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A SIMPLE PAPER TEST FOR ISONIAZID IN URINE*

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INTRODUCTION

Tests for the presence of chemotherapeutic drugs or their metabolites in urine play an important part in the management of the treatment of tuberculosis (Dixon *et al.*, 1957; Fox, 1958). A previous report from this Centre (Gangadharam *et al.*, 1958) presented a comparison of a number of methods for detecting isoniazid in urine including the direct naphthoquinone-mercuric chloride (N-M) test (Short and Case, 1957), and also a modification of this test which employed alkaline hydrolysis to liberate isoniazid from its conjugated forms. The direct-and hydrolysis N-M tests have been employed in this Centre for the past four years to control the self-administration of isoniazid used in the domiciliary treatment of pulmonary tuberculosis. The effect of irregularity in taking isoniazid as detected by these tests on the response to treatment has been reported elsewhere (Tuberculosis Chemotherapy Centre, 1960). Since this method has the disadvantage that it requires a certain amount of equipment and trained personnel, it is not suitable for routine use in all chest clinics or under field conditions.

An attempt was therefore made in this Centre to simplify the direct N-M test by incorporating the reagents in absorbent papers; Though impregnation of the paper with the pHl0 buffer and naphthoquinone reagent was successful, impregnation with the aqueous solution of the mercuric chloride was unsatisfactory. In 1960, Cattaneo, Fantoli and Belasio published details of a paper test modification of the N-M test in which this difficulty was overcome by impregnating absorbent papers with a solution of mercuric chloride in ether. Since then this modification has been adopted for the preparation of the test-paper developed in this Centre.

Since a lower concentration of the naphthoquinone reagent and a shorter period of exposure was used in the preparation of the test-paper developed in this Centre than described by Cattaneo *et al.* (1960), both the paper tests have been compared with the direct and combined N-M tests described previously (Gangadharam *et al.*, 1958). This paper presents the results of the comparison and of an investigation of the specificity of the paper test.

MATERIALS AND METHODS

Specimens of urine from patients

In all, 1455 specimens of urine, collected from patients who were receiving isoniazid in a daily dosage that ranged from approximately 4 to 16 mg/kg. body-weight were tested in a series of 32 experiments, 18 to 64 urine specimens being tested in each experiment.

Control urine specimens

A total of 116 specimens of urine, 84 collected from among 28 staff members and 32 collected from patients who were not receiving isoniazid or PAS, were tested in 28 of the 32 experiments as a check on the occurrence of false positive results.

REAGENTS

Direct and hydrolysis naphthoquinone-mercuric chloride tests (Direct and hydrolysis N-M tests)

(i) Borate buffer pH10: 12.369 g. of boric acid and 14.991 g. of potassium chloride were dissolved in 1000 ml. of distilled water and the solution was added to 860 ml. of 0.2 N sodium hydroxide.

(ii) Sodium 1:2 *naphthoquinone-4-sulphonate:* 0.1 per cent (w/v) aqueous solution. This was stored in a dark bottle at 6°C for upto one month.

(iii) Mercuric chloride: 5.0 per cent (w/v) aqueous solution.

(iv) Sodium hydroxide: 40 per cent (w/v) aqueous solution.

(v) Hydrochloric acid: 1.5 per cent (w/v) aqueous solution.

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Naphthoquinone-mercuric chloride paper tests

(a) Tuberculosis Chemotherapy Centre (TCC) method

(*i*) *Borate buffer pH10:* Same as above.

(*ii*) *Sodium* 1:2 *naphthoquinone-4-sulphonate:* Same as above.

(*iii*) *Mercuric chloride:* 0.5 per cent (w/v) solution in ether.

PREPARATION OF THE PAPER

A mixture (5:1) of the pHl0 borate buffer and the 0.1 per cent (w/v) aqueous solution of naphthoquinone reagent was prepared and thoroughly mixed. Sheets of locally obtained blotting paper (this was found to be as suitable for this purpose as the more expensive filter papers) 30 cm x 15 cm in size, were soaked in this mixture for one to two minutes and air-dried overnight in a darkened room at approximately 23°C. When dry, they were dipped into the solution of mercuric chloride for 10-15 seconds, and then air-dried at approximately 23°C for 10 minutes. They were then cut into small squares (4 cm x 4 cm) and stored in a light-proof box at 6°C.

