Advances in Tuberculosis Research

Fortschritte der Tuberkuloseforschung

Progrès de l'Exploration de la Tuberculose

16

Advances in Tuberculosis Research

Fortschritte der Tuberkuloseforschung
Progrès de l'Exploration de la Tuberculose

Editor: GEORGES CANETTI, Paris

Co-Editors: HANS BIRKHÄUSER, Basel, and HUBERT BLOCH, Basel

16

Contributors – Mitarbeiter – Collaborateurs

GILLES CONGE, Paris – R. M. EHRLICH, New York – L. EIDUS, Ottawa (Canada) – COLETTE FILLASTRE, Paris – A. A. GROVER, Madison (Wis.) – J. K. LATTIMER, New York – F. M. LÉVY, Paris – R. MANDE, Paris – EVELYNE ORSSAUD, Paris – D. W. SMITH, Madison (Wis.) – E. WIEGESHAUS, Madison (Wis.)

11 figures, 63 tables

19 68

BASEL (Switzerland) S. KARGER NEW YORK

Index

Perspectives for BCG Standardization

F.M. Lévy, R. Mande, Gilles Conge, Colette Fillastre and Evelyne Orssaud, Paris

Nonliving Immunogenic Substances of Mycobacteria

D.W. Smith, A.A. Grover and E. Wiegeshaus, Madison, Wis.

Adv. Tuberc. Res., vol. 16, pp. 1–31 (Karger, Basel/New York 1968)

Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada

Detection of Minor Antituberculosis Drugs in the Urine

L. Eidus

Index

I. Introduction

The discovery of effective antituberculosis drugs opened the way for domiciliary treatment. The number of ambulatory patients treated, after an initial period of hospitalization or entirely at home, is steadily increasing. The problem caused in the past by the lack of the necessary sanatorium beds is now replaced by another one, namely the responsibility for assuring adequate surveillance and hospital standards in ambulatory chemotherapy.

Trials in India [60] as well as in the USA [21, 46, 65] indicate that not all patients will consistently follow the medical advice and prescriptions. It is a common experience in long-term chemotherapy that after amelioration of the acute symptoms, the patient often tends to become irregular in self-administration of the medication. Fox [23] refers to a number of publications, showing that in chronic diseases, requiring regular treatment for an extended period, the rate of patients not taking the drugs or taking them irregularly is quite high.

In developed countries the continued personal contact of the physician with the patient and the indoctrination of the latter concerning the importance of regular medication stimulates the patient's cooperation. However, this relationship does not ensure that the patient may not become indifferent after a while and that objective methods of control should be neglected. A reliable test for checking the regularity of self-administered medication may also show the patient's attitude towards his illness, his responsibility towards himself and his environment in the case of communicable disease. It may, however, indicate that the patient did not receive the correct medicine or that the drug had deteriorated for some reason, as has been observed during our laboratory experience. The urine test should therefore not be regarded as a control of the patient alone but as a routine procedure for the physician's personal information. When it is considered that the alternative to this control test might be prolonged hospitalization or failure of the therapy, an adequate supervision of the self-medication is certainly justified. In countries where the treatment of tuberculosis is compulsory the test indicates whether self-administration by the patient is warranted or whether, due to his forgetfulness or indolence, he needs closer supervision. The physician, knowing the patient's circumstances and social conditions, will use this information for the benefit of the patient.

In developing countries, due to the lack of medical staff, the personal contact between the physician and the patient is often minimal. The enormous difficulties encountered in carrying out effective chemotherapy in these countries are not only due to insufficient financial resources, but also to the general tendency of the patients to discontinue their medication too soon (CANETTI [9]). A simple control test, easy to perform by the paramedical team, will assist in the surveillance of the patient. It will give warning signals of the patient's flagging cooperation and will enable the staff to apply personal and social persuasion towards continuation of the treatment. Except for a few well organized studies, many of the control and eradication programmes of these countries are poorly managed or suffer from difficulties in transferring pilot studies to large scale projects. The supervised administration of drugs may even fail due to insufficient organization. GILROY [27] reports that in a supervised campaign for malaria prophylaxis, surprisingly, only 56% of the urine samples examined gave positive results, for which he blames the drug distributors as well as the recipients. Moreover, our own experience with cycloserine showed that in poverty-stricken communities patients treated in sanatoria managed to outwit reliable supervision in order to sell their drugs on the black market [47]. If urine tests had not been carried out this would never have been detected. Under these circumstances the value of urine tests in the checking of self-medication cannot be stressed enough (FOX [23]). It will help to detect mistakes and to improve an inadequate standard of efficiency. Under certain circumstances we may even be confronted with the suggestion that streptomycin, intramuscularly administered, should also be checked by a urine test, in order to avoid misuse of the government supply and to ensure that the patient has received it.

