PROGRESS IN MEDICAL LABORATORY TECHNIQUE – 2

F. J. BAKER

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PROGRESS IN
MEDICAL LABORATORY
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2

Edited by

F. J. BAKER

F.I.M.L.T., F.I.S.T., F.R.M.S.

Chief Technician, Department of Pathology, Brompton Hospital; Associate Lecturer in Bacteriology, Paddington Technical College, London

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PREFACE

It was very gratifying to find such a good response to the first *Progress in Medical Laboratory Technique* and it is hoped that this, the second number in the series, will be as well received.

The format of the book now seems to fall into a definite pattern. Techniques of some of the newer methods have been tried, and when found practical have been included in this book—many of them given in detail. With other subjects, a more broader view has been taken, and details of techniques omitted. Some of the material included is not new, but because of its omission in many standard textbooks it was felt that its inclusion in this book was justified.

The inclusion of a subject such as extracorporeal circulation was also felt to be warranted as in many hospitals today the routine laboratories are becoming more and more involved in these operations, and that an account of the type of operations performed would be of great interest.

I would like to thank all those who were most helpful in their constructive comments on the first of this series, and hope that similar comments will help to maintain the standard of subsequent issues. I would also like to thank the contributors, whose high standard made my task easier, and of course our publishers who also helped to make my Editorship a pleasure to perform.

F. J. B.

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CHAPTER 1

ADVANCES IN CHEMICAL PATHOLOGY

D. Kilshaw

INTRODUCTION

This chapter describes in detail some of the chemical pathology techniques and methods which have recently become available for use in routine laboratories.

ANALYSIS OF PORPHYRINS IN URINE

δ-Amino-laevulinic acid (ALA), a precursor of porphobilinogen (PBG), is excreted in the urine in increased amounts in lead intoxication, even though the excretion of PBG is normal. The estimation of both compounds has become important in the investigation of abnormal porphyrin metabolism. ALA excretion is indeed a more sensitive indicator of lead poisoning than urinary coproporphyrin estimation or basophilic stippled cell counts (Haeger, 1960). Both of these tests are less tedious technically than that of urinary lead, and are thus preferential routine procedures. The urinary excretion of ALA is also raised in acute porphyria (Ackner and colleagues, 1961), porphyria cutanea tarda and some forms of porphyria occurring in South Africa (Haeger, 1960). These are the only diseases in which the urinary ALA is known to be raised, but in these porphyrias ALA is always accompanied by PBG increase.

Quantitative Determination of δ-Amino-Laevulinic Acid and Porphobilinogen in Urine

Principle

The PBG is separated from ALA on an ion-exchange column; the PBG is estimated directly with Ehrlich's reagent and ALA with the same reagent after condensation with acetyl-acetone to form a monopyrrolic compound (Mauzerall and Granick, 1956; Haeger, 1960; With, 1961).

Reagents

(1) Dowex 2-x8, 200–400 mesh.* The resin is placed in water, stirred well and the supernatant decanted until clean. This removes all the

* V. A. Howe Ltd., 46 Pembridge Rd., London W. 11.

finer particles. It is then converted to the acetate form by placing in a chromatography column and washing with 3·0 M sodium acetate, until the eluate is chloride free (test with $AgNO_3$). After washing the column with water until the washings are neutral, the resin is stored in twice its own volume of water in a closed container.

(2) Dowex 50-x8, 200–400 mesh. The finest particles are separated off as before, and the resin converted to the sodium form by allowing it to stand overnight in twice its own volume of 2 N NaOH. After decanting the supernatant fluid, the resin is stirred with twice the volume of distilled water, using a mechanical stirrer. This process is repeated until the decanted washings are neutral. The resin is reconverted to the acid form by treating it with 1 volume of 4 N HCl and 6 volumes of 2 N HCl, 1 N HCl and distilled water. It is stored in twice its own volume of distilled water. Both resins will keep for at least 3 months at room temperature but, because of bacterial growth resulting in considerably lowered values, it is essential to wash the resins with distilled water before use.

(3) Acetic acid 1 N and 0·2 N.

(4) Sodium acetate 0·5 M.

