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Guidelines on Standard Operating Procedures for Clinical Chemistry

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Contents

Page

Foreword vii
Acknowledgements ix
Preface xi

SECTION A: GENERAL INTRODUCTION

1. Introduction 1
Quality manual 1
Standard operating procedures 2
Laboratory errors 3
Preparation of QC pool 5
Construction of Levey Jennings chart 7
External quality assessment 10

2. Introduction to SOP 12
Specifications 12

SECTION B: BIOCHEMICAL MEASUREMENTS IN PLASMA/SERUM

3. Glucose –Glucose Oxidase Method 15
Introduction 15
Principle of the method 15
Specimen type, collection and storage 15
Reagents 16
Equipment, glassware and other accessories 17
Procedure 17
Calculation and calibration graph 18
Analytical reliabilities 18
Hazardous materials 19
Reference range and clinical interpretation 19
Limitations 19
References 19

4. Urea –Diacetyl Monoxime Method 21
Introduction 21
Principle of the method 21
Specimen types, collection and storage 21
Reagents 21

Equipment, glassware and other accessories 22
Procedure 22
Calculation and calibration graph 23
Analytical reliabilities 24
Hazardous materials 24
Reference range and clinical interpretation 24
Limitations 24
References 25

5. Creatinine – Jaffe's Method 26

Introduction 26
Principle of the method 26
Specimen type, collection and storage 26
Reagents 26
Equipment, glassware and other accessories 27
Procedure 27
Calculation and calibration graph 28
Analytical reliabilities 29
Hazardous materials 29
Reference range and clinical interpretation 29
Limitations 29
References 30

6. Cholesterol – Cholesterol Oxidase Method 31

Introduction 31
Principle of the method 31
Specimen type, collection and storage 31
Reagents 31
Equipment, glassware and other accessories 32
Procedure 32
Calculation and calibration graph 32
Anaytical reliabilities 33
Hazardous materials 33
Reference range and clinical interpretation 33
Limitations 34
Reference 34

7. Bilirubin – Jendrassik and Grof Method 35

Introduction 35
Principle of the method 35
Specimen type, collection and storage 35
Reagents 35
Equipment, glassware and other accessories 36
Procedure 36
Calculation and calibration graph 37
Analytical reliabilities 39
Hazardous materials 40
Reference range and clinical interpretation 40
Limitations 40
Reference 40

8. Total Protein – Biuret Method 41

Introduction 41
Principle of the method 41
Specimen type, collection and storage 41
Reagents 41

Equipment, glassware and other accessories 42
Procedure 42
Calculation and calibration graph 43
Analytical reliabilities 43
Hazardous materials 44
Reference range and clinical interpretation 44
Limitations 44
Reference 44

9. Albumin - BCG Dye Binding Method 45

Introduction 45
Principle of the method 45
Specimen type, collection and storage 45
Reagents 45
Equipment, glassware and other accessories 46
Procedure 46
Calculation and calibration graph 47
Analytical reliabilities 48
Hazardous materials 48
Reference range and clinical interpretation 48
Limitations 48
Reference 48

10. Transaminases - Colorimetric End-point Method 49

Introduction 49
Principle of the method 49
Specimen type, collection and storage 49
Reagents 49
Equipment, glassware and other accessories 50
Procedure 50
Calculation and calibration graph 51
Analytical reliabilities 53
Hazardous materials 53
Reference range and clinical interpretation 54
Limitations 54
Reference 54

11. Alkaline Phosphatase - P-nitrophenol Method 55

Introduction 55
Principle of the method 55
Specimen type, collection and storage 55
Reagents 55
Equipment, glassware and other accessories 56
Procedure 56
Calculation and calibration graph 57
Analytical reliabilities 58
Hazardous materials 59
Reference range and clinical interpretation 59
Limitations 59
Reference 59

12. Calcium-O-Cresolphthalein Complexone Method 59

Introduction 59
Principle of the method 59
Specimen type, collection and storage 59
Reagents 59

Equipment, glassware and other accessories 60
Procedure 60
Calculation and calibration graph 61
Analytical reliabilities 61
Hazardous materials 62
Reference range and clinical interpretation 62
Limitations 62
Reference 62

13. Phosphorus - Stannous Chloride Reduction Method 63

Introduction 63
Principle of the method 63
Specimen type, collection and storage 63
Reagent 63
Equipment, glassware and other accessories 64
Procedure 64
Calculation and calibration graph 65
Analytical reliabilities 66
Hazardous reagents 66
Reference range and clinical interpretation 66
Limitations 66
Reference 67

14. Sodium and Potassium - Flame photometry 69

Introduction 69
Principle of the method 69
Specimen type, collection and storage 70
Reagents 70
Equipment, glassware and other accessories 71
Procedure 71
Calculation 71
Analytical reliabilities 72
Hazardous materials 72
Reference range and clinical interpretation 73
Limitations 73

SECTION C: BIOCHEMICAL MEASUREMENTS IN CSF

15. Cerebrospinal Fluid (CSF) 75

Introduction 75

16. CSF glucose –Glucose Oxidase Method 77

Principle of the method 77
Reagents 77
Equipment, glassware and other accessories 78
Procedure 78
Calculation and calibration graph 79
Analytical reliabilities 80
Hazardous materials 80
Reference range and clinical interpretation 80
Limitations 80
References 80

17. CSF Protein - Pyrogallol Dye Binding Method 81
Principle of the method 81
Reagents 81
Equipment, glassware and other accessories 82
Procedure 82
Calculation and calibration graph 82
Analytical reliabilities 83
Hazardous materials 84
Reference range and clinical interpretation 84
Limitations 84
Reference 84

18. CSF protein –Turbidimetry Method 85
Principle of the method 85
Reagents 85
Equipment, glassware and other accessories 86
Procedure 86
Calculation and calibration graph 86
Analytical reliabilities 87
Hazardous materials 88
Reference range and clinical interpretation 88
Limitations 88
Reference 88

SECTION D: URINALYSIS

19. Introduction 89
Collection of specimen 89

20. Qualitative Tests 91
Appearance 91
pH 91
Ketone bodies - Rothera's test 92
Urobilinogen –Erhlich ŝ test 93
Bilirubin - (Harison spot test) Fouchet's test 94

21. Semi-Quantitative Tests 97
Specific Gravity (Mass Density) 97
Proteins –Heat and acetic acid method 98
Protein –Sulphosalicylic acid method 99
Sugar: Benedict's test 100

22. Dipstick Technology 103
Introduction 103
Glucose 103
Proteins 104
Multiple reagent strips 105

Index **..... 107**

Foreword

There has been a significant increase in the use of clinical laboratories in health services in recent past. Though all disciplines of laboratory sciences are contributing in mitigating the misery and mortality of human beings, clinical chemistry has been in use for the longest period and is accessible even in the peripheral areas of most of the developing countries. This has been possible because most of the tests conducted to assess the biochemical status of the human body are simple, easy to perform, rapid, economical and do not demand a sophisticated infrastructure. However, in the absence of uniformity in the performance of these tests, the results generated would not meet the desired purpose and might have an adverse impact on the diagnosis as well as treatment. To obviate this problem, and to ensure quality of results, Standard Operating Procedures (SOPs) play a vital role. Every laboratory has to develop SOPs and use them in-house.

Quality assurance in health laboratories has been one of the important areas for WHO. As a part of technical support to Member Countries, WHO has undertaken to develop model guidelines for the development of SOPs in various specialties of health laboratory services. The Guidelines for Development of Standard Operating Procedures in Clinical Chemistry is an effort in this direction. These guidelines are intended for various laboratories that are performing tests for clinical chemistry, to enable them to develop appropriate SOPs that suit their requirements and are in consonance with their mandate as well as infrastructure.

I am sure the readers will find these useful in strengthening the quality assurance system of their laboratories and thus improving the quality of health care in their setting.

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Regional Director

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Preface

Laboratory investigations pertaining to clinical chemistry have been yielding useful information to clinicians, both in the diagnosis of illness as well as the monitoring of treatment. These have been extensively used throughout the world. People in the developing countries are also deriving benefit from these simple and economical tests. The utility of these tests has been especially recognized in the management of lifestyle diseases such as diabetes mellitus and other cardiovascular ailments - the incidence of which is increasing steadily even in developing nations.

The extensive use of these tests has resulted in the marketing of a variety of test reagents with variable technology. The absence of standard operating procedures (SOP) makes it very difficult for many laboratories to generate reliable results. The World Health Organization has been advocating the use of standard operating procedures in all laboratory activities. To achieve this objective, a series of subject-specific guidelines to develop SOPs suiting the requirements of particular laboratories have been developed.

The Guidelines for Standard Operating Procedures in Clinical Chemistry were prepared to strengthen the quality assurance component of clinical chemistry laboratories for the commonly performed tests by conventional technology that is still in use in wide areas of Member Countries of the South-East Asia Region.

It is hoped that these guidelines will be useful to laboratory professionals in improving the quality of their tests.

1. General Introduction

Health care delivery is no longer a simple process of examining the patient and giving him a prescription. Over the years there has been rapid expansion in the various branches of health care services. As part of this expansion process and explosion of scientific medical knowledge, laboratory diagnosis has gained tremendous importance in today's practice. Through the use of quality control (QC) the laboratory can ensure that the results being issued by it are reliable enough to allow decisions to be taken with confidence. QC is the study of those errors which are the responsibility of the laboratory, and of the procedures used to recognize and minimize them. Incorrect laboratory results may lead to wrong management decisions with possible fatal results. The reliability of laboratory results is therefore most important. It is not sufficient to *think 'that* my 'results are satisfactory. This has to be proved with scientific evidence. Laboratory personnel must know that QC is an obligation to the patient, that it is designed to give the analyst confidence in the methods used and that its purpose is not to find scapegoats or to punish those making mistakes.

Quality Manual

Towards achieving quality, international accreditation programmes strongly recommend the production of a quality manual by the laboratory.

The quality manual of a laboratory is a document or a set of documents describing the organizational structure, responsibilities, procedures and processes by which the laboratory achieves its objectives and gains confidence in its work. The manual is indispensable for achieving and maintaining good overall quality. Furthermore, the preparation of a quality manual may induce the laboratory to improve quality. Even a non-mandatory quality manual may be a valuable document for a clinical laboratory in demonstrating to clinicians and the hospital administration a commitment to quality[(1)].

"The laboratory shall define and document its policies and objectives for, and its commitment to, good laboratory practice. The hospital management shall ensure that these policies and objectives are documented in the quality manual and communicated to, understood by, and implemented by all laboratory personnel concerned. The quality manual contents are as follows[2] ":

Contents of quality manual

These are:

- Quality Policy and Quality System
- Organization
- Quality Control
- Personnel

- Accommodation and Environment
- Equipment
- Reference Materials
- Test Procedures
- Handling of Reagents
- Sample Collection, Storage and Disposal
- Maintenance of Records
- Laboratory Reports and Despatch of Reports

Quality policy

The aim of the laboratory is to provide clinically useful information through laboratory measurement of samples from patients, taking into account the allocated resources.

The quality policy is implemented by the following means;[2]

- Proper sample collection, stabilization, transport, sample preparation and identification.
- Reliable analytical work so that systematic and random errors do not exceed specified limits.
- Turn-around time within specified limits for routine and emergency measurements, and for rare routine measurements.
- Data reported in a clear form and supplemented with relevant information, including reference intervals to allow reliable clinical interpretation.
- Appropriate communication to the clinicians so that the results will be interpreted correctly and logically integrated into further (clinical and laboratory) evaluation of the patients, and that the clinicians become aware of unexpected problems and errors.

Standard operating procedures

The preparation of test procedures comes under the broad heading of Standard Operating Procedures (SOPs). SOP is a clear, concise and comprehensive written instruction of a method or procedure which has been agreed upon and authorized as the operating policy of the department.

In general, SOPs, which mainly contain detailed descriptions of each analytical method, are essential for maintaining the same analytical quality over a long period of time. The procedures are a prerequisite to correct transfer of methods from one laboratory to another. The contents of SOP are as follows:

(1) Introduction
(2) Principle of method
(3) Specimen types, collection and storage
(4) Reagents, standards and control - preparation and storage
(5) Equipment, glassware and other accessories
(6) Detailed procedure

(7) Calculations, calibration curve
(8) Analytical reliabilities –(QC and Statistical assessment)
(9) Hazardous reagents
(10) Reference range and clinical interpretation
(11) Limitations of method (e.g. interfering substances and troubleshooting)
(12) References
(13) Date and signature of authorization
(14) (Effective date + Schedule for review)

Laboratory errors

Analytical errors are classified into random errors and systematic errors. It is clear that random errors indicate poor precision while systematic errors indicate poor accuracy. A few examples of random errors are pipetting error, transcription error, wrong sample numbering and labelling, and fluctuating readings on the colorimeter. Systematic errors could occur due to wrong procedure, incorrect standard and calibration procedure.

Errors can occur in any of the limb of the cycle of events taking place in a hospital, starting from the physician examining the patient and back to the physician (pre-analytical/ analytical/post-analytical).

The physician, after examining the patient, decides and orders a test, and collects and transports the patient's samples; this constitutes the pre-analytical limb of the cycle of events. In the analytical limb the sample is received by the laboratory and analysed. The post-analytical limb consists of the transfer of the result to the physician and a meaningful interpretation of the laboratory data by the physician, followed by necessary action.

Definition:

Accuracy *is the degree of agreement between a measured value and its 'true/consensus' value. On the contrary, inaccuracy, which is represented by analytical bias, is defined as the % of the difference between the measured value and the 'true' value over the true value. Therefore, good accuracy means least analytical error.*

Precision *refers to reproducibility. It refers to the agreement between replicate measurements. It is quantitatively expressed as the standard deviation (SD) or more precisely as percent coefficient of variation (CV), which is defined as SD times 100 divided by the mean value of the results in a set of replicate measurements. Therefore, good precision means least CV.*

Pre-analytical

The pre-analytical system shall take care of the following aspects[3], as each can have a major effect on the accuracy of the result:

- Patient preparation
- Request forms
- Specimen collection, containers, labelling and phlebotomy equipment and procedure
- Specimen transport

- Specimen preparation
- Specimen storage

Analytical

The following aspects[3] shall be monitored, evaluated, implemented and maintained to ensure the accuracy and precision of the test carried out:

- Quality of distilled water
- Calibration of measuring and testing instruments including balances, thermometers, incubators, waterbaths, autoclaves, centrifuges and semi-automatic pipettes, and regular servicing and maintenance of equipment.

It is essential to use a standard calibrator which is traceable to national/international reference material. The laboratory shall obtain evidence of traceability to the reference material from the supplier. Precision can be maintained through the use of suitable QC material, either commercial or prepared in-house. The QC material should be analysed at predetermined intervals along with patient samples to monitor systematic and random errors. Such QC material shall also be traceable to a national/international certified reference material so that the accuracy of measurements can be monitored.

All data relating to the laboratory's internal QC practices and performance in external quality assessment schemes (scoring, ranks, etc.) shall be recorded, reviewed and corrective actions implemented.

Stability of reagents

Laboratory personnel should be aware that the stability of all reagents kept at room temperature will go down from the stated values if the temperature exceeds 35^0C.

Use of calibration graphs

A fresh standard curve should be carried out for the analyses described in this manual whenever:

- the calibrator is changed
- new reagents are introduced
- problems with QC are encountered

Post-analytical

In order to avoid transcriptional errors in the results of the test, the reporting/signatory technicians shall verify the results entered manually or through on-line instrument interfaces before the results are reported or despatched.

Rectification of laboratory errors

It is therefore essential to continually ask the following questions.

(1) Is there an analytical error?

(2) If so, what type of error is this?

(3) What could have been the causes for this error?

(4) How to rectify this error?

It is important to identify analytical errors and classify them as either random or systematic errors. Towards this end, the laboratory should implement internal QC procedures. This involves preparation of a QC pool, either human or bovine, quantification of unavoidable laboratory errors, construction of Levey-Jennings chart and daily analysis of QC along with every batch of patients 'samples.

Preparation of QC pool

Ethanediol stabilized liquid serum QC pool has been established in the authors' laboratory [4] based on the WHO method [5]. This procedure is applicable to both pooled human serum as well as bovine serum. This preparation is economical and appropriate for use in the laboratories in developing countries.

Use of patients 'sera

A serum pool can be prepared by salvaging the extra serum from leftover patients' samples after analysis. Samples that are significantly haemolysed or lipemic or icteric should be excluded. Similarly, samples that show positive tests for HIV antibodies and Hbs antigen should also be excluded. In view of the dangers in handling infectious blood samples, use of animal-based QC pool is recommended.

Use of bovine serum

Collect about 2-3 litres of fresh bovine blood in a 5-litre clean plastic bucket. Allow to clot at room temperature for about 30 minutes.

Slice the clot into small pieces using a sharp knife and leave the bucket at 2-8^{0}C for 12 hours to enable the serum to ooze out. Decant the crude serum into a one litre beaker or flask.

Transfer this crude serum into several glass centrifuge tubes (size 15 x 120 mm) and then centrifuge for 10 minutes at 3500 rpm and decant the clear serum into a clean bottle.

Transfer one litre serum into a one-litre plastic bottle.

Mix the contents well and store the container at –20^0 C for 12 hours or until frozen.

While monitoring day-to-day laboratory performance with internal QC, it is preferable to use different levels of QC materials to cover the entire pathological ranges. Therefore, methods of preparation of three levels of QC (low, normal and high) are described below.

If the preparation of all three levels of QC pool is not possible, it is essential to make use of at least one level, viz. normal level.

The procedures described below for the preparation of all three QC levels are applicable to both human serum and bovine serum.

(a) Preparation of normal-level QC serum

Remove the container from the freezer and fix it upside down over a one-litre plastic measuring cylinder. Collect the first 830 mg, which will be rich in all constituents. Add 150 ml of ethanediol to this and mix well. Take an aliquot and measure the levels of

various analytes. Use 20 ml distilled water to dissolve the various substances that will be added to the serum in order to increase the levels of these to the desired normal levels. Total volume = 830+150+20 = 1000 ml

The QC serum thus prepared will contain 15% (V/V) ethanediol.

(b) Preparation of high-level QC serum

Freeze one litre of clear serum at -20^{0}C. Remove the container from the freezer and fix it upside down over a one-litre plastic measuring cylinder. Collect the first 700ml, which will be more concentrated and rich in all constituents. Add 127.5 ml of ethanediol to this and mix well. Take an aliquot and measure the levels of various analytes. Use 22.5 ml distilled water to dissolve the various substances that will be added to the serum in order to increase their levels to the desired levels. Total volume = 700 +127.5+22.5 = 850 ml.

The QC serum thus prepared will contain 15% (V/V) ethanediol.

(c) Preparation of low-level QC serum

Start with 500ml of clear serum. Measure potassium level. Calculate the final volume, to which 500 mg of this serum must be diluted to adjust the potassium level to 3.5 mmol/L.

Initial volume	500 ml
Final volume	(x) ml
Initial K+ value (e.g)	6.3 mmol/L
Final K+	3.5 mmol/L
Therefore,	500 x 6.3 = 3.5 x (x)
x= 500 x 6.3/3.5 = 900 ml	
15% of 900 ml	= 135 ml
Extra volume to be) added (900-500	= 400 ml
Ethanediol	= 135 ml
Therefore, distilled water to be added (400-135)	= 265 ml.

In actual practice, although potassium concentration will be diluted to 3.5 mmol/L, the levels of some other constituents will become too low. Therefore the levels of these need to be raised to some extent so that these will be maintained at low level but not too low. Out of the 265 ml distilled water, 25 ml may be used for dissolving desired quantities of such of those constituents that need to be added to the serum to raise their amount to the desired low levels.

Adjustment of analytes

Glucose, urea, creatinine, sodium (NaCl) and potassium (KCl): -

Dissolve each of these in 5 ml distilled water and add to the main bulk of serum.

Calcium :- Use dried $CaCO_3$. Dissolve in 5 ml in 1N HCL and add to the serum.

After adding all the analytes mix the contents well and centrifuge the serum once again for 10 minutes at 3500 rpm. Collect the supernatant serum in a sterile one-litre flask. Mix the contents well and dispense the serum into 5 ml sterile penicillin vials, seal the vials and store at –20^{0}C. Laboratories can decide on the volume of aliquots depending on their requirements.

Construction of Levey Jennings Chart

On each day when analyses are performed a fresh sample is thawed, thoroughly mixed and analysed. (**Remember:** Ethanediol-treated serum may not freeze completely at –20^{0}C; however, the constituents are quite stable). The QC serum is analysed for a period of 20 days or so. [**Important to note:** Analysis should not be carried out by only one person; all staff should participate in this exercise to determine the true unavoidable error in the laboratory]. From these data, mean and SD are calculated. Levey Jennings chart is then constructed with $x \pm 2SD$ as warning limits and $x \pm 3$ SD as control limits.

Calculate the %CV for each analyte to ascertain whether this is within the acceptable limit (Ideal = $\leq$ 5%. Must be definitely $\leq$ 8%). If % CV is found to be high, this will indicate that between-day laboratory precision (variation) is high and the data cannot be used to construct a Levey Jennings chart. It is then essential to identify the causes for this, correct these and then repeat the whole exercise and confirm that the %CV is well within the acceptable limit.

Table 1 shows precision data obtained by a WHO trainee from a developing country for routine biochemical analytes through analysis of ethanediol-treated QC serum for 20 days, while undergoing training at the author's laboratory recently.

Table 1

Analyte	Autoanlyser Hitachi 912		Manual Method	
	Mean	**%CV**	**Mean**	**%CV**
Glucose	167	1.2	166	4.2
Total protein	9.8	1.2	9.6	3.3
Albumin	3.5	1.4	3.6	4.2
Urea	50	1.7	55	3.8
Calcium	10.3	1.9	10.5	4.0
Cholesterol	185	1.4	180	4.5
Creatinine	1.7	0.7	1.8	3.0

Interpretation of QC data

A. According to WHO[6] an analytical system is 'out of control' if one of the four criteria is met. That is:

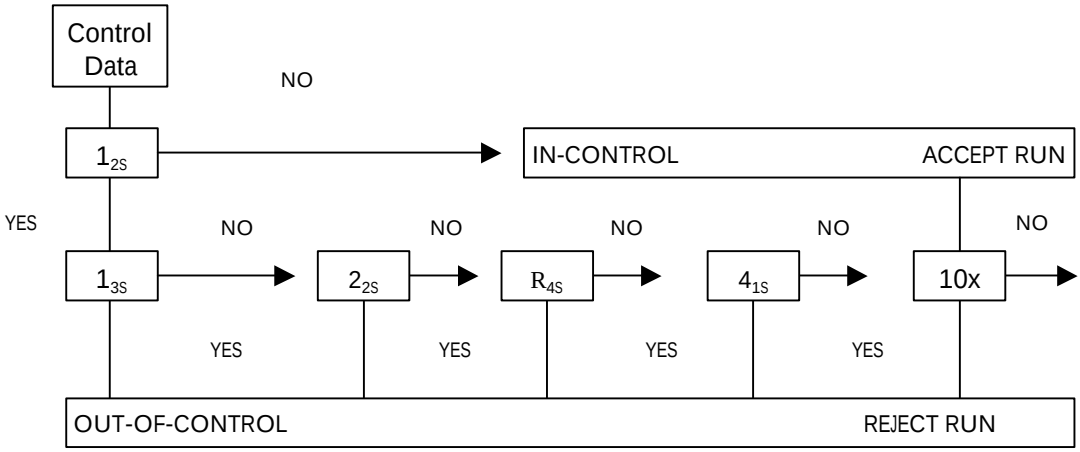
- a value lies entirely outside the control limits
- seven consecutive values show a rising tendency
- seven consecutive values show a falling tendency
- seven consecutive values lie on the same side of the mean

If one of these situations arises, the patients 'results must be discarded, the cause of the error sought and removed, and then the batch repeated with a QC serum.

