AN ENZYMATIC METHOD FOR THE DETERMINATION OF PREDNISOLONE PHOSPHATE IN PHARMACEUTICAL PREPARATIONS

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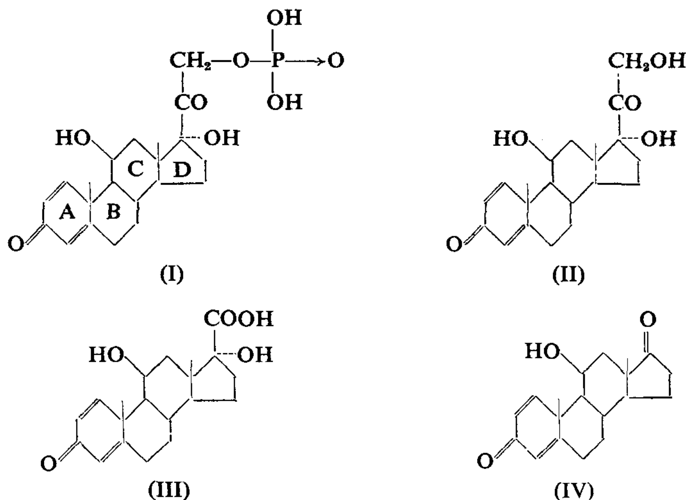
A method proposed for determining prednisolone phosphate in pharmaceutical preparations is based on enzymatic hydrolysis and ultra-violet spectroscopy; it is applicable to preparations that have undergone partial decomposition from prolonged or unsatisfactory storage.

The introduction of the 21-monophosphoric ester of prednisolone (I) in various pharmaceutical formulations has necessitated the development of a generally applicable analytical procedure, capable of distinguishing between the steroid ester and its decomposition products. Decomposition of prednisolone phosphate may occur by either of two mechanisms. The conjugated system in ring A is photolabile[1], undergoing drastic modifications of rings A and B; this change is accompanied by a reduction in ultra-violet absorption. The 21-phosphate ester linkage is susceptible to hydrolysis, liberating the relatively insoluble prednisolone (II), which further is degraded to the 17-aetio-acid (III) and 17-ketone (IV)[2–4]. Preparations undergoing accelerated storage tests may therefore contain (II), (III) and (IV), in addition to products of the photochemical reaction.



No specific analytical methods for phosphoric esters appear to have been reported. A recent colorimetric procedure for the determination of steroid esters[5], based on hydroxamic acid formation, is not applicable to phosphoric esters. Other methods for the steroid alcohols depend on either the dihydroxyacetone side chain or the conjugated system in ring A,

both of which are closely associated with physiological activity. Methods based on the side chain include a number of widely used colour reactions, such as reduction of tetrazolium salts[6], reaction with a phenylhydrazine reagent[7] (the Porter-Silber reaction) or determination of the formaldehyde[8] produced by periodate oxidation, none of them directly applicable to the esterified alcohol. The second group of methods, based on the structure of ring A, includes ultra-violet absorption procedures or requires colour formation with sulphuric acid; these methods are applicable to simple aqueous solutions of the steroid ester but, without modification, frequently fail with the more complex preparations, especially if partial decomposition has taken place.

Ideally an analytical procedure should incorporate a measure of both functional groups and give no response to decomposition products. In one possible approach, prednisolone and its degradation products are extracted from the aqueous sample by a suitable immiscible solvent and the residual water-soluble steroid ester is then determined by ultra-violet spectroscopy. This procedure requires a fore-knowledge of all degradation products and is limited in application to formulations containing no ultra-violet absorbing components that are not extracted by the selected solvent. A more promising approach involves measurement of either the steroid alcohol or the inorganic phosphate produced by deliberate hydrolysis. Determination of the steroid alcohol by ultra-violet spectroscopy after isolation by solvent extraction would be particularly useful, since the complete determination would then depend on both physiologically functional groups.