(5) Acetate buffer pH 4·6. 57 ml glacial acetic acid and 136 g sodium acetate trihydrate ($CH_3COONa.3H_2O$) diluted to 1,000 ml with distilled water. Check pH.

(6) Acetyl-acetone (redistilled).

(7) Ehrlich's reagent I. 2 g of *para*-dimethylaminobenzaldehyde AR in 100 ml 6 N HCl.

(8) Ehrlich's reagent II. 1 g of *para*-dimethylaminobenzaldehyde AR is dissolved in 30 ml glacial acetic acid and 8 ml 70 per cent perchloric acid AR. When completely in solution transfer to 50 ml volumetric flask and dilute to the mark with glacial acetic acid. This solution should be prepared fresh. The perchloric acid in this reagent is 2 N and should never exceed 4 N as spontaneous decomposition will occur.

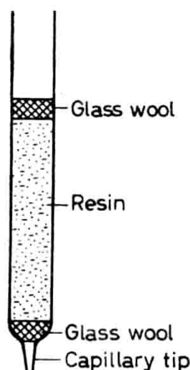

Figure 1. Chromatography column

Apparatus and preparation of columns

(1) SP 600 spectrophotometer or similar instrument.

(2) Chromatography columns of 1 × 10 cm dimension are required. These can easily be constructed from a 25 ml burette or from glass tubing, by drawing out one end (plugging with glass wool to hold the resin) and attaching a plastic burette tap* to regulate the rate of flow. A slurry of resin is added to give a $2 \pm 0{\cdot}1$ cm of settled material. To prevent the resin being disturbed during the addition of fluids, another glass wool plug is placed on top of the column. Adjust the rate of flow to about 6 drops per min and wash the resin with about 25 ml of distilled water before use.

Stability of porphobilinogen and δ-amino-laevulinic acid

PBG is labile and therefore the analysis should be carried out on a fresh specimen. However, PBG can be preserved at pH 7–8 by collecting the specimen in a dark bottle containing sodium carbonate and keeping at a temperature of approximately 4°C. ALA keeps for at least 20 days at pH 4–6 and at a temperature of above 4°C. If a fresh specimen of urine cannot be obtained the above precautions must be adopted.

Method

One ml of urine or suitable aliquot, adjusted to pH 5–7, is placed on to a Dowex 2 column. The column is washed with 2 × 2 ml of distilled water and the combined eluates containing ALA and urea are collected (A). PBG is then eluted from the column by adding 2 ml 1 N acetic acid, allowing the column to drain and then adding 2 ml 0·2 N acetic acid. The combined eluates are transferred to a 10 ml volumetric flask and diluted to the mark with distilled water. A 2 ml aliquot of PBG solution is treated with 2 ml of Ehrlich's reagent II and the colour allowed to develop for 15 min. Two ml of distilled water plus 2 ml Ehrlich's reagent II is used as a blank. The optical density is measured at 555 mμ, using cells of 1 cm light path. If the test is positive, a pink to cherry red colour rapidly develops. The combined eluate (A) is transferred to a Dowex 50 column and the urea eluted with 30 ml distilled water (checked by testing eluate with equal volume of Ehrlich's reagent I, yellow colour obtained with urea). After adding 3 ml of 0·5 M sodium acetate the ALA is eluted with 7 ml acetate buffer pH 4·6, collecting the eluate into a 10 ml volumetric flask. 0·2 ml acetyl-acetone is added and the solution diluted to the mark with acetate buffer pH 4·6. Mix well, transfer contents to a 15 ml stoppered test tube and place in a boiling water bath for 10 min and then cool to room

* Townsen and Mercer.

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temperature. As a blank, 7 ml 0·5 M acetate buffer is treated in the same way as the eluate from the Dowex column. A 2 ml aliquot of ALA solution is estimated colorimetrically as described above, using 2 ml of Ehrlich's reagent II.