B. Use of two different levels of QC simultaneously in every batch of analysis provided valuable information on the type of errors – whether these are random (precision) errors or systematic (accuracy) errors.

Westgard's rules for interpreting QC data obtained using a single-level QC as well as two different-level QCs are schematically presented in Fig.1.[7]

***Figure 1**. Quality Control Rule Diagram*



Warning Rule

$^{1}2S$ One observation > $\bar{x} \pm 2$ SD

Rejection Rules (used if warning rule is exceeded; run rejected if any of the following rules are violated) **R= Random error; S = Systematic error**

R $^{1}3S$ One observation > $\bar{x} \pm 3$ SD

S $^{2}2S$ Two observations > same limit, that is $\bar{x}$ + 2 SD or $\bar{x}$ - 2SD (same control- two consecutive runs, or two different controls – same run)

R $^{R}4S$ Difference between two observations within run > 4SD (two different controls – one > $\bar{x}$ + 2SD and the other > $\bar{x}$ – 2SD)

S $^{4}1S$ Four consecutive observations > same limit, that is $\bar{x}$ + 1SD or $\bar{x}$ - 1SD (same control four consecutive runs)

S ^{10}X Ten consecutive observations on same side of mean (same control, ten consecutive runs, or two different controls, five consecutive runs)

Remedial action

A well-run internal QC system makes possible immediate intervention in the release of patients 'results. In the event of a control system alert, it is advisable to proceed through the following steps in that order.

(a) Decision: Immediate decision whether action is necessary.

(b) Investigation: Check to locate the error.

(c) Repair: Action to eliminate the error.

If the decision is taken that the method is out of control, the first action is to withhold patients 'results in that batch. Then the analyst should start by checking for the simplest and most frequent faults, and then continue as necessary in a logical order depending on the method and equipment involved.

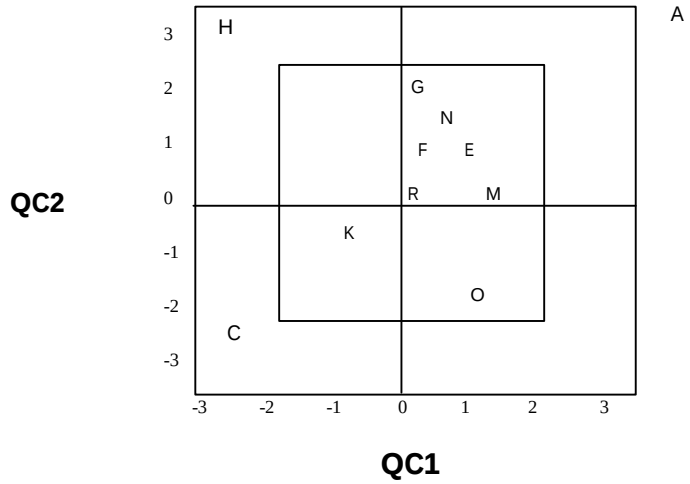
It is good practice to start by excluding gross errors such as mix-up of control materials, reagents or pipettes, misuse of measuring instruments [wrong filter or aged lamp in the photometer], or failure to follow instructions for a step in the method.

The results obtained on a control specimen may be in error for several reasons, including deterioration due to age, incorrect storage or contamination, wrong identification and mistake in preparation or constitution.

Further action will depend on whether the alert is due to a change in accuracy or in precision. If accuracy has deteriorated, attention should be focused on the possibility of systematic sources of error such as incorrect reaction temperature, calibration errors and faulty devices. If precision has deteriorated, steps in the analytical procedure should be checked, for instance, deproteinization, composition of reagents and reaction mixtures, measuring systems [photometer, flame photometer, etc].

To easily differentiate between systematic and random errors, laboratories are encouraged to construct Youden charts. These are constructed by having $\bar{x} \pm 2SD$ & $\bar{x} \pm 3SD$ of one level QC in the x axis and that of another level QC in the y axis. If an analyst analyses both levels of QC and plots the data in the Youden chart, a single plot will be obtained. If this falls within the inner square, it means that the value obtained for both QC are well within the acceptable limit, i.e. $\bar{x} \pm 2SD$. On the other hand, if a plot appears outside this limit, it could mean that the data are outside the acceptable limit and the error could be either systematic or random. Fig.2 shows sample data plotted in Youden chart.

Figure 2.



It can be inferred that :-

(a) Plots E, F, G, K, M, N, O & R are acceptable

(b) Plot A values for both QC are > +2SD
Plot C values for both QC are > - 2SD

These data indicate $^{2}2S$ error, i.e. systematic error

(c) Plot H Values for QC_1 (x axis) is > - 2SD
while value for QC2 (y axis) is > + 2SD

This indicates R_{4s} error i.e. random error

Prevention of systematic errors

To prevent or minimize systematic errors, the laboratory should adhere to the following points:

(1) Use of proper calibration technique. Use of pure chemicals, precision balance, quality distilled water. Proper preservative and storage

(2) Regular checking of photometric filter, bulb, tubing, etc.

(3) Use of recommended analytical methods

(4) Calibration of pipettes at regular intervals

(5) Instrument calibration – photometric check

(6) Use of calibrated cuvette

(7) Regular preventive maintenance of equipment – daily, weekly and monthly.

While it is easy to identify systematic errors, it is quite difficult to pinpoint random errors. In order to minimize this possibility, it is important to educate the staff on various aspects that could lead to random errors.

External quality assessment

Participation in External Quality Assessment Schemes is important for interlaboratory comparison and the maintenance of long-term accuracy and precision of the analytical systems in the laboratory. This gives the laboratory an opportunity to have an appraisal of the methods employed and switch over to better methods.

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2. Introduction to SOP

(1) The methods described in this manual have been developed in the author's laboratory taking into account the facilities available in the intermediate laboratories in developing countries, the technical level of laboratory personnel and the availability of chemicals/reagent kits at affordable cost. The validity of these methods with reference to accuracy, precision, linearity, etc. has been well established. These methods have been tried and tested and are being employed in a large number of laboratories in India.

(2) Numerous kits are available today manufactured by several commercial firms, which contain all the reagents needed for various biochemical tests. Details are given in the kit leaflet as to the preparation and storage of reagents. It is important to follow the manufacturer's instructions carefully. With the ready availability of the kits, very few laboratories are preparing their own reagents from the basic chemicals. Even those laboratories using in-house reagents have the choice of preparing the standard in their laboratories or buy from commercial companies. It is very important to remember that whatever is the source, the standard should be properly prepared. Use of improperly prepared standard will drastically affect the quality of patients' results.

(3) (a) Generally the intermediate laboratories are equipped with either a filter photometer or spectrophotometer to carry out the analysis as a manual method. However, some laboratories employ semiautomatic methods for which automated photometric systems are used.

(b) Two types of such systems are available for use in the laboratory. A comparison of the specifications of these two types is given in the following Table.

Comparision of Semiautomated Systems

Specifications	Coventional Suction Cuvette	Flow-Through Sipper Systems
	Example: BM 4010 (Boehringer Mannheim, Germany)	Example: BM 4020 (Boehringer Mannheim Germany)
Programmable channels	None, Chemistries to be set as and when tests are done	Chemistries can be programmed into a defined number of channels and can be recalled as and when tests are done.

Specifications	Coventional Suction Cuvette	Flow-Through Sipper Systems
Measuring temperature	25, 30, 37^{0}C	25, 30, 37^{0}C
Type of chemistries done	Absorbance, end point, two point, kinetic.	Absorbance, end point, two point, kinetic.
Modularity	Yes, the instrument has separate photometer, suction device and incubating waterbath.	No, integrated. All the hardware is integrated into one compact unit to save space.
Temperature maintenance	By circulating waterbath	Peltier element
Cuvette volume required for measurement	500µl	Ranging from 20-80µl facilitating the use of small volumes
Total reaction volume	1000µl	Ranging from 175µl to 500µl.
Cuvette material	Quartz	Quartz.
No of wavelength (nm)	340, 405, 546, 578, 623	340, 405, 505, 546, 570, 623, 700
Wavelength positioning	Manual rotation. Choice depends on the chemistry.	Automatic. Instrument automatically sets the wavelength when the operator selects the chemistry to be done.

(c) General (common) laboratory glassware like pipette, beaker, volumetric flask, measuring cylinder and test tube are essential for carrying out the assays.

(d) Semiautomatic (push button) pipettes are now employed in several laboratories. If a laboratory employs such pipettes, regular maintenance must be carried out as per instructions provided by the manufacturer. Calibration of pipettes should also be checked at definite intervals.

(4) The following Table shows a set of sample data related to calibration of three different pipettes with volumes 50, 100 & 200 µl. The calibration procedure consists of weighing water delivered through these pipettes into a preweighed beaker for at least 10 times. From the data obtained, mean, SD, %CV and % error have been calculated. %CV expresses the precision and % error expresses accuracy. These data are well within the acceptable limit, i.e. 5%.

S. No	Weight of 50 ml of water (g)	Weight of 100 ml of water (g)	Weight of 200 ml of water (g)
1	0.0505	0.0899	0.1900
2	0.0455	0.1000	0.1965
3	0.0455	0.0970	0.1957
4	0.0500	0.0958	0.1948

S. No	Weight of 50 ml of water (g)	Weight of 100 ml of water (g)	Weight of 200 ml of water (g)
5	0.0466	0.0945	0.1960
6	0.0467	0.0954	0.1968
7	0.0500	0.0974	0.1956
8	0.0506	0.0993	0.1863
9	0.0477	0.0973	0.1897
10	0.0480	0.0976	0.1869
Mean	**0.0481**	**0.0964**	**0.1928**
SD	**0.002**	**0.0028**	**0.0041**
%CV	**4.2**	**2.9**	**2.1**
% error	**1.8**	**3.6**	**3.6**

(5) Protection of laboratory personnel from infectious diseases transmitted by blood, body fluids and tissue

A biohazardous condition is a situation involving infectious agents, biological in nature such as hepatitis B virus, human immunodeficiency virus (HIV), or the tuberculosis bacterium. These infectious agents are present in human blood and blood products and in other body fluids.

The major sources of contamination when handling potentially infectious agents are:-

- Hand-to-mouth contact
- Hand-to-eye contact
- Direct contact with superficial cuts, open wounds, and other skin conditions that permit absorption into subcutaneous skin layer
- Splashes or aerosol contact with skin and eyes.

Specimens from patients known to have these infectious diseases (Biohazard specimens) should be handled using special precautions. However, since it is not possible to identify all carriers it is also sensible to introduce a system of universal precautions whereby high-risk procedures are identified as well as high-risk specimens. If laboratory staff use the recommended precautions this should increase their protection.

Strictly adhere to the following procedures: -

- Wear gloves when in contact with body fluids, such as serum, plasma, urine or whole blood
- Wash your hands when gloves are removed or changed
- Perform procedures carefully to minimize aerosol formation
- Wear protective clothing such as laboratory coats or aprons when working with specimens
- Keep your hands away from your face
- Cover all superficial cuts before starting any work
- Dispose of specimens and other contaminated materials according to your laboratory's biohazard control procedure
- Keep your work area disinfected, disinfect tools and other items that have been in any contaminated area.

- ➢ Do not eat or drink or apply cosmetics while in the laboratory
- ➢ Do not mouth pipette any liquid, including water.

3. Glucose –Glucose Oxidase Method

Introduction

Glucose is a reducing monosaccharide that serves as the principal fuel of all the tissues. It enters the cell through the influence of insulin and undergoes a series of chemical reactions to produce energy.

Lack of insulin or resistance to its action at the cellular level causes diabetes. Therefore, in diabetes mellitus the blood glucose levels are very high. Some patients with very high blood glucose levels may develop metabolic acidosis and ketosis caused by the increased fat metabolism, the alternate source for energy. Hyperglycaemia is also noted in gestational diabetes of pregnancy and may be found in pancreatic disease, pituitary and adrenal disorders.

A decreased level of blood glucose, hypoglycaemia is often associated with starvation, hyper insulinaemia and in those who are taking high insulin dose for therapy.

Principle of the method

Glucose present in the plasma is oxidized by the enzyme glucose oxidase (GOD) to gluconic acid with the liberation of hydrogen peroxide, which is converted to water and oxygen by the enzyme peroxidase (POD).

4 aminophenazone, an oxygen acceptor, takes up the oxygen and together with phenol forms a pink coloured chromogen which can be measured at 515mm.

$$\text{Glucose} \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{POD}} H_2O + [0]$$

$$[0] + \text{4 aminoophenazone} + \text{Phenol} \longrightarrow \text{Chromogen (pink)}$$

Specimen type, collection and storage

Plasma is the specimen of choice for glucose estimation. Plasma glucose levels have been checked to be quite stable for 6 hours at room temperature (25 -35^{0}C) in the author's laboratory. It is important that plasma should be separated from the cells soon after collection, preferably within 1 hour.

About 2 ml of the patient's blood should be collected by venipuncture into a tube containing a mixture of potassium ethylene diaminetetraacetate (EDTA) sodium fluoride at a ratio 1:2 (W/W). Five mg of the mixture is adequate for 2 ml of blood. The tube should be gently but thoroughly shaken for complete mixing.

Preparation of the anitcoagulant mixture: 100 g of potassium EDTA and 200 g of sodium fluoride should be mixed and ground into a fine powder using a blender. This should preferably be done in a fume cupboard. The mixture should be stored in a clean container.

A thin, long spatula that can scoop 5 mg when levelled, can be used for delivering the mixture into the tube.

Reagents

All chemicals must be Analar grade

(a) Phosphate Buffer : 100 mmol/L. pH 7.0

To 800 ml of distilled water add the following in the order:

Disodium hydrogen phosphate dihydrate [Na2HPO4 2H2O]	12.95 g
Anhydrous potassium dihydrogen phosphate [KH2PO4]	4.95 g
Sodium azide [NaN3]	0.5 g

Add one by one, dissolve and finally make up to 1 litre with distilled water. Stable for 3-4 months, at 2-8^{0}C. Check that the final pH is 7.0 $\pm$ 0.05 with a pH meter.

(b) Colour Reagent

To 100ml of the above phosphate buffer add the following in the order and then mix to dissolve:

4 amino phenazone	16 mg
GOD [Sigma G 7016]	1800 units
POD [Sigma P 8250]	100 units
Phenol	105 mg
Tween 20 [Sigma P 1359]	50µl

Reconstitute the purchased GOD & POD powder with phosphate buffer. Dispense separately into vials so that each vial represents the requisite number of units. Store the vials frozen. Stable for 2 weeks at 2-8^{0}C. Store in a brown bottle.

(c) Benzoic acid 1g/l.

Dissolve 1.0g of benzoic acid in water and make up to 1 litre with water. This solution is stable indefinitely at room temperature.

(d) Stock glucose solution, 1 g/l.

Before weighing, dry the glucose at 60-80^{0}C for 4 hours. Allow to cool in a dessicator. Dissolve 1g of glucose in benzoic acid solution and make up to 100 ml in a volumetric flask. Stable for six months at room temperature (25-35^{0}C).
DO NOT FREEZE THE STANDARD

(e) Working glucose standard 100 mg/dl.

Dilute 10 ml of stock glucose (use either a volumetric pipette or a burette) to 100 ml with benzoic acid in a 100 ml volumetric flask. Mix well. Stable for 6 months at room temperature (25-35^{0}C).

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP

Procedure

The protocol of the procedure is described below.

(a) Dilution of standards (S1-S5), Test & QC

Pipette the following into appropriately labelled 13 x 100 mm tubes

	S1	S2	S3	S4	S5	Test	QC
Distilled Water (ml)	1.9	1.8	1.7	1.6	1.5	1.9	1.9
100 mg/dl glucose (ml)	0.1	0.2	0.3	0.4	0.5	-	-
Test sample /QC (ml)	-	-	-	-	-	0.1	0.1
Mix well							

(b) Colour development

Pipette the following into another set of appropriately labelled tubes.

	Blank	S1	S2	S3	S4	S5	Test	QC
Colour reagent (ml)	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Distilled water (ml)	0.1		-	-	-	-	-	-
Diluted Standards (ml)		0.1	0.1	0.1	0.1	0.1	-	-
Diluted Test Sample/QC (ml)	-	-	-	-	-	-	0.1	0.1

Mix all tubes well. Incubate at 37^{0}C in a waterbath for 15 minutes.

Remove from waterbath and cool to room temperature. Set the spectrophotometer/ filter photometer to zero using blank at 510 nm/ green filter and measure the absorbance of Standards, Test and QC.

This protocol is designed for spectrophotometers / filter photometer that require a minimum volume of reaction mixture in the cuvette of 1 ml or less. Economical use of reagents is possible with this protocol, thus the cost per test can be kept to the minimum. However, if a laboratory employs a photometer requiring a large volume of the reaction mixture for measurement, viz. 5 ml, it is advisable to increase the volume of all reagents mentioned under Tabulation "(b) Colour development" proportionately.

Calculation and calibration graph

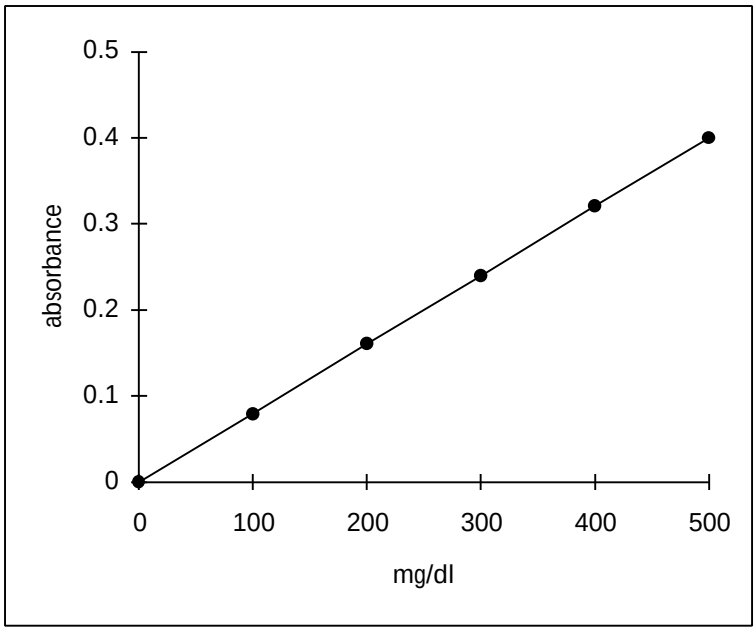
Since the protocol for standard tube S1 and test is identical, the standard S1 will represent a concentration of 100 mg/dl. The glucose concentrations represented by other standard tubes are S2 =200; S3 = 300; S4 =400 & S5 = 500 (mg/dl).

Plot the absorbance values of standards against their respective concentrations. The measurable range with this graph is from 10 to 500 mg/dl.

Plot absorbance values of Test/QC on the calibration graph and read off the concentrations.

Once linearity is proved, it is not necessary to prepare the standard graph every time that patients 'samples are analysed. It will be adequate if standard S2 is taken every time and patients 'results are calculated using the formula :

$$\frac{\text{Test absorbance}}{\text{Standard absorbance}} \times 200 \text{ mg/dl}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients 'results).

Since glucose is the most common analyte measured in a laboratory, it is advisable to include an internal QC (normal QC pool) with every batch of samples analysed in the day, irrespective of the number of samples in a batch. Further, even when a single sample is analysed as an "emergency" sample at any time of the day or night, it is essential to include an internal QC. From the QC results obtained for the day, mean,

standard deviation and %CV can be calculated to ensure that ***within-day precision*** is well within the acceptable limit, i.e, 5%.

The mean value of internal QC for the day can be pooled with the preceding 10 or 20 mean values obtained in the previous days, and ***between-day precision*** can be calculated and expressed as % CV. Ensure that this is well within the acceptable limit, i.e, 8%.

At least once a day analyse another QC serum from either a low QC or high QC pool.

"Assayed" QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

This procedure uses sodium azide and phenol, which are poisonous and caustic. Do not swallow, and avoid contact with skin and mucous membranes

Reference range and clinical interpretation

Plasma glucose:	Fasting:	70 – 110 mg/dl
	Post-prandial:	80 – 140 mg/dl
	Random:	60 – 140 mg/dl

Elevated plasma glucose levels are expected in a variety of clinical conditions, especially diabetes mellitus, Cushing's syndrome and hyperadrenalism. Decreased plasma glucose levels are observed in hyper-insulinism, anti-diabetic treatment and hypoadrenalism.

Limitations

Any sample that gives a glucose value > 500 mg/dl should be diluted 1:2 with 0.9g% sodium chloride solution and the correct value obtained by multiplying the result by 3.

At high plasma levels, uric acid, glutathione and bilirubin may interfere with the assay by causing a decrease in glucose values. Ascorbic acid will decrease glucose values by retarding colour development. Do not report results from specimens with suspected interference. Inform the requesting physician of the problem.

References

(1) Trinder, P. (1969). Annals of Clin. Biochem. 6: 24 – 27.

(2) Barham D and Trinder P. (1972). Analyst 97: 142 – 145

4. Urea – Diacetyl monoxime method

Introduction

Urea contributes most of the body's non-protein nitrogen, accounting for about 45% of the total. It is the major end-product of protein catabolism in humans. It is synthesized in the liver, released into blood circulation and excreted by the kidneys. Measurement of urea in blood is a useful indicator of renal and hepatic integrity.

Principle of the method

Urea reacts directly with diacetyl monoxime under strong acidic conditions to give a yellow condensation product. The reaction is intensified by the presence of ferric ions and thiosemicarbazide. The intense red colour formed is measured at 540nm/ yellow green filter.

Specimen types, collection and storage

Serum is the specimen of choice. Store samples for no longer than 8 hours at room temperature (25-35^{0}C) and 7 days at 2-8^{0}C. For a longer duration, store in the freezer. If the samples show evidence of bacterial contamination, do not use these for urea estimation. Plasma could also be used for urea estimation.

Reagents

All chemicals must be Analar grade.

(a) Stock acid reagent

Dissolve 1.0g of ferric chloride hexahydrate in 30 ml of distilled water. Add 20 ml orthophosphoric acid and mix. Store in a brown bottle at room temperature (25-35^{0}C).Stable for 6 months.

(b) Mixed acid reagent

Add slowly 100 ml of Conc. H2S04 to 400 ml distilled water taken in a 1-litre flat-bottom conical flask kept in an icecold waterbath. Mix well and add 0.3ml of stock acid reagent. Mix and store in a brown bottle at room temperature (25-35^{0}C). Stable for 6 months.

(c) Stock colour reagent – A

Dissolve 2g diacetyl monoxime in distilled water and make the volume up to 100 ml in a volumetric flask. Store in a brown bottle at room temperature (25-35^{0}C). Stable for 6 months.

(d) Stock colour reagent - B

Dissolve 0.5 g thiosemicarbazide in distilled water and make up to 100 ml in a volumetric flask. Store in a brown bottle at room temperature (25- 35^{0}C). Stable for 6 months.

(e) Mixed colour reagent

Mix 35 ml of stock colour reagent A with 35 ml of stock colour reagent B and make up to 500 ml with distilled water. Store in a brown bottle at room temperature (25- 35^{0}C). Stable for 6 months.