Hydrolysis of prednisolone phosphate by chemical means necessitates severe treatment and causes much degradation of the liberated alcohol. Consideration was therefore given to the possibility of hydrolysing under mild conditions using a suitable enzyme, of which several are commercially available. An enzymatic method has the particular advantage that interfering substances can, if necessary, be removed by extraction before hydrolysis, under the same conditions as are subsequently used for extraction of the hydrolytic product. High specificity is thus assured. However, since enzymatically catalysed reactions are reversible, they seldom reach completion unless one of the hydrolytic products is removed continuously; further, enzyme poisons must be absent, or at least present in quantities insufficient significantly to alter the reaction rate. These and other matters bearing on the hydrolysis of prednisolone phosphate were therefore studied more closely.

Experimental

An "alkaline intestinal phosphatase" and a "wheat-germ acid phosphatase" were obtained from a commercial source. Hydrolyses were carried out by incubating mixtures of standard prednisolone phosphate solution, buffer and enzyme solution for different times. The extent of hydrolysis was determined after isolating the liberated alcohol by extraction with ether or chloroform, evaporation to dryness, solution of the residue in ethanol and ultra-violet spectroscopy. The ultra-violet

absorption of prednisolone disodium phosphate in water shows a maximum at 247 mμ, E(1 per cent, 1 cm.) 312.

Variables likely to influence hydrolysis are pH, temperature and the substrate: enzyme concentration ratio. Preliminary experiments showed the wheat-germ phosphatase to have a much slower reaction rate and a less favourable equilibrium position than the other enzyme. It was therefore discarded, and the alkaline phosphatase was used for all subsequent experiments. This had diminished activity below pH 8·0; above pH 9·0 loss of liberated alcohol by degradation of the dihydroxyacetone structure became significant. Moreover, the reaction was retarded by ionisable phosphate, which may be present in the sample as buffer. This interference was eliminated by precipitation of phosphate with calcium chloride, but to obtain adequate removal of phosphate a pH of not less than 8·6 was required. The hydrolysis rate did not vary significantly over the temperature range 25° to 50°. The speed of reaction and its completion were facilitated by high enzyme or low substrate concentrations or both together. The liberated alcohol may be extracted by ether or chloroform, the latter being preferred because of its greater efficiency. Prolonged or unsatisfactory storage may lead to the formation of the steroid alcohol and its decomposition products which may be removed by a preliminary solvent extraction.

METHOD

Reagents

Glycine buffer. Dissolve 7·505 g. of glycine and 5·85 g. of sodium chloride in sufficient water to produce 500 ml. Adjust to pH 8·6 with 0·2N sodium hydroxide. *Calcium-magnesium solution.* Dissolve 1 g. of calcium chloride and 1 g. magnesium chloride in sufficient glycine buffer to produce 20 ml. *Purified alkaline phosphatase solution.* Dissolve sufficient alkaline phosphatase in 20 ml. water to produce an enzyme concentration of 1,000 units per ml. Shake gently for 1 minute with an equal volume of chloroform. After separation discard the chloroform.

Procedure

Transfer an aliquot of the sample containing 2·5 mg. of prednisolone phosphate to a 250 ml. glass-stoppered separator, dilute to 15 ml. with water, add 25 ml. of chloroform, stopper, shake for 1 minute, and set aside for 2 minutes. Transfer the lower chloroform layer to a second separator, and wash it with 2 ml. of water. Reject the chloroform, and transfer the washings to the first separator. Repeat the extraction and washings with a second 25 ml. portion of chloroform. Transfer the aqueous phase and washings to a 25 ml. volumetric flask, dilute to volume with water, and mix. Transfer a 5.0 ml. aliquot to a glass-stoppered boiling tube. Add 5 ml. of *glycine buffer*, 1 ml. of *calcium-magnesium solution*, 1 ml. of *purified alkaline phosphatase solution* and mix by swirling. Stopper the tube, bring to 37° by immersion in warm water, and then maintain at 37° for 4 hours. Cool to room temperature. Transfer the contents of the tube to a 250 ml. stoppered separator with the aid of two

2 ml. portions of water. Add 25 ml. of chloroform to the separator by the tube. Stopper, shake for 1 minute, and set aside for 2 minutes. Transfer the lower chloroform layer to a second separator, and extract the aqueous phase with two further 25 ml. portions of chloroform. Combine the chloroform extracts, wash with 2 ml. of water, and transfer quantitatively to a 250 ml. glass-stoppered flask. Distil to near dryness on a steam bath, removing the last 1 or 2 ml. of chloroform in a current of air. Cool to room temperature. Add 25·0 ml. of ethanol, stopper, and swirl to dissolve the residue.

