Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 7, pp. 893–898, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00

Short Communication

Detection and estimation of pyrimethamine in urine

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Keywords: *Pyrimethamine; colour test; detection in urine.*

Introduction

Pyrimethamine [**I**; 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine] has been in use as a suppressive in anti-malarial therapy for several years, often in combination with a sulphonamide such as sulphadoxine. Although pyrimethamine has been fairly well studied, the growing phenomenon of *P. falciparum* resistance to the available anti-malarial drugs has brought about the need for constant *in vivo* clinical assessment of the drug sensitivity of plasmodia to the drugs in areas of endemic malaria. Simple and sensitive field methods for the detection of the drugs are required during such assessments [1].

A colour test has been described for pyrimethamine based on the dye Bromophenol Blue [2]. This test is not specific for pyrimethamine as there could be interference from chloroquine and amodiaquine, two other anti-malarials for which a test based on the same dye has been described [2]. The colour change undergone by the dye in the presence of pyrimethamine may also be affected by many other basic nitrogenous compounds. Furthermore, the quantitative estimation of the drug could not be achieved using this method.

A procedure is described here for the specific detection, and spectrophotometric determination, of pyrimethamine in urine. The method is based on the reaction of pyrimethamine with salicylaldehyde and zinc acetate to give a yellowish-green fluorescent derivative.

Experimental

Reagents

Pyrimethamine (Roche Products Ltd, UK), Daraprim® tablets (Wellcome) were obtained from commercial sources. Other reagents (obtained from BDH) were: acetic

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acid, salicylaldehyde, zinc acetate dihydrate and chloroform. Silica gel for thin layer chromatography (TLC) was obtained from Merck (Darmstadt, FRG).

Instrumentation

Absorbance measurements were recorded on a Pye-Unicam SP-26-200 single beam spectrophotometer using a glass cell with a pathlength of 1 cm.

Reaction of pyrimethamine with salicylaldehyde

A sample of 0.249 g (0.001 mol) of pyrimethamine was dissolved in 30 ml of chloroform and treated with 5 ml of a 5% (v/v) solution of salicylaldehyde in chloroform and 5 ml of a 2% (v/v) solution of acetic acid in the same solvent.

The reaction mixture was kept at room temperature for 15 min, after which it was warmed in a water bath at 60°C until the volume was reduced to about 10 ml. A 1 ml portion of a 10% (m/v) solution of sodium metabisulphite was added and the mixture warmed for a further 10 min with vigorous shaking at intervals to remove excess salicylaldehyde.

The aqueous layer was removed with a Pasteur pipette and the chloroform shaken with a little anhydrous sodium sulphate to dry it.

On removal of the solvent, the pyrimethamine–salicylaldimine was obtained as a pale yellow solid (m.p. = 226–228°C). (Found: C = 64.18%, N = 15.06%, H = 4.60%; expected: C = 64.68%, N = 15.89%, H = 4.80%.)

Thin layer chromatography of the salicylaldimine was performed on silica gel and with two different solvent systems composed of chloroform/methanol (3:1, v/v) and of dichloromethane/methanol (7:3, v/v), respectively.

Reaction of pyrimethamine with salicylaldehyde and zinc acetate

Pyrimethamine was reacted with salicylaldehyde as described above. After removing excess salicylaldehyde with sodium metabisulphite and the chloroform solution of the salicylaldimine dried as described, the chloroform solution was treated with 0.183 g of zinc acetate dihydrate and a further 20 ml of chloroform added. The mixture was evaporated to dryness on a water bath (60°C) over a 10-min period. A bright yellow solid was obtained (m.p. = 156–158°C).

The zinc content of the compound was determined by heating 60 mg of the compound with 2 ml of a 4:1 (v/v) mixture of concentrated hydrochloric acid and concentrated nitric acid in a 100 ml beaker, using a hot plate. After heating to dryness the residue was triturated with 20 ml of water and the solution filtered into a 50-ml volumetric flask. The beaker was rinsed twice with 5 ml of water and the rinsings filtered into the volumetric flask. After making up to volume, the concentration of zinc in the solution was determined by titration with standardized 0.001 M EDTA solution.

Elemental analyses for hydrogen and nitrogen were also carried out. (Found: N = 14.42%, H = 4.19%, Zn = 8.34%; expected, N = 14.58%, H = 4.16%, Zn = 8.51%.)

A solution of the yellow solid in chloroform or methanol has a yellowish-green fluorescence in daylight.