(f) Stock urea standard

Weigh 1.0g of analytical-grade urea and dissolve in 100ml of benzoic acid (1g/dl). Use a 100ml of volumetric flask for preparing this. Store at room temperature (25- 35^{0}C). Stable for 6 months.

(g) Working standard 50mg/dl

Dilute 5.0ml of stock urea standard to 100 ml with benzoic acid. Store at room temperature (25- 35^{0}C). Stable for 6 months.

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP

Procedure

The protocol of the procedure is described below.

(a) Dilution of Standards (S1-S3), Test & QC

Pipette the following into appropriately labelled 13 x 100 mm tubes

	S1	S2	S3	Test	QC
Distilled Water (ml)	1.9	1.8	1.7	1.9	1.9
50 mg/dl Urea (ml)	0.1	0.2	0.3	-	-
Test sample /QC (ml)	-	-	-	0.1	0.1
Mix Well					

(b) Colour Development

The colour reagent is prepared fresh at the time of analysis by mixing distilled water, mixed acid reagent and mixed colour reagent in the ratio 1:1:1.

Pipette the following into another set of appropriately labelled 18 x 150 mm tubes.

	Blank	S1	S2	S3	Test	QC
Colour reagent (ml)	3.1	3.0	3.0	3.0	3.0	3.0
Respective diluted standard ml)	-	0.1	0.1	0.1	-	-
Diluted test /QC (ml)	-	-	-	-	0.1	0.1

Mix all tubes well. Keep them in a boiling waterbath for 15 minutes. Remove from waterbath and cool the tubes for 5 minutes. Set the spectrophometer/filter photometer to zero with blank at 540nm/yellow green filter and measure the absorbance of the other tubes.

Calculation and calibration graph

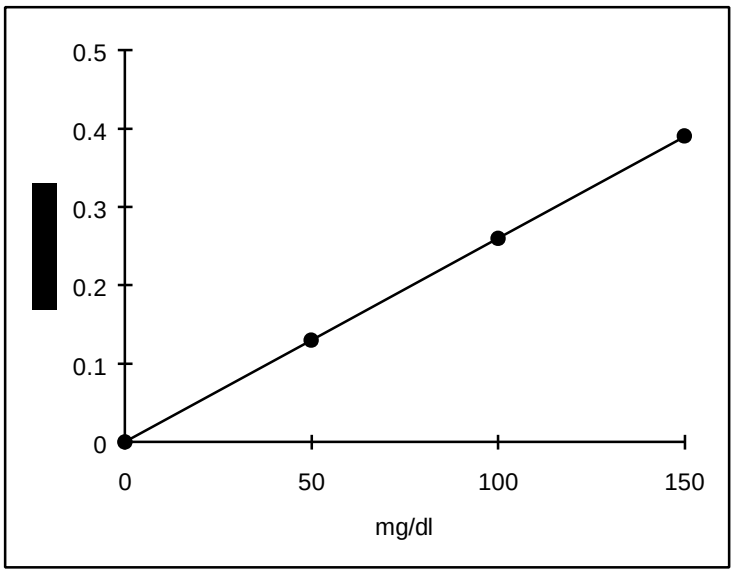
Concentration of standards:

S1 = 50 mg/dl
S2 = 100 mg/dl
S3 = 150 mg/dl

Plot the absorbance values of standards against their respective concentrations. The measurable range with this graph is from 10 to 150 mg/dl. A calibration graph should be constructed whenever a new set of reagents is prepared. Plot absorbance values of test/QC on the calibration graph and read off the concentrations.

Once linearity is proved, it will be enough if S3 is set up every time that patients ' samples are analysed and the results calculated using the formula:

$$\text{Urea in test sample} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times 150 \text{ mg/dl}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** for the use of internal QC and interpretation of daily QC data (for releasing patients 'results).

Since urea is one of the most common analytes measured in a laboratory after glucose, it is recommended that an internal QC (normal QC pool) be included with every batch of samples analysed in the day, irrespective of the number of samples in a batch. Further, even when a single sample is analysed as an "emergency" sample at any time of the day or night, it is essential to include an internal QC. From the QC results obtained for the day, mean, standard deviation and %CV can be calculated to ensure that ***within-day precision*** is well within the acceptable limit, i.e. 4%.

The mean value of internal QC for the day can be pooled with the preceding 10 or 20 mean values obtained in the previous days and between–day precision can be calculated and expressed as % CV. Ensure that this is well within the acceptable limit, i.e. 8%.

At least once a day analyse another QC serum from either a low QC or high QC pool.

"Assayed" QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

Most of the chemicals used in this method are acids. Care should therefore be taken to avoid mouth pipetting and contact with skin.

Reference range and clinical interpretation

Serum/ Plasma Urea ... .15 – 40 mg/dl

Elevated serum urea levels may be due to pre-renal, renal or post-renal etiology. Pre-renal causes could be cardiac related or due to increased protein catabolism, and dehydration. Renal causes include glomerulonephritis, chronic nephritis, nephrotic syndrome and other kidney disease. Post-renal causes include obstruction of the urinary tract.

Decreased serum urea levels could be due to pregnancy, intravenous infusion, low antidiuretic hormone secretion, low protein intake, severe liver diseases, inborn errors of urea cycle and SIADH (Syndrome of inappropriate ADH secretion).

Limitations

Specimens with gross icterus cannot be assayed as it will cause falsely elevated urea values. Do not report results from specimen with suspected interference. Inform the requesting physician of the problem.

References

(1) Wybenga, D.R., Di Glorgio, J.& Pileggi, V.J. (1971). Clinical Chem., 17, 891-895.

(2) Seaton, B & Ali. A (1984) Med. Lab. Sciences 41, 327 –336.

5. Creatinine – Jaffe's method

Introduction

Creatinine is a waste product formed in muscle from a high-energy storage compound, creatine phosphate. Creatine phosphate can be stored in muscle at approximately four times the concentration of adenosine triphosphate. In muscles it spontaneously undergoes degradation to form a cyclic anhyride-creatinine. The blood concentration of creatinine and its excretion in urine are remarkably constant in normal individuals. Therefore serum creatinine level is used as an indicator for assessing kidney function.

Principle of the method

Creatinine present in serum or plasma directly reacts with alkaline picrate resulting in the formation of a red colour, the intensity of which is measured at 505nm/green filter. Protein interference is eliminated using sodium lauryl sulphate. A second absorbance reading after acidifying with 30% acetic acid corrects for non-specific chromogens in the samples.

Specimen type, collection and storage

Serum or plasma can be used. Avoid using haemolysed or lipaemic samples. Stable for 12 hours at room temperature (25-35^{0}C), one week at 2-8^{0}C and for 3 months at –20^{0}C.

Reagents

All chemicals must be Analar grade

(a) Reagent A

Into 400ml of distilled water taken in a 500 ml beaker add 4.4g of NaOH. Mix to dissolve, then add 9.5g trisodium phosphate [$Na_3PO412H_2O$], dissolve and then add 9.5g of sodium tetraborate [$Na_2B_4O_710H_2O$]. After dissolving check that the pH is above 10, adjust if necessary by the dropwise addition of 1M NaOH. Transfer to a 500 ml volume flask and make up to 500ml with distilled water. Mix well. Stable for 3 months at 2-8^{0}C.

(b) Reagent B

Dissolve 20g sodium lauryl sulfate in a final volume of 500ml distilled water. Stable for 6 months at room temperature (25-35^{0}C).

(c) Reagent C

Picric acid supplied commercially contains 50% by weight of water to ensure safety in transit. Therefore the amount of picric acid weighed out should be proportionally more than the amount of the required anhydrous picric acid.

For reagent C, 4.6g of anhydrous picric acid is required. Therefore weigh approximately 7.0g but not less than 6.0g moist picric acid and add to 500ml of distilled water taken in a volumetric flask, mix and leave overnight at 37^0 C. Then filter and store in brown glass bottle at room temperature (25-35^{0}C). Stable for 1 year.

(d) Working reagent

At the time of analysis freshly mix equal volumes of the above three reagents. After use discard any leftover working reagent.

(e) Stock creatinine standard 100mg/dl

Dissolve 100 mg of pure creatinine in 0.1 M HCl and make up to 100 ml with 0.1 M HCl in a volumetric flask. Stable for 6 months at 2-8^0 C.

(f) Working creatinine standard

Dilute 2, 4, 6 and 8 ml of stock creatinine standard each to 100 ml with 0.1 M HCl to get creatinine concentrations of 2, 4, 6 and 8 mg/dl, respectively. Stable for 6 months at 2-80C.

(g) 30% (V/V) Acetic acid

Dilute 30ml of glacial acetic acid to 100ml with distilled water. Stable for 3 months at room temperature (25-35^0 C).

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

Pipette the following into appropriately labelled 18 x 150 mm tubes

(Standards:S2 = 2mg/dl, S4 = 4mg/dl, S6 = 6mg/dl & S8 = 8mg/dl)

	Blank	S1	S2	S3	Test	QC
Working reagent (ml)	3.0	3.0	3.0	3.0	3.0	3.0
Distilled Water (ml)	0.2	-	-	-	-	-

	Blank	S1	S2	S3	Test	QC
Standard (ml)	-	0.2	0.2	0.2	-	-
Test sample /QC (ml)	-	-	-	-	0.2	0.2
Mix Well						

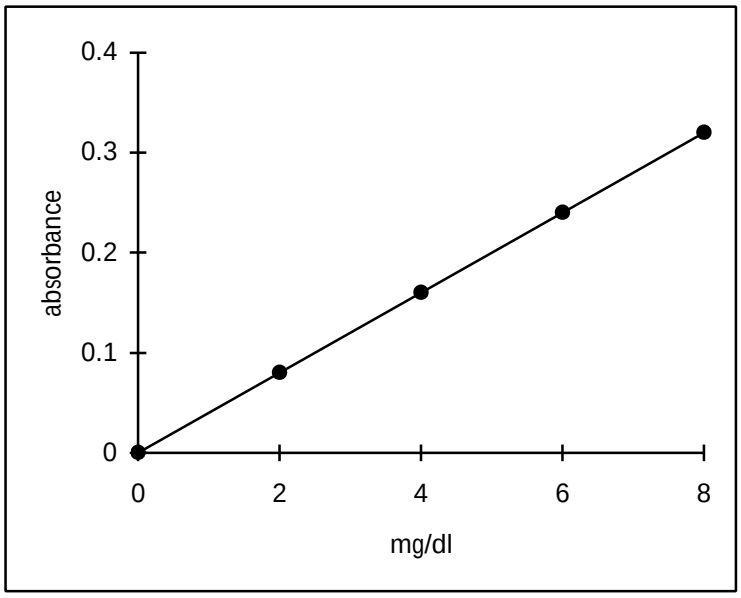
Leave at room temperature (25-35^{0}C) for 30 minutes. Set the spectrophotometer/ filter photometer to zero with blank at 505 nm/green filter and measure the absorbance of the other tubes. After measuring the absorbance pour the solutions back into the respective tubes. Then add 0.2 ml of 30% acetic acid to the test and QC tubes, mix well and leave at room temperature (25-35^{0}C) for 5 minutes. Again set the spectrophotometer/filter photometer to zero with blank at 505nm/green filter and measure the absorbance of test and QC.

Calculation and calibration graph

Subtract the second absorbance values of test and QC from the first set of values. Draw a calibration graph by plotting the absorbance values of standards against their respective concentrations. The measurable range with this graph is from 0.2 to 8.0 mg/dl. Plot the corrected absorbance of test and QC and read off the values of creatinine.

Once linearity is proved, it is enough if a single standard such as S6 is taken each time when patients ' samples are analysed and the results are calculated using the following formula

$$\text{Serum Creatinine} = \frac{\text{Test absorbance}}{\text{Standard absorbance}} \times 6 \text{ mg/dl}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients 'results).

Since creatinine is one of the most common analytes measured in a laboratory, inclusion of an internal QC (normal QC pool) with every batch of samples analysed in the day, is recommended, irrespective of the number of samples in a batch. Further, even when a single sample is analysed as an "emergency" sample at any time of the day or night, it is essential to include an internal QC. From the QC results obtained for the day, mean, standard deviation and %CV can be calculated to ensure that ***within-day precision*** is well within the acceptable limit, i.e, 5%.

The mean value of internal QC for the day can be pooled with the preceding 10 or 20 mean values obtained in the previous days and ***between-day precision*** can be calculated and expressed as % CV. Ensure that this is well within the acceptable limit, i.e, 8%.

At least once a day analyse another QC serum from either a low QC or high QC pool.

"Assayed" QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

Picric acid is poisonous and sodium hydroxide is caustic – avoid contact with skin and mucous membranes.

Reference range and clinical interpretation

Serum/Plasma Creatinine: Male 0.7 – 1.4 mg%
Female 0.4 – 1.2 mg%

Serum creatinine concentration is related to muscle mass and the values are lower in children. Increased serum creatinine is associated with decrease in glomerular filtration rate (GFR), whether the cause is pre-renal, renal or post- renal. Pre-renal factors include conditions such as congestive heart failure, shock, diarrhoea, uncontrolled diabetes mellitus, use of diuretics, etc. Renal factors involve mainly damage to the glomeruli. Post-renal factors may be prostatic hypertrophy, calculi blocking the ureters or neoplasms compressing the ureters. The serum creatinine concentration is monitored closely after a renal transplantation because a rising concentration, even though small, may be an indication of graft rejection.

Limitations

Ascorbic acid, uric acid, glucose, ketones and cephalosporin antibiotics, if present at high concentrations, may interfere in the assay causing falsely high values. Do not report the

result from specimens with suspected interference. Inform the requesting physician of the problem.

References

(1) Slot C. (1965) Scand J. Clin. Lab Invest. 17, 381 –387.

(2) Seation B. & Ali A (1984) Med. Lab Sci., 41, 327- 336.

6. Cholesterol – Cholesterol oxidase method

Introduction

The major constituents of plasma lipids are cholesterol and triglycerides. Cholesterol is an important compound of cell membrane and precursor for the synthesis of bile salts and steroid hormones.

Cholesterol is synthesized in the liver and transported in the blood mainly in the form of LDL and HDL. In blood, cholesterol is present in free as well as esterified form.

Over the decades serum cholesterol has been measured by methods employing Liebermann-Burchard reaction. The enzymatic method has become popular in recent years. The percentage of participants in the External Quality Assessment Scheme conducted from the author's laboratory, employing the enzymatic method, has increased significantly from 10 to 85 in the last decade.

Principle of the method

Cholesterol esters in serum are hydrolysed by cholesterol esterase. The free cholesterol is then oxidized by cholesterol oxidase to the corresponding ketone liberating hydrogen peroxide, which is then converted to water and oxygen by the enzyme peroxidase. Para aminophenazone (4 aminophenazone) takes up the oxygen and together with phenol forms a pink coloured quinoneimine dye, which can be measured at 515nm/ yellow green filter.

Specimen type, collection and storage

Serum or plasma can be used. A fasting blood sample is preferred for lipid profile test. However, if cholesterol alone has to be analysed, a random sample can also be used.

The specimen is stable for a week at 2 - 8^{0}C and at least for 3 months at - 20^{0}C.

Reagents

All chemicals must be Analar grade

General note: Several companies provide compact kits for the measurement of

cholesterol by the enzymatic method. These kits are most economical and readily available and therefore practically in most of the laboratories cholesterol is measured by using kits. Laboratories using kits are advised to follow carefully the instructions given in the leaflet. Commercial companies generally provide a single reagent consisting of the following chemicals:

4 –aminophenazone	Cholesterol esterase
Phenol	Cholesterol oxidase
Peroxidase	Sodium azide

The reagent is provided in the lyophilysed form and proper instructions are given for reconstitution and use in the assay. The reagent is generally stable for one week when stored at 15 to 25^{0}C and one month at 2- 8^{0}C.

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

Pipette the following into appropriately labelled 13 x 100 mm tubes

	Blank	**Standard**	**Test**	**QC**
Reagent solution (ml)	2.0	2.0	2.0	2.0
Standard (ml)	-	0.02	-	-
Test sample /QC (ml)	-	-	0.02	0.02

Mix well. Incubate at 37^{0}C in a waterbath for 5 minutes or at room temperature (25- 35^{0}C) for 15 minutes. Remove from waterbath and cool to room temperature. Set spectrophotometer / filter photometer to zero using blank at 510 nm / yellow green filter and measure the absorbance of standard, test and QC.

This protocol is designed for spectrophotometers / filter photometers that require a minimum volume of reaction mixture in the cuvette of 1ml or less. Since economical use of the reagent is possible with this protocol, the cost per test can be kept to the minimum. However, if a laboratory employs a photometer requiring a large volume of reaction mixture for measurement, viz. 5 ml, it is advised that the volumes of reagent, standard, and test sample/QC mentioned under #6 be increased proportionately.

Calculation and calibration graph

Linearity for calibration graph has been well documented by several kit companies. In the author's laboratory it is from 20 to 500 mg/dl.

Therefore a single standard (viz. 200 mg/dl) can be used and cholesterol in patients ' samples can be calculated using the formula.

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc. of Std} \; (200) \; \text{mg/dl}$$

Anaytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients 'results).

Include one internal QC in every batch of samples analysed every day irrespective of the number of samples in a batch. Since cholesterol is analysed in a single batch in a day in an intermediate laboratory, it will not be possible to analyse several QC samples and calculate within-day precision. However, even if a single QC sample is analysed in a day, this value can be pooled with the preceding 10 or 20 values obtained in the previous days and ***between-day precision*** can be calculated and expressed as %CV. Ensure that this is well within the acceptable limit, i.e. 8%.

Once a week it will be good to analyse another QC serum from either a low QC or a high QC pool.

"Assayed " QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

This procedure uses phenol, which is caustic. Do not swallow, and avoid contact with skin and mucous membranes.

Reference range and clinical interpretation

Serum Cholesterol - 150-250 mg/dl

Serum cholesterol is increased in hypothyroidism, diabetes mellitus, nephrotic syndrome and in various hyperlipidaemias especially those causing xanthomatosis. Elevated serum cholesterol is a serious risk factor for the development of coronary artery disease. Decreased serum cholesterol is seen in severe hepatoclellular disease, hyperthyroidism and anaemia.

Limitations

Haemolysis and lipaemia cause elevated cholesterol levels.

Serum bilirubin >5mg/dl and ascorbic acid > 10 mg/dl also cause elevated cholesterol levels. Do not report results from specimens with suspected interference. Inform the requesting physician of the problem.

Reference

(1) Allain CC, Poon LS, Chan CSG et al. (1974) Clin Chem 20 : 470

7. Bilirubin – Jendrassik & Grof method

Introduction

Bilirubin is formed from the haem fragment of haemoglobin released by aged or damaged red blood cells. Liver, spleen and bone marrow are the sites of bilirubin production. Bilirubin formed in spleen and bone marrow is transported to the liver. In the liver it is converted into bilirubin conjugates – bilirubin mono and diglucuronides. Any liver disease affects the above systems, and hence bilirubin accumulates in serum leading to jaundice.

Principle of the method

Conjugated (direct) bilirubin in serum is coupled with diazotised sulphanilic acid to form a red coloured compound. Ascorbic acid is used to stop the coupling reaction, and to eliminate interference by haemoglobin. Caffeine benzoate solution is used to split the unconjugated bilirubin protein complex releasing the bilirubin so that it can react with diazotised sulphanilic acid.

The tartrate buffer makes the mixture alkaline and converts the red acid bilirubin to a green coloured compound which shows peak absorbance at 607 nm. At this wavelength the absorbance due to haemoglobin or carotene is minimal.

Specimen type, collection and storage

Use only clear, non-haemolysed samples of serum. Bilirubin is unstable and light sensitive and therefore the assay should be carried out within 2 hours of sample collection. If a longer delay is unavoidable, refrigerate the sample. Samples can be frozen at –20^{0}C, to keep bilirubin stable for 2 months.

Reagents

All chemicals must be Analar grade

(a) Caffeine-benzoate

Dissolve 100g caffeine sodium benzoate and 25 g sodium benzoate together in 800 ml of distilled water. Heat the solution to 60^{0}C and then add 125g of hydrated sodium acetate and 1g of EDTA. Mix to dissolve and then make up to 1 litre with distilled water. Filter the solution. Store at room temperature (25-30^{0}C). Stable for 6 months.

(b) Sulphanilic acid

Dissolve 5 g sulphanilic acid in 500ml distilled water with heating. Add 15ml of conc. HCl and when cool make up to 1 litre. Store at room temperature (25-30^{0}C). Stable for 6 months.

(c) Sodium nitrite

Dissolve 500 mg sodium nitrite in about 80ml distilled water and then make up to 100 ml. Store at 2-8^{0}C. Prepare fresh once a month.

(d) Diazo reagent

Mix 10ml sulphanilic acid with 0.25ml sodium nitrite. The solution is stable for approximately 3 hours at room temperature (25-30^{0}C) and 24 hours at 2-8^{0}C.

(e) Alkaline tartrate

Dissolve 100g NaOH and 350 g sodium potassium tartrate in distilled water and make up to 1 litre. Store at room temperature (25-30^{0}C). Stable for 6 months.

(f) Ascorbic acid (4 g/dl)

Dissolve 200 mg of ascorbic acid in 5ml of distilled water. This solution must be freshly prepared each day.

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

Add reagents, standards and test samples/QC in the order indicated into appropriately labelled tubes (18 x 150mm)

	Standard Blank	Std	Test/QC Blank	Test/QC Direct Bil.	Test/QC Total Bil.
Distilled water (ml)	0.8	0.8	0.8	0.8	0.8
Bilirubin Std (ml)	0.2	0.2	-	-	-
Test sample /QC (ml)	-	-	0.2	0.2	0.2
Diazo reagent (ml)	-	0.5	-	0.5	0.5
Caffeine sodium benzoate (ml)	-	2.0	-	-	2.0
Mix and wait for 10 minutes at room temperature (25-30^{0}C)					

	Standard Blank	Std	Test/QC Blank	Test/QC Direct Bil.	Test/QC Total Bil.
Ascorbic (ml)	0.1	0.1	0.1	0.1	0.1
Diazo reagent (ml)	0.5	-	0.5	-	-
Caffeine sodium benzoate (ml)	2.0	-	2.0	2.0	-
Alkaline Tartrate (ml)	1.5	1.5	1.5	1.5	1.5

Mix all tubes. Set the spectrophotometer/ filter photometer to zero with distilled water at 607nm/ orange filter and read the absorbance in the order of assay tubes mentioned in the Table.

Preparation of bilirubin standard

Commercially available bilirubin is water insoluble but the addition of a small amount of dimethyl sulphoxide (DMSO) and NaOH will dissolve it. As diluent, non-icteric and non-lipaemic human pooled serum tested negative for HIV antibodies and Hbs antigen could be used. However, there is always a risk of infection from this material.

Pool daily leftover normal human sera until about 120 ml are collected. Take an aliquot for screening for the presence of HIV antibodies and Hbs antigen and ensure that both are negative. Centrifuge the pooled serum twice at 3500 rpm for 10 minutes and collect the serum in a clean container. Measure the total bilirubin of the pooled serum to ensure that it is < 0.5 mg/dl.

Weigh accurately 10mg bilirubin in a small stoppered glass tube. Add 2.0 ml DMSO and 0.5 ml of 0.4M NaOH and shake the tube in dim light until the bilirubin has dissolved (It may be necessary to warm the tube in a 37^0C water-bath to speed up this step).

