THE ASSAY AND IDENTIFICATION OF PYRIMETHAMINE AND ITS PREPARATIONS

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A gravimetric method of assay for pyrimethamine, using phosphotungstic acid as precipitant, is described. Assay procedures are given for three preparations of the drug, viz. Tablets of pyrimethamine, Tablets of pyrimethamine and quinine, and Powder of pyrimethamine and sulphaguanidine. Tests of identity for pyrimethamine, additional to those described in the British Pharmaceutical Codex, are also given.

Pyrimethamine (2:4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine) is one of a series of substituted 2:4-diaminopyrimidines synthesised by Hitchings and his co-workers in the course of a study of folic acid antagonists[1–4].

The compound is active against a number of micro-organisms; in particular it is a powerful inhibitor of *Plasmodium falciparum* and of *Eimeria tenella*, and is thus both an antimalarial and a coccidiostatic agent.

Pyrimethamine is the subject of a monograph in the British Pharmaceutical Codex 1954. Goodwin[5] and Schmidt, Hughes and Schmidt[6] estimated trace amounts of the drug in biological materials by a colorimetric method depending on complex formation with a suitable dye. A search of the literature failed to elicit any other description of the analytical chemistry of pyrimethamine.

The present investigation arose from a need for a method of assay for certain preparations of the drug; at the same time the opportunity was taken of developing a gravimetric method of assay and of supplementing the tests of identity given in the British Pharmaceutical Codex.

Purity of Reagents

All reagents were of "Analar" or B.P. grade, or other suitable standard of purity. Two samples of pyrimethamine B.P.C. were used for the determination of the gravimetric factor and the specific extinction coefficients (1, loss on drying to constant weight at 105° nil; 2, recrystallised from ethanol and dried to constant weight at 60°). Both gave identical results.

Gravimetric Assay of Pyrimethamine

Preliminary Experiments. As in earlier work with polymyxin[7], preliminary experiments were made to find the most suitable reagent for the gravimetric assay. Of the precipitants examined, phosphotungstic acid gave the best results. In particular it was found that (i) the complex formed is virtually insoluble in dilute acid medium, (ii) the complex is readily obtained in a form suitable for filtering and washing free from excess precipitant; and (iii) the results are highly reproducible and are independent of the batch of precipitant used.

To establish optimum conditions of precipitation, washing and drying of the phosphotungstate complex, the effect of systematic changes in

the following variables was studied: (i) acidity of precipitation medium; (ii) optimum quantity of precipitant; (iii) volume of liquid to be filtered; (iv) conditions of heating before addition of precipitant; (v) acidity of wash-liquid; (vi) volume of wash-liquid required; (vii) optimum drying conditions for the phosphotungstate complex.

The solubility of pyrimethamine phosphotungstate in water at 20° is 0·0005 per cent, whilst in acid medium it is virtually nil; the complex is sparingly soluble in cold ethanol and readily soluble in methanol, acetone and hot ethanol. The complex is readily obtained in the anhydrous state, being unaffected by heating for prolonged periods at 100° or 110°.

As a result of these experiments, the following procedure was adopted.

Assay Process. Weigh out accurately about 50 mg. of pyrimethamine and dissolve in 80 ml. of 5 per cent w/v sulphuric acid. Add 40 ml. of water, heat to boiling and add slowly, with constant stirring, 8 ml. of a 5 per cent w/v aqueous solution of Analar phosphotungstic acid, previously filtered through a Whatman No. 5 paper. Heat gently for a further two minutes, stirring constantly, then allow to stand for one hour and cool to room temperature. Filter through a tared No. 4 sintered glass crucible and transfer the precipitate completely on to the filter with three 20 ml. portions of 2 per cent w/v sulphuric acid. Finally wash the residue on the filter with three 20 ml. portions of water. Dry at 50° for two hours, or over phosphorus pentoxide *in vacuo* for not less than four hours, then heat at atmospheric pressure at 110° for 1½ hours, cool and weigh.

Each g. of residue is equivalent to 0·2040 g. of $C_{12}H_{13}N_4Cl$.