Within the last two decades a number of urine tests were introduced for the detection of primary antituberculosis drugs. These tests were discussed in an extensive study by HOBBY [32] and in a short review by KENT [38]. Today we have rapid and simple tests for the detection of PAS in the urine [14, 43, 52, 57, 58, 67]. There were however up to 1961 only a few procedures available for isoniazid, these [11, 25, 26, 56] were moderately sensitive, reacting only with free isoniazid. The presence of PAS and a number of common drugs interfered with these tests. Recently more sensitive and specific methods have been developed, which are suitable for fast inactivators, determining the main metabolites of isoniazid, such as isonicotinic acid

[3, 37, 40] and acetylisoniazid [16, 17]. VENKATARAMAN *et al.* [61] have evaluated these tests in a comprehensive study.

With the introduction of new procedures for isoniazid, our repertory for the major antituberculosis drugs seems to be quite complete. On the other hand, not sufficient attention has been paid to the detection of minor antituberculosis drugs in the urine. This is probably due to the fact that these drugs were only occasionally used in domiciliary treatment. The increased incidence of acquired resistance to the major antituberculosis agents enhanced the importance of the second line drugs. With their more extensive use, the need for reliable urine tests became an urgent problem. The unpleasant side effects of the minor antituberculosis agents often caused reluctance or refusal by patients to take these drugs, even in hospitals. The toxic effect which is frequently encountered, as well as the increasing impatience of the sufferer towards his prolonged or renewed treatment, may lead to discouragement and negligence concerning his prescribed regimen. A thorough supervision of the patient and the regular performance of the urine test are therefore most important, especially when this medication may be the last chance to achieve quiescence of the patient's tuberculosis process.

Chemotherapeutic investigations with the aim of evaluating the efficiency of minor antituberculosis drugs are another reason for introducing urine tests. In such studies, it is most important to know whether the patients, included in the investigation, are following their drug regimen regularly. A comparative study of this kind will give a true picture of the effectiveness of the regimens under consideration only when each of the drugs administered is separately checked by suitable urine tests.

The present paper deals with methods for checking the regularity with which the patient takes the minor antituberculosis drugs, such as pyrazinamide, cycloserine and ethionamide. Before describing and evaluating the laboratory methods recommended for detection of the particular drugs in the urine, a short account is also given of non-specific procedures useful in the control of drug administration.

II. Non-specific Procedures

1. Count of the Patient's Drug Supply

The patient's stock of antituberculosis drugs can be checked during an unexpected visit to his home if the amount given to him per week

is known [22]. An excessive number of tablets would prove that the patient is not taking his drugs regularly or has stopped taking them altogether. A deficit may indicate that the patient has not been following the prescription precisely. A correct stock may show that the patient is either taking his medicine regularly or is disposing of it according to the prescription, so that the balance of the stock is exact at any one time. Urine tests periodically performed should give meaning to the stock count. Stock counting is a drastic method for control of self-medication. It casts aspersions on the patient's integrity and may impair his relationship with the medical profession. It has been mainly applied under special conditions in underdeveloped countries.

2. Time-recording Pill Dispenser

MOULDING [41] recommended the use of pill dispensers, in which a timing mechanism had been incorporated. It consists of a wooden or plastic block with holes for the drugs which are arranged in two circles. The block is covered by a plastic top with two holes for the removal of tablets. They are arranged in such a way that only one hole releases a tablet at any one time. The cover is attached to the block by means of a central bar. A spring-lock mechanism will permit the cover to move only in one direction, one notch at a time after a button has been pressed and in total not more than one full turn. The days of the week are printed around the main block corresponding to the notches for medication.

In the centre of the block a 16 mm photographic film, protected from light, is fitted into a cylindrical depression. Attached to the cover is a source of radioactive material containing approximately 0.1 microcuries of chlorine-36, sealed in plastic to a depth of 1 mm. With each movement of the cover a different section of the photographic film is exposed to the radioactive source.