Transfer about 75ml of the pooled serum into a 100 ml volumetric flask. Add the bilirubin solution slowly with continuous mixing to the serum. Rinse the tube with a few drops of DMSO and then with pooled serum and add to the flask. Make up to the mark with the pooled serum. Froth can be dispersed by touching with a glass rod minimally smeared with silicone or by adding a trace of capryl alcohol (octan –2-ol). Wrap the flask with carbon paper and store it at 2-8^0C until the standarization procedure is complete. This bilirubin standard will have a concentration of about 10-11 mg/dl. The exact value will be determined after carrying out the procedure outlined in 3.5.7

Calculation and calibration graph

Extrapolation method

Prepare standards (S_1-S_5) as shown below

	S1	S2	S3	S4	S5
Bilirubin standard (ml)	0.02	0.05	0.1	0.15	0.2
Pooled serum (ml)	0.18	0.15	0.1	0.05	-
Theoretical Concentration of bilirubin (mg/dl)	1.0	2.5	5.0	7.5	10

Proceed with bilirubin estimation in the usual way as for total bilirubin. Measure the absorbance of all standards against distilled water in a spectrophotometer at 607 nm/ orange filter in a filter photometer. Construct a calibration graph by plotting the theoretical bilirubin concentrations against the corresponding absorbance values. This graph will not pass through the origin, instead it will intercept the 'y' axis at a specific point, indicating that the blank tube contains a certain amount of bilirubin corresponding to the 'y' intercept absorbance value. Upon extrapolation of the calibration graph, this will read a specific value of bilirubin on the negative side of the x axis (concentration axis). In the sample graph given below (graph I), the bilirubin content of the blank is shown as 0.5 mg/dl. This value must be added to the amount of bilirubin dissolved in 100 ml of pooled serum. Therefore, in this example the actual bilirubin content in the standard S5 will be 10 + 0.5 = 10.5 mg/dl. Hence the levels of bilirubin in the diluted standards from S4 down to S1 will vary accordingly. This is explained in the Table given below.

Standard	**Theoretical bilirubin value (mg/dl)**	**Actual bilirubin value after correcting for blank (mg/dl)**
S5	10.0	10.50
S6	7.5	7.85
S3	5.0	5.25
S2	2.5	2.60
S1	1.0	1.05

The calibration graph with corrected bilirubin values should then be plotted against absorbance values as shown in graph II. The test/ QC absorbance values should be plotted on this graph and concentrations read off. The measurable range with this graph is from 0.2 to 20.0 mg/dl.

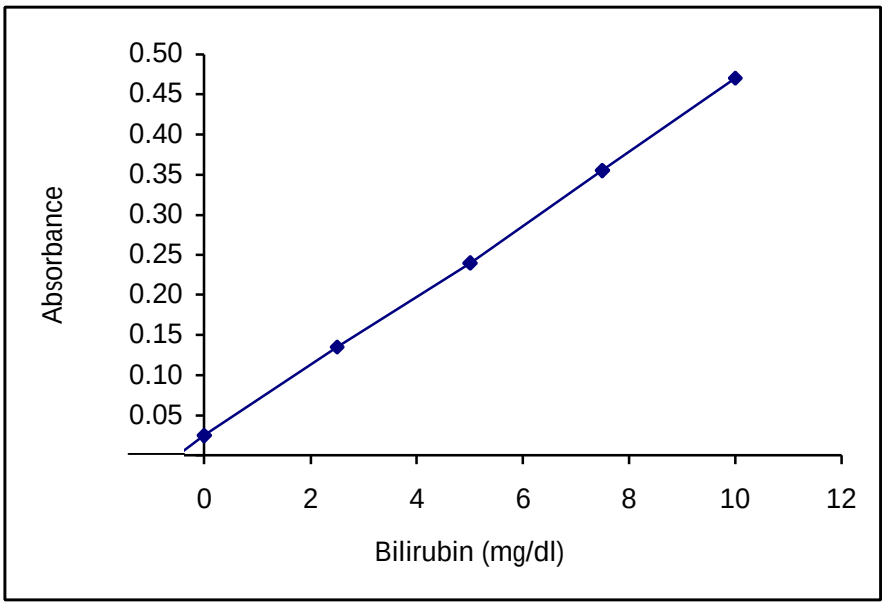
Once linearity is proved, it will be enough if a single standard is set up every time that patients 'samples are analysed and the results are calculated using the formula:

$$\frac{\text{Test absorbance - Test blank absorbance (TBK}}{\text{Std. Absorbance – Std blank absorbance (SBK)}} \times \text{Concentration of Bilirubin standard}$$

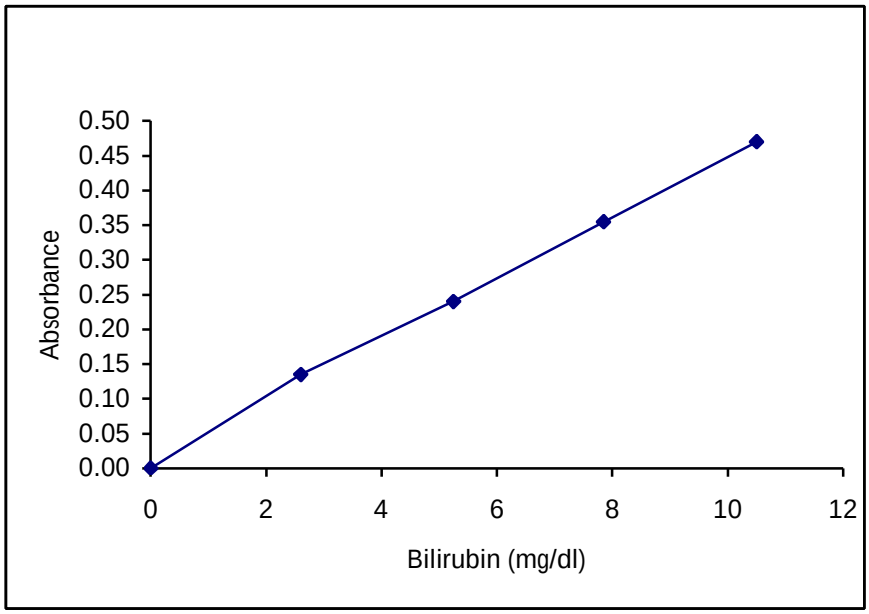
$$\text{1. Total Bilirubin mg/dl} = \frac{\text{Test (total) absorbance-TBK}}{\text{Standard absorbance – SBK}} \times \text{Concentration of Bilirubin standard}$$

$$\text{2. Direct Bilirubin mg/dl} = \frac{\text{Test (direct) absorbance -TBK}}{\text{Standard absorbance – SBK}} \times \text{Concentration of Bilirubin standard}$$

Graph 1



Graph 2



Storage of standard

Aliquot small volumes of standard into screw-capped vials and store in the freezer on the same day it is prepared. Stable for 2 months at - 20^0C. ***Do not refreeze leftover standard after use.***

Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients 'results).

Include one internal QC in every batch of samples analysed every day irrespective of the number of samples in a batch. Since bilirubin is analysed in a single batch in a day in an intermediate laboratory, it will not be possible to analyse several QC samples and calculate within-day precision. However, even if a single QC sample is analysed in a day, this value can be pooled with the preceding 10 or 20 values obtained in the previous days and ***between-day precision*** can be calculated and expressed as %CV. Ensure that this is well within the acceptable limit, i.e, 10%.

Once a week it is good to analyse another QC serum from either a low QC or high QC pool.

"Assayed " QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

This method uses sulphanilic acid and sodium hydroxide. Avoid contact with eyes, skin and mucous membranes.

Reference range and clinical interpretation

Serum Direct Bilirubin - up to 0.5 mg/dl

Serum Total Bilirubin - 0.2 –1.0 mg/dl

Hyperbilirubinaemia is characteristic of jaundice. Increase in unconjugated bilirubin is observed in haemolytic and neonatal jaundice.

In viral and toxic hepatitis there is impaired hepatocellular conjugation and excretion of bilirubin with a major rise in conjugated and a lesser rise in unconjugated bilirubin in serum. In cirrhosis there is overall damage to liver cells and hence the ability of the liver to form conjugated bilirubin, resulting in an increase in unconjugated bilirubin in serum. In obstructive jaundice there is an increase in predominantly conjugated bilirubin in serum.

Limitations

Samples with bilirubin concentrations higher than 20mg/dl should be diluted with an equal volume of distilled water and the result obtained should be multiplied by 2. There is no interference in the assay by haemoglobin up to a concentration of 1.0g/dl; however, strong haemolysis will interfere negatively with measurement. Do not report results for specimens with suspected interference. Inform the requesting physician of the problem.

Reference

(1) Doumas BT, Kwok-Cheung PP, Perry BW, et al. Clin. Chem.

(2) (1985) 31 : 1779-89.

(3) Tietz. NW. Fundamentals of Clinical Chemistry.

(4) Published by WB Saunders Company. 1986. page 1388-1390.

8. Total Protein –Biuret method

Introduction

The serum-total protein, as its name implies, represents the sum total of numerous different proteins, many of which vary independently of each other. Proteins are present in all body fluids but the protein concentration is normally high (> 3g/dl) only in plasma, lymphatic fluids and some exudates. Protein concentration in the cerebro-spinal fluid of normal subjects is < 45 mg/dl, whereas the urine contains only a trace. Measurement of serum-total protein is useful in conditions relating to changes in plasma or fluid volumes, such as shock and dehydration. In these conditions concentration of serum-total protein is elevated indicating hemoconcentration. Haemodilution is reflected as relative hypoproteinemia, which occurs with water intoxication or salt retention syndrome, during massive intravenous infusions.

Principle of the method

Proteins form a purple coloured complex with cupric ions in alkaline solution. The reaction takes its name from the simple compound biuret which reacts in the same way. The intensity of the purple colour is measured at 540 nm / yellow green filter and compared with a standard serum of known protein concentration.

Specimen type, collection and storage

Either serum or plasma may be used, but serum is preferred. A fasting specimen is not required but may be desired to decrease lipaemia. Avoid hamolysis. Tightly stoppered samples are stable for 24 hours at room temperature (25-35^{0}C), one week at 2-8^{0}C and for 3 months at -20^{0}C.

Reagents

All Chemicals must be Analar grade

(a) Sodium chloride diluent 0.9% W/V

Dissolve 4.5g sodium chloride in about 400ml of distilled water and then make up to 500ml with distilled water. Stable at room temperature (25-35^{0}C). Make a fresh solution once in 6 months.

(b) Biuret reagent

Dissolve 4.0g sodium hydroxide in about 400 ml of distilled water. Add 4.5 g sodium potassium tartrate. Mix to dissolve. Then add 1.5g copper sulphate followed by 4.5g potassium iodide. Transfer the solution into a 500 ml volumetric flask and make up to the mark with distilled water. Store in a tightly stoppered polyethylene bottle at room temperature (25-35^{0}C). Stable for 6 months.

(c) Standard

In many biochemical estimations direct standardization is employed; for example, glucose, urea and creatinine are available in pure forms, and can be directly weighed and used. In the case of serum total proteins this is not possible since serum proteins consist of several protein fractions. However, an acceptable procedure is to make use of bovine albumin to prepare the standard. Alternatively, the laboratory can obtain protein standard from commercial firms or prepare standard in-house.

The method of in-house preparation of standard is described below :

The laboratory can use either leftover patients 'sera or bovine serum to prepare the standard. Note that there is a risk of infection from pooled patients 'specimens. Prepare pooled serum as described in Section 1 General Introduction under Preparation of QC pool ' After aliquoting and freezing the pooled serum, analyse total protein in the adequots daily for a period of 20 days using a reliable protein standard (commercial source or non-commercial source such as WHO). Calculate the mean value and assign this as the standard value.

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

Pipette the following into appropriately labelled 18 x 150 mm tubes (Standard = S1, S2 & S3)

	Blank	**S1**	**S2**	**S3**	**Test**	**QC**
Sodium Chloride diluent (ml)	2.5	2.45	2.4	2.35	2.4	2.4
Standard (ml)	-	0.05	0.1	0.15	-	-
Test Sample /QC (ml)	-	-	-	-	0.1	0.1
Mix well						
Biuret reagent (ml)	3.0	3.0	3.0	3.0	3.0	3.0
Mix well						

Incubate at room temperature (25-35^{0}C) for 15 minutes. Set the spectrophotometer / filter photometer to zero using blank at 540 nm/ yellow green filter and measure the absorbance of standards, test & QC.

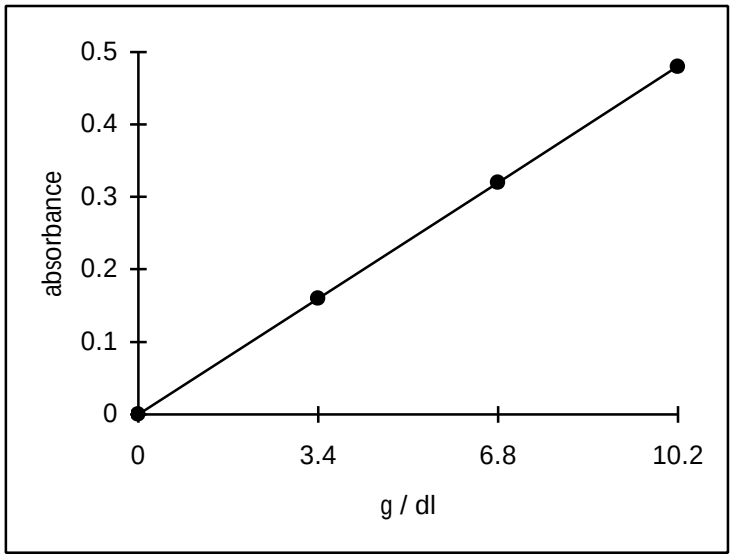
Calculation and calibration graph

Since the protocol for standard tube S2 and the test is identical, standard S2 will represent the actual concentration of the pooled serum used as standard. Standard S1 contains half the volume of S2 and S3 has 1½ times the volume of S2. Therefore protein concentrations represented by S1 and S3 will be ½ and 1½ times of S2 concentration, respectively. For example, if S2 concentration is 6g/dl, S1 will be 3g/dl and S3 will be 9g/dl.

Plot the absorbance values of standards against their respective concentrations. The measurable range with this graph is from 0.5 to10.0g/dl. Plot the absorbance values of test/QC on the calibration graph and read off the concentrations.

Once linearity is proved, it is not necessary to prepare standard graph every time when patients 'samples are analysed. It will be adequate if standard S2 is taken every time and patients 'results are calculated using the formula:

$$\frac{\text{Test absorbance}}{\text{Standard absorbance}} \times \text{Concentration of S2 ..... g/dl}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients 'results).

Include one internal QC in every batch of samples analysed every day irrespective of the number of samples in a batch. Since total protein is analysed in a single batch in a

day in an intermediate laboratory, it will not be possible to analyse several QC samples and calculate within-day precision. However, even if a single QC sample is analysed in a day, this value can be pooled with the preceding 10 0r 20 values obtained in the previous days and ***between-day precision*** can be calculated and expressed as %CV. Ensure that this is well within the acceptable limit, i.e, 6%.

Once a week it will be good to analyse another QC serum from either a low QC or high QC pool.

"Assayed " QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

Sodium hydroxide used in this procedure is a strong alkali and is caustic. Do not swallow, and avoid contact with skin and mucous membranes.

Reference range and clinical interpretation

Serum Total Protein ... ... 6.5 –8.5 g/dl

In addition to dehydration and diarrhoea, increased serum-total protein levels are observed in multiple myeloma. Hypoproteinemia is generally seen in conditions associated with hypoalbuminemia.

Limitations

Haemolysed and lipaemic sera interfere strongly with the measurement of proteins. Set up appropriate blank for such samples by adding 01.ml of serum to 5.5ml of sodium chloride diluent. The absorbance obtained against distilled water is then subtracted from the test absorbance before the test result is calculated.

Reference

(1) Reinhold. J.G. (1953). Standard methods of clinical chemistry 1:p :88

9. Albumin - BCG dye binding method

Introduction

Serum albumin consists of a single species of proteins representing approximately 60% of the total protein. It is synthesized exclusively in the liver and functions as a regulator of blood oncotic pressure, as a carrier for many cations and water insoluble substances, and as a pool of aminoacids for caloric or synthetic purposes.

Principle of the method

Albumin binds quantitatively with bromocresol green at pH 4.15 resulting in the formation of a green colour which can be measured at 630nm/red filter.

Specimen type, collection and storage

Either serum or plasma may be used, but serum is preferred. A fasting specimen is not required but may be desired to decrease lipaemia. Avoid haemolysis. Tightly stoppered samples are stable for 24 hours at room temperature (25-35^{0}C), one week at 2-8^{0}C and for 3 months at -20^{0}C.

Reagents

All chemicals must be Analar grade

(a) Sodium hydroxide 1 M :

Weigh out 4.0 g of sodium hydroxide (NaOH), dissolve and make up to 100ml with distilled water. This solution is stable for several months at room temperature (25-35^{0}C) in a polypropylene container.

(b) Brij - 35..... 30g/dl

Readily available at the above concentration from S.D Fine chemicals or Loba Chemical Company, in India.

Solid Brij can also be obtained from Sigma Co. In this case, warm 30g solid Brij in a beaker in a small volume of distilled water to dissolve and make up to 100ml with distilled water.

(c) Bromo Cresol Green (BCG) dye solution:

Transfer 25ml of I M NaOH into a one-litre volumetric flask containing 600ml distilled water. Add 5.6g succinic acid and then add 56 mg of BCG powder. Mix and then make up to 1 litre with distilled water. Check the pH. If it is less than 4.15, adjust to 4.15 $\pm$ 0.05 by the dropwise addition of 1 M NaOH.

Add 100 mg sodium azide and 3.5ml 30 g/dl Brij-35 to the reagent. Check the absorbance of the reagent at 630 nm/ red filter against distilled water. It should be less than 0.2. If it is greater than 0.2, add some more Brij to bring down the absorbance. Store in a polyethlyene container. Stable for 6 months at room temperature (25-35^{0}C).

(d) Standard

In many biochemical estimations direct standardization is employed; for example, glucose urea and creatinine are available in pure forms, and can be directly weighed and used, whereas, in the case of human albumin, this is not readily available. The laboratory can obtain albumin standard from commercial firms or prepare the standard in-house.

The method of in-house preparation of the standard is described below:

Prepare pooled serum as described in **Section 1 General Introduction** under **Preparation of QC Pool '**. The laboratory should pool daily leftover patients' sera. For albumin standard, only the use of human serum is recommended, since it has been documented that the affinity of BCG to bovine albumin is different from its affinity to human albumin beyond 3g/dI. After aliquoting and freezing the pooled serum, analyse albumin in the aliquots daily for a period of 20 days using a reliable albumin standard (commercial source or non-commercial source such as WHO). Calculate the mean value and assign this as the standard value.

Note: There is always a risk of infection from this material.

Equipment, glassware and other accessories

Refer Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

(a) Dilution of Standards (S1 -S4), Test & QC

Pipette the following into appropriately labelled 13 x 100mm tubes

	S1	S2	S3	S4	Test	QC
Distilled water (ml)	1.9	1.8	1.7	1.6	1.8	1.8
Standard (ml)	0.1	0.2	0.3	0.4	-	-
Test sample/QC (ml)	-	-	-	-	0.2	0.2
Mix well						

(b) Colour Development

Pipette the following into another set of appropriately labelled 18 x 50mm tubes

	Blank	S1	S2	S3	Test	QC
Distilled Water (ml)	0.1	-	-	-	-	-
Diluted Standard (ml)	-	0.1	0.1	0.1	-	-
Diluted Test Sample /QC (ml)	-	-	-	-	0.1	0.1
BCG Solution (ml)	2.5	2.5	2.5	2.5	2.5	2.5

Mix all tubes well. Incubate at room temperature (25-35^{0}C) for 10 minutes. Set the spectrophotometer /filter photometer to zero using blank at 630 nm/ red filter and measure the absorbance of standards, test & QC.

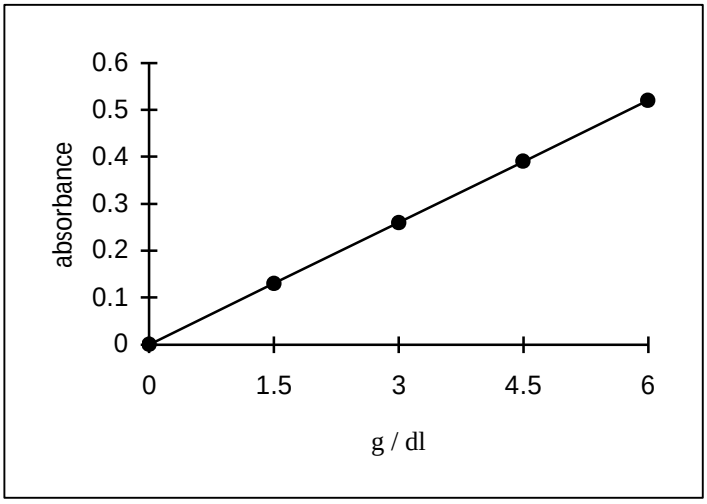
Calculation and calibration graph

Since the protocol for standard tube S2 and the test is identical, standard S2 will represent the actual concentration of the pooled serum used as standard. Standard S1 contains half the volume of S2 , S3 has 1½ times and S4 has twice the volume of S2. Therefore albumin concentrations represented by S1, S3 and S4 will be ½ , 1 ½ and 2 times of S2 concentration respectively. For example, if S2 concentration is 3g/dl, SI will be 1.5g/dl, S3 will be 4.5g/dl and S4 will be 6g/dl.

Plot the absorbance values of standards against their respective concentrations. The measurable range with this graph is from 0.5 to 6.0 g/dl. Plot the absorbance values of test/QC on the calibration graph and read off the concentrations.

Once linearity is proved, it is not necessary to prepare the standard graph every time when patients' samples are analysed. It will be adequate if standard S2 is taken every time and patients' results are calculated using the formula :

$$\frac{\text{Test absorbance}}{\text{Standard absorbance}} \times \text{Concentration of S2 g/dl}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients' results).

Include one internal QC in every batch of samples analysed every day irrespective of the number of samples in a batch. Since albumin is analysed in a single batch once a day in an intermediate laboratory, it will not be possible to analyse several QC samples and calculate within-day precision. However, even if only a single QC sample is analysed in a day, this value can be pooled with the preceding 10 or 20 values obtained in the previous days and ***between-day precision*** can be calculated and expressed as %CV. Ensure that this is well within the acceptable limit, i.e, 6%.

At least once a week analyse another QC serum from either a low QC or high QC pool.

"Assayed" QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

Sodium hydroxide used in this procedure is a strong alkali and is caustic. Do not swallow, and avoid contact with skin and mucous membranes.

Reference range and clinical interpretation

Serum albumin - 3.5- 5.0 g/dl

Serum levels of albumin are used to assess nutritional status and have important influences on the metabolism of endogenous substances such as calcium, bilirubin and fatty acids and on the effect of drugs and hormones. Hyperalbuminemia has little diagnostic significance except in dehydration. Hypoalbuminemia is very common in many illnesses like impaired synthesis (liver disease), increased catabolism, reduced aminoacid absorption, protein loss in urine, malnutrition and protein losing enteropathy, and in hospital patients with acute illness.

