.6
	5	132.0	133.2	100.9	0.6
	6	96.0	95.8	99.8	0.5
	7	99.3	98.5	99.2	1.1
Prednisone	1	101.0	100.4	99.4	0.9
	2	92.9	93.3	100.4	0.1
	3	89.9	88.8	98.8	1.5
	4	85.8	86.1	100.3	0
	5	106.4	106.0	99.6	0.7
	6	119.4	120.9	101.3	1.0
	7	116.7	117.6	100.8	0.5
Triamcinolone	1	91.7	92.1	100.4	0.1
	2	100.1	99.4	99.3	1.0
	3	104.2	105.1	100.9	0.6
Triamcinolone acetonide	1	83.2	83.0	99.8	0.5

Mean % found = 100.3 SD = 0.7
Average deviation from mean = 0.6%

the formulation, it uses a colorimetric procedure based on a condensation of the steroid with isoniazid. The USP method (2), for both the bulk drug and the formulation, uses alkaline phosphatase to hydrolyze the phosphate ester, followed by extraction of the liberated prednisolone into methylene chloride and measurement of the absorbance of the steroidal solution at 241 mμ.

EXPERIMENTAL

Spectra were obtained at 60 Mc.p.s. using an analytical NMR spectrometer (Varian A-60A). 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 milligauss (nominal dial setting) was found to give the maximum integral amplitude (3) and was used for the integrations. Tetramethylsilane in chloroform was used as an external reference to measure chemical shifts.

Procedure (Water-Insoluble Compounds)—*Bulk Drug*—Approximately 100 mg. of steroid was accurately weighed and dissolved in 1 ml. of dimethyl sulfoxide containing fumaric acid (25.40 mg./ml.). The NMR spectrum was integrated 5 times in each direction through the region shown (Fig. 1).

Dosage Forms—Tablets—Twenty 5-mg. or ten 8-mg. tablets were powdered and refluxed for 15 min. with 50 ml. of 95% ethanol. The mixture was filtered, the residue and filter pad were washed with 50 ml. of 95% ethanol, and the combined filtrate was evaporated to dryness by flash evaporation. The residue was dissolved in 1 ml. of dimethyl sulfoxide containing fumaric acid (25.40 mg./ml.). The NMR spectrum was integrated as for the bulk drug.

Injections—Four milliliters (25 mg./ml.) or 2 ml. (40 mg./ml.) of aqueous suspension was carefully measured into a 50-ml. round-bottom flask and evaporated to dryness. One milliliter of dimethyl sulfoxide containing fumaric acid (25.40 mg./ml.) was added to the residue, and the whole was carefully mixed to effect dissolution of the steroid in the solvent. Some of the residue (preservative, suspending agents) was not soluble in the dimethyl sulfoxide and remained adhered to the wall of the flask. A clear solution was obtained, and the NMR spectrum was determined as for the bulk drug.

Procedure (Water-Soluble Compounds)—*Bulk Drug*—Approximately 100 mg. of PSP, previously dried *in vacuo* at 105°, was accurately weighed in a glass-stoppered flask, to which was added about 10 mg. of pure triethylamine hydrochloride, also accurately weighed. The mixture was dissolved in the minimum amount of D_2O (0.5–1.0 ml.). The NMR spectrum was obtained in the usual way (Fig. 2), the signals of interest being integrated 5 times in each direction. The signals at 2.42τ (for PSP) and 6.78τ (for triethylamine hydrochloride) were used as a basis for the assay.

Formulation—Four milliliters of injection of PSP was accurately measured into a 50-ml. round-bottom flask, and the solvent was removed by flash evaporation. Approximately 10 mg. of triethylamine hydrochloride (accurately weighed) was added to the flask, and the mixture was dissolved in the minimum amount of D_2O. The NMR spectrum was obtained and integrated as for the bulk drug.

Calculations—*Bulk Drug*—

amount of steroid (mg.) =

$$\frac{\text{E.W. (steroid)}}{\text{E.W. (standard)}} \times \frac{\text{I (steroid)}}{\text{I (standard)}} \times \text{weight of standard} \quad \text{(Eq. 1)}$$

where E.W. is the molecular weight divided by the number of protons in the signal chosen and I is the integral height.