Determine the extinction of this solution against a blank of ethanol in 1 cm. cells at λ max near 242 mμ. Calculate the prednisolone phosphate

TABLE I

REPLICATE DETERMINATIONS

Determination	Prednisolone phosphate per cent w/v	
No.	Lotion	Injection
1	0·267	2·05
2	0·272	2·08
3	0·275	2·02
4	0·278	2·02
Mean	0·273	2·04
Range	0·267–0·278	2·02–2·08

content of the sample, taking the E(1 per cent, 1 cm.) value of prednisolone alcohol as 415 and 1·344 as the conversion factor for alcohol to ester disodium salt.

RESULTS

Recovery experiments were made on aqueous solutions of a recrystallised specimen of the steroid phosphate disodium salt. This derivative is hygroscopic hence solutions of approximately known strength were prepared and their equivalent steroid alcohol contents determined from the ultra-violet absorption of direct aqueous dilutions. The alcohol recovered after hydrolysis by the proposed method was calculated as a percentage of the figure obtained by direct dilution. The mean of 10 such determinations was 97·9 per cent and the standard deviation 1·0 per cent. The precision attainable is shown (Table I) by replicate determinations on a production lotion and injection expected to contain 0·27 and 2·0 per cent w/v of active agent respectively.

The proposed method has been applied to preparations for which the direct solvent-extraction method fails. The prednisolone phosphate content of fresh preparations containing *p*-hydroxybenzoic acid esters as preservative may be determined by the rapid solvent-extraction method. However, this procedure is rendered inapplicable by decomposition of the preservative on prolonged storage, resulting in formation of a non-extractable ultra-violet absorbing substance. The proposed enzymatic procedure, by virtue of its "before and after" extractions, achieves a

complete separation of steroid from both the preservative and its decomposition products.

Experimental preparations containing monothioglycerol gave unexpectedly low results. Initially this was thought to be due to enzyme poisoning by the thiol group; however, a four-fold increase in enzyme concentration produced the same result, and subsequent work indicated chemical interaction with the prednisolone phosphate. The proposed method failed on application to an experimental injection product containing nicotinamide, which is only slowly extracted from aqueous solutions by the usual solvents. The slow rate of extraction made it impracticable to attempt a complete preliminary extraction, but the amounts extracted after hydrolysis were sufficient to invalidate the ultra-violet procedure. This difficulty was overcome by applying the tetrazolium reduction colour reaction to the

TABLE II

RESULTS OF ANALYTICALLY PREPARED SAMPLES

Preparation	Prepared strength per cent	Strength found per cent
1. Enema containing *p*-hydroxybenzoates	0·033 w/v	*0·033 w/v
2. Suppository	0·50 w/w	0·49 w/w
3. Lotion containing neomycin and nicotinamide ..	0·26 w/w	**0·25 w/w
4. Injection containing nicotinamide	3·3 w/v	**3·3 w/v
5. Injection containing phenol and sodium formaldehyde sulphoxylate	2·9 w/v	2·9 w/v

* In this determination the initial extraction was acidified.
** In these determinations final measurement was made by the tetrazolium reduction colour reaction.

extracted alcohol. Experimental results are summarised in Table II. No enzyme poisons have been encountered, the method has been successfully applied to preparations containing phenol, *p*-hydroxybenzoic esters, phenylethanol, merthiolate and neomycin. Determinations giving unexpectedly low results, however, should be repeated with an increased enzyme concentration as a check against inhibition. The proposed method is also applicable to the 21-monophosphoric esters of hydrocortisone and dexamethasone.

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After Mr. Boon presented the paper there was a DISCUSSION.