MICROANALYSIS IN MEDICAL BIOCHEMISTRY

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FIFTH EDITION

With 32 Illustrations

CHURCHILL LIVINGSTONE

Edinburgh and London 1974

CHURCHILL LIVINGSTONE

Medical Division of Longman Group Limited

Distributed in the United States of merica by Longman Inc., New York, and by associated compames, branches and representatives throughout the world.

First Edition	1946
Reprinted	1947
Spanish Translation	1948
Italian Translation	1951
Second Edition	1951
Serbo-Croat Translation	1953
Third Edition	1956
Reprinted	1959
Italian Translation	1958
Arabic Translation	1960
Fourth Edition	1964
Fifth Edition	1974

ISBN 0 443 01022 6
Library of Congress Catalog Card Number
73 – 94318

Printed in Great Britain by
Willmer Brothers Limited, Birkenhead

Preface

During the ten years that have passed since the last edition of this book, the subject has continued to advance at a fast pace. It has therefore been necessary almost completely to rewrite this edition so that it may once more describe our current practice in the Department of Chemical Pathology at the Royal Postgraduate Medical School. Inevitably, the size of the book increases with successive editions, but we have tried to limit this growth so that the book remains a practical laboratory manual.

One major change has been the treatment of automatic analysis, which has become universally adopted in all but the smallest laboratories. Descriptions of the familiar continuous flow apparatus and of discrete analysers are still included in a separate chapter, but applications to individual problems are now included as appropriate throughout the chapters describing the estimation of the various substances. We have however also included manual methods suitable for smaller numbers of tests, so that the book may be useful to a wide range of laboratories.

Other topics which have recently become more important include the investigation of electrolytes and acid-base status of the blood, the detection and estimation of drugs, the examination of proteins and lipids of the blood plasma and the use of thin layer chromatography, particularly in screening for inborn errors of metabolism. All these have received more detailed treatment than previously.

The units adopted throughout are the 'classical' ones, familiar to clinical chemists at the time of writing, although one knows that these will be replaced in the near future by the S.I. units based on the mole and the litre. A chapter has therefore been included explaining the new units and providing conversion tables and normal values so as to make the future change-over as easy as possible.

More than ever before, this book now depends on the contributions provided by my present and past colleagues at the Royal Postgraduate Medical School to whom I extend my grateful thanks. They are primarily responsible for the good features; for the errors and omissions, I must take the blame. The contributors include:

Dr. J. D. Acland (Electrolytes and Drugs)

Mr. M. Fisher (Faecal Analysis and Metabolic Studies)

Dr. K. Fotherby (Steroids)
Dr. Ruth Haslam (Organic Constituents and Function Tests)
Dr. Elizabeth Hughes (Thin Layer Chromatography)
Dr. B. Lewis (Lipids)
Dr. D. W. Moss (Enzymes and Automatic Analysis)
Miss Beryl Schumer (Specimen Preparation and Data Handling)
Dr. Brenda Slavin (Proteins)
Dr. R. Tupper (S.I. Units)
Dr. J. Whitfield (C.S.F. and Qualitative Tests)

I have received further essential, expert and cheerful help from Miss Claire Johnstone and Miss Frederika Phillips in the preparation of the manuscripts and from Miss Genevieve Leballeur, who drew the figures. Acknowledgement is made to the *British Medical Bulletin* for Figures 1 and 2 and to W. B. Saunders Ltd. for Figures 24 and 25.

London, 1974. I. D. P. Wootton

Contents

1
Normal or Reference Values

By normal value is meant the amount of a constituent present in the body fluid or excretion of a healthy human being. In fact, this amount varies over a range, and is affected by a number of factors, including age, sex, season, race, diet and genetic constitution. Thus, while most healthy persons can be included in a class having the accepted 'normal' amount, some individuals are found to show divergent figures. Such exceptional individuals may be entirely normal in all other investigated respects, and hence it is now recommended that the range should be termed 'reference' rather than 'normal'.

The values given in this chapter are taken from our own accumulated results and from the literature. In most cases, they apply to the methods of analysis given here, or to a closely similar procedure. In some instances, we have carried out comparisons between the method given and a method which was used when the standards for normal human beings was being determined. Blood values are for fasting persons.