Calculation

Porphobilinogen—As PBG is not available in a relatively pure state the relationship of optical density to concentration cannot be easily checked. According to Mauzerall and Granick (1956), optical densities above 0·700 do not obey Beer's law. Thus, when the optical density is below 0·700, assuming molar extinction coefficient = $6{\cdot}1 \times 10^4$ (using 2 N $HClO_4$)

$$1 \text{ mMol}/100 \text{ ml} = \frac{6{\cdot}1 \times 10^4}{10^2} = 610$$

$$\text{i.e.} \quad 226 \text{ mg}/100 \text{ ml} = 610 \text{ (mol. wt. of PBG} = 226)$$

$$\text{i.e.} \quad 1 \text{ mg}/100 \text{ ml} = \frac{610}{226}$$

Urine is diluted 20 times

$$\therefore \text{ mg of PBG}/100 \text{ ml urine} = OD \times \frac{226}{610} \times 20 = OD \times 7{\cdot}4$$

δ-Amino-laevulinic acid—The relationship of optical density to concentration is linear for an optical density up to about 0·8. This can easily be checked for the instrument in question by calibrating with concentrations of ALA up to 5 mg/100 ml using ALA HCl.* Ten mg ALA HCl = 7·82 mg ALA. Thus, if Beer's law is obeyed, assuming molar extinction coefficient = $5{\cdot}52 \times 10^4$ (using 2 N $HClO_4$)

$$1 \text{ mMol}/100 \text{ ml} = \frac{5{\cdot}52 \times 10^4 \times 10}{10^3} = 552$$

$$\text{i.e.} \quad 131 \text{ mg}/100 \text{ ml} = 552 \text{ (mol. wt. of ALA} = 131)$$

$$\text{i.e.} \quad 1 \text{ mg}/100 \text{ ml} = \frac{552}{131}$$

Urine is diluted 20 times

$$\therefore \text{ mg ALA}/100 \text{ ml urine} = OD \times \frac{131}{552} \times 20 = OD \times 4{\cdot}74$$

Normal range (after Haeger, 1960)

ALA in mg/100 ml urine = 0·01–0·57, mean 0·29
PBG in mg/100 ml urine = 0·00–0·20, mean 0·10

* L. Light and Co. Ltd.

Note—With (1961) pointed out that to obtain greater accuracy in weaker solutions, the optical density is best compared in cells of 4 cm light path, because a normal excretion of PBG (0·1 mg/100 ml) would be only equivalent to an optical density of 0·008 in a cell of 1 cm light path.

In the PBG estimation, if the urine is preserved with sodium carbonate, the effervescence which occurs during the addition of the acetic acid to the column will temporarily disturb the resin until the CO_2 has been liberated. The resin will then settle down to its normal position.

SCREENING TEST FOR PORPHYRINS IN URINE

A number of methods have been described for the quantitative estimation of porphyrins in urine, most of which involve the use of organic solvents for extraction. These techniques are not very adaptable as a routine screening test, for many urine specimens are often highly coloured because of the increasing use of a wide variety of drugs. These pigmented urines absorb light in the visible and near ultra-violet region of the spectrum as can be shown by the high optical density readings obtained by the direct spectrophotometric technique of Rimington and Sveinsson (1950). One way of overcoming the difficulty of estimating porphyrins in pigmented urines is to use an anion-exchange resin.

Dowex Column Method

Principle

Urine is treated with iodine solution to oxidize the colourless porphyrinogens to porphyrins and then allowed to pass through an anion-exchange column. After washing the column with water, the porphyrins are eluted with 3 N HCl and estimated quantitatively in the spectrophotometer (Mills, 1961).

Reagents

(1) Dowex 1-x8 anion-exchange resin (20–50 mesh, chloride form) is used without any further treatment.

(2) 3 N HCl.

(3) 0·02 per cent iodine solution, prepared freshly.

Apparatus

(1) SP 600 spectrophotometer or similar instrument using cells of 1 cm light path.

(2) Chromatography columns of 1×15 cm dimension. These can be constructed as previously described on page 3.

Collection of Specimen for Analysis

For routine screening tests, the analysis of a single urine specimen would be justifiable, but it is better to collect a 24 h specimen into a dark bottle containing 5 g of sodium carbonate as preservative.