In 22 determinations using three samples of Analar phosphotungstic acid (loss on drying to constant weight at 110° = 6·00, 7·79 and 10·78 per cent, respectively), the standard error of the mean ($100s/\bar{x}\sqrt{n}$) was found to be 0·094 per cent. Results of comparative assays by the B.P.C. method and the gravimetric method are given in Table I.

TABLE I

ASSAY OF PYRIMETHAMINE BY GRAVIMETRIC AND B.P.C. METHODS

	Per cent $C_{12}H_{13}N_4Cl$ recovered	
	Gravimetric method	B.P.C. method
Sample A, determination 1 ..	100·5	99·3
" " " 2 ..	99·97	99·2
" " " 3 ..	99·77	98·7
" " " 4 ..	100·2	99·0
Sample B, determination 1 ..	99·86	98·6
" " " 2 ..	100·0	99·2
" " " 3 ..	100·2	98·9
" " " 4 ..	100·2	98·4

ASSAY OF PREPARATIONS OF PYRIMETHAMINE

Tablets of Pyrimethamine

Assay Process. Weigh and powder 20 tablets. Weigh out accurately a quantity of powder, equivalent to about 50 mg. of pyrimethamine and add 40 ml. of 5 per cent w/v sulphuric acid. Heat to approximately

50° and maintain at this temperature for 30 minutes, stirring intermittently. Cool, filter through a Whatman No. 2 paper and wash the filter with two 20 ml. portions of 5 per cent w/v sulphuric acid. Complete the determination as described under the assay for pyrimethamine, commencing with the words ". . . add 40 ml. of water, heat to boiling and . . ."

Results of assays of two production batches of tablets of pyrimethamine, each tablet containing 25 mg. of pyrimethamine and 100 mg. of a mixture of inert (i.e. non-nitrogeneous) tablet bases, are given in Table II.

TABLE II

ASSAY OF TABLETS OF PYRIMETHAMINE (25 MG.)

Tablet sample No.	Sample of phosphotungstic acid used	Weight of pyrimethamine found per average tablet (mg.)	Per cent of stated strength
Batch I, container 1 ..	A	25·08	100·3
" " " " ..	"	25·28	101·1
" " " 2 ..	"	24·92	99·7
" " " " ..	B	25·13	100·5
Batch II, " 1 ..	"	25·22	100·9
" " " " ..	"	25·27	101·1
" " " 2 ..	"	25·34	101·4
" " " " ..	A	25·41	101·6

Tablets of Pyrimethamine and Quinine

The tablets for which a method of assay was required contained: pyrimethamine 5 mg.; quinine dihydrochloride B.P. 300 mg.; tablet bases 8 mg. The tablets were available both in a sugar-coated and an uncoated form.

Preliminary Experiments. The proposed method of assay is based on the observation that in 0·1 N hydrochloric acid solution the wavelength of maximum absorption for pyrimethamine coincides closely with the wavelength of minimum absorption for quinine dihydrochloride, whilst the latter compound has a characteristic absorption band at 347 mμ, at which wavelength pyrimethamine is virtually transparent. The absorption curves are shown in Figure 1.

The extinction coefficients with reference to the anhydrous compounds are as follows:—

	270·5 mμ	347 mμ
Pyrimethamine B.P.C.	317	0·06
Quinine Dihydrochloride B.P.	17·1	134·6
equivalent to Quinine	20·95	164·8

The values obtained for the specific extinction coefficient of quinine alkaloid at 270·5 mμ and 347 mμ (minimum and maximum, respectively), may be compared with the corresponding values given in the literature[8]: Quinine 270 mμ, 21·12; 347 mμ, 164·1.

The extraction of quinine dihydrochloride from the tablets with 0·1N hydrochloric acid presents no difficulty. On the other hand the extraction of pyrimethamine with this solvent is a slow process in the cold, since the solubility at 20° is only 0·10 per cent. By heating to about 50°, solution is greatly facilitated and it was found that so heating the tablets afforded a

convenient means of extraction. Prolonged heating at 55° had a negligible effect on the ultra-violet absorption characteristics of standard solutions of both drugs in 0·1N hydrochloric acid.