At a very early stage in this work, Wootton, King and Maclean Smith (1951) and Wootton and King (1953) investigated about 80 normal adults aged 20 to 50 years. These workers found that it was not always possible to express their results as an average normal figure with a standard deviation because only in a minority of cases could the values be fitted by a symmetrical 'normal' distribution of Gaussian type (Fig. 1). Most of the remaining skewed distributions were satisfactorily fitted by lognormal curves (Fig. 2) of a type which has often been used for biological data (Gaddum, 1945a, 1945b). It is thus necessary to give these normal values in terms of a range, and we have chosen to calculate the range which encloses 90 per cent of the population (Table 1) so that the chances of a normal individual falling below the lower limit of the range is 1 in 20—a conventional statistical chance. Similarly, there is the same chance that a normal person will have a value above the upper limit. Other tables of normal values are included in Tables 2 and 3, constructed from other data not suitable for detailed mathematical treatment in which an attempt has been made to estimate ranges with the same significance as the calculated ones. Further information is often given in the individual sections for each substances.

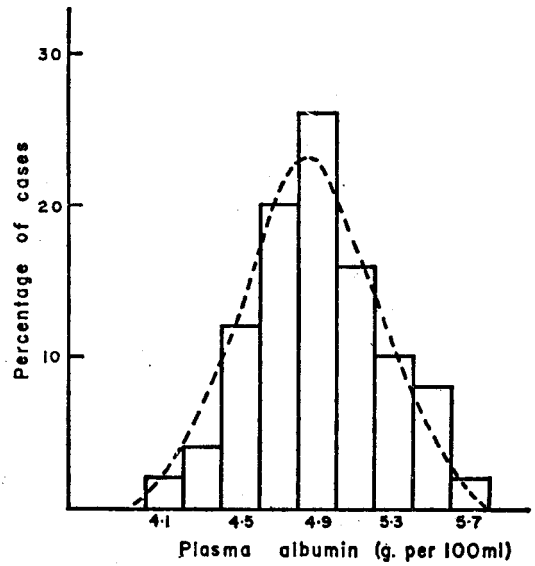

Figure 1. Distribution of serum albumin levels in the population. This is a symmetrical Gaussian distribution.

Variations in health and disease

In many cases, blood levels of certain constituents are raised (or occasionally lowered) as a manifestation of a disease state. Such changes are used for diagnostic and prognostic purposes and their detection makes up a large part of routine clinical chemistry (Table 4). The extent of the deviation from the normal range which is needed to justify a confident diagnosis of abnormalities depends, of course, on the stability of the normal figure. In this respect, blood constituents are not all the same. Thus serum calcium, for example, almost never falls outside the range 9 to 11 mg per 100 ml unless there is something amiss; similarly, a tartrate labile acid phosphatase higher than 0.8 K.A. units per 100 ml is almost diagnostic of prostatic carcinoma, provided a suitable serum specimen is used. On the other hand, constituents such as glucose and urea vary widely even in a single individual as a result of dietary and hormonal influences, and similar fluctuations during the day also occur in the levels of serum potassium and inorganic phosphate.

Control of laboratory accuracy

The relevance of reference values, and also their range, will depend

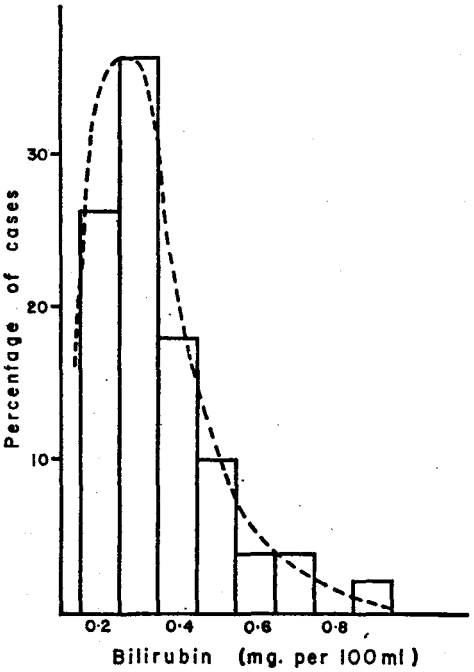

Figure 2. Distribution of serum bilirubin levels. This distribution is 'lognormal' and hence not symmetrical.

on the accuracy achieved by the laboratory. Repeated surveys performed by distributing identical samples to a number of laboratories have nearly always demonstrated a poor overall standard of laboratory performance (e.g. Wootton and King, 1953; Belk and Sunderman, 1947; Hendry, 1963). It is now generally accepted that a good standard of accuracy can only be maintained if a continuous effort is made and it is standard practice for a laboratory to monitor its own performance continuously by applying one or more quality control systems.