(b) Cattaneo et al. (1960) (Cattaneo) method

(i) Borate buffer pH10: Same as above.

(ii) Sodium 1:2 *naphthoquinone sulphonate:* 0.3 per cent (w/v) aqueous solution.

(iii) Mercuric chloride: 0.5 per cent (w/v)

PREPARATION OF THE PAPER

Sheets of locally obtained blotting paper were immersed for 1 hour in a mixture of equal volumes (1:1) of the pHl0 borate buffer and the 0.3 per cent (w/v) aqueous solution of the naphthoquinone reagent. The subsequent stages in the preparation of the paper were the same as described for the TCC paper.

URINE TESTS

(i) Direct naphthoquinone-mercuric chloride test (Direct N-M test)

To 5.0 ml. of urine in a large test-tube (150mm x20 mm), 2.5 ml. of borate buffer and 0.5 ml. of the naphthoquinone reagent were added with thorough shaking after each addition. After 5 to 10 minutes, 1.0 ml. of mercuric chloride solution was added. The tube was centrifuged for one minute at 1000 r.p.m. to allow the precipitate formed to settle at the bottom. The test was read by observing the colour of the precipitate; a purple colour indicated the presence of free isoniazid.

(ii) Hydrolysis naphthoquinone-mercuric chloride test (Hydrolysis N-M test)

To 5.0 ml. of urine in a large test-tube (150mm x 20mm) exactly 1.0ml. of the sodium hydroxide was added with a 2.0 ml. syringe fitted with a long needle. The tube was placed in a boiling water bath for exactly 10 minutes after which it was cooled under the tap and about 95 per cent of the volume of hydrochloric acid necessary to neutralise the 1.0 ml. of sodium hydroxide was added. The urine sample was then tested for the presence of free isoniazid as in the direct N-M test. The use of the direct followed by the hydrolysis N-M test if the former gave a negative result is referred to as the combined N-M test.

(iii) Naphthoquinone-mercuric chloride paper test (N-M paper test)

Test papers prepared by the Tuberculosis Chemotherapy Centre (TCC) method and by the method of Cattaneo *et al.* (1960), were used in the same way. With a Pasteur pipette, three drops of the urine were added to the test paper placed on a glazed tile. The development of a purple ring when the urine had dried (5-10 minutes) indicated the presence of isoniazid.

Reading of tests

The tests were read independently by two observers who were unaware of the source of the specimens of urine. The result of each test was recorded as negative, trace, 1-plus positive or 2-plus positive. If one observer read a trace and the other a trace or a positive, the test was regarded as positive; if the other observer recorded a negative result, the test was regarded as negative. For purposes of calculating the observer error, scores of 0, 1, 2 and 3 were given for the negative, trace, 1-plus positive and 2-plus positive readings, respectively.

RESULTS

Control urines

A total of 116 control urine specimens from volunteers and patients who had not taken

isoniazid or PAS were included in 28 of the 32 experiments in which the urine specimens from patients under treatment with isoniazid were tested. Both the TCC paper test and the direct N-M test gave positive results with three specimens, one specimen from a volunteer being positive by both tests. Nineteen of these control specimens of urine were tested by the Cattaneo paper test; none gave positive results.

Specificity of the tests for isoniazid

The specificity of the TCC and Cattaneo paper tests and the direct N-M test for isoniazid was studied by performing these tests on specimens of urine obtained from staff members at 0, 2 and 6 hours after they had taken one of the following drugs: 'Aspirin' (acetyl salicylic acid 500 mg.); 'Anacin' (acetyl salicylic acid 194 mg, phenacetin 194 mg, caffeine 16 mg, and quinine 15 mg); 'Saridon' (phenyl-dimethyl isopropyl pyrazalon 150 mg); 'Codopyrine' (acetyl salicylic acid 260 mg, codeine phosphate 10 mg).; pyrazinamide 500 mg; cycloserine 250 mg; thiacetazone 50 mg; ethionamide 250 mg; p-amino salicylic acid (PAS) 1000 mg; phthalyl-sulpha thiazole 500 mg; sulphadiazine 500 mg; chlorpromazine 25 mg. or ephedrine 32 mg.