Solutions of pyrimethamine in 0·1N hydrochloric acid obey Beer's law over the range of concentration investigated (0 to 1·5 mg./100 ml. for pyrimethamine) so do solutions of quinine in 0·1N hydrochloric acid[8–10]. To test the validity of the assumption that in a solution of pyrimethamine and of quinine dihydrochloride in 0·1N hydrochloric acid the contributions to the total absorption made by the respective components are additive, a number of solutions containing known amounts of pyrimethamine and of quinine dihydrochloride in the ratio of about 1:60 were prepared. These solutions were suitably diluted and their optical densities at 270·5 mμ and 347 mμ determined. Results of two determinations are given in Table III.

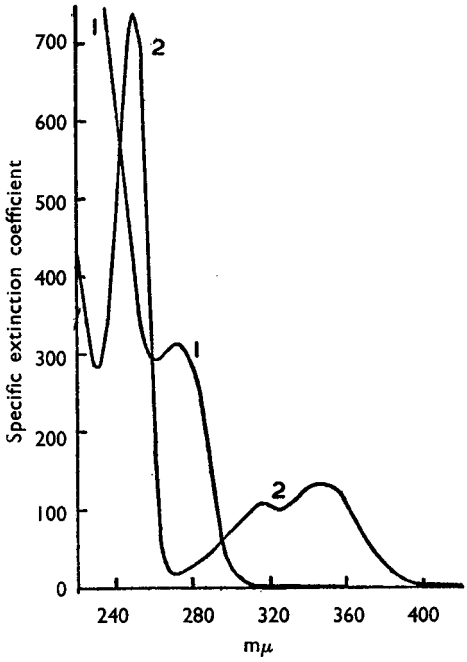

FIG. 1. Ultra-violet absorption spectra of pyrimethamine and of quinine dihydrochloride in 0·1N hydrochloric acid.

1. Pyrimethamine B.P.C.
2. Quinine dihydrochloride B.P.

Tablet bases and the coating material (sugar) had no effect on the spectrophotometric determinations; thus the specific extinction coefficient of sucrose B.P. in 0·1 N hydrochloric acid at the respective wavelengths was found to be:

Wavelength	*E*(1 per cent, 1 cm.)
270·5 mμ	0·0028
347 mμ	0·0003

Thus, the spectrophotometric assay should be applicable to both coated and uncoated tablets, and this was proved in practice.

Assay Process. To 20 tablets add approximately 300 ml. of 0·1N hydrochloric acid. Heat to about 50°, and maintain at this temperature until the tablets have disintegrated, then continue heating still at 50° for a further 1½ hours. Cool and filter through a sintered glass filter of No. 3 porosity. Transfer all the residual solid matter from the flask on to the filter with small portions of 0·1N hydrochloric acid. Wash the filter with the same acid and dilute to 500 ml.

TABLE III

ASSAY OF STANDARD SOLUTIONS OF PYRIMETHAMINE AND QUININE DIHYDROCHLORIDE

	Solute	Taken (mg./100 ml.)	Found (mg./100 ml.)	Per cent Recovered
Determination 1.	Pyrimethamine	0·3856	0·3864	100·2
	Quinine dihydrochloride	23·63	23·69	100·2
Determination 2.	Pyrimethamine	0·39105	0·3865	98·86
	Quinine dihydrochloride	23·58	23·56	99·98

Dilute 10 ml. of filtrate to 500 ml. with the same acid and determine the optical density of this solution at 270·5 mμ in 1 cm. quartz cells, using 0·1N hydrochloric acid as reference liquid.

Let the optical density at this wavelength be d_1.

Transfer 25 ml. of the diluted solution to a graduated 200 ml. flask and make up to volume with 0·1N hydrochloric acid. Determine the optical density at 347 mμ in 1 cm. quartz cells.

Let the optical density at this wavelength be d_2.

Since the contribution made by the pyrimethamine to the total absorption at 347 mμ is negligible, the weights per average tablet of quinine dihydrochloride and of pyrimethamine are given by the following expressions:—

Weight of quinine dihydrochloride (anhydrous), in mg. per average tablet $= 742{\cdot}9\ d_2$.