General laboratory practice

A good analyst is aware of the importance of his results and his procedures will be based on common sense and good scientific practice. Thus when setting up a new method in the laboratory, it is advisable to spend some time analysing sets of standard solutions, until one can define over what range the optical density (or other measurement) is linear with the concentration. Such repeated runs

TABLE 1. *Calculated normal values for blood, serum or plasma*

Sodium	mEq/l	136 to 149	Normal
Potassium	mEq/l	3.8 to 5.2	Lognormal
Chloride	mEq/l	100 to 107	Normal
Bicarbonate	mEq/l	24 to 30	Normal
Calcium	mg/100 ml	9.4 to 11.0	Normal
Magnesium	mEq/l	1.4 to 1.8	Normal
Inorganic phosphate	mg P/100 ml	2.8 to 4.2	Normal
Urea	mg/100 ml	14 to 38	Lognormal
Glucose	mg/100 ml	63 to 100	Normal
Creatinine	mg/100 ml	0.1 to 1.4	Lognormal
Bilirubin	mg/100 ml	0.1 to 0.5	Lognormal
Cholesterol	mg/100 ml	170 to 250	Lognormal
Total protein	g/100 ml	6.1 to 7.7	Normal
Albumin	g/100 ml	3.6 to 4.8	Normal
Fibrinogen	mg/100 ml	150 to 450	Normal
Alkaline phosphatase	K.A. units/100 ml	4 to 11	Lognormal
Total acid phosphatase	K.A. units/100 ml	1.1 to 3.5	Lognormal
Amylase	Units/100 ml	80 to 180	Lognormal

TABLE 2. *Approximate composition and 24-hour output*

Urine	*mg/100 ml*	*g/24 hours*
Water	–	1500
Urea	1500	25
Ammonia	50	0.8
Creatinine	130	2
Amino acids	40	0.6
Uric acid	25	0.4
Total nitrogen	1000	15
Phosphate (as P)	100	1.5
	mEq/l	*mEq/24 hours*
Sodium	130	200
Potassium	45	70
Calcium	7	10
Magnesium	10	15
Chloride	140	200

Faeces	*per 24 hours*
Wet weight	60 to 250 g
Dry weight	10 to 50 g
Fat	less than 5 g
Nitrogen	1.5 g
Phosphate (as P)	0.5 g
Sodium	3 mEq
Potassium	10 mEq
Calcium	30 mEq
Magnesium	10 mEq

TABLE 3. *Estimated normal values*

Blood, serum or plasma		
Tartrate labile acid phosphatase	K.A. units/100 ml.	0 to 0.8
Glutamic oxaloacetic transaminase	iu/l	2 to 20
Glutamic pyruvic transaminase	iu/l	2 to 15
Lactate dehydrogenase	iu/l	50 to 170
Hydroxybutyrate dehydrogenase	iu/l	40 to 100
5′-nucleotidase	iu/l	2 to 17
Serum iron	μg/100 ml	110 to 130
Total iron-binding capacity	μg/100 ml	250 to 400
Uric acid	mg/100 ml	2 to 7
Haemoglobin	g/100 ml	13.5 to 14.5
pH	at 38°	7.35 to 7.42
P_{CO_2}	mmHg	34 to 45
Cerebrospinal fluid		
Protein	mg/100 ml	15 to 45
Lange		0000000000
Glucose	mg/100 ml	50 to 70
Chloride	mEq/litre	120 to 126

will also show whether the results are reproducible from batch to batch and from day to day. After this, a number of specimens from normal subjects should be analysed to confirm the normal range of the method. In some instances, it will also be advisable to add known quantities of a constituent to normal serum, perform the analysis and calculate the 'recovery' to show that significant quantities are not being lost in protein precipitation or at some other stage of the method. Finally, if the method is intended to replace one already in use in the laboratory, it is necessary to make a careful comparison of the results produced by the old and new methods; several dozen comparisons would normally be sufficient. The reasons for serious discrepancies should be sought, and if the new results are significantly different from the old, it will probably be necessary to warn the clinical staff of the change in method.