Visual detection of pyrimethamine in standard aqueous, urine and saliva solutions

A standard solution of 100 $\mu g\ ml^{-1}$ of pyrimethamine was prepared by dissolving 10 mg of pyrimethamine in 100 ml of chloroform. Aliquots (0.01–1.0 ml) of the chloroform solution were placed in stoppered test tubes and the solution evaporated by

warming in a water bath (60–70°C). To the residues, 2 ml of water (or drug-free urine or saliva) was added to give standard aqueous (or urine, or saliva) solutions containing 1.0–100 μg of pyrimethamine. The solutions were made alkaline with 0.5 ml of 2 M sodium hydroxide solution and extracted with 5.0 ml of chloroform by shaking by hand for 1–2 min. After removing the aqueous layers with a Pasteur pipette, the chloroform extracts were shaken with a few milligrams of anhydrous sodium sulphate and then transferred to a set of 10 × 2 cm test tubes. After adding 0.5 ml of a 5% (v/v) solution of salicylaldehyde in chloroform, a few milligrams (about 20 grains) of zinc acetate dihydrate crystals were added and the chloroform rapidly evaporated by heating in a water bath (70°C). The residues were re-dissolved in 0.5 ml of chloroform and the solutions observed for the appearance of a yellowish-green colour. The solutions were also examined under UV light.

Blank water (or urine or saliva) were similarly treated for comparison.

Visual detection of pyrimethamine in urine from dosed volunteers

A single oral dose of Daraprim® equivalent to 25 mg of pyrimethamine was administered to each of four volunteers from whom pre-dose urine had been obtained. All urine samples were collected separately within the following 12–18 h. Thereafter, sample collection continued on a daily basis, the first urine to be voided in the morning being collected.

For the detection of pyrimethamine, 5 ml of urine samples were placed in stoppered test tubes, made alkaline with 0.5 ml of 2 M sodium hydroxide solution and extracted with 5 ml of chloroform, as described above for standard urine solutions. Subsequent drying of the chloroform extract with anhydrous sodium sulphate and reaction with salicylaldehyde and zinc acetate, evaporation to dryness and re-constitution of the residue in 0.5 ml of chloroform were also as described above.

Spectrophotometric determination of pyrimethamine in urine. Calibration in chloroform, water and urine

A standard solution containing 100 μg ml^{-1} of pyrimethamine was prepared as described above. Aliquots (0.1–1.0 ml) of the solution were placed in test tubes and enough fresh chloroform added to obtain 5.0-ml chloroform solutions containing 10–100 μg of pyrimethamine. These solutions were reacted with salicylaldehyde and zinc acetate as described above. After evaporating the reaction mixtures to dryness, the residues were re-dissolved in 5.0 ml of chloroform and the absorbance of the solutions measured at 480 nm using blank chloroform which had been similarly treated as a reference.

Standard aqueous (or urine) solutions containing 5–100 μg of pyrimethamine were prepared as described for visual detection of standard solutions above. The solutions were also made alkaline with sodium hydroxide, extracted with 5.0 ml of chloroform and the dried chloroform extracts reacted with salicylaldehyde and zinc acetate as described above. After evaporating the reaction mixtures to dryness the residues were re-dissolved in 5.0 ml of chloroform and the absorbances of the solutions measured at 480 nm using drug-free water (or urine) which had been similarly treated as reference.

Results and Discussion

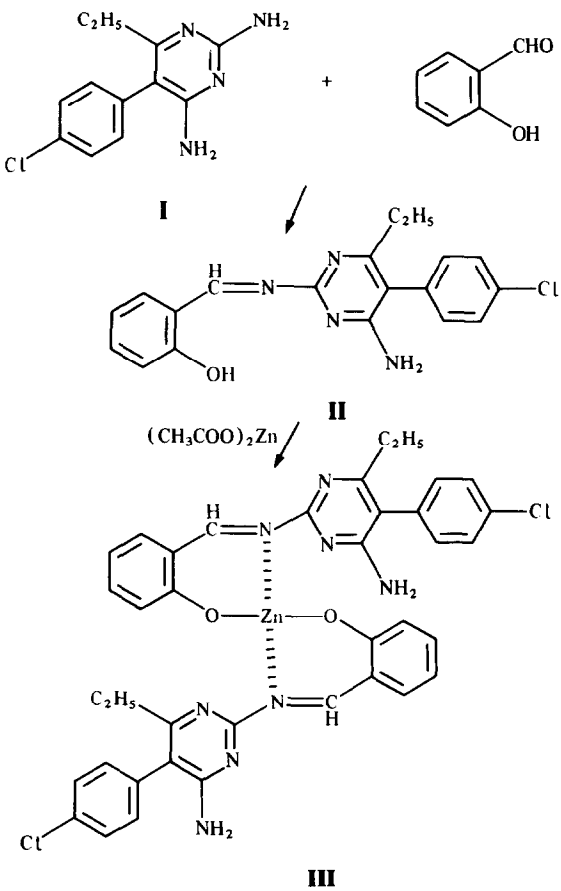
Except for the reported reaction of 2-amino-4-hydroxypyrimidine with benzaldehyde

[3], simple Schiff-bases derived from 2-, 4-, or 6-aminopyrimidines are unknown. Preferential anil formation at the position 5 has been observed in the reaction of 2,5-diamino- and 4,5-diaminopyrimidines, in amine–aldehyde condensation reactions [4].