The dispenser is set up for patients taking a drug according to the calendar markings at a certain time once a day. In this particular case the cover with its radiation source will advance daily and the film will be exposed for the same length of time in each position, producing uniform spots in the developed film. If the patient does not take the drug on a certain day, one spot will naturally receive an excess amount of radiation; whereas the portion of the film passed over quickly in

order to catch up to the proper day of the week will remain almost unexposed.

According to MOULDING [41] the radioactive material used in this recording system does not present a health hazard to the patient.

This device is certainly superior to mere pill counting. It requires, however, staff for filling the device and developing the film. Once the patient understands the mechanism of this time-recording pill dispenser, he can easily defeat its purpose if he so wishes. It may therefore be helpful if periodic urine tests are performed, as these will be useful in checking and interpreting the photographic records.

3. Tracer Substances

Tracer substances would be most valuable for the control of drug administration in chronic diseases, but the properties required of such tracers are so complex that it is difficult to find a substance which will satisfy all requirements. It should not be toxic during prolonged administration, nor produce unpleasant side effects. It must be palatable, readily absorbed and easily detected in low concentration in the urine; yet not be interfered with by foodstuffs or commonly used drugs. It should not counteract the accompanying antituberculosis drug, antagonize or decompose it, or even aggravate the disease itself. It should be relatively inexpensive. Dyes such as methylene blue [28] may distress the patient by discolouring his urine and clothes.

HOBBY and DEUSCHLE [31] used riboflavin-tagged isoniazid for control of isoniazid medication. The reliability of employing this tracer substance has been further confirmed by DEUSCHLE [13] and HUEMPHNER *et al.* [34]. The riboflavin tracer can also be combined with other antituberculosis drugs. One disadvantage of this tracer substance is that vitamin preparations administered in large doses may contain riboflavin in interfering quantities. Furthermore, the method depends on a relatively costly photofluorometer and technically skilled personnel to operate it [34]. On the other hand, it provides us with a method suitable for drug investigation in countries where equipment and trained staff are readily available and where the cost of tracer substances can be afforded. Such a procedure has the advantage that the detection period of the tracer substance in the urine can be adjusted according to the plan of investigation by augmenting or decreasing

the riboflavin content of the tablets. This cannot be done with the antituberculosis drug itself when given in therapeutic doses to the patient and thus we may find that the laboratory methods available for the particular drugs are either too sensitive (giving a positive result for a period of over 30 hours) or not sensitive enough (yielding positive reactions for too short a time only after drug administration).

III. Specific Procedures

1. Detection of Pyrazinamide or its Metabolite in the Urine

A. Determination of Pyrazinoic Acid

Pyrazinamide is excreted in the urine unaltered as well as in hydrolyzed form as pyrazinoic acid. The method of ALLEN *et al.* [1] determines pyrazinoic acid. This substance forms with crystals of Mohr's salt an orange-red colour at pH 4–7 with an optical density maximum at 460 mμ.

Reagents. 1. Sulphuric acid solution 50%; 2. Mohr's salt (Ferrous ammonium sulphate crystals); 3. Saturated lead acetate.

Procedure. To 10 ml urine, 1 ml saturated lead acetate is added and shaken in order to remove the interfering urochromes. After centrifuging this mixture and decanting the supernatant, 8 ml of the clear solution is treated with 0.1 ml of 50% sulphuric acid to remove the excess lead. The insoluble lead sulphate will precipitate and has to be removed by centrifugation. The solution is then divided into two aliquots, each containing 3 ml. The acidity of the first aliquot is adjusted with the help of indicator paper to a pH between 4 and 7. A few crystals of Mohr's salt are added to this solution which produces an orange-red colour in the presence of pyrazinoic acid.

According to ALLEN *et al.* [1] pyrazinamide is to a large extent hydrolyzed in the body. This procedure could therefore be used for checking pyrazinamide ingestion. If, however, the amount of pyrazinoic acid is insufficient to give positive reaction in the above test, then the second aliquot (3 ml) may be used for hydrolysis of the unaltered pyrazinamide. By adding one drop of 50% potassium hydroxide to the urine sample and heating the tube for one hour at 100° C. pyrazinamide will be broken down to pyrazinoic acid and can be tested as above.