Once the method has become routine, the main requirement is to maintain a satisfactory performance. A good practice is to renew standard solutions at intervals which are not too long and to compare old and new standards at each change. Occasionally a test on a specimen should be repeated on the following day and the results compared. A close eye should be kept on the general level of values found. In this respect, small changes in the apparent average value are much more easily detected if the analysis is a common one carried out in large batches which are to some extent self-checking. It is much more difficult to maintain acceptable accuracy if the analysis is only done occasionally, and it may help to include one or two known

TABLE 4. *Common variations of plasma constituents in disease*

Acid phosphatase	High in carcinoma of the prostate
Alkaline phosphatase	High in bone disease, obstructive jaundice, in children
Amylase	High in acute pancreatitis
Bicarbonate	High in metabolic alkalosis (pyloric stenosis, potassium depletion), respiratory failure
Bilirubin	High in jaundice
Calcium	High in hyperparathyroidism, invasive bone tumours, sarcoids, myelomatosis, carcinoma of the breast
	Low in tetany, parathyroidectomy, rickets, osteomalacia, malabsorption, renal failure, hypoproteinaemia
Cholesterol	High in obstructive jaundice, nephrotic syndrome, diabetes, pregnancy, myxoedema
	Low in thyrotoxicosis
Creatinine	High in renal failure
Glutamic-oxaloacetic-transaminase	High in cardiac infarction, hepatitis
Glutamic-pyruvic transaminase	High in hepatitis
Iron	Low in iron-deficiency anaemia
	High in haemochromatosis
Lactate dehydrogenase	High in cardiac infarction, hepatitis
5′-nucleotidase	High in obstructive jaundice
Protein	High in myelomatosis
	Low in nephrotic syndrome
Urea	High in renal failure, intestinal obstruction, cardiac failure, haematemesis
Uric acid	High in renal failure, gout

normal specimens with each unknown. Probably this should always be done in assays of enzymes, such as serum amylase. It is the activity of the enzyme which determines the enzyme units which are reported, and enzyme activity is especially sensitive to minor changes of conditions and the presence of unsuspected traces of inhibitors.

QUALITY CONTROL SYSTEMS

Several different types of system are in successful use. Many laboratories use a combination of types.

Internal control samples. This is probably the commonest practice. With each batch of unknown specimens is included a control sample (or more than one) which is put through the analytical process. The result obtained is compared with the known value of the control sample and the batch is only accepted if the result is acceptably close to the correct value.

Control samples may be prepared by the laboratory, e.g. by pooling sera together and carefully analysing them to assign the 'correct' value. Pooled sera, carefully mixed, dispensed into small volumes, stoppered and preserved in a frozen state at −20°C are often stable for many months but this procedure has become less popular with the realization that such human material may carry hepatitis virus. The alternative is to use commercial products which are now marketed with the 'correct' values already determined.

It is an advantage if the analyst concerned does not himself know the correct value, since otherwise he is subject to conscious or unconscious bias. It is therefore necessary to have several alternative control samples available from which the daily selection can be made.

The results are best plotted on a 'cusum' graph (Woodward and Goldsmith, 1964). For each analysis a fixed value is chosen, which is close to the expected result for the control sample but is not necessarily identical to it. The difference between the value obtained and the fixed value is calculated every time the control sample is analysed and this difference is added to the cumulative sum of differences (hence the name cusum) which has accumulated from the previous occasions. The new cumulative sum is then plotted (Fig. 3).