Limitations

Haemolysed and lipaemic sera interfere strongly with the measurement of albumin. Set up appropriate blank for such samples by adding 0.1 ml of serum to 4.5 ml of sodium chloride diluent (Refer Total Protein). The absorbance obtained is then subtracted from its test absorbance before calculating the test result.

Reference

(1) Spencer K and Price C.P. (1977). Ann. Clin. Biochem 14, 105-115.

10. Transaminases - Colorimetric end-point method

Introduction

The two transaminases of diagnostic importance are:

(1) serum glutamic oxaloacetate transaminase (SGOT) or aspartate amino transferase (AST), and

(2) serum glutamic pyruvate transaminase (SGPT) or alanine amino transferase (ALT). While AST is found in every tissue of the body, including red blood cells, and is particularly high in the cardiac muscle, ALT is present in moderately high concentration in liver and low in cardiac, skeletal muscle and other tissues. Both AST and ALT measurements are useful in the diagnosis and monitoring of patients with hepatocellular disease.

Principle of the method

Transamination is the process in which an amino group is transferred from amino acid to an α-keto acid. The enzymes responsible for transamination are called transaminases. The substrates in the reaction are α-ketoglutaric acid (α KG) plus L-aspartate for AST, and αKG plus L-alanine for ALT. The products formed by enzyme action are glutamate and oxaloacetate for AST and glutamate and pyruvate for ALT. Addition of 2,4, dinitrophenyl hydrazine results in the formation of hydrazone complex with the ketoacids. A red colour is produced on the addition of sodium hydroxide. The intensity of colour is related to enzymic activity.

Specimen type, collection and storage

Serum or EDTA/heparinized plasma can be used in this assay.

Transaminases are stable in serum for 6 hours at 25-35^{0}C, 7 days at 2-8^{0}C and for one month when stored at - 20^{0}C.

Reagents

All chemicals must be Analar grade

(a) Phosphate buffer, pH 7.4 :

Dissolve 14.9 g disodium hydrogen phosphate dehydrate ($Na_2HPO4\ 2H_20$) and 2.2g anhydrous potassium dihydrogen phosphate (KH_2PO_H) in distilled water and make up to one litre. Check the pH, and, if necessary, adjust to 7.4 using small amounts of either KH_2PO_4 or Na_2HPO_4- Stable for 3 months when stored at 2-8°C.

(b) AST Substrate

Dissolve 2.66 g DL- aspartic acid and 30 mg α-keto glutarate in 20.5 ml of 1 M NAOH. Adjust the pH to 7.4 by adding I M NAOH drop wise while stirring. Make up to 100 ml with phosphate buffer. Add 1 ml of chloroform as preservative. Stable for 2 months when stored at 2- 8°C. Discard if it becomes turbid.

(c) ALT Substrate

Dissolve 1.78 g DL-alanine and 30 mg α-keto glutarate in 20 ml of phosphate buffer containing 1.25 ml of 0.4 M NAOH. Make up to 100 ml with buffer and adjust to pH 7.4 if necessary. Add 1 ml chloroform as preservative. Stable for 2 months when stored at 2-8°C. Discard if it becomes turbid.

(d) Pyruvate standard 2 mmol/ml

Dissolve 220 mg sodium pyruvate in phosphate buffer and make up to 100 ml. Dilute 10 ml of this solution to 100 ml with phosphate buffer to obtain the working standard containing 2 µmol pyruvate per ml. The remaining 90 ml of the first solution should be discarded. The working standard should be stored in small aliquots of 2 ml in the freezer. One aliquot of working standard should be used for preparing a calibration graph. Discard the leftover standard in the vial.

(e) Colour reagent

Dissolve 200 mg 2,4 dinitro-phenylhydrazine (2,4 DNPH) in hot 1M HCI and make up to 1 litre with 1M HCI. Stable for 6 months when stored at 2- 8^0C.

(f) 0.4 M Sodium hydroxide

Dissolve 16 g sodium hydroxide in about 800 ml of distilled water and make up to 1 litre with distilled water. Store in a polyethylene container at 25-35^0C. Stable for 6 months.

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

a) AST

The protocol of the procedure is described below.

Pipette the following into appropriately labelled 18 x 150mm tubes

TBK = Test Blank & QCBK = QC Blank

	TBK	QCBK	TEST	QC
AST Substrate (ml)	0.5	0.5	0.5	0.5
Test sample/QC (ml)	-	-	0.1	0.1
Mix and incubate at 37 ^{0}C in a waterbath for 1 hour				
2,4 DNPH (ml)	0.5	0.5	0.5	0.5
Mix and remove the tubes from the waterbath				
Test sample/QC (ml)	0.1	0.1	-	-
Mix and leave the tubes for 20 minutes at room temperature (25-35^{0}C)				
0.4M NaOH (ml)	5.0	5.0	5.0	5.0
Mix and leave the tubes for 5 minutes at room temperature (25-35^{0}C)				

Set the spectrophotometer/filter photometer to zero using distilled water at 5 1 0 nm/yellow green filter and measure the absorbance of TBK, QCBK, Test and QC in the order.

(b) ALT

Pipette the following into appropriately labelled 18 x 150 mm tubes.

	TBK	QCBK	TEST	QC
ALT Substrate (ml)	0.5	0.5	0.5	0.5
Test sample/QC (ml)	-	-	0.1	0.1
Mix and incubate at 37 ^{0}C in a waterbath for 30 minutes				
2,4 DNPH (ml)	0.5	0.5	0.5	0.5
Mix and remove the tubes from the waterbath				
Test sample/QC (ml)	0.1	0.1	-	-
Mix and leave the tubes for 20 minutes at room temperature (25-35^{0}C)				
0.4M NaOH (ml)	5.0	5.0	5.0	5.0
Mix and leave the tubes for 5 minutes at room temperature (25-35^{0}C)				

Set the spectrophotometer/filter photometer to zero using distilled water at 5 1 0 nm/yellow green filter and measure the absorbance of TBK, QCBK, Test and QC in the order.

Calculation and calibration graph

In the measurement of both serum AST & ALT, only pyruvate is used as the standard. Theoretically speaking, oxaloacetate should be used as the standard for AST assay and pyruvate as the standard for ALT assay. Oxaloacetate formed in the AST assay is unstable and immediately gets converted into pyruvate; hence the use of pyruvate standard for AST assay. One unit/L of AST or ALT is defined as the liberation of 1μmol of pyruvate per

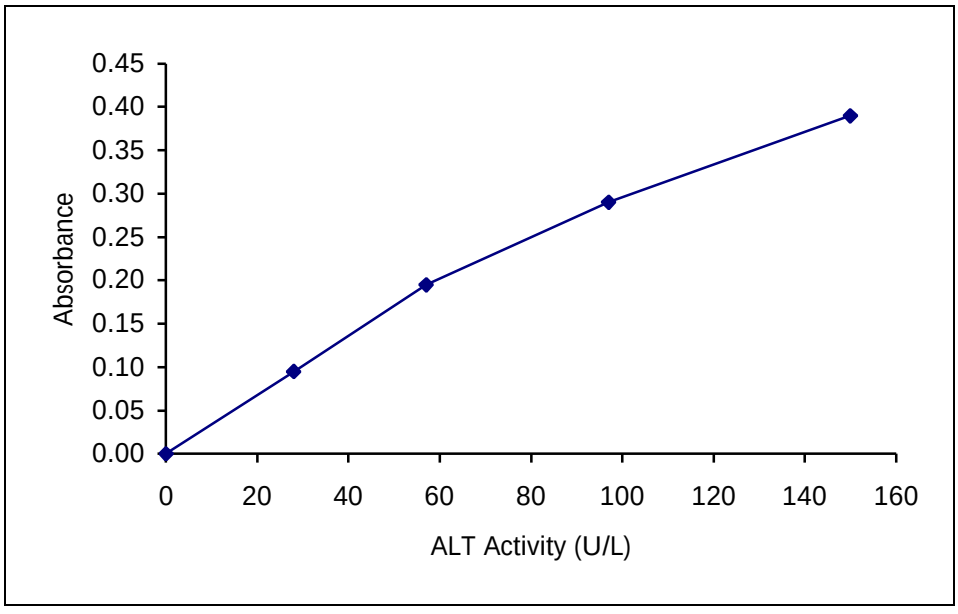
minute at 37^{0}C incubation per litre of serum. As α-keto glutarate-cosubstrate in the assay contributes to the final absorbance, the change in absorbance is not linearly related to the theoretical value of pyruvate produced and hence the enzyme activity. This is evident from the sample calibration graph shown.

	Blank	**S1**	**S2**	**S3**	**S4**
Pyruvate Standard (ml)	-	0.1	0.2	0.3	0.4
ALT (or AST) Substrate (ml)	1.0	0.9	0.8	0.7	0.6
Distilled water (ml)	0.2	0.2	0.2	0.2	0.2
24, DNPH (ml)	1.0	1.0	1.0	1.0	1.0
Mix and leave the tubes for 20 min at room temperature(25-35^{0}C)					
0.4 M NaOH (ml)	10.0	10.0	10.0	10.0	10.0
Mix and leave the tubes for 20 minutes at room temperature (25-35^{0}C)					
Equivalent AST in U/L serum	-	24	61	114	190
Equivalent ALT in U/L serum	-	28	57	97	150

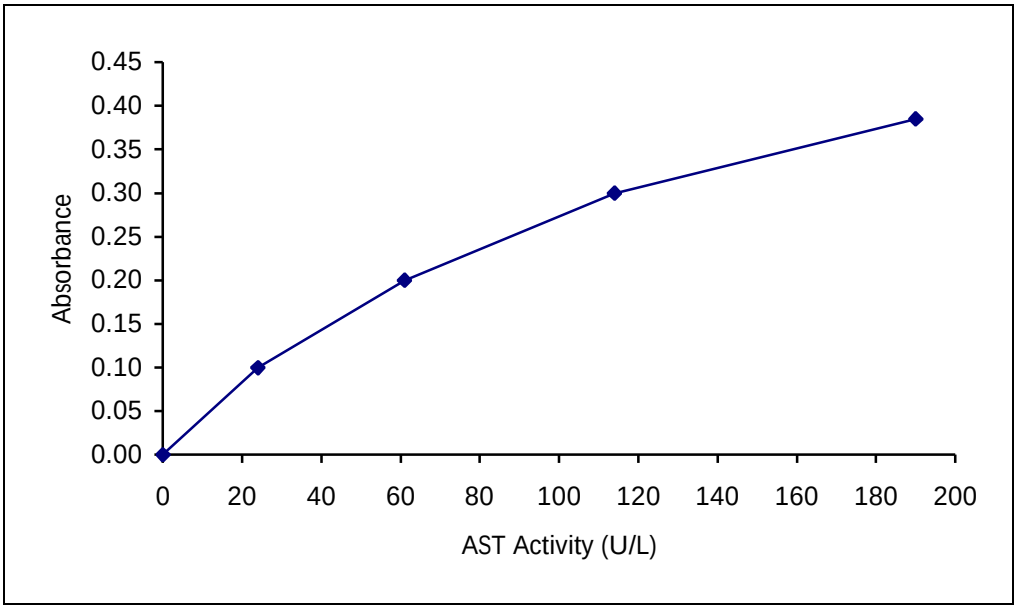
Construct a calibration curve by plotting the corresponding absorbance of standards against their respective AST/ALT activities. The measurable ranges with these graphs are from 5.0 to 150 U/L for ALT and 5.0 to 190 U/L for AST.

Plot the difference in the absorbance between test and TBK as well as QC and QCBK for AST & ALT and read off the enzyme activities on the calibration graphs; otherwise refer to the Table recommended by standard clinical chemistry textbooks like Tietz or Varley relating pyvurate values to AST/ALT activities (if it is available).

ALT



AST



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients' results).

Include one internal QC in every batch of samples analysed each day irrespective of the number of samples in a batch. Since AST or ALT is analysed in a single batch in a day in an intermediate laboratory, it will not be possible to analyse several QC samples and calculate within-day precision. However, even if only a single QC sample is analysed in a day, this value can be pooled with the preceding 10 or 20 values obtained in the previous days and ***between-day precision*** can be calculated and expressed as %CV. Ensure that this is well within the acceptable limit, i.e, 10%.

At least once a week analyse another QC serum from either a low QC or high QC pool**.**

"Assayed " QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

This procedure uses NaOH, which is caustic. Do not swallow, and avoid contact with the skin and mucous membranes.

Reference range and clinical interpretation

The reference ranges by this method are:

AST 8 - 40 U/L

ALT 5 - 35 U/L

High levels of serum AST activity are seen in the heart, liver, skeletal muscle and kidney tissues. Increased activity of AST in serum is observed in myocardial infarction after 20-36 hours of onset and hence used as a supporting evidence in the diagnosis of myocardial infarction. Values are usually less than 10 times the upper limit of normal (ULN). Increased activities are also observed in viral/toxic hepatitis, muscular dystrophy and in pulmonary embolism.

ALT is distributed mainly in the liver and to a lesser extent in the kidney and muscles. Increased ALT activity is observed in hepatitis and cirrhosis. Values may be increased to >10 times –100 times ULN in hepatitis.

Limitations

For samples with enzyme activity greater than 150 U/L for ALT and greater than 190 U/L for AST, dilute the specimen 1 in 10 with 0.9 % saline. Some specimens may require a further 1 in 10 dilution to give a final dilution of 1 in 100. Multiply the final result by the dilution factor.

Avoid using the haemolysed sample as this will cause falsely elevated values. In this case inform the requesting physician and ask for another specimen.

Reference

(1) Reitman, S & Frankel, S. (1957) Am J Clin Pathol., 28, 56-63.

11. Alkaline phosphatase - p-nitrophenol method

Introduction

Phosphatases are enzymes which catalyse the splitting of a phosphate from mono-phosphoric esters. Alkaline phosphatase (ALP), a mixture of isoenzymes from liver, bone, intestine and placenta, has maximum enzyme activity at about pH 10.5. Serum ALP measurements are of particular interest in the investigation of hepatobillary and bone diseases.

Principle of the method

Paranitrophenyl phosphate, which is colourless, is hydrolysed by alkaline phosphatase at pH 10.5 & 37^0C to form free paranitrophenol, which is coloured yellow. The addition of NaOH stops the enzyme activity and the final colour shows maximum absorbance at 410 nm.

Specimen type, collection and storage

Serum or heparinized plasma can be used. Stable for 7 days at 2- 8^0C. The activity increases if samples are left at room temperature (25-35^0C) for several hours.

Reagents

All chemicals must be Analar grade**.**

(a) 2-amino 2- methyl 1-propanol (AMP) buffer pH 10.5

Add 116 ml of AMP to 600 ml of distilled water. Mix and adjust the pH to 10.5 with 6 M HCI and then make up to 1 litre with distilled water. Stable for 6 months at 2- 8^0C.

(b) Magnesium chloride (1.5 mmol/1).

Dissolve 300 mg of magnesium chloride hexahydrate in distilled water and make up to 1 litre. Stable for 6 months at room temperature (25- 35^0 C)

(c) Substrate

Dissolve 83.5 mg of disodium paranitrophenyl phosphate in 1.0ml magnesium chloride solution. Stable for 24 hours at 2- 8^{0}C. This solution should be colourless; do not use it if the OD at 410nm > 0.800.

(d) Sodium hydroxide 0.25 M

Dissolve 10 g of NaOH in about 800 ml of distilled water and then make up to 1 litre with distilled water. Store in a polythene bottle at room temperature (25- 35°C). Stable for 6 months.

(e) Stock paranitrophenol (PNP) 10.8 mmol/l.

Weigh out 150 mg of PNP and dissolve in about 80ml of NaOH (0.25M) and then make up to 100 ml with the same NaOH solution. Store in a brown glass bottle at room temperature (25- 35^{0}C). Stable for 3 months.

(f) Working PNP 54 mmol/l

Pipette 0.5 ml of the PNP stock solution into a 100ml volumetric flask and make up to the mark with NaOH solution (0.25 M). Prepare fresh before use.

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

(a) Preparation of standards (SI -S6)

Pipette the following into appropriately labelled 18 x 150mm tubes

	S1	S2	S3	S4	S5	S6
Working PNP solution (ml)	0.5	1.0	2.0	3.0	4.0	5.0
NaOH solution (ml)	4.5	4.0	3.0	2.0	1.0	-
Activity U/L	40	80	160	240	320	400
Mix Well						

Set the spectrophotometer / filter photometer to zero absorbance at 410 nm / violet filter against 0.25M NaOH and measure the absorbance of the above standards.

(b) Enzyme measurement in test / QC

Pipette the following into another set of appropriately labelled 18 x 150 mm tubes.

	Blank	**Test**	**QC**
AMP buffer (ml)	1.4	1.4	1.4
Mix and Incubate at 37^{0}C for 5 minutes			
Test Sample/QC (ml)	-	0.05	0.05
Substrate (ml)	0.1	0.1	0.1
Mix and Incubate at 37^{0}C for 15 minutes			
NaOH (ml)	4.0	4.0	4.0

(Note: NaOH should be added to each tube in sequence maintaining timed intervals)

Mix and cool the tubes to room temperature (25- 35^{0}C). Measure the absorbance of test / QC at 410nm /violet filter, setting the spectrophotometer /filter photometer to zero with the blank.

Calculation and calibration graph

The working PNP concentration is 54 µmol/L. Standard SI contains 0.5ml PNP

$$\text{Concentration of PNP in SI} = \frac{54}{1000} \times 0.5 = 0.027\mu\text{mol.}$$

ALP activity in U/L = Liberation of 1 µmol of PNP per minute at 37^{0}C incubation per litre serum.

In the assay protocol, 0.05 ml serum is mixed with reagent and incubated for 15 minutes and the total volume is made up to 5.55ml. But the total volume in the case of each standard (SI to S6) is 5.0 ml.

PNP in umol/L or ALP activity in U/L in the test sample =

$$\frac{\text{Test absorbance}}{\text{Std absorbance}} \times \frac{0.027}{15} \times \frac{5.55}{5.0} \times \frac{1000}{0.05}$$

$$= \frac{\text{Test absorbance}}{\text{Std absorbance}} \times 40$$

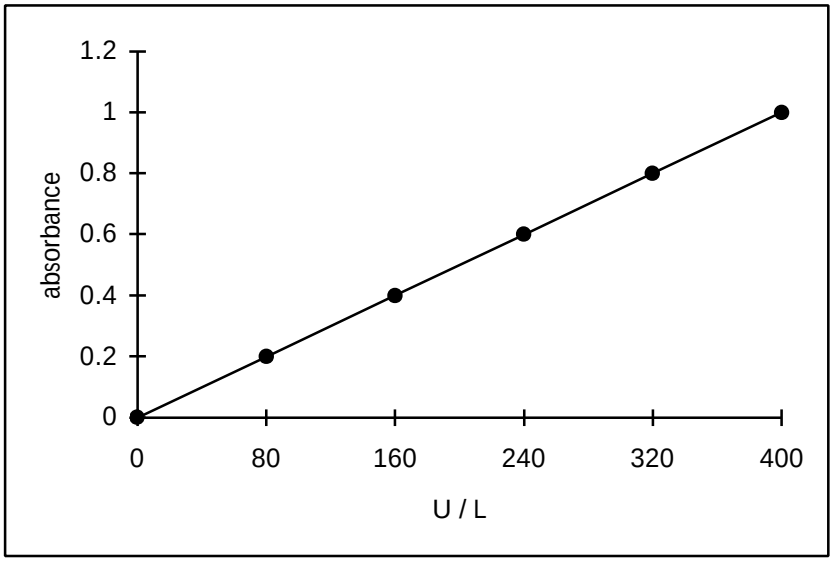
i.e, ALP activity equivalent for S I = 40 U/L. Similarly the ALP activities represented by other standards are : S2 = 80, S3 = 160, S4 = 240, S5 = 320 and S6 = 400 U/L.

Construct a calibration graph by plotting the equivalent activity of ALP of the standards against their corresponding absorbance values. The measurable range with this graph is from 10 to 400 U/L.

Plot the absorbance values of test /QC on the calibration graph and read off the concentrations.

Once linearity is proved, it will be enough if a single standard is set up every time that patients' samples are analysed. Use standard S6 in the assay and calculate the results using the formula :

$$\frac{\text{Test Absorbance}}{\text{Std Absorbance}} \times 400..... \text{U/L.}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients' results).

Include one internal QC in every batch of samples analysed every day, irrespective of the number of samples in a batch. Since alkaline phosphatase is analysed in a single batch in a day in an intermediate laboratory, it will not be possible to analyse several QC samples and calculate within-day precision. However, even if a single QC sample is analysed in a day, this value can be pooled with the preceding 20 values obtained in the previous days and ***between day precision*** can be calculated and expressed as %CV. Ensure that this is well within the acceptable limit, i.e. 10%.

Once a week it is good to analyse another QC serum from either a low QC or high QC pool.

"Assayed" QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

This procedure uses NaOH, which is caustic. Do not swallow, and avoid contact with the skin and mucous membranes. 4 nitro phenol is toxic. Do not pipette by mouth. Use a dispenser.

Reference range and clinical interpretation

Serum alkaline phosphatase (adults) - 40- 125 U/L. (Levels up to 3 times this may be normal in children)

Liver, bone and placenta contain very high concentrations of ALP. Therefore, increase in ALP activity is usually related to hepatobiliary and bone disorders. Increased ALP levels are observed in liver diseases, osteomalacia, rickets and bone disorders. Moderate elevations are sometimes noted in congestive heart failure, intestinal disease and intra-abdominal bacterial infections.

Limitations

Avoid exposure of the freshly dissolved substrate to strong sunlight, since the reagent is light sensitive. The change in absorbance will increase with an increase in temperature, since the pH of the reagent will be different at different temperatures. Haemolysed specimens are not suitable for analysis. Inform the requesting physician of the problem and ask for another specimen.

Any sample that gives a value > 400 U/L should be diluted either 1:2 or 1:3 with 0.9 g% sodium chloride solution and the correct value obtained by multiplying it by the appropriate dilution factor.

Reference

(1) Bomers GN, McComb RB (1975). Clin.Chem. 21: 1988- 1995.

12.Calcium-O-Cresolphthalein complexone method

Introduction

Calcium is the mineral present in the largest amount in the body (1150g). Approximately 99% of total body calcium is deposited in the skeleton. A higher proportion of non-skeletal calcium is present within cells than in extracellular fluids, and most of this intracellular calcium is bound to proteins in the cell membrane. Intracellular ionized calcium is physiologically active and functions as an intracellular messenger by binding to or being released from specific intracellular proteins, a process that changes protein conformations and hence its activity or function.

Principle of the method

Calcium forms a purple-coloured complex with ortho-cresolphthalein complexone in an alkaline medium. The inclusion of HCl helps to release calcium bound to proteins and 8 hydroxy-quinoline eliminates the interference by magnesium. 2-amino, 2-methyl, 1-propanol (AMP) provides the proper alkaline medium for the colour reaction. The intensity of the colour is measured at 540nm/yellow green filter.

Specimen type, collection and storage

Serum is the preferred specimen. Haemolysed and heparinised samples are unsuitable for this method. Similarly, plasma prepared using EDTA, oxalate or citrate must not be used as these preservatives cause removal of calcium by chelation.

Calcium in serum is stable for 12 hours at room temperature (25- 35^0C), one week at 2- 8^0C and for a longer period up to 3 months at - 20^0C.

Reagents

All chemicals must be Analar grade

(a) AMP Buffer pH 10. 7

Measure 37.8 ml of AMP reagent and add 150 ml of distilled water and mix. Adjust the pH to 10.7 with 6NHCI and make up to 250ml with distilled water. Store in the refrigerator in a brown coloured glass bottle. Stable for 3 weeks.