Weight of pyrimethamine (anhydrous), in mg. per average tablet $= 39{\cdot}432\ d_1 - 40{\cdot}076\ d_2$.

Assay results for three samples of Tablets of Pyrimethamine and Quinine are given in Table IV.

TABLE IV

ASSAY OF TABLETS OF PYRIMETHAMINE AND QUININE

	Constituent determined	Weight of constituent found per average tablet (mg.)	Per cent of stated strength
Sample 1 (uncoated)	Pyrimethamine	5·02	100·4
	Quinine dihydrochloride	296·7	98·9
Sample 2 (sugar-coated)	Pyrimethamine	5·08	101·6
	Quinine dihydrochloride	300·9	100·3
Sample 3 (sugar-coated)	Pyrimethamine	5·07	101·4
Determination 1	Quinine dihydrochloride	293·5	97·8
Sample 3 (sugar-coated)	Pyrimethamine	5·08	101·6
Determination 2	Quinine dihydrochloride	295·6	98·5

If a smaller number of tablets is to be assayed, or if the tablets are of a different strength, or have a different pyrimethamine: quinine dihydrochloride ratio, it will be necessary to modify the dilutions. The appropriate expressions for relating the assay results to the optical densities can be readily computed from the extinction coefficients at 270·5 mμ and 347 mμ.

Powder of Pyrimethamine and Sulphaguanidine

This preparation is used in veterinary practice as a coccidiostatic agent in admixture with a suitable diluent. The powder is made up of

pyrimethamine 6 parts (1·186 per cent w/w), sulphaguanidine B.P. 500 parts (98·814 per cent w/w).

Preliminary Experiments. The spectral characteristics of the constituents of the mixture are not sufficiently distinct to permit their estimation by measurement of total ultra-violet absorption at two wavelengths. Pyrimethamine could not be assayed without at least a partial preliminary separation of the constituents of the mixture. A quantitative separation can be made by direct extraction of the pyrimethamine from the powder with chloroform, in which approximate solubilities at room temperature are: pyrimethamine, 0·75 per cent; sulphaguanidine B.P. 0·0010 per cent.

TABLE V

ASSAY OF PYRIMETHAMINE IN POWDER OF PYRIMETHAMINE AND SULPHAGUANIDINE

	Pyrimethamine found (per cent)	Per cent of stated strength
Sample A, determination 1 ..	1·194	100·7
" " " 2 ..	1·200	101·2
Sample B, determination 1 ..	1·150	97·0
" " " 2 ..	1·144	96·5

TABLE VI

ASSAY OF SULPHAGUANIDINE IN POWDER OF PYRIMETHAMINE AND SULPHAGUANIDINE

	Titration with 0·1M sodium nitrite		Micro-determination of S		
	Sulphaguanidine B.P. found (per cent)	Per cent of stated strength	S found (per cent)	Equivalent to sulphaguanidine B.P. (per cent)	Per cent of stated strength
Sample A, determination 1	97·68	98·9	13·75	99·59	100·8
" 2	97·92	99·1	13·58	98·38	99·6
Sample B, determination 1	99·11	100·3	13·54	98·08	99·3
" 2	98·17	99·4	13·70	99·24	100·4

Assay for pyrimethamine. Weigh out accurately approximately 0·6 g. of powder into a glass-stoppered flask and shake mechanically for 30 minutes with 40 ml. of previously dried and redistilled chloroform. Filter through a dry Whatman No. 1 paper and wash the flask and filter with one 20 ml. and one 10 ml. portion of redistilled chloroform. Combine the filtrates and evaporate to dryness. Take up the residue in a mixture of 100 ml. of 0·1N hydrochloric acid and 10 ml. acetic acid B.P., and dilute to 500 ml. with 0·1N hydrochloric acid. Determine the optical density of the resulting solution at 272·5 mμ in 1 cm. quartz cells, using 0·1N hydrochloric acid as reference liquid. The specific extinction coefficient of pure pyrimethamine in 0·1N hydrochloric acid is E(1 per cent, 1 cm.) (272·5 mμ) = 320.