B. Pyrazinamide Spot Test

CACCIA [8] reported in 1957 the use of sodium nitritopentacyanoferroate for the quantitative estimation of pyrazinamide in biological fluid. The method, however, was not described. In our experience, sodium nitritopentacyanoferroate reagent $Na_4[Fe(CN)_5NO_2]$ proved to be very reliable for the detection of pyrazinamide and was therefore incorporated in our procedures [47].

Reagent. Sodium nitritopentacyanoferroate (SNP) reagent: solution of 1% sodium nitroprusside $Na_2[Fe(CN)_5NO].2H_2O$ in 1 *N* sodium hydroxide.

The reagent can be stored for only one hour. If longer storage of the reagent is required a 2% aqueous solution of sodium nitroprusside should be prepared and freshly mixed with an equal volume of 2 *N* sodium hydroxide prior to the test. The aqueous solution may be stored for one or two weeks in a dark bottle at 4° C.

Procedure. It is recommended that the spot test should be performed in a white porcelain plate with hemispherical depressions so that results can be read easily and a number of urine specimens conveniently examined at the same time.

Three drops of urine and one drop of reagent are placed in a depression of the plate and mixed by moving the plate gently back and forth on the work bench. In the presence of pyrazinamide the bright yellow colour of the reagent changes to orange in three minutes.

Remarks. In mass examinations, the urine may be stored and the tests performed once a day. To facilitate the preparation of SNP reagent, finely ground sodium nitroprusside may be distributed in either gelatine capsules or small paper envelopes containing amounts of 50 mg. When a fresh reagent is required the content of a capsule or envelope is poured into 5 ml of 1 *N* sodium hydroxide and dissolved. 1 *N* sodium hydroxide is commercially available in sealed polyethylene tubes or it can be prepared by dissolving 4 g of sodium hydroxide pellets in 100 ml of distilled water. Before preparation of the alkaline solution, the distilled water should have been boiled, cooled and hermetically sealed, to keep it carbonate-free.

If the test is to be performed several times in a day, e.g., during home visits in which only a few tests will be made, a preparation of 2% aqueous solution of sodium nitroprusside may be preferred. From this solution the SNP reagent can be made available instantly in the required amounts.

C. Acetest Tablet[1] *for Detection of Pyrazinamide*

PINES and RICHARDSON [44] reported the use of Acetest tablets for the detection of pyrazinamide in the urine. These tablets contain sodium nitroprusside, aminoacetic acid and disodium phosphate.

[1] Product of Ames Co.; Stocke Pages U.K. for detection of ketone bodies in urine.

Procedure. One Acetest tablet is placed on a clean white tile and one drop of urine is added to the surface of the tablet allowing it to be absorbed. In the presence of pyrazinamide a dull pinkish brown colour develops within a period of a few seconds up to two minutes.

If the urine is not fresh, the colour reaction may require up to ten minutes. The pinkish brown colour of pyrazinamide is quite different from the purple chromogen produced by acetone and can therefore be distinguished easily from the latter.

2. Detection of Cycloserine in the Urine

A. Tube Test

This procedure was introduced by JONES [36] for the determination of cycloserine in body fluids. This simple method may also be used as a qualitative test. The reagent develops a blue colour with cycloserine under acidic conditions.

Reagents. 1. 4% sodium nitroprusside solution in distilled water; 2. 4 *N* sodium hydroxide; 3. 3 *N* acetic acid.

Sodium nitritopentacyanoferroate reagent is prepared by mixing an equal volume of reagents 1 and 2. This solution should be used within fifteen minutes of preparation.

Procedure. 3 ml of urine are pipetted into a test tube, followed by 1 ml of 3 *N* acetic acid and 1 ml of sodium nitritopentacyanoferroate reagent. If less than 3 ml of urine are used, water should be added to bring it up to this volume. In the presence of cycloserine a blue colour develops. Urine with high pigmentation and a low concentration of cycloserine may be decolourized by activated charcoal. According to CACCIA [8], charcoal absorbs pyrazinamide but does not influence cycloserine. In the qualitative test, however, the colour of the urine practically never interferes.

B. Cycloserine Spot Test

Reagents. 1. SNP reagent: a fresh solution of 1% sodium nitroprusside in 1 *N* sodium hydroxide. This solution is prepared as instructed for the pyrazinamide spot test (see page 8); 2. 1 *N* hydrochloric acid solution.

Procedure. In a semi-circular depression of a white porcelain tile, three drops of urine are placed, followed by one drop of SNP reagent. After mixing the urine with the reagent, two drops of 1 *N* hydrochloric

solution are added. In the presence of cycloserine a blue colour appears.