Tablets—As for the bulk drug, then:

$$\text{amount of steroid/tablet} = \frac{\text{total weight (steroid)}}{\text{no. of tablets in sample}} \quad \text{(Eq. 2)}$$

Table II—Recovery of Steroids from Excipient Mixtures

Mixture	Steroid	Amount Added, mg.	Amount Found, mg.	%
1	Prednisone	97.6	96.3	98.7
2	Prednisone	137.3	135.9	98.9
3	Prednisone	137.0	135.0	98.6
4	Triamcinolone	124.4	125.2	100.6
5	Triamcinolone	106.3	106.9	100.6

Injections—As for bulk drug, then:

$$\text{amount of steroid (mg./ml.)} = \frac{\text{total weight (steroid)}}{\text{no. of ml. in sample}} \quad \text{(Eq. 3)}$$

RESULTS AND DISCUSSION

The NMR method, based on the 60 Mc.p.s. spectrum and using an internal standard, has been shown (4) to be suitable as an assay procedure for meprobamate and chemically related substances. The spectrum of a steroidal 1,4-dien-3-one is rather complicated, however, and an internal standard must be chosen with great care, since there are few spaces in the spectrum into which a reference signal might fit. To be of use as a standard, a substance should be an easily purified solid, freely soluble in the solvent of choice. It should not react with the solvent or the substrate and should possess a recognizable, isolated signal in a suitable region of the spectrum. Fumaric acid was chosen as an internal reference standard for use with water-insoluble 1,4-dien-3-ones, because its NMR spectrum in dimethyl sulfoxide shows a single sharp signal at 3.1τ, which fits well into the spectrum of the steroids. Figure 1 shows the part of the spectrum utilized in the analysis; the signals occur at approximately 2.1τ (doublet, C-1 proton), 3.6τ (doublet, C-2 proton), and 3.7τ (singlet, C-4 proton).

Also shown in Fig. 1 is the position of the signal from the vinylic proton in a steroidal 4-en-3-one (4.1τ). Partial spectra were obtained from prepared mixtures containing 2, 5, and 10% of 4-en-3-one in 1,4-dien-3-one to show the intensity of the signal from the impurity. This signal can be seen to be well removed from the primary assay signal (2.1τ) and the standard signal (3.1τ).

The results obtained from the analysis of eight reference steroids are shown in Table I. In two cases, there was very little material available, and only one analysis could be carried out on each. Nevertheless, the results obtained from fluocinolone acetonide and triamcinolone acetonide are both within the average deviation from the mean, which for 30 samples is 0.6%. Eleven of the 30 results are outside the average deviation, which is the equivalent of one standard deviation. This does not appear to be important for practical purposes, however, since the overall range of results is 98.8–101.6%. The NMR method thus appears to be suitable for the assay of bulk steroids of the 1,4-dien-3-one type.

Table II shows that recovery of prednisone and triamcinolone from mixtures with lactose and starch is reproducible and almost complete. The extraction procedure thus appears to be suitable for use in the determination of steroids in dosage forms.

Table III shows the results obtained from some commercial dosage forms of the steroids under consideration. The NMR procedure for tablets is simpler than the colorimetric method because no weighing of the tablets is involved. Recovery of the steroid from the powdered tablets in the colorimetric method involves several extractions of an aqueous suspension of the powder with chloroform, followed by dilution of the chloroform solution. An aliquot is taken, and the chloroform is removed by evaporation on a steam bath; then the residue is dissolved in ethanol. The NMR procedure consists of refluxing with 95% ethanol, filtration, and removal of the solvent by flash evaporation. The residue is dissolved in 1 ml. of the stock solution of fumaric acid in dimethyl sulfoxide. The stock solution was stored in a cool, dark place for several months without deterioration. One problem frequently encountered in extracting drugs from powdered tablets into chloroform is that of emulsion formation. Such emulsions are sometimes very stable, and separation of all the chloroform solution is difficult. This problem does not enter into the NMR procedure at all.

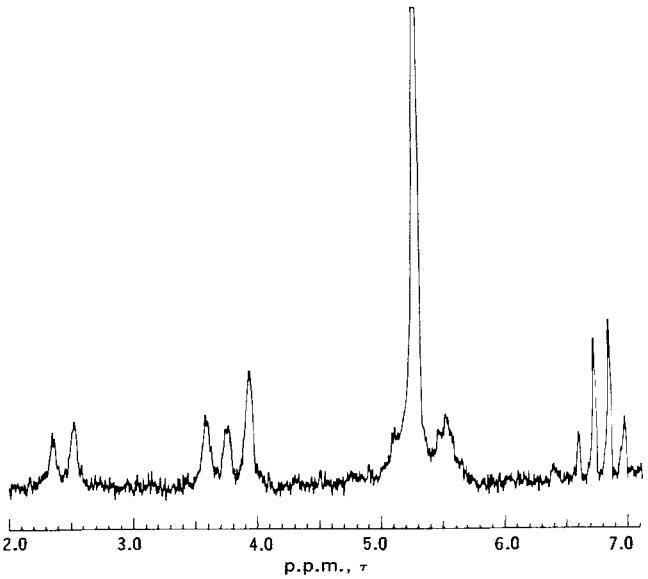

Figure 2—*Partial NMR spectrum of PSP + triethylamine hydrochloride in D_2O.*

The result obtained from Brand C, a prednisone tablet, requires some comment.The colorimetric method was carried out 8 times on this product, using a variety of water–chloroform ratios in the extraction. However, the results showed only 90% of the labeled amount, whereas the NMR procedure showed 98%. The reason for this low result may be that some very bad emulsions were obtained, even when a large excess of chloroform over water was used. The pairs of results obtained from the other five types of tablets compare very well.