Of these drugs only PAS gave any reaction and this only with the Cattaneo test-papers which became pink with the two-hour specimen. The effect of a higher dose of PAS on the efficiency of the three tests in detecting isoniazid was therefore studied in six volunteers who were given 5 g. of PAS and 100 mg. of isoniazid in cachets. Specimens of urine were collected at zero and six hours after taking the cachets. None of the specimens collected before the drugs were taken gave a positive result with any of the tests. The TCC paper and the direct N-M test detected isoniazid in all the. six-hour specimens of urine. On the other hand, with the Cattaneo papers all the six-hour specimens gave a deep pink colour and no purple ring could be detected. In summary, of all the drugs tested, only PAS produced a colour and this only with the Cattaneo test papers; the pink colour which was produced interfered with the detection of the purple colour produced by isoniazid.

Stability of the TCC test-paper on storage

The stability of the TCC test-papers on storage at room temperature (30°C) and at 6°C was studied by comparing the number of positive results obtained with test-papers after they had been stored for different periods of time.

It was found that TCC test-papers which had been stored protected from light at 6°C for up to eight weeks, detected as positive all of six urines which were positive for isoniazid by the direct N-M test. On the other hand, when the test-papers were stored protected from light at room temperature (30°C) they rapidly deteriorated. Thus, the proportion of positive results was 10 out of 10 after two days of storage, 8 of 10 after four days, 5 of 6 after one week, 2 of 7 after two weeks and none of 6 after four weeks.

Stability of the colour developed by the test papers

The stability of the colour developed by the test papers was studied by reading test papers from tests carried out on 20 specimens of urine, as soon as they were dry and 1, 3, 5 and 24 hours later. To avoid bias, the papers were arranged in a different random order for each reading.

It was found that the purple ring produced with the TCC papers could be read with equal precision up to 24 hours. On the other hand the colour of the Cattaneo papers darkened progressively and it was very difficult to differentiate a positive from a negative after one hour. The colour of the precipitate in the direct N-M test also persisted unchanged for at least 24 hours.

Comparison of the TCC paper test with the Direct and Combined N-M test

Table I presents the results obtained with the TCC paper and the direct and combined N-M tests on a total of 1455 urine specimens obtained from patients who were receiving isoniazid. The specimens were tested in 32 experiments. Of the 1455 urine specimens, 1070 (73.5 per cent) gave a positive result for isoniazid with the TCC paper test compared with 1030 (70.8 per cent) with the direct N-M test and 1335 (91.8 per cent) with the combined N-M test. (Of the 120 urine specimens which were negative by the combined N-M test 6 were positive by the TCC paper test.) The TCC paper test was thus, if anything, slightly more sensitive than the direct N-M test but considerably less sensitive than the combined N-M test. The difference between the results obtained with

TABLE I

Comparison of the TCC paper test with the direct and combined N-M tests

Method	Total number of specimens tested	Specimens positive for isoniazid*	
		No.	%
TCC Paper ...	1455	1070	73.5
Direct N-M ...	1455	1030	70.8
Combined N-M ...	1455	1335	91.8

* For definition of positivity see text.

the TCC paper test and the combined N-M test attains statistical significance ($P<0.01$).

The TCC and Cattaneo paper tests compared with the Direct and Combined N-M tests

The TCC paper test was compared with the Cattaneo paper test using test-papers prepared in this laboratory according to the method described by Cattaneo *et al.* (1960) and with the direct and combined N-M tests in 305 of the 1455 urine specimens obtained from patients who were receiving isoniazid. These 305 specimens were those tested in the 19th, 20th, 29th, 30th, 31st and 32nd experiments. Of these 305 specimens, 231 (75.7 per cent) were positive by the TCC paper test, 213 (70.0 per, cent) by the Cattaneo paper test, 222 (70.3 per cent) by the direct N-M test and 281 (92.1 per cent) by the combined N-M test. Two of the 36 specimens that were negative by the combined N-M test were positive by both the TCC and the Cattaneo paper tests. Thus, the TCC paper test detected slightly more positives than either the Cattaneo paper test or the Direct N-M test. However, only the differences between the combined N-M test and the other tests attain statistical significance.