(b) Colour reagent

Add 15 ml conc. HCI to a 250 ml volumetric flask containing about 25ml of distilled water. Transfer with washing 25 mg 0-cresolphthalein complexone powder into it, mix to dissolve. Then add 250 mg of 8 hydroxy-quinoline and dissolve and then make up to 250 ml with distilled water. Store in a brown coloured glass bottle at room temperature (25-35^{0}C). Stable for about one month.

(c) Stock calcium standard 50 mg/dl

Before weighing, dry calcium carbonate at 100^{0}C for 2 hours. Allow to cool in a dessicator. Dissolve 625 mg of dried calcium carbonate in 50 ml of distilled water taken in a 500 ml volumetric flask and add 3.5ml conc. HCl. Mix to dissolve and make up to 500 ml with distilled water. Store in a brown bottle at room temperature (25-35^{0}C). Stable for about 6 months.

(d) Working standards

Into four 100 ml volumetric flasks transfer 10, 15, 20 and 25 ml of stock calcium standard and dilute each to 100 ml with benzoic acid to get working standards containing 5, 7.5, 10, and 12.5 mg/dl calcium, respectively. Store in brown bottles at room temperature (25-35^{0}C). Stable for 2 months.

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

All glassware used in this method must be scrupulously cleaned, soaked overnight in 3% (V/V) HCl to remove traces of calcium, thoroughly rinsed with distilled water and dried before use.

Procedure

The protocol of the procedure is described below.

Add reagents, standards (S5= 5mg/dl, S7.5 = 7.5mg/dl, S10= 10 mg/dl, S12.5 = 12.5mg/dl) and test sample /QC in the order indicated into appropriately labelled tubes (18 x 150mm)

	Blank	**S5**	**S7.5**	**S10**	**S12.5**	**Test**	**QC**
Distilled water (ml)	0.1	-	-	-	-	-	-
Standard (ml)	-	0.1	0.1	0.1	0.1	-	-
Test samples/QC (ml)	-	-	-	-	-	0.1	0.1
Colour reagent (ml)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Mix Well							
Buffer	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Mix Well							

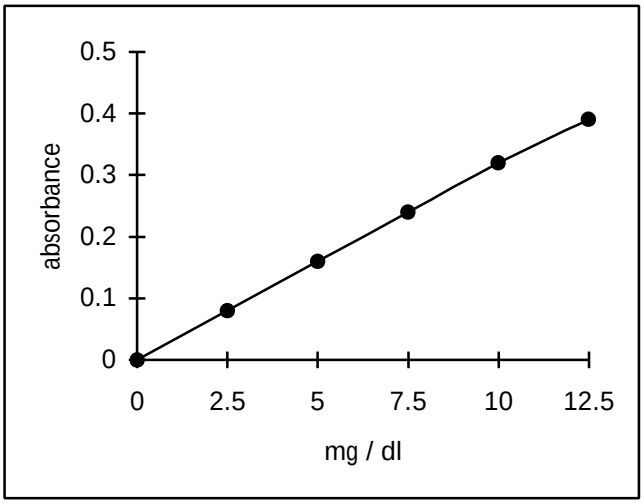
Incubate at room temperature (25-35^{0}C) for 15 minutes. Set the spectrophotometer /filter photometer to zero using blank at 540 nm/ yellow green filter and measure the absorbance of standards, test & QC.

Calculation and calibration graph

Construct a calibration graph by plotting the absorbance of the standards against their respective concentrations. Plot the absorbance values of test/QC on the calibration graph and read off the concentrations. The measurable range with this graph is from 1.0 to 12.0 mg/dl. It is advisable to plot a calibration graph whenever the reagents are freshly prepared.

Once linearity is proved, it is just enough if a single standard such as S_{10} (10 mg/dl) is used and the conentration in the patient's sample is calculated using the formula :

$$\frac{\text{Test absorbance}}{\text{Standard absorbance}} \times 10 \text{..... mg/dl.}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients' results).

Include one internal QC in every batch of samples analysed each day irrespective of the number of samples in a batch. Since calcium is analysed single batch in a day in an intermediate laboratory, it will not be possible to analyse several QC samples and calculate within-day precision. However, even if only a single QC sample is analysed in a day, this value can be pooled with the preceding 10 or 20 values obtained in the previous days and ***between-day precision*** can be calculated and expressed as %CV. Ensure that this is well within the acceptable limit, i.e, 8%.

At least once a week analyse another QC serum from either a low QC or high QC pool.

"Assayed" QC sera with stated values (ranges) are available from several commercial sources, viz Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

Concentrated hydrochloric acid, which is corrosive, is used in this procedure. Do not mouth pipette or swallow, and avoid contact with skin and mucous membranes.

Reference range and clinical interpretation

Serum Calcium..... . 8.5 - 10.4 mg/dl

Calcium measurements are used in the diagnosis and treatment of parathyroid diseases, a variety of bone diseases, chronic renal failure and tetany. Increased serum calcium levels are associated with primary hyperparathyroidism, multiple myeloma, metastatic bone lesions and hypervitaminosis D. Hypocalcaemia is associated with hypoparathyroidism, nephrotic syndrome, and rickets and renal failure.

Limitations

Haemolysed and lipaemic sera interfere with the measurement of calcium. Set up an appropriate blank for such samples by adding 0.1ml of serum to 4ml of distilled water. The absorbance obtained against distilled water is then subtracted from the test absorbance before the test result is calculated.

Reference

(1) Gitelman H.(1967) Anal.Biochem 20 : 521.

(2) Gindler EM & King JD (1972) Am JCIin Pathol 58: 376.

13.Phosphorus - Stannous chloride reduction method

Introduction

Eighty per cent of body phosphorus is laid down in bone matrix as insoluble salts. The organic phosphate esters are primarily confined within cells, associated with nucleoproteins, hexoses and purines. Phosphate forms high energy bonds in ATP, GTP and creatine phosphate. Inorganic phosphate ions are mostly confined to the extracellular fluid where they are part of buffer systems.

The plasma phosphate concentration is regulated by parathyroid hormone (PTH) and vitamin D3. PTH stimulates the kidney to excrete phosphate while conserving calcium. In chronic renal disease, phosphate retention occurs because of impaired glomerular filtration.

Principle of the method

Phosphorus in serum reacts with ammonium molybdate to form phosphomolybdate, which is then reduced by stannous chloride and hydrazine sulphate to molybdenum blue. The intensity of the colour is measured at 640 nm.

Specimen type, collection and storage

Use only clear, non-haemolysed serum separated from erythrocyte as soon as possible, as cells contain organic phosphate which can be enzymatically cleaved thereby increasing the serum concentration of phosphorus.

Phosphorus in serum is stable for 12 hours at room temperature (25-35^{0}C), one week at 2-8^{0}C and for a longer period up to 3 months at –2^{0}C.

Reagent

All chemicals must be Analar grade

(a) Trichloroacetic acid 3 g/dl

Dissolve 3 g trichloroacetic acid (TCA) in distilled water and make up to a final volume of 100 ml. Store in a brown glass bottle at room temperature (25-35^{0}C). Stable for 3 months.

(b) Ammonium molybdate solution

Into a 100 ml volumetric flask add 70 ml of distilled water and then add slowly 3.5 ml of conc. H2SO4. Add 1g of ammonium molybdate. Mix well and make up to the mark with distilled water. Stable for one month at 2-8^{0}C.

(c) Hydrazine sulphate solution

Into a 100 ml volumetric flask add 70 ml of distilled water and add slowly 2.8 ml of conc. H2SO4 followed by 0.2 g hydrazine sulphate and 0.02g stannous chloride. Mix well and make up to the mark with distilled water. Store in a brown glass bottle. Stable for one month at 2-8^{0}C.

(d) Stock phosphorus standard 100 mg/dl

Weigh 2.19 g anhydrous KH2PO4 and dissolve in distilled water and then make up to 500 ml with distilled water. Stable for one month at 2 -8^{0}C.

(e) Working phosphorus standard 10 mg/dl

Dilute 10 ml of stock phosphorus standard to 100 ml with distilled water. Stable for one month at 2-8^{0}C.

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

Pipette the following into appropriately labelled 13 x 100 mm tubes.

(a) Dilution of Standards S1- S4; Test & QC.

	S1	S2	S3	S4	Test	QC
3g% TCA (ml)	1.95	1.9	1.85	1.8	1.8	1.8
10mg/dl standard (ml)	0.05	0.1	0.15	0.2	-	-
Test sample / QC (ml)	-	-	-	-	0.2	0.2
Mix well. Leave for 5 minutes at room temperature (25 -35^{0}C) Centrifuge test & QC only for 10 minutes at 3000 rpm.						

(b) Colour development

Pipette the following into appropriately labelled 18 x 150 mm tubes

	Blank	S1	S2	S3	S4	Test	QC
3g/dl TCA (ml)	1.0	-	-	-	-	-	-
Diluted standard (ml)	-	1.0	1.0	1.0	1.0	-	-
Supernatant (ml)	-	-	-	-	-	1.0	1.0
Amm.Molybdate (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Hydrazine Sulphate (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Mix and leave for 5 minutes at room temperature (25-35 °C).

Set the spectrophotometer/filter photometer to zero using blank at 640 nm/red filter and measure the absorbance of standards, test and QC.

Calculation and calibration graph

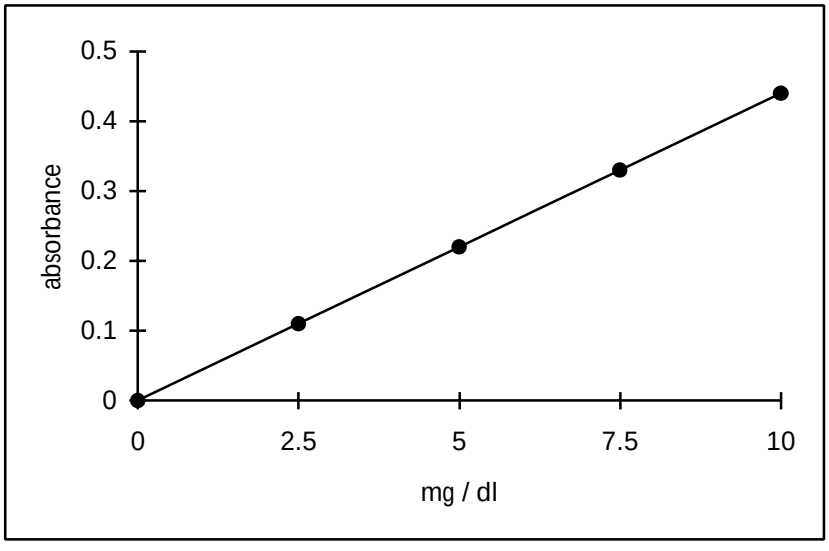
Since the protocol for standard tube S4 and the test is identical, the Standard S4 will represent a concentration of 10 mg/dl. The phosphorus concentrations represented by other standards are SI = 2.5, S2 = 5.0 & S3 = 7.5 mg/dl.

Plot the absorbance values of standards against their respective concentrations. The measurable range with this graph is from 0.5 to 10.0 mg/dl.

Plot the absorbance values of test/QC on the calibration graph and read off to the concentrations.

Once linearity is proved, it is not necessary to prepare the standard graph every time that patients' samples are analysed. It will be adequate if standard S2 is taken every time and patients' results are calculated using the formula:

$$\frac{\text{Test absorbance}}{\text{Standard absorbance}} \times 5 \text{.....mg/dl}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients' results).

Include one internal QC in every batch of samples analysed each day irrespective of the number of samples in a batch. Since phosphorus is analysed in a single batch in a day in an intermediate laboratory, it will not be possible to analyse several QC samples and calculate within-day precision. However, even if only a single QC sample is analysed in a day, this value can be pooled with the preceding 10 or 20 values obtained in the previous days and ***between-day precision*** can be calculated and expressed as %CV. Ensure that this is well within the acceptable limit, i.e, 8%.

At least once a week analyse another QC serum from either a low QC or high QC pool.

"Assayed" QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous reagents

The reagents include conc. H_2SO_4, which is corrosive. Avoid contact with skin and eyes. Wash with copious amounts of water if it comes into contact with skin or eyes.

Reference range and clinical interpretation

Serum phosphorous 3.5 - 5.0 mg/dl

An increase in serum phosphorous is found in chronic nephritis progressing with increased renal failure. A moderate increase is observed in hypoparathyroidism and vitamin D excess.

A decrease in serum phosphorous is observed in rickets or osteomalacia and also in hyperparathyroidism. Hypophosphatemia may result due to disorders of renal tubular reabsorption.

Limitations

Contaminated glassware is the greatest source of error. Therefore all glassware used in this method must be scrupulously cleaned, soaked overnight in 3% (V/V) HCl, thoroughly rinsed with distilled water and dried before use.

Haemolysed and lipaemic sera interfere with the measurement of phosphorous. Set up an appropriate blank for such samples by adding 0.2ml serum to 3ml of distilled water. The absorbance obtained against distilled water is then subtracted from the test absorbance before the test result is calculated

Serum must be separated by centrifugation as soon as possible after collection of the patient's blood sample, preferably <2 hours, otherwise, phosphate present in erythrocytes will be released into the serum causing falsely elevated values.

Reference

(1) Harold Varley "Practical Clinical Biochemistry 6th Edn 1988 page 448- 449.

14. Sodium and potassium - Flame photometry

Introduction

Sodium, the major extracellular cation, plays a role in fluid distribution among body compartments. The ingested sodium is filtered in the renal glomerulus and approximately 70% is reabsorbed in the proximal tubule. Further reabsorption occurs in the loop of Henle and <5% is reabsorbed distally under the influence of aldosterone.

About 65-70% of the total body sodium is in its exchangeable form. The exchangeable sodium is made up of extracellular and intracellular sodium. The intracellular sodium concentration is about 10 mmol/L and the extracellular, i.e. the plasma sodium concentration, is about 140 mmol/L. Sodium maintains the osmotic pressure of the extracellular fluid and helps in retaining water in the extracellular compartment. Along with other cations it is also involved in neuromuscular irritability, acid base balance, maintenance of blood viscosity and resting membrane potential.

A high plasma sodium concentration of more than 145 mmol/L is referred to as hypernatremia. This can occur due to simple dehydration, excess sodium intake, steroid therapy as well as in diabetic insipidus. Hyponatremia, with plasma sodium concentration less than 130 mmol/L, can occur due to diuretic medication, kidney disease, excessive sweating, congestive heart failure or gastrointestinal disorder.

Potassium is the major intracellular cation. It is widely distributed in the body in muscle tissue, nerve tissue, blood cells and plasma. It is filtered in the glomerulus, absorbed in the proximal tubule and finally excreted by exchange for sodium in the distal tubule. Potassium influences muscular activity, cardiac function and nerve conduction process.

In hyperkalemia the plasma potassium concentration exceeds 5.5 mmol/L. Acute hyperkalemia is a medical emergency. In hypokalemia the plasma potassium level will be less than 3.5 mmol/L. This can occur due to excessive loss in gastrointestinal secretions and urine, and also in renal tubular acidosis.

Principle of the method

When a solution of an inorganic salt such as sodium chloride is sprayed into the flame, the elements in the compound are partly converted into the atomic state. Due to the heat energy of the flame a very small proportion of these atoms is excited and the

electrons move to a higher energy level. The proportion of the atoms that are excited depends upon the concentration of the particular element and on the temperature of the flame. In the excited state the electrons are unstable and they rapidly revert back to their former lower energy level. As they change from the excited state or higher energy level back to the lower energy level, they emit the light in the form of a fixed wavelength, to produce a spectrum. Under carefully controlled conditions the amount of light emitted is directly proportional to the number of atoms that are excited, which in turn is proportional to the concentration of the substance in the sample.

Specimen type, collection and storage

Both sodium and potassium are stable in serum for several hours at 25- 35°C and for 3 months at - 20°C. Anticoagulants containing sodium or potassium salts are not suitable, but lithium heparin may be used as an anticoagulant.

If whole blood is left unseparated for >3hours or refrigerated, potassium will leak out of the red cells giving falsely increased values.

Reagents

All chemicals must be Analar grade

Sodium chloride (NaCl) and potassium chloride (KCl) should be dried for 2-3 hours at about 100°C before use. Before weighing, the chemicals must be allowed to cool to room temperature either in a desiccator or in a container with a tight-fitting lid with a small air space.

(a) Stock Sodium 1000 mmol/L

Weigh out 29.25 g dried NaCl, dissolve in about 400 ml of distilled water taken in a 500 ml volumetric flask and then make up to 500ml with distilled water. Store in a pyrex glass bottle at 25-35°C. Stable for one year.

(b) Stock Potassium 100 mmol/L

Weigh out 0.746 g dried KCl, dissolve in about 80 ml of distilled water taken in a 100 ml volumetric flask and then make up to 100 ml with distilled water. Store in a pyrex glass bottle at 25-35^{0}C. Stable for one year.

(c) Working Standards

- Low standard for Sodium 100mmol/L: Dilute 10 ml of stock sodium to 100 ml with distilled water. Stable for 6 months at 25- 35^{0}C.
- Combined standard for sodium and potassium 140Na^+/5K^+ mmol/L: Dilute 14 ml of stock sodium and 5 ml of stock potassium together to 100 ml with distilled water. Store in a pyrex bottle at 25- 35^{0}C. Stable for 6 months.
- Aspiration standard for sodium - 1.0 mmol/L: Dilute 1.0 ml of working standard to 100 ml with distilled water. Prepare fresh each time.
- Combined aspiration standard for sodium 1.4 mmol/L and potassium 0.05 mmol/L. Dilute 1.0 ml of working standard (combined standard for Na^+/K^+140/5mmol/L) to 100ml with distilled water. Prepare fresh each time.

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

(a) Sample dilution

Dilute each serum sample 1:100 with distilled water by mixing 0. 1ml sample with 9.9 ml distilled water.

(b) Procedure for simultaneous measurement of Na+ & K+ in the flame photometer (digital flame photometer)

- Switch on the flame photometer. Digital display should turn on.
- Turn the set (full scale) F.S. coarse and fine controls 'into maximum clockwise position.
- Select appropriate filter with the help of filter selector wheel (Na^+ on the left side and K^+ on the right side).
- Switch on the compressor and check the air pressure. Adjust it to read between 0.4 and 0.6 k g/cm^2.
- Open the gas cylinder, remove the trapper at the rear of the flame photometer and ignite the flame.
- Adjust the gas regulator to get a maximum height non-luminous blue flame with 10 distinct cones (5 on each side of the burner head).
- Feed distilled water to the atomizer and wait for at least 30 seconds.
- Adjust the 'Set Ref Coarse' and Fine controls' to zero digital readout for K^+ only.
- Aspirate 1.0 mmol/L Na^+ solution. Wait at least 30 seconds and then adjust the Set Ref Coarse and Fine controls' to a digital read out of I 00 for Na^+ only.
- Aspirate the combined standard solution (1.4/0.05, Na^+/K^+) and wait at least for 30 seconds. Adjust 'F.S control' on Na^+ side for readout 140 and that on K^+ side for a digital readout of 50.
- Repeat steps 9 and 10 once again. The flame photometer now stands calibrated.
- Now feed diluted test sample / QC to the atomizer for at least 30 seconds before recording the readings for Na^+ and K^+.

Calculation

After aspirating the standard solution, the digital reading for Na^+ is adjusted to 140 and that of K^+ to 50. This is done in order to represent Na^+ and K^+ values in undiluted serum. Since the test sample/QC is diluted initially 1: 100 and then aspirated, the initial standard values for Na^+ & K^+ (1.4 & 0.05 mmol/L) must be multiplied by 100 to

represent 140 mmol/L Na^+ and 5 mmol/L K^+. In the case of K^+, in order to improve the sensitivity of the assay the digital reading for the standard is further multiplied by 10 to show a reading of 50.

In essence, the test sample/QC digital readings are compared with the standard readings for Na^+ and K^+. The digital reading appearing for Na' of the test sample/QC is read as mmol/L value straightaway. On the other hand, the test sample /QC K^+ value represents $1/10^{th}$ of the digital reading.

For example, digital reading for "140 Na^+ = 140 mmol/L Na^+; digital reading for 45 K^+ = 4.5 mmol/L K^+

Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients' results).

Since Na^+, K^+ are very commonly analysed parameters in a laboratory, it is recommended that internal QC (normal QC pool) be included with every batch of samples analysed in a day, irrespective of the number of samples in a batch. Further, even when a single sample is analysed as an "emergency" sample at any time of the day or night, it is essential to include an internal QC. From the QC results obtained for the day, mean, standard deviation and % CV can be calculated to ensure that ***within-day precision*** is well within the acceptable limit, i.e. 4%.

The mean value of internal QC for the day can be pooled with the preceding 10 or 20 mean values obtained in the previous days and ***between-day precision*** can be calculated and expressed as % CV. Ensure that this is well within the acceptable limit, i.e, 8%.

At least once a week analyse another QC serum from either a low QC or high QC pool.

"Assayed" QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

However, care should be taken when operating the flame photometer as the technician will be using liquefied petroleum gas. Leakage of either air or gas during operation will cause explosion. Apply soap solution at the connecting point to check such leakage.

Hazardous materials

The reagents used are made up of only sodium chloride and potassium chloride. Therefore no precautionary measures are required.

Reference range and clinical interpretation

The reference ranges by this method are:

- Serum sodium 130 - 145 mmol/L
- Serum potassium 3.5 - 5.0 mmol/L

Elevated levels of serum sodium occur in conditions such as severe dehydration, hyperadrenalism and brain injury.

Low serum sodium values are noticed in metabolic acidosis, salt- losing nephritis, Addison's disease, etc. Increased serum potassium level is observed in anoxia, metabolic renal tubular acidosis and shock or circulatory failure.

Low serum potassium values are observed due to low intake of dietary potassium over a period of time or increased loss through kidney, vomiting or diarrhoea. Increased secretion of adrenal steroids or some diuretics may also promote the loss of potassium.

Limitations

Avoid using haemolysed serum. This will cause elevated K^+ level.

Reliability of the results depends on the proper maintenance of the flame photometer, salient features of which are listed below.

(a) Requirements

- Non-luminous blue flame
- Supply of dry air at a controlled pressure, viz. 10- 1 5 Kg /cm2
- Regular availability of liquid petroleum gas

(b) Maintenance

- Disconnect power and gas supply before proceeding to do maintenance.
- Turn the control in both Na+/K+ display fully anti-clockwise.
- Disconnect the drain outlet and the gas and air inlets.
- Remove the top panel and disconnect the photocell.
- Remove the side panel and take out the atomizer.
- Disconnect the air line at the pressure gauge.
- Disconnect the air and gas inlets to the mixing chamber.
- Remove the burner head and remove mixing chamber
- Wash the above well with tap water and distilled water.

- Clean the atomizer with a thin wire and adjust its spray by passing compressed air. This can be done by screwing / unscrewing the two knurled nuts in the atomizer.
- Remove the air tube and flush out any water remaining in the tube due to the cooling of compressed air.
- Do not use oxyacetylene or highly explosive mixture as fuel.
- After cleaning, refix everything carefully.

(c) Cleaning of various units in a flame photometer

- *Atomizer and capillary tube*: Flushing with copious amount of distilled water is adequate. If blockage occurs, remove the atomizer from its seating and flush with dry air or clean it using a thin wire.