Method

The Dowex resin is added to the column which is partially filled with water until a height of 6 cm of settled material is obtained. A glass wool plug is placed on top of the resin adjusting the rate of flow so as not to exceed 4 ml/min. Due to the difficulty in removing urinary pigments from the resin even with conc. HCl a fresh resin bed is prepared each time. To 5 ml of urine add 0·5 ml of freshly prepared iodine solution. After mixing well, the mixture is added to the column and allowed to pass through the resin. The column is washed with 20 ml of water and, if the eluate is still coloured, continue washing with more water until the eluate is colourless. The washings are discarded and the porphyrins eluted with 30 ml of 3 N HCl. The eluate is well mixed and the optical density measured at 380, 403, 406 and 430 mμ. For most instruments the maximum absorption for coproporphyrin in 3 N HCl would be 403–404 mμ and for uroporphyrin 406–407 mμ, but this should be checked by taking extra readings to ensure the maximum absorption has been observed.

Calculation

To correct for impurities having a spectral absorption in the Soret band region the following formula is used (*see* Rimington and Sveinsson, 1950; With, 1955; Rimington, 1960; Mills, 1961).

If V = volume of eluate and U = volume of urine taken

(*1*) *Coproporphyrin excretion*

$$D\ \text{corr.} = 2D_{max} - (D_{380} + D_{430}) \times 806 \times V/U = \mu\text{g/l. of urine}$$

(*2*) *Uroporphyrin excretion*

$$D\ \text{corr.} = 2D_{max} - (D_{380} + D_{430}) \times 937 \times V/U = \mu\text{g/l. of urine}$$

where D_{max} is the highest optical density for either coproporphyrin or uroporphyrin. The factors 806 and 937 are derived in the following manner (Mills, 1961).

(*1*) *806.* 1μg of coproporphyrin in 3 N HCl has a maximum absorption of 0·674 in cells of 1 cm light path. Correction divisor $K = 1{\cdot}84$.

$$\text{Therefore } \mu\text{g coproporphyrin/ml} = \frac{D\ \text{corr.}}{1{\cdot}84 \times 0{\cdot}674}$$

$$= D\ \text{corr.} \times 0{\cdot}806$$

$$\mu\text{g coproporphyrin/l.} = D\ \text{corr.} \times 806$$

(*2*) *937*. Likewise 1 μg of uroporphyrin in 3 N HCl has a maximum absorption of 0·580 in cells of 1 cm light path.

For estimating total urinary porphyrins, neither of these equations is applicable, since one is dealing with mixtures of porphyrins.

Results

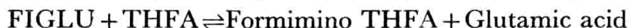
Normal values for coproporphyrin range from 60–280 μg/day (Watson, 1959) and uroporphyrin values from 5–30 μg/day (Schwartz, 1953; Lockwood, 1953; Dresel, Rimington and Tooth, 1956).

Note—(1) If the urine is preserved with sodium carbonate, effervescence will occur when the acid is added to the column, thereby disturbing the column temporarily until all the CO_2 has been liberated when the resin will settle down to its normal position. (2) This technique will probably eliminate about 95 per cent of the urine specimens from further consideration. However, if the results obtained fall outside the ranges quoted, it is essential that more accurate estimations should be carried out by using a technique described by Rimington (1961). If urinary pigments are present in the specimen they must be removed by the Dowex resin beforehand.

DETERMINATION OF FORMIMINOGLUTAMIC ACID IN URINE

The investigation of folic acid deficiency was until recently carried out by a time consuming bio-assay of folic or tetrahydrofolic acid in serum or urine. The introduction of electrophoretic methods for the detection of formiminoglutamic acid (FIGLU) in urine now enables folic acid deficiency to be more easily investigated in a routine laboratory.

FIGLU is a metabolite of histidine which is normally converted to glutamic acid by reacting with tetrahydrofolic acid (THFA)



$$\text{FIGLU} + \text{THFA} \rightleftharpoons \text{Formimino THFA} + \text{Glutamic acid}$$

In folic acid deficiency, THFA is unavailable for formimino group transfer and therefore FIGLU accumulates in the urine. It may be detected in the urine of such patients, directly or after a loading dose of histidine monohydrochloride. Since the histidine loading technique is quantitative and the direct method is in the process of being developed as a quantitative method, it is intended to give a description of both procedures in detail.