Assay for sulphaguanidine. Determine the sulphaguanidine content of the powder either by the B.P. method for sulphaguanidine (each ml. of 0·1M sodium nitrite is equivalent to 0·02323 g. of $C_7H_{10}O_2N_4S,H_2O$), or from the sulphur content of the powder (each g. of S is equivalent to

7·243 g. of $C_7H_{10}O_2N_4S,H_2O$). Pyrimethamine does not interfere in either determination.

Results of assays for two samples of Powder of pyrimethamine and sulphaguanidine are given in Tables V and VI.

IDENTIFICATION

The following tests of identity, in addition to those in the British Pharmaceutical Codex, are applicable to the characterisation of pyrimethamine.

Action of Alkaloidal Precipitants

A 0·2 per cent w/v solution of pyrimethamine in 5 per cent w/v sulphuric acid gives a positive reaction to the usual alkaloidal precipitants

Ammonium reineckate is particularly useful for characterising the drug, since it affords a crystalline derivative from dilute acid solution. The test is conveniently carried out as follows:—

Dissolve 10 mg. of pyrimethamine in 30 ml. of 0·5N sulphuric acid and add 10 ml. of previously-filtered 1 per cent w/v aqueous solution of ammonium reineckate. The complex crystallises out slowly in the form of fine pale red needles. Allow to stand for one hour, filter at the pump and wash the crystals with two 20 ml. portions of water. The complex decomposes at about 138°.

The phosphotungstate and picrate complexes of pyrimethamine may be useful in the identification of pyrimethamine in biological and toxicological specimens. These complexes are appreciably less soluble in water and in dilute mineral acids than the reineckate and are thus suited for the identification of small amounts of the drug; the approximate decomposition temperatures are: phosphotungstate 211° (no charring); picrate 239°.

2:4-*Diacetyl Derivative*

Heat under reflux for 30 minutes 1 g. of pyrimethamine with 2 ml. of a 1:1 glacial acetic acid-acetic anhydride mixture. Cool, add 50 ml. of water, shake, allow to stand for five minutes and decant the supernatant liquid. To the residual solid in the flask add 40 ml. of a 2 per cent w/v aqueous solution of sodium bicarbonate and heat gently for a few minutes. Cool thoroughly and allow to stand for not less than thirty minutes. Filter at the pump, wash well with water and recrystallise from 50 per cent ethanol.

Melting point of the crystals after drying at 100°: 172°. Found: C, 57·75; H, 5·17; N, 16·88; Cl, 10·66; $C_{16}H_{17}O_2N_4Cl$ requires C,57·74; H, 5·15; N, 16·84; Cl, 10·66 per cent.

Identification of Pyrimethamine in Preparations

Tablets of pyrimethamine. Powder one or more tablets and weigh out a quantity of material equivalent to 10 mg. of pyrimethamine. Add 30 ml. of 0·5 N sulphuric acid and heat at 50° for 30 minutes. Cool, filter and complete the test as described for pyrimethamine, commencing

with the words ". . . add 10 ml. of a previously-filtered 1 per cent w/v aqueous solution of ammonium reineckate. . . ."

Powder of pyrimethamine and sulphaguanidine. Extract 0·85 g. of powder (equivalent to 10 mg. of pyrimethamine) as described in the assay for pyrimethamine. Treat the residue obtained on evaporation with 1 per cent w/v ammonium reineckate solution and isolate the complex as described for pyrimethamine.

No method has so far been developed for the identification of the drug in tablets of pyrimethamine and quinine beyond computation of the ratio of the optical densities at 270·5 mμ and 347 mμ in the assay process, viz.

$$\frac{d_1}{d_2} = 1{\cdot}33$$

Chromatography

Pyrimethamine may be identified by its R_F value on a paper chromatogram. The system *n*-butanol (50 ml.)—water (50 ml.)—citric acid (1 g.), used with Whatman No. 1 papers impregnated with a 5 per cent w/v aqueous sodium dihydrogen citrate solution, proposed by Curry and Powell[11] for the toxicological examination of alkaloidal extracts, is suitable for this purpose and may be used to separate pyrimethamine from quinine. The spots are revealed by dipping the papers in a tartaric acid solution of potassium iodobismuthate[12].

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