3. Tests for the Combined Detection of Pyrazinamide and Cycloserine

These tests determine the presence of both antituberculosis agents in the same urine sample by a joint procedure. As shown above, the same reagent, viz., sodium nitritopentacyanoferroate, produces chromogens with both pyrazinamide and cycloserine. This observation was used by KRAUS *et al.* [39] for the introduction of a paper strip test for the simultaneous detection of pyrazinamide and cycloserine. The colour reagent is incorporated in a filter paper and striped afterwards with a solution of 20% citric acid. The alkaline sodium nitroprusside reacts directly with pyrazinamide, while the acidic lines produce a blue colour with cycloserine.

BJOERNESJOE [7] and later RAO *et al.* [47] have shown that the development of the chromogens in the presence of organic acid is a more complex process, since not only cycloserine reacts under this condition with the SNP reagent, but pyrazinamide also forms a pink to red colour. In the presence of both antituberculosis compounds a violet colour appears as a mixture of blue and red. The chromogen of pyrazinamide can be eliminated from the mixture by adding mineral acid to it [47]. This means that the use of mineral acid renders the test for cycloserine more specific. Based on this finding RAO *et al.* [47] introduced a combined spot test which selectively detects pyrazinamide and cycloserine in consecutive steps.

Another sodium nitroprusside derivative, sodium pentacyanoaminoferroate $Na_3[Fe(CN)_5NH_3]$ was also introduced for the combined detection of antituberculosis drugs. This reagent was originally studied by FEIGL [20] for qualitative spot tests. Investigations by SCARDI [53], SCARDI and BONAVITA [54, 55] as well as PICARD [45], showed that this compound exhibits colour reactions with isoniazid, cycloserine and ethionamide. These findings were utilised by HOLIČEK and HERLIK [33], to develop a rapid screening test for the detection of pyrazinamide, cycloserine, isoniazid and ethionamide in the urine. According to the authors, these drugs produce in this test different colours and the antituberculosis compounds can even be recognized in various combinations. Thus one single reaction may verify whether the patient had been taking the prescribed medication.

A. Paper Strip Test [39]

Reagents. 1. Alkaline sodium nitroprusside: 1 g of sodium nitroprusside dissolved in 100 ml of 4% potassium hydroxide; 2. Solution of 20% citric acid in distilled water.

Preparation of the strips. Filter paper impregnated with freshly prepared alkaline sodium nitroprusside solution is dried at 50–60° C. Thick (4–6 mm) parallel lines are drawn 6–8 mm apart on the impregnated filter paper with the 20% citric acid solution. After drying, the lines of citric acid turn light grey. The dry filter paper is cut into strips perpendicular to the citric acid streaks.

Procedure. The strips should be dipped for a few seconds into the urine which is to be tested. In the presence of pyrazinamide, the yellow colour of the filter paper changes in 2–10 min to a red colour. A positive reaction for cycloserine is indicated by the grey transverse lines turning blue within an interval of 2–10 min. In the absence of cycloserine, the grey lines will fade away.

B. Combined Spot Test [47]

This method determines the presence of pyrazinamide and cycloserine in three stages. The first stage detects pyrazinamide; the second stage confirms it and indicates cycloserine; and the third one verifies cycloserine and differentiates it from pyrazinamide.

Reagents. 1. SNP reagent-fresh solution of 1% sodium nitroprusside in 1 *N* sodium hydroxide prepared as instructed for the pyrazinamide spot test (see page 8); 2. 6 *N* acetic acid prepared by mixing 1 part of glacial acetic acid with 2 parts of distilled water. This solution remains stable for several months; 3. 1 *N* hydrochloric acid

Procedure. First stage: In a semicircular depression of a porcelain tile, three drops of urine are placed by means of a Pasteur pipette followed by one drop of SNP reagent. The tile should be shaken gently to mix the two components. In the presence of pyrazinamide, the bright yellow colour of the reagent changes to orange in about 2–3 min. At this stage no other antituberculosis drugs produce chromogens.

Second stage: By adding one drop of acetic acid, the orange colour of pyrazinamide changes to pink within a few seconds. If both pyrazinamide and cycloserine are present in similar amounts, a violet colour develops. A blue color indicates cycloserine alone, or in such a high concentration as to mask the pink colour of pyrazinamide.