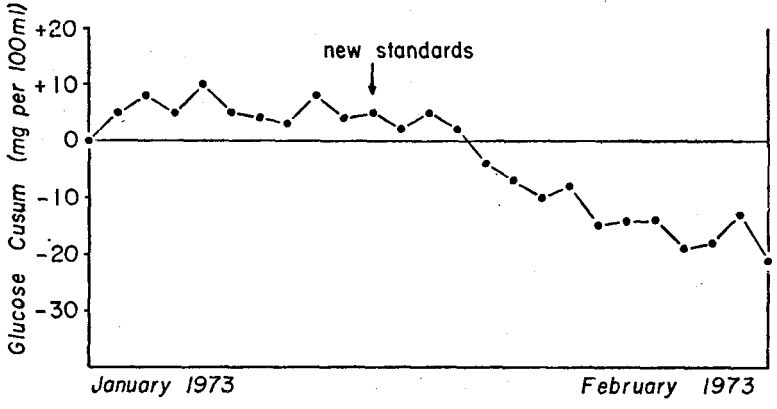

Figure 3. Cusum plot of routine glucose analyses.

A method which is working well is shown by a graph in which the individual points are close to a straight line which may ascend or descend uniformly. The slope of the line is related to the particular fixed value chosen and is irrelevant; what is important is any large deviation from the straight line or change in its slope. This graphical display will show up small but continuing disturbances (such as a slight deterioration in standard solution) which are not sufficiently

large in themselves to be detected by inspection of a single control sample result. The method also does not rely on an accurately-known true value for the control sample so that it is particularly useful for home-produced controls.

External control samples. This type of control is not sufficient in itself, but provides a useful support for a system based on internal samples. The external control samples are prepared by some outside body and circulated to the laboratories in their scheme at regular intervals. Commercial firms are often involved but sometimes the scheme is run by a national body such as the National Health Service of the United Kingdom.

The samples may be liquid or freeze-dried. In each case, results are reported by the participating laboratories and are collated by the organizers. Usually, the collected results are published to the laboratories concerned as a histogram, so that each participant can see how his result compares with those of the other laboratories and can thus gain an idea of whether his results are consistently higher or lower than the mode. Sometimes more elaborate calculations are performed on the results so that a 'variance index' can be produced to express the quality of the laboratory's results in a quantitative fashion.

An external comparison of different laboratory results is especially valuable in the case of enzyme assays, whch are so dependent on the reaction conditions and hence tend to vary widely from laboratory to laboratory.

Mathematical methods. When a laboratory workload increases until batches of at least 40 to 60 are analysed simultaneously, another method of quality control becomes available. It depends on the observation that, for any individual constituent, large batches of patients' results closely resemble each other, particularly if the very-grossly abnormal values are eliminated. This applies even when the values retained include moderately abnormal ones in addition to those falling within the normal range. It is a reflection of the fact that a very similar 'mix' of specimens is submitted each day to the busy laboratory and a correspondingly constant distribution of results can be expected if the analytical method is working satisfactorily.

In its most elementary form, this method involves noting all the laboratory results produced in a single batch (or on a single working day), deleting those few which fall outside a certain range ('truncation limits') and calculating the average of the results left. This 'batch mean' is surprisingly constant from day to day and will therefore provide a sensitive index of drift in the analytical procedure.

The batch mean to be expected and the appropriate truncation

limits must be established by experience for each laboratory. As guidance, the limits used in our own laboratories for certain common constituents are given in Table 5.

TABLE 5. *Truncation limits for batch means of plasma/serum constituents*

Sodium	mEq/l	90 to 180
Potassium	mEq/l	3.0 to 6.0
Bicarbonate	mEq/l	19.0 to 31.0
Urea	mg/100 ml	10 to 80
Creatinine	mg/100 ml	0.1 to 6.5
Total protein	g/100 ml	4.0 to 8.0
Albumin	g/100 ml	2.5 to 6.0
Calcium	mg/100 ml	4.5 to 12.0
Phosphorus	mg/100 ml	1.0 to 7.0
Uric acid	mg/100 ml	3.0 to 10.0
Cholesterol	mg/100 ml	80 to 300
Bilirubin	mg/100 ml	0 to 7.5
Alkaline phosphatase	K.A. units/100 ml.	2 to 25
Glutamic oxaloacetic transaminase	iu/l	1 to 50

A considerable amount of mathematical analysis of patient results has been undertaken and elaborate treatment of batch parameters has been recommended (Hoffmann, 1972; Hoffmann and Waid, 1965). At the present moment, anything further than the simple calculation outlined here is not widely accepted.

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