  If cleaning of atomizer is done with a wire before and after using it, blockage will rarely occur. If all the above fails, a new atomizer is to be fixed.
- *Mixing chamber:* Flushing with distilled water is adequate. Do not use detergent or soap solution because it will remain inside if washing is not done properly out and will give erratic reading due to the presence of Na^+ / K^+ in the soap solution.

(d) Fault diagnosis

Symptom	Diagnosis	Remedy
1. Unstable reading	Excessive vibration	Provide shock-proof base (e.g.) glass plate on foam rubber.
	Air supply blocked	Check air supply and clear blockage
	Atomizer low gas pressure	Remove, wash and dry burner
	Filter dirty	Clean with isopropanol.
2. Intermittent reading	Blocked atomizer	Clean atomizer using a thin wire
	Faulty photocell	Change photocell
	Dirty photocell	Clean photocell
3. Low sensitivity	Blocked atomizer	Clean blockage
	Low gas pressure	Check gas pressure
	Faulty photocell	Change photocell.

15. Cerebrospinal fluid (CSF)

Introduction

CSF originates from the blood. The choroid plexes in the 1st , 2nd and 3rd ventricles of the brain are the sites of CSF production. CSF is formed from plasma by the filtering and secretary activities of the choroid plexus and lateral ventricles. CSF circulates around the brain and the spinal cord.

CSF nourishes the tissues of the central nervous system (CNS) and helps to protect the brain and the spinal cord from injury. It primarily acts as a water shock absorber. It totally surrounds the brain and the spinal cord and thus absorbs any blow to the brain. CSF also acts as a carrier of nutrients and waste products between the blood and the CNS.

Specimen type, collection and storage. Only a physician or a specially trained nurse must collect the specimen. Laboratory personnel should be present, however, so that the specimen is delivered to the laboratory immediately after collection. Spinal puncture is a process by which a long needle is inserted into the subarachnoid space between LI and L5 and about 2ml of CSF is withdrawn. The specimen is then transferred into a clean penicillin vial containing about 8mg of a mixture of EDTA and sodium fluoride in the ratio of 1:2. Protein estimation can also be carried out on this specimen.

Glucose and protein estimations should be performed as soon as possible after drawing the CSF specimen. If testing is delayed, the specimen may be frozen at - 20^0C up to 3 days. Centrifuge specimens containing red blood cells or particulate matter.

Do not delay testing the CSF because cells and trypanosomes are rapidly lysed once the CSF is removed from the body. Glucose will be rapidly destroyed in the absence of preservatives.

Remember: CSF is the most precious biological material. Often, only small volumes of CSF are available for analysis due to difficulty in collection. Hence handle this with care.

The specimen may contain virulent organisms. Avoid mouth pipetting.

16. CSF glucose –glucose oxidase method

Principle of the method

Glucose present in the CSF is oxidized by the enzyme glucose oxidase (GOD) to gluconic acid with the liberation of hydrogen peroxide, which is converted into water and oxygen by the enzyme peroxidase (POD). 4 aminophenazone, an oxygen acceptor, takes up the oxygen and together with phenol forms a pink coloured chromogen which can be measured at 515nm.

$$\text{Glucose} \xrightarrow{\text{GOD}} \text{4 Gluconic acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{POD}} 4\ H_2O + [O]$$

$$[O] + \text{4 aminophenazone} + \text{phenol} \dashrightarrow \text{chromogen (pink)}$$

Reagents

All chemicals must be Analar grade

(a) Phosphate buffer : 100 mmol/L. pH 7.0

To 800 ml of distilled water add the following in the order:

Disodium hydrogen phosphate dihydrate [$Na_2HPO_4\ 2H_2O$]....12.95 g;
Anhydrous potassium dihydrogen phosphate [KH_2PO_4].....4.95 g;
Sodium azide [NaN_3].....0.5 g

Add one by one, dissolve and finally make up to one litre with distilled water. Stable for 3-4 months, at 2-8^{0}C. Check final pH with a pH meter.

(b) Colour reagent

To 100ml of the above phosphate buffer add the following in the order and then mix to dissolve :

4 amino phenazone	16 mg
GOD [Sigma G 7016]	1800 units
POD [Sigma P 8250]	100 units
Phenol	105 mg
Tween 20 [Sigma P 1359]	50µl

Reconstitute the GOD and POD powder with phosphate buffer. Dispense separately into vials so that each vials represents the requisite number of units. Store the vials frozen.

Stable for 2 weeks at 2-80C. Store in a brown bottle.

(c) Benzoic acid 1g/l.

Dissolve 1.0g of benzoic acid in water and make up to one litre with water. This solution is stable indefinitely at room temperature.

(d) Stock Glucose solution, 1 g/l.

Before weighing, dry the glucose at 60-80^{0}C for 4 hours. Allow to cool in a dessicator. Dissolve 1g of glucose in benzoic acid solution and make up to 100 ml in a volumetric flask. Stable for six months at room temperature (25-35^{0}C). **Do not freeze the standard**

(e) Working glucose standard 100 mg/dl.

Dilute 10 ml of stock glucose (use either a volumetric pipette or a burette) to 100 ml with benzoic acid in a 100 ml volumetric flask. Mix well. Stable for 6 months at room temperature (25-35^{0}C).

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

(a) Dilution of standards (S1-S5), test & QC

Pipette the following into appropriately labelled 13 x 100 mm tubes

	S1	S2	S3	S4	S5	Test	QC
Distilled Water (ml)	1.9	1.8	1.7	1.6	1.5	1.9	1.9
100 mg/dl glucose (ml)	0.1	0.2	0.3	0.4	0.5	-	-
Test sample /QC (ml)	-	-	-	-	-	0.1	0.1
Mix well							

(b) Colour development

Pipette the following into another set of appropriately labelled tubes.

	Blank	S1	S2	S3	S4	S5	Test	QC
Colour reagent (ml)	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Distilled water (ml)	0.1	0.1	-	-	-	-	-	-
Diluted Standards (ml)	-	-	0.1	0.1	0.1	0.1	-	-
Diluted Test sample/QC (ml)	-	-	-	-	-	-	0.1	0.1

Mix all tubes well. Incubate at 37^0C in a waterbath for 15 minutes. Remove from waterbath and cool to room temperature. Set the spectrophotometer/ filter photometer to zero using blank at 510 nm/ green filter and measure the absorbance of standards, test and QC. This protocol is designed for spectrophotometers / filter photometers that require a minimum volume of reaction mixture in the cuvette of one ml. or less. Since economical use of reagents is possible with this protocol, the cost per test can be kept to the minimum. However, if a laboratory employs a photometer requiring a large volume of the reaction mixture for measurement, viz. 5 ml, it is advised that the volume of all reagents mentioned under Tabulation "(b) Colour development ", be increased proportionately.

Calculation and calibration graph

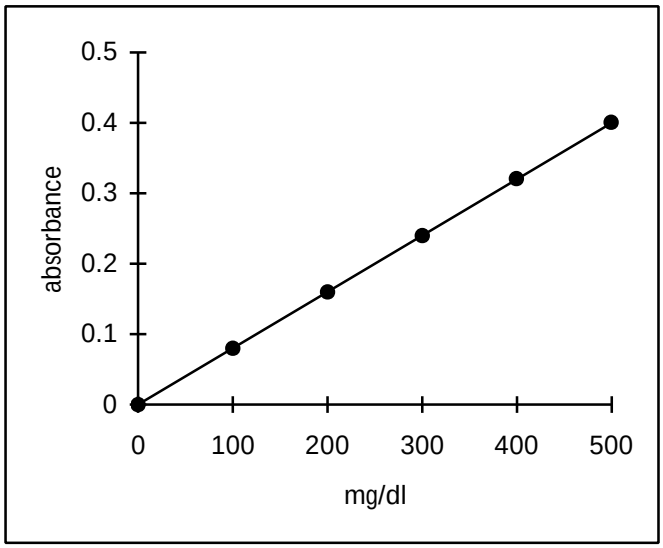
Since the protocol for standard tube S1 and test is identical, the standard S1 will represent a concentration of 100 mg/dl. The glucose concentrations represented by other standard tubes are S2 = 200; S3 = 300; S4 = 400 and S5 = 500 mg/dl.

Plot the absorbance values of standards against their respective concentrations. The measurable range with this graph is from 10 to 500 mg/dl.

Plot absorbance values of test/QC on the calibration graph and read off the concentrations.

Once linearity is proved, it is not necessary to prepare the standard graph every time that patients 'samples are analysed. It will be adequate if standard S2 is taken every time and patients 'results are calculated using the formula :

$$\frac{\text{Test absorbance}}{\text{Standard absorbance}} \times 200 \text{ ... ... mg/dl}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients 'results).

Since CSF analysis is carried out infrequently in intermediate laboratories, one QC for glucose should be included as and when CSF glucose is analysed. Hence it will not be possible to analyse several QC samples and calculate within-day precision. However, even if only a single QC sample is analysed in a day, this value can be pooled with the preceding 10 or 20 values obtained in the previous days and ***between-day precision*** can be calculated and expressed as % CV. Ensure that this is well within the acceptable limit, i.e, 8%.

At least once a day analyse another QC serum from either a low QC or high QC pool.

"Assayed " QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

This procedure uses sodium azide and phenol, which are poisonous and caustic. Do not swallow, and avoid contact with skin and mucous membranes

Reference range and clinical interpretation

Concentrations of analytes in the CSF should always be compared with those in plasma. Normal CSF glucose is about 60% of the plasma value.

Normal range for CSF glucose 50-80 mg/dl

Decreased CSF glucose levels are observed in tuberculosis, benign lymphocytic chronic meningitis and in hypoglycemia. Increased levels are observed in encephalitis, poliomyelitis and in cerebral abscess.

Limitations

Grossly bloody CSF may give spuriously elevated values for glucose. Undue delay in analysis may give low values. The report to the requesting physician should include the appearance of the CSF before and after centrifugation.

References

(1) Trinder, P. (I 969). Annals of Clin. Biochem. 6: 24 - 27.

(2) Barham D and Trinder P. (1972). Analyst 97: 142-145.

17. CSF Protein - Pyrogallol dye binding method

Principle of the method

Protein molecules present in CSF bind quantitatively with pyrogallol red-molybdate complex at pH 2.0 to form a violet complex, the intensity of which is measured at 600nm and compared with the colour given by a set of human protein standards.

Reagents

All chemicals must be Analar grade

(a) Pyragallol red dye

Dissolve 10 mg of disodium molybdate, 5.9g of succinic acid, 134mg of sodium oxalate and 430mg of sodium benzoate in about 800 ml of distilled water taken in a one-litre volumetric flask. To this add 25 mg of pyrogallol red dye and mix well till it is completely dissolved. Make up to the mark with distilled water. Store in an amber bottle. Stable at 2-80C for 3 months.

(b) Standards :

Refer Total Protein (page 40 (c) Standard) for the preparation of protein standard from pooled serum. Note that there is always a risk of infection from pooled serum. After determining the concentration of the protein standard, dilute this to several levels as described below.

For example, if the total protein value of the pooled serum is 6.7g/dl, then the volume of pooled serum to be diluted to 100ml to get a protein concentration of 20 mg/dl is found out using the dilution formula.

IV x IC = FV x FC

IV = Initial Volume of pooled serum.

IC = Initial Concentration of total protein (mg/dl).

FV = Final Volume.

FC = Final Concentration

IV x 6700 = 100 x 20

$$IV = \frac{100 \times 20}{6700} \qquad \frac{20}{67} = 0.3 \text{ ml}$$

i.e. Dilute 0.3 ml of the pooled serum to 100ml with 0.9 g/dl sodium chloride containing 0.1 g/dl sodium azide. Similarly, 40, 80 and 120 mg/dl standards are prepared by diluting 0.6, 1.2 and 1.8 ml of the pooled serum each to 100 ml with the diluent. The standards are stable at 2-8°C for one month.

The following table summarizes the preparation of diluted standards.

Concentration of protein in pooled serum is taken as 6.7g/dl.

Standard	Pooled serum (ml)	Final diluted volume (ml)	Concentration of Standard (mg/dl)
S1	0.3	100	20
S2	0.6	100	40
S3	1.2	100	80
S4	1.8	100	120

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

Pipette the following into appropriately labelled 13 x 100 mm tubes

	Blank	S1	S2	S3	S4	Test	QC
Pyrogallol red (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Distilled water (ml)	0.05	-	-	-	-	-	-
Standard (ml)	-	0.05	0.05	0.05	0.05	-	-
Test sample (ml)	-	-	-	-	-	0.05	-
QC serum(1:100) (ml)		-	-	-	-	-	0.05

Mix all tubes well. Leave at 25-35^{0}C for 15 minutes. Set the spectrophotometer /filter photometer to zero using blank at 600 nm/ red filter and measure the absorbance of standards, test and QC.

Calculation and calibration graph

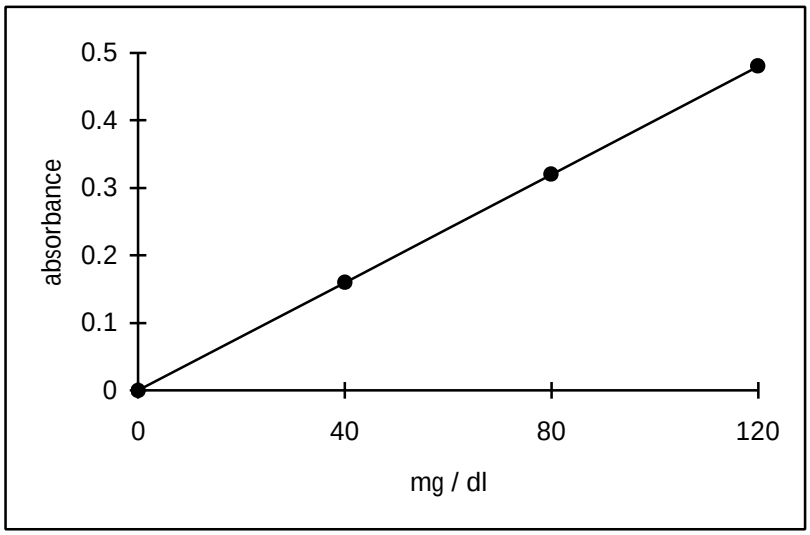
Since standards and test/ QC procedures are identical, the absorbance values of standards are plotted against their respective concentrations. The calibration curve should be linear up to 120 mg/dl, with a lower limit of 4-5mg/dl.

Plot the absorbance values of test on the calibration graph and read off protein concentrations in patients ' CSF. As 1: 100 diluted QC serum is analysed, read off the

protein concentration in QC on the calibration graph and multiply the value by 100 to get the correct protein value in QC serum.

Once linearity is proved, it is not necessary to prepare the standard graph every time that patients' samples are analysed. It will be adequate if standard S4 is taken every time and patients' results are calculated using the formula.

$$\frac{\text{Test absorbance}}{\text{Standard absorbance}} \times 120 \text{ mg/dl.}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients 'results).

Include one internal QC every time a patient specimen is measured, irrespective of the number of samples in a batch. However, even if only a single QC sample is analysed in a day, this value can be pooled with the preceding 10 or 20 values obtained in the previous days and the ***between-day precision*** can be calculated and expressed as %CV. Ensure that this is well within the acceptable limit, i.e, 8%.

At least once a week analyse another QC serum from either a low QC or high QC pool.

"Assayed" QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera from a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

This procedure uses sodium azide. Do not swallow, and avoid contact with skin and mucous membranes.

Reference range and clinical interpretation

CSF protein - 15-45 mg/dl

Normally the protein present in CSF is entirely albumin, but in many disease states, CSF contains a mixture of albumin and globulins. Increase in proteins up to 400 mg/dl is observed in meningitis and up to several grams in spinal tumour. In inflammatory lesion, increase in protein is associated with increase in cells. A marked increase is also observed in paralysis and in disseminated sclerosis.

Abnormally increased total CSF protein may be found in conditions where there is an increased permeability of the capillary endothelial barrier through which ultrafiltration occurs. Examples of such conditions include; bacterial, viral and fungal meningitis, traumatic tap, multiple sclerosis, obstruction, neoplasm and cerebral infarction.

Limitations

Any CSF protein value > 120 mg/dl should be diluted with 0.9% saline either 1:2 or 1:3 and reassayed, and the value obtained is multiplied by the appropriate dilution factor to get the correct value. The presence of blood and pus will increase CSF protein. The report to the requesting physician should include the appearance of CSF before and after centrifugation.

Reference

(1) Watanabe et al (1986) Clin. Chem 32 : 1551-1554

18. CSF protein – Turbidimetry method

Principle of the method

Protein present in CSF is measured by precipitating the proteins with 3g% sulphosalicylic acid and comparing the absorbance of the turbidity at 640 nm with that of protein standards.

Reagents

All chemicals must be Analar grade

(a) Sodium chloride diluent

Dissolve 9g of sodium chloride and 0.5 g of sodium azide together in a final volume of one litre of distilled water in a volumetric flask. Store the solution in an amber coloured bottle. Stable for one year at 25-35^{0}C.

(b) Sulphosalicylic acid 3g/dl

Dissolve 30 g sulphosalicylic acid in a final volume of one litre distilled water. Store in an amber coloured bottle at 25-35^{0}C. Stable for 6 months.

(c) Standards

Refer Total Protein (page 40, (c) Standard for the preparation of protein standard from pooled serum. Note that there is always a risk of infection from pooled serum.

After determining the concentration of the protein standard, dilute this to several levels as described below.

For example, if the total protein value of the pooled serum is 6.7g/dl, then the volume of pooled serum to be diluted to 100ml to get a protein concentration of 20 mg/dl is found out using the dilution formula.

IV x IC = FV x FC

IV = Initial Volume of pooled serum.

IC = Initial Concentration of total protein (mg/dl).

FV = Final Volume.

FC = Final Concentration.

IV x 6700 = 100 x 20

$$IV = \frac{100 \times 20}{6700} = \frac{20}{67} = 0.3 \text{ ml}$$

i.e. Dilute 0.3 ml of the pooled serum to 100ml with 0.9 g/dl sodium chloride containing 0.1g/dl sodium azide. Similarly, 40, 80 and 120 mg/dl standards are prepared by diluting 0.6, 1.2 and 1.8 ml of the pooled serum each to 100 ml with the diluent. The standards are stable at 2-80C for one month.

The following table summarizes the preparation of diluted standards.

Concentration of protein in pooled serum is taken as 6.7g/dl.

Standard	Pooled serum (ml)	Final diluted volume (ml)	Concentration of Standard (mg/dl)
S1	0.3	100	20
S2	0.6	100	40
S3	1.2	100	80
S4	1.8	100	120

Equipment, glassware and other accessories

Refer to Section A (2), Introduction to SOP.

Procedure

The protocol of the procedure is described below.

Pipette the following into appropriately labelled 13 x 100 mm tubes

	Blank	S1	S2	S3	S4	Test	QC
Protein standard (ml)	-	1.0	1.0	1.0	1.0	-	-
Test sample (ml)	-	-	-	-	-	1.0	-
QC serum(1:100) (ml)	-	-	-	-	-	-	1.0
Sodium chloride (ml)	1.0	-	-	-	-	-	-
Sulphosalicylic acid (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0

Mix all tubes well. Leave at 25-35^{0}C for 5 minutes. Set the spectrophotometer /filter photometer to zero using blank at 640 nm/ red filter and measure the absorbance of standards, test and QC.

Calculation and calibration graph

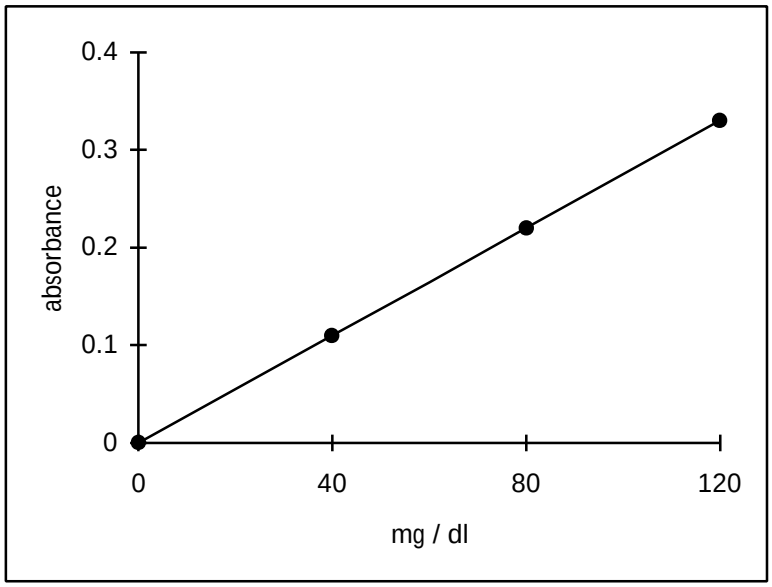
Since standards and test/ QC procedures are identical, the absorbance values of standards are plotted against their respective concentrations. The calibration curve should be linear up to 120 mg/dl with a lower limit of 10 mg/dl.

Plot the absorbance values of test on the calibration graph and read off protein concentrations in patients 'CSF. As 1: 100 diluted QC serum 's analysed, read off protein

concentration in QC on the calibration graph and multiply the value by I 00 to get the correct protein value in QC serum.

Once linearity is proved, it is not necessary to prepare the standard graph every time when patients ' samples are analysed. It will be adequate if only standard S4 is taken every time and patients' results are calculated using the formula.

$$\frac{\text{Test absorbance}}{\text{Standard absorbance}} \times 120 \text{..... mg/dl.}$$



Analytical reliabilities

Refer to **pages 7-9 of section 1 (General Introduction)** on the use of internal QC and interpretation of daily QC data (for releasing patients 'results).

Include one internal QC every time a patient specimen is measured, irrespective of the number of samples in a batch. However, even if only a single QC sample is analysed in a day, this value can be pooled with the preceding 10 or 20 values obtained in the previous days and ***between-day precision*** can be calculated and expressed as %CV. Ensure that this is well within the acceptable limit, i.e, 8%.

At least once a week analyse another QC serum from either a low QC or high QC pool.

"Assayed" QC sera with stated values (ranges) are available from several commercial sources, viz. Boehringer Mannheim, BioRad & Randox.

If a laboratory uses QC sera rom a commercial source, it is important that the company certifies that their QC materials are traceable to international reference materials.

Hazardous materials

This procedure uses sodium azide and sulphosalicylic acid. Do not swallow, and avoid contact with skin and mucous membranes.

Reference range and clinical interpretation

CSF protein - 15-45 mg/dl

Normally the protein present in CSF is entirely albumin, but in many disease states CSF contains a mixture of albumin and globulins. Increase in proteins up to 400 mg/dl is observed in meningitis and up to several grams in spinal tumour. In inflammatory lesion, increase in protein is associated with increase in cells. A marked increase is also observed in paralysis and in disseminated sclerosis.

Abnormally increased total CSF protein may be found in conditions where there is an increased permeability of the capillary endothelical barrier through which ultrafiltration occurs. Examples of such conditions include; bacterial, viral and fungal meningitis, traumatic tap, multiple sclerosis, obstruction, neoplasm and cerebral infarction.

Limitations

Any CSF protein value > 120 mg/dl should be diluted with 0.9% saline either 1:2 or 1:3 and re-assayed, and the value obtained is multiplied by the appropriate dilution factor to get the correct value. The presence of blood and pus increases CSF total protein. The report to the requesting physician should include the appearance of CSF before and after centrifugation.

Reference

(1) Varley's Practical Clinical Biochemistry. 6th edition, published by Heinemann Medical Books, London (1988) page 447-448.