Reproducibility of sampling was the chief problem in assaying the injectable preparations, which were both aqueous suspensions; however, the results are all well within the official limits (1, 2). No emulsion problems were encountered with these suspensions, in spite of the presence of suspending agents in the formulations. In the NMR procedure, not all of the dried formulation was soluble in dimethyl sulfoxide, but this apparently did not present any

Table III—Analysis of Dosage Form by NMR and Colorimetric Methods

Sample	Steroid	Labeled Amount, mg.	Found, mg.[a] NMR	Found, mg.[a] Colorimetric
Brand A, tablet	Prednisone	5	5.1	5.0
Brand B, tablet	Prednisone	5	4.9	4.9
Brand C, tablet	Prednisone	5	4.9	4.5[b]
Brand D, tablet	Prednisolone	5	5.1	5.0
Brand E, tablet	Prednisolone	5	4.8	4.9
Brand F, tablet	Triamcinolone	8	8.2	8.2
Brand G, injection	Prednisolone acetate	25/ml.	26.3/ml.	24.9/ml.
Brand H, injection	Triamcinolone acetonide	40/ml.	39.8/ml.	41.5/ml.

[a] Each result is the average of two determinations. [b] This result is the average of eight determinations.

Table IV—Determination of PSP (Bulk Drug) by NMR

Determination No.	Weight $Et_3N \cdot HCl$, mg.	Weight PSP, mg. Added	Found	%
1	10.17	106.2	106.0	99.8
2	12.80	140.8	141.6	100.6
3	13.20	90.6	91.1	100.6
4	9.72	104.1	104.6	100.5
5	10.53	150.3	148.5	98.8
6	9.01	120.2	121.9	101.4
7	11.99	101.5	100.2	98.7
8	12.62	119.2	119.9	100.6
	Mean % = 100.1		SD = 0.95	

problems of recovery based on a comparison of the two pairs of results. It was found, for the formulations studied, that the excipients did not interfere with the NMR method; hence a minimum of cleanup is required.

The NMR procedure, as described, is specific for steroidal 1,4-dien-3-one in the presence of related 4-en-3-ones. This cannot be said of the colorimetric method, which works equally well with other steroids containing the α-ketol side chain. Indeed, it has been shown in this laboratory that such common pharmaceutical aids as ascorbic acid and lactose also produce the color under the reaction conditions used. Since it has been shown (5) that samples of corticosteroids of the 1,4-dien-3-one type sometimes contain significant amounts of related 4-en-3-one precursors, a more specific assay procedure is perhaps desirable.

PSP is a water-soluble corticosteroid and, therefore, the solvent of choice for NMR is D_2O. The almost inevitable presence of a strong signal of 5.3τ (H_2O/HOD) with its attendant spinning side bands presents a considerable problem, since it reduces the space available in which to put a reference signal. Triethylamine hydrochloride was found to be a suitable internal standard, fulfilling all the conditions described for such substances.

The 60-Mc.p.s. spectrum of PSP in D_2O (Fig. 2) shows a broad doublet at 2.42τ (J = 10 c.p.s.), being the signal from the vinylic proton at C-1 in PSP. This signal was compared with that produced by the six equivalent methylene protons of triethylamine hydrochloride at 6.78τ (quartet, J = 7.5 c.p.s.).

In addition to the steroid, the commercial sample of PSP injection contains nicotinamide, phenol, sodium hydroxide, sodium bisulfite, and disodium edetate. Some interference is caused with the signal at 2.42τ (C-1 proton), making accurate integration impossible. However, the signals at 3.66τ (doublet, J = 10 c.p.s., C-2 proton) and 3.93τ (singlet, C-4 proton) are unaffected. The total integral for the two protons was used in the determination, with the necessary correction being made to the calculation.

Results obtained from the analysis of eight samples of PSP are shown in Table IV.

A sample of injection of PSP labeled to contain the equivalent of 20 mg. of prednisolone/ml. was analyzed twice by the NMR method and each time found to contain 18.8 mg./ml., and once by the USP method (2), giving 19.1 mg./ml.

For the injectable sample, the NMR method requires much less manipulation than the official UV method (2) and is also relatively more specific. Comparison of the results obtained for analysis of the commercial preparation showed the two methods to be in good agreement.

CONCLUSION

Synthetic corticosteroids of the 1,4-dien-3-one type can be assayed satisfactorily by means of NMR spectroscopy, even in the presence of related 4-en-3-one precursors. Because of the sensitivity of present NMR spectrometers, the method is chiefly of value for raw material analysis; it is, however, easily adapted for composite tablet assays and for the assay of injectable preparations.

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