Pyrimethamine, a 2,4-diaminopyrimidine derivative was, however, found to react readily with salicylaldehyde to give the salicylaldimine, 2-(2-hydroxybenzylideneamino)-4-amino-5-(*p*-chlorophenyl)-6-ethylpyrimidine (**II**) as shown in Scheme 1.

A solution of the pyrimethamine–salicylaldimine in chloroform or methanol exhibits a pale blue fluorescence under UV light. Following TLC, however, the spot of the pyrimethamine–salicylaldimine showed intense yellowish-green fluorescence under UV light. This enhancement of the fluorescence of the salicylaldimine was thought to be due to the minimization of rotational and vibrational non-radiative dissipation of absorbed energy when the molecules are adsorbed on silica gel, and that a similar effect may be achieved through chelation of the salicylaldimine with a suitable metal ion.

The pyrimethamine–salicylaldimine was found to react readily with zinc acetate to give the zinc chelate, bis[N-(4-amino-5-*p*-chlorophenyl-6-ethylpyrimid-2-yl)-salicyl-aldiminato] zinc(II) as shown in Scheme 1. The zinc chelate (**III**), which on solution in



chloroform (or methanol) exhibits a yellowish-green fluorescence in daylight, also forms readily when a chloroform solution of milligram amounts of pyrimethamine is treated with salicylaldehyde and the mixture shaken with a few milligrams of zinc acetate at ambient temperature. With a 1 μg amount of the drug, however, the reaction mixture had to be warmed briefly for 20–30 s at 60–70°C for the fluorescence of the zinc chelate to appear.

Visual detection of pyrimethamine in standard aqueous, urine and saliva solutions

When the reaction was applied to a series of standard solutions of pyrimethamine in chloroform, visual detection of the drug was possible down to a level of 1 μg.

With aqueous and urine standard solutions, however, visual detection was only possible down to a level of 2 μg. Although the drug could also be detected in saliva, problems encountered included emulsion formation during extraction of these solutions and subsequent interference from oral debris.

No interference was observed from amodiaquine, chloroquine and sulphadoxine. A chloroform solution of *p*-aminophenol (a metabolite of paracetamol), containing 500 μg of the compound, gave a pale yellow non-fluorescent colour on reaction with salicylaldehyde and zinc acetate. However, aqueous or urine solutions of *p*-aminophenol gave no colour reaction on being treated as described for solutions of pyrimethamine in these fluids.

Visual detection of pyrimethamine in dosed volunteers

Following the administration of a single oral dose of 25 mg of pyrimethamine to each of four volunteers, the drug could be detected in the urine from three of the volunteers for 2 weeks after drug administration. The drug could only be detected for 10 days in the urine of the fourth volunteer.

It was found that the drug was much more readily detected when the urine sample used was the first urine to be voided in the morning of each day. Subsequent urine collections on the same day often gave a faint colour in the test.

Table 1
Calibration data for pyrimethamine in chloroform, water and urine

Pyrimethamine taken (μg)	Absorbance* Chloroform†	Water‡	Urine§
5	—	0.028 (26.65)	0.042 (31.50)
10	0.040	0.048 (18.72)	0.073 (19.67)
20	0.183	—	—
40	0.370	0.134 (2.28)	0.242 (4.91)
60	0.598	—	—
80	0.848	0.225 (4.10)	0.304 (4.07)
100	—	0.380 (8.21)	0.392 (1.95)

*Each value is the mean of two determinations for chloroform, five for water and four for urine solutions. The RSDs (%) are in parentheses.

Regression equations: where y is the absorbance of the zinc chelate of pyrimethamine–salicyladimine and χ(μg) is the amount of pyrimethamine reacted.

†$y = 0.0113(\pm 0.001)\,\chi - 0.065(\pm 0.050)$.

‡$y = 0.00327\ (\pm 0.000364)\,\chi + 0.007(\pm 0.021)$; $r = 0.9694$.

§$y = 0.00344(\pm 0.000418)\,\chi + 0.05(\pm 0.026)$; $r = 0.9724$.

Spectrophotometric determination of pyrimethamine in chloroform, aqueous and urine solutions

Calibration graphs of absorbance of the zinc chelate of pyrimethamine–salicylaldimine (**III**) against the amount of pyrimethamine in chloroform, water and urine showed good linearity over the range 0–100 μg (Table 1).

Conclusion

A specific colour test for pyrimethamine is described which permits visual detection of the drug in urine for up to 2 weeks following the oral administration of a single 25 mg dose. The test is based on the reaction of pyrimethamine with salicylaldehyde and zinc acetate to give a fluorescent yellowish-green derivative. Spectrophotometric measurement of the derivative has also been applied to the determination of the drug in urine with a detection limit of 0.27 μg in 2 ml of urine.

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[*Received for review 3 May 1988; revised manuscript received 15 November 1988*]