19. Introduction

Urine is one of the most easily obtained specimens examined in the laboratory, and examination of the urine not only provides information about the functioning of the kidneys and possible abnormalities of the urinary tract, but may also lead to the diagnosis of various systemic diseases of the human body which are reflected by the presence of several substances in the urine.

Collection of specimen

(a) Early morning urine

This is the best urine specimen for routine analysis and is collected soon after the patient awakens. It is usually concentrated and has an acid pH. Casts and cells are poorly preserved in dilute or alkaline urine and traces of dissolved substances such as protein and sugar can be missed if the urine is very dilute.

(b) Random urine

This specimen is collected at any time and is convenient for the patient and is suitable for most screening purposes.

(c) Preservative used

For routine analysis, no preservative is required but the urine is best examined fresh. Bacterial growth will ruin a specimen if analysis is delayed for more than 3 hours. Refrigeration is the best way to preserve it if analysis is delayed. Refrigeration for more than 24 hours is not recommended.

(d) Container for urine collection

The container used must be thoroughly clean and free from any detergent or disinfectant residue since the oxidants contained in such cleaning agents may cause the test areas for glucose and blood to indicate false positive results. After the urine is collected, the container should preferably be sealed.

20. Qualitative tests

Appearance

Normal urine colour varies from light yellow to deep amber. Urine colour sometimes may vary depending upon the drink, if any, consumed by the patient. The colour of urine is sometimes related to a pigment called "urochrome". The degree of colour also depends on whether the specimen is concentrated or dilute.

Normal urine is usually clear. If the pH is alkaline, turbidity may be observed due to the precipitation of phosphates. Such urine should be centrifuged before analysis. Turbidity due to the presence of chyle (chylomicrons) cannot be centrifuged, but requires filtration using a special cellulose filter having <0.1 μm diameter.

pH

Use a narrow range pH paper or a pH meter.

In some clinical situations, measurements of approximate pH within $\pm$ 0.5 pH units using a narrow-range pH paper or exact pH using a pH meter may be very helpful.

(a) Procedure

Using pH paper

Put a drop of urine on a portion of pH indicator paper. The colour obtained is compared with a standard chart. For checking the reliability of the pH paper cross check the pH of buffer solutions of known pH values having acidic and alkaline pH ranges.

(b) Using a pH meter

Calibrate the pH meter using standard buffers, one having an acidic pH and the other an alkaline pH, preferably less than 9.0. Pour about 40-50ml of urine into a 100ml beaker, calibrate the pH meter once again using the buffer of pH 7.0 and measure the pH of the urine.

Result

Normal urine pH ranges from 4.5 to 8.0.

The urine pH values are reported for example as 6.0 if pH paper is used or as 6.1 if a pH meter is used.

Interpretation and quality control

Urine pH is usually acidic in normal people, especially non-vegetarians, and is usually alkaline in vegetarians.

An early morning urine pH <5.5 indicates that renal tubular acidification mechanism is intact.

As a quality control measure, use certified reference buffers (commercial source), one in acidic range, say, pH 4.0 and the other in alkaline range, preferably pH 9.2 to check the reliability of the pH paper used or to assess the performance of the pH meter.

Always use a pH indicator paper before the date of expiry. Do not use outdated pH papers. Always close the bottle containing the pH paper tightly.

The pH meter must be calibrated against a correct calibration buffer.

Ketone Bodies - Rothera's test

The three main ketone bodies are acetone , acetoacetic acid (diacetic acid) and beta-hydroxybutyric acid. Testing for ketone bodies should be done on fresh urine or the specimen kept at 4 ^{0}C.

(a) Principle

Acetone and acetoacetic acid react with sodium nitropruside in the presence of alkali to produce a purple colour.

(b) Reagents

Rothera's Reagent : Dry mixture

Pulve210rize 7.5g sodium nitropruside with 200g ammonium sulphate. Store in a clean amber bottle at 25^0-35^{0}C. Stable for 6 months.

Ammonia concentrated, specific gravity 0.91

(c) Procedure

To about 5ml of urine taken in an 18 x 150mm glass tube, add about one teaspoon of the mixture, mix well, then add 0.5 to 1.0 ml of concentrated ammonia down to the side of the tube so that it layers on top of the urine. Observe for any colour change within 30-60 seconds.

(d) Result

If acetone and diacetic acid are present, then a purple (permanganate calomel red) colour will form at the junction of the two layers within 30-60 seconds. The result can be graded from trace to 3+ based on the intensity of the colour formed, as detailed below.

No change in colour	- Negative
Pinkish ring	- +
Red ring	- + +
Deep purple ring	- + + +

(e) Interpretation and quality control

Ketone bodies are intermediary products of fat metabolism and their presence in the blood and then in the urine are indications that the metabolism is disordered or incomplete. This is associated with metabolic acidosis. This occurs in poorly controlled diabetes mellitus and also in starvation.

Normal urine does not contain methyl ketone. Weak false positive reactions may occur if the urine contains L-dopa and phenyl pyruvic acid.

If there is suspicion of a false positive test, heat the urine in a test tube in a bunsen burner flame for one minute, allow to cool and repeat the Rothera's test. Heated urine will not give a positive Rothera's due to ketone bodies.

As a quality control measure, the reagent should be checked frequently using a positive control (1-2 drops of acetone is added to 5ml of urine). The use of distilled water in place of urine for negative control is recommended.

Urobilinogen –Erhlich s test

(a) Principle

Erhlich's reagent in conc HCI. reacts with urobilinogen to form a pink coloured aldehyde complex in chloroform.

(b) Reagents

Erhlich's reagent

Dissolve 2 g of P-dimethyl aminobenzaldehyde in 100ml of 20% HCI. Store at 25- 35^{0}C in an amber coloured bottle. Stable for 3 months.

10g/dl Barium chloride

Dissolve 10g barium chloride in 100ml of distilled water. Store at 25- 35^{0}C. Stable for 6 months.

Saturated ammonium sulphate

Chloroform AR or GR quality

(c) Procedure

To 12 ml of urine taken in a 25ml or 50 ml measuring cylinder add 3 ml of barium chloride followed by 3 drops of saturated ammonium sulphate. Mix well. Transfer a portion of it into a 15 x 120mm glass tube. Centrifuge for 5 minutes at 3500 rpm. Transfer about 5ml of the supernatant into an 18 x 150 mm glass tube and add 0.5 ml of Erhlich's reagent. Mix well. Then add 3ml chloroform and shake well. Allow to stand one minute. Observe for any colour change in the chloroform layer (bottom layer).

(d) Result

Urobilinogen

Colourless	:	Not detected
Faint red colour	:	Normal
Red or bright red	:	Positive or highly positive depending upon the intensity of colour.

(e) Interpretation and quality control

Urobilinogen is normally excreted in trace and a normal urine will always show a faint red colour in the chloroform layer. It is always a good practice to run a normal urine as control whenever an urobilinogen test is done. Excess urobilinogen is seen in urine in haemolytic jaundice, viral hepatitis and cirrhosis and is absent in obstructive jaundice.

Bilirubin - (Harison spot test) Fouchet's test

(a) Principle

When ferric chloride in acid solution is added to a precipitate (Ref : Urobilinogen procedure) of urine containing bilirubin, a green colour is produced as the bilirubin in the urine is oxidized to biliverdin.

(b) Reagent

Fouchet's reagent.

Dissolve 25g of trichloroacetic acid in about 50ml of distilled water, then add 1g ferric chloride, mix to dissolve and then make up to 100ml with distilled water. Store at 25 – 35^0C. Stable for 6 months.

(c) Procedure

To 12 ml of urine taken in a 25 ml or 50 ml measuring cylinder add 3 ml of barium chloride followed by 3 drops of saturated ammonium sulphate. Mix well. Transfer a portion of it into a 15 x 120mm glass tube. Centrifuge for 5 minutes at 3500 rpm. Decant the supernatant and add 1or 2 drops of Fouchet's reagent to the precipitate. Examine for any colour change.

(d) Result

No colour change in the precipitate	: Negative
Appearance of a green or blue colour	: Positive

(e) Interpretation and quality control

Bilirubin is not present in normal urine. For a positive control, a few drops of either a bilirubin standard or an icteric serum are added to a normal urine sample and the specimen is analysed for the presence of bilirubin. Any bilirubin present in the urine is conjugated and indicates excess in the serum due to cholestasis.

21. Semi-Quantitative Tests

Specific Gravity (Mass Density)

Urinometer method

(a) Principle

Specific gravity is a function of the number, density and weight of the solute particles present and is used as a measure of the concentrating power of the kidney. The specific gravity of urine is its density compared with the density of distilled water that is conveniently fixed as 1.000 at 20^0C.

It is measured using a weighted cylinder called "urinometer", which floats in the urine and which is calibrated against distilled water at 20°C. Check the working of the urinometer by floating it in distilled water to see if the reading is 1.000. As the specific gravity varies with temperature, apply temperature correction before reporting.

(b) Procedure

- Pour about 40 - 50ml of urine into a 100ml glass measuring cylinder.
- lower the urinometer gently into the urine, rotate and release (avoid frothing).
- Wait for the urinometer to settle (make sure that the urinometer does not come into contact with the sides or bottom of the cylinder).
- Read the specific gravity given on the scale at the surface of the urine (use the lower point of the meniscus for reading).
- Observe the temperature of the urine.

(c) Result

Check the temperature at which the urinometer is calibrated. It is usually at 20^0C. For every 3^0C that the urine temperature is above the calibration temperature, add 0.001 to the measured specific gravity and for every 3^0C that the urine temperature is below the calibration temperature, subtract 0.001 from the measured specific gravity.

Example

Urinometer is calibrated at 20^0C
Urine temperature 23^0C
The measured specific gravity 1.023
The temperature of urine is 3^0C higher than the calibration temperature.
∴Specific gravity to be added to the measured specific gravity =
3/3 x 0.001 = 0.003/3 = 0.001.
∴The actual specific gravity = 1.023+0.001=1.024.

(d) Interpretation and quality control

The normal urine-specific gravity is 1.010 –1.030

The presence of an increased amount of protein affects the specific gravity by 0.001 for every 0.4g/dl protein level in urine. As a quality control measure, the functioning of the urinometer must also be checked by floating in other liquids whose densities are greater than distilled water. There is also an adjustment for glucose Subtract 0.001 for every 270 mg/dl glucose in the urine.

Proteins –Heat and acetic acid method

(a) Principle

Proteins in urine are coagulated by heat and the degree of coagulation is directly proportional to the amount of proteins present. Coagulation can be further enhanced when drops of acetic acid are added.

(b) Procedure

Pour 2-3 ml of urine into a 13 x 100mm glass tube and hold it using a tube holder. Check the urine pH; if it is >pH 7 or <3, adjust to between 4-5 using 3% acetic acid. Heat the upper half of the column of urine in a flame until it boils. Look for the appearance of cloudiness in the heated portion and contrast it with the lower portion of the tube. Appearance of cloudiness in the upper portion indicates the presence of proteins. Add 2-3 drops of 3% acetic acid to the precipitate and observe. If the precipitate disappears, it indicates the presence of phosphates and carbonate (later produces effervescence when the precipitate disappears). Persistence of the precipitate shows the presence of albumin. On adding 2-3 drops of conc. $HN0_3$ if the precipitate disappears, the presence of mucin or nucleoprotein is suggested.

(c) Result

This test may be used as semi-quantitative, as follows;

Colour change	Result
No cloudiness	Negative
Faint cloudiness (may be observed only if the tube is held against a black background).	Trace
Definite nongranular cloud without flocculation	1+
Heavy and granular cloud without flocculation	2+
Dense cloud with marked flocculation	3+
Thick curdy flocculation & coagulation	4+

(d) Interpretation and quality control

This test is sensitive enough to detect protein down to a concentration of 2-3 mg%.

For quality control, dilute 22g% of human albumin solution to get a concentration of 5 mg/dl. Use this as a test and check the reliability and sensitivity of this method.

Note: If an alkaline urine is boiled, the protein may be converted into the so- called "alkaline metaprotein", which is not coagulated by heat. Therefore it is always better to acidify the urine before doing this test.

If too much acetic acid is added, the protein may be converted to the so-called "acid metaprotein", which is also not coagulated by heat. Therefore the urine should be only mildly acidic.

Protein –Sulphosalicylic acid method

(a) Principle

Urine proteins are precipitated by sulphosalicylic acid, which gives a white precipitate, and the degree of the precipitate is proportional to the protein level.

(b) Reagent

3g % sulphosalicylic acid (SSA)

Weigh 7.5 g of sulphosalicylic acid and dissolve it in about 200ml of distilled water and then make up to 250 ml with distilled water. Store at 25 - 35^0C. Stable for 6 months.

(c) Procedure

To 2ml of urine taken in a 13 x 100mm glass tube, add 2 ml of 3g% SSA. Mix gently. Leave for 5 minutes at room temperature. Compare the degree of the precipitate with 4ml of SSA taken in a similar test tube.

(d) Result

Same as given on page 99 "(c) Result "

(e) Interpretation and quality control

The sulphosalicylic acid method will not detect protein in a normal urine, but will be sensitive enough to detect protein present down to 20mg%. As a quality control measure, a 22g/dl albumin solution can be diluted appropriately with 0.9 g/dl sodium chloride to get standards containing 20, 50, 200, 500 and 2500 mg/dl proteins. These standards are stable for one month when stored at 2-8^{0}C. When they are subjected to the same procedure as urine, the results can be interpreted as follows:

Concentration of proteins	Reported as
20 mg/dl	Trace
50 mg/dl	1+
200 mg/dl	2+
500 mg/dl	3+
2500 mg/dl	4+

Sugar: Benedict's test

(a) Principle

Urinary sugars when boiled in Benedict's reagent reduce copper sulphate to a reddish cuprous oxide precipitate in hot alkaline medium, the intensity of which is proportional to the amount of sugar present in the urine. The results are reported as I+,2+, etc. depending upon the colour and intensity of the cuprous oxide precipitate.

(b) Reagent

Dissolve 17.3g of crystalline copper sulphate in about 800ml of distilled water, then add 100 g of sodium carbonate, mix to dissolve and finally add 175g of sodium citrate. Mix well to dissolve and then make up to one litre with distilled water. Store in an amber coloured bottle at 25- 35^{0}C. Stable for one year.

(c) Procedure

To 5 ml of Benedict's reagent taken in an 18 x 150mm glass tube, add 8 drops (0.5 ml) of urine, mix well and boil for 2.3 minutes, preferably in a boiling waterbath. Cool the tube and observe for any colour change.

(d) Result

The results are reported as follows:

Observation	Inference
No change in the original colour of Benedict's solution	Negative
Solution appears pale green and slightly cloudy	Trace
Definite cloudy green	1+
Yellow to orange precipitate	2+ (1 g/dl)
Orange to red precipitate	3+ (2 g/dl)
Brick red precipitate & clear supernatant	4+ (>2 g/dl)

(e) Interpretation and quality control

Normal urine does not contain any reducing sugar. If protein is present in large amounts, it may interfere with the precipitation of the cuprous oxide.

To overcome this problem, precipitate the proteins using 3% SSA filter using a Whatman filter paper and use the filtrate to test the amount of sugar present.

As a quality control measure, standards containing known amounts of glucose are prepared in saturated benzoic acid and one of the standards is used every day to check the reliability of the patient's results. The standard results may be transformed in the following semi-quantitative way.

100mg/dl	Trace
250mg/dl	1+
500mg/dl	2+
750mg/dl	3+
2 g/dl	4+

False positive reactions are known to occur due to the presence of non-carbohydrate substances like ascorbic acid, homogentisic acid, creatinine and uric acid. Reducing sugars like lactose, galactose, fructose and pentoses will also give a positive reaction.

The dipstick technique is specific for glucose and eliminates the false positive reaction due to the substances mentioned above.

22. Dipstick Technology

Introduction

Although easily adaptable manual methods are available for urine analysis, not all intermediary-level laboratories have facilities to prepare their own reagents. Sugar, albumin, urobilinogen and bilirubin are the four biochemical substances tested in a random urine sample. Although the heat and acetic acid test detects the presence of proteins such as albumin, only a semiquantitative test will be really useful. In the same way, Benedict's test, which is commonly used, detects only the total reducing substance and does not predict the amount of glucose present. The state-of-the-art technology is the use of dipstick to detect biochemical substances in a convenient way. Many companies are now manufacturing test strips based on the basic wet chemistry reactions of the respective biochemical substances.

This section describes the routine screening tests done using dipstick technology, highlighting the principal reactions and limitations.

Correct storage of strips

Protect the strips from moisture and excessive heat and light but do not refrigerate. Replace the top on the storage container immediately after removing a strip.

Glucose

Compared to Benedict's test, which detects the total sugar present in urine, the strip test detects semi-quantitatively the amount of glucose present in urine. It is a fast and convenient way of testing urine to determine the amount of glucose present. Two types of dipstrips are available, viz. Clinistix and Diastix . These are fast and convenient ways of testing urine to determine the amount of glucose excreted in urine.

(a) Principle

Clinistix

The strip is impregnated with the enzymes glucose oxidase and peroxides, and the indicator substance O-toluidine. The 0-toluidine is oxidized to a blue-green substance (Schiff s base) with varying shades of colour, which is then compared with the standard chart provided in the kit to report the approximate level of glucose present in the urine.

Diastix

This strip has an area impregnated with the above enzymes together with potassium iodide and a blue background dye. The oxygen liberated in the final reaction binds with the dye to produce a series of colour changes 30 seconds after wetting the strip with urine.

(b) Procedure

Completely immerse the reagent area of the strip in fresh urine for 1-2 seconds and remove. Gently tap the edge of the strip against the side of the urine container to remove excess urine. Compare the test area closely with a colour chart exactly 30 seconds after dipping the strip in the urine. Hold the strip close to the colour chart and match carefully.

(c) Results

The results are expressed as either negative or varying degrees of positive, indicating different amounts of glucose present.

Factors affecting sensitivity

Follow the instructions provided in the kit insert carefully regarding the handling of strips so that the sensitivity of the test will remain good. Exposure to atmospheric air reduces the sensitivity. pH and temperature are unimportant factors. Darkening of the enzyme-coated area indicates loss of sensitivity. Hence discoloured strips should not be used.

False positive

Contamination of glassware with oxidizng agents such as sodium Hypochlorite and bleaching powder and detergents like sodium phosphate will oxidize and change the colour of chromogen in Clinistix.

False negative

As ascorbic acid is an oxygen acceptor and most likely to be present in large amounts in the urine of pregnant women, this will cause a false-negative result.

These procedures will not detect the presence of other reducing sugars, fructose, galactose, etc. in the urine as they are specific for glucose.

Proteins

Several rapid screening tests are in routine use. The majority of the test strips have been developed to detect albumin and may be negative in the presence of other proteins, such as Bence Jones Proteins.

(a) Principle

It is based on the protein error of a pH indicator. At a constant pH any colour change that happens to an indicator is due to protein. The test area of the reagent strip is impregnated with an indicator, tetrabromophenol blue, buffered to pH 3.0. At this pH it is yellow in the absence of protein. Protein forms a complex with the dye turning the colour of the dye to green or bluish green.

(b) Result

The colour is compared with the colour chart provided, which indicates the approximate protein concentration.

A false-positive result may occur if:

- the specimen is contaminated with vaginal or urethral secretions
- a strongly alkaline urine is used
- the urine container is contaminated with disinfectants such as chlorohexidine

False-negative results will be observed if acid has been added to the urine as a preservative (for example in the estimation of urinary calcium)

Multiple Reagent Strips

Test for glucose, bilirubin, ketones, specific gravity, blood, pH, protein and urobilinogen

Using a single strip an array of semi-quantitative tests listed above can be done.

(a) Principle

It is a firm plastic strip to which are affixed several separate reagent areas. Depending on the reagents being used, these strips are employed for tests indicated above.

- Glucose: It makes use of the same principle as described for Diastix, the final colour ranging from green to brown.
- Bilirubin: It is based on the coupling of bilirubin with diazotized dichloronaniline in a strongly acid medium. The colour ranges through various shades of tan.
- Ketone: It is based on Rothera's reaction principle and on the development of colours, ranging from buff-pink for a negative reading to purple when acetoacetate reacts with nitropruside. It also detects acetone but not beta-hydroxybutyrate.
- Specific gravity : In the presence of an indicator the polyelectrolytes present in urine give colours ranging from deep blue green in urine of low ionic concentration through green to yellow green in urine of increasing ionic concentration.

- pH: This test is based on the double indicator principle that gives a broad range of colours covering the entire urinary pH range. Colours range from orange through yellow and green to blue.
- Protein: It is based on the protein error of the pH indicator. At a constant pH, the presence of protein leads to the development of any green colour. Colours range from yellow for "negative" through yellow green and green to green blue for "positive" reactions.
- Uroblilinogen: This test is based on a modified Ehrlich reaction, in which p-dimethyl amino benzaldehyde in conjunction with a colour enhancer reacts with urobilinogen in a strongly acid medium to produce a pink-red colour.

(b) Procedure

Do not use discoloured strips. Do not touch the test areas. Dip the test areas of the strip in urine completely, but briefly, to avoid dissolving out the reagents. Read the test results carefully at the time specified in good light and with the test area held near the specimen appropriate colour chart on the bottle label. Do not read the strips in direct sunlight.

Interference

Glucose:	Ascorbate and Ketones may cause a false-negative result.
Bilirubin:	Indican (ureloxyl sulfate) will cause a false-positive result, while ascorbate will cause a false-negative result.
Ketone:	Pigmented urine or urine containing levodopa metabolites/sulph-hydroxyl drugs may cause a false-positive result.
Protein:	Refer to page 105 under Dipstick - Protein, (b) Result
Quality Control:	Dipstix for glucose and proteins are generally recommended for intermediate as well as peripheral laboratories for routine urine screening. However, it is good to cross-check occasionally the performance of the strips by comparing with conventional methods described under Section 5.3. Urinalysis –Semiqualitative tests. It is also good to check the strip results with the patient s other relevant biochemical results.

Index

Accuracy 3

Calibration graph
(See relevant section of each SOP)

Dipstick Technology - Urine Screening
Glucose 103
Multiple reagent strips 105
Proteins 104

Flame Photometer
Instructions on use 69
Maintenance.....73

Hazardous materials
(See relevant section of each SOP)

Interpretation of results
In Serum
Albumin 44
Alkaline phosphatase 56
Bilirubin.....36
Calcium.....59
Cholesterol 29
Creatinine.....26
Glucose 18
Phosphorus.....63
Potassium 69, 70
Sodium.....69, 70
Total protein 40
Transaminases.....47
Urea.....22

In CSF
Glucose 75
Protein 81, 84

In Urine
Bilirubin.....94
Ketone Bodies 92
pH.....91
Specific Gravity (Mass Density) 97
Sugar 100
Urobilinogen 93

Laboratory Error
Analytical 4
Post-analytical 4
Pre-analytical..... 3
random..... 3
Systematic..... 3

Levey Jennings Chart 7
Precision 3
Quality
External quality assessment..... 10
Interpretation of QC data..... 7
Preparation of QC pool..... 5
Quality Manual 1
Quality policy..... 2

Reference ranges
(See in individual section on interpretation of results)

Safety in laboratories
Preventive measures against laboratory infections 13

Semiautomated
Pipette Calibration 12
Systems..... 11

Specimen type, collection and storage
(See relevant section of each SOP)

Standard operating procedures..... 2
Westgard's rules 7