Observer error in reading the tests

The error between observers was studied from the results of two experiments in which 103 urine specimens were tested by the TCC paper test, the Cattaneo paper test and the direct N-M test, and read independently by two observers. After converting the results into numerical values using the scoring system described on page 220, they were examined by analysis of variance; in none of the three tests was there a significant difference between the reading of the two observers.

Duration urine remained positive after a single dose of isoniazid

The period for which urine specimens were positive by the TCC paper test after a single oral dose of approximately 6.7 mg. isoniazid per kg. body-weight (300 mg. for a volunteer weighing 100 lb.) was studied in 20 volunteers. Urine specimens were collected before the isoniazid was taken and at 1, 2, 3,4, 5, 6, 7, 8, 10 and 12 hours thereafter. All the specimens were tested together in a randomised order. None of the specimens collected from the 20 volunteers before they had taken the isoniazid was positive. The numbers of positive specimens were 20 of 20 from one to six hours inclusive, 17 of 20 at seven hours, 15 of 20 at eight hours, 14 of 20 at ten hours and 13 of 20 at twelve hours.

DISCUSSION

The investigations reported here have shown that the paper modification of the naphthoquinone-mercuric chloride (N-M) test developed in this Centre (TCC paper test) was of about the same sensitivity as the direct N-M test previously described by Short & Case (1957) and Gangadharam *et al.* (1958). However, the TCC paper test was considerably less sensitive than the combined N-M test (Gangadharam *et al.*, 1958), but this was to be expected since the TCC paper test was performed only on unhydrolysed urine.

Both the TCC paper and the direct N-M tests gave false positive results in three (2.6 per cent) of the 116 control specimens of urine tested concurrently with the urine specimens from patients who were receiving isoniazid. In addition, the TCC paper test gave positive results in 6 of the 120 specimens obtained from patients which were' negative by the combined N-M test. Corresponding figures for the direct N-M test are not available since specimens were not retested after hydrolysis if they gave positive results in this test. In

consequence the two tests cannot be compared in this respect.

This study has shown that the TCC paper test was slightly more sensitive than the Cattaneo paper test and had 2 minor advantages. The Cattaneo test-papers darkened progressively after use and this masked the purple ring produced by the isoniazid. In consequence the accuracy of reading the Cattaneo papers decreased if they were read. after 1 hour. This could be of importance in a domiciliary chemotherapy service under which condition it might be desirable to test the patients' urine in the home and read or check the results some hours later in a central clinic. Secondly, PAS was found to interfere with the detection of isoniazid by the Cattaneo test-papers, but not by the TCC test-papers. Since essentially similar findings to those found with the Cattaneo test-papers prepared in this laboratory with locally bought blotting paper were obtained with test-papers kindly supplied by Prof. Cattaneo, it is most likely that these differences between the TCC and Cattaneo test-papers were due to the only other important dissimilarity between them, namely, the higher concentration of naphthoquinone reagent, used in the preparation of the Cattaneo test-papers.

Since our attempts to simplify the hydrolysis procedure have so far been unsuccessful, the only practical approach under field conditions seems to be to examine urine specimens by the paper test during the period in which they could be expected to be positive if the isoniazid had been taken. Under the climatic conditions of Madras, this period has been shown to be from about one to six hours following a dose of approximately 6.7 mg. of isoniazid per kg. body-weight. In chest clinics where facilities to carry out the hydrolysis procedure are available it is suggested that the paper test could be used to replace the direct N-M test with a consequent saving in time and expense.

SUMMARY

A paper test modification of the direct naphthoquinone-mercuric chloride (N-M) test developed in this Centre has been compared with a similar paper test described by Cattaneo *et al.* (1960), and with the previously described direct and combined N-M tests. The paper test was, if anything, slightly more sensitive than the Cattaneo paper test and the direct N-M test but considerably less sensitive than the combined N-M test. However, the paper test detected isoniazid in all the urine specimens obtained from one to six hours from 20 volunteers who had taken a single dose of approximately 6.7 mg. isoniazid per kg. body-weight. The application of the paper test for use in chest clinics and under field conditions has been discussed.

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