Estimation of FIGLU after Histidine Loading

Principle

Following an oral load of histidine monohydrochloride, an aliquot of the patient's urine is treated with alkali, thus converting the

FIGLU to glutamic acid. The glutamic acid is separated from other amino acids by electrophoresis on cellulose acetate paper and located by spraying with ninhydrin. It is estimated quantitatively by comparison with standards similarly treated (Kohn, Mollin and Rosenbach, 1961; Zalusky and Herbert, 1962a; Hayward, 1962).

Reagents

(1) Pyridine-acetic acid buffer pH 5·4. Pyridine 50 ml glacial acetic acid 20 ml made up to 4 l. with distilled water.

(2) Ninhydrin location reagent. 200 mg ninhydrin dissolved in 6 ml ethanol and 94 ml diethyl ether.

(3) 2·5 N potassium hydroxide.

(4) 2·5 N hydrochloric acid.

(5) Standards are prepared by adding known amounts of FIGLU or *L*-glutamic acid (ranging from 50–1,000 μg/ml) to normal acidified urine. FIGLU in acidified urine will keep for many months at 4°C if preserved with a few crystals of thymol.

(6) Solvent for dissolving the cellulose acetate strips. Chloroform 90 parts ethanol 10 parts v/v.

Materials

(1) Oxoid cellulose acetate strips* 36 × 5 cm. These are cut into 12 × 5 cm strips for use.

(2) Shandon Universal electrophoresis apparatus with a bridge of 10 cm.

(3) Power supply to deliver 300 V and at least 25 mA.

Preparation of patient

The patient must fast overnight. In the morning give 15 g of *L*-histidine monohydrochloride by mouth (Kohn, Mollin and Rosenbach, 1961), washing down all the histidine with more water. After giving the histidine, the patient must not eat for another hour. Three hours after giving the dose, the bladder is emptied and the urine discarded. During the next 5 h, the urine is collected into a bottle containing 1 ml concentrated hydrochloric acid and a few crystals of thymol. The volume is measured and an aliquot taken for electrophoresis. The urine is kept frozen or at 4°C.

Procedure

Test—To 0·3 ml of urine add 0·1 ml of 2·5 N KOH, mix well and allow to hydrolyse at room temperature for 2 h, then add 0·1 ml 2·5 N HCl. Mix well.

Blank—To 0·3 ml of urine add 0·2 ml distilled water. Mix well.

* Oxo Ltd., London.

Electrophoresis

A 12 × 5 cm cellulose acetate strip is marked as shown with a soft pencil after examining for the absence of ridges, oblique lines and blotches.

The strips are impregnated by floating them on the surface of the buffer in a shallow dish. After letting the buffer soak in from underneath, the strip is finally submerged. A quick immersion will trap air and prevent complete saturation. The excess buffer is blotted off lightly between sheets of filter paper. Too heavy a blotting will create opaque areas. The strip is placed in the electrophoresis tank and pulled taut. Ten µl. are applied to the same point of the strip in several successive aliquots, thereby keeping the area at the point of application as small as possible. An aliquot of urine containing glutamic acid is put up at the same time to serve as a marker.

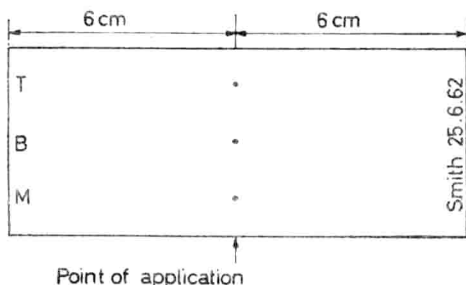


Figure 2. Markings on a cellulose acetate strip. T, test; B, blank; M, marker

The strips are pulled tight and the lid replaced. A current of 200 V and 6–8 mA is applied for 30 min. The time may vary, and must be established for the apparatus being used. The strips are dried at 80–100°C for 10–15 min.

Staining

The strip is passed slowly through the ninhydrin solution in a small dish, then immediately placed between two sheets of filter paper lying between two pieces of cardboard. The pieces are clipped together with bulldog clips and heated at 90–100°C for 5 min. A longer time of heating will cause a background colour to appear. The strip is removed from the cardboard and left for 30 min (*Figure 3*).

The glutamic acid spot in both the test and blank is cut out in equivalent areas, dissolved in 2·5 ml of chloroform/ethanol mixture

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