MICROTECHNIQUES IN THE MODERN LABORATORY OF CLINICAL CHEMISTRY

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INTRODUCTION

"Clinical chemistry encompasses the study of the fundamental principles of chemistry as applied to an understanding of the functioning of the human in health and disease."1 Note that in this discipline the focus of man's activity is directed toward understanding himself. It is therefore understandable that this branch of chemistry has received the prime attention of many scientists since the alchemists searched in blood, urine and tissue for the philosopher's stone that would give them eternal life. In modern times this search has manifested itself in an approach to this same problem with techniques which have become increasingly sophisticated as developments in related disciplines have taken place.

Modern organic chemistry may be said to have had its birth when urea, a major constituent of human urine, was synthesized. This achievement was followed by intensive study which resulted in the elucidation of the structures of the purines, pyrimidines, proteins, porphyrins and numerous other substances isolated from human sources. These workers were faced with the problem of examining and identifying limited amounts of material. It was therefore only natural that Emich, Pregl and others were stimulated to develop microtechniques for solving these problems.

These developments have continued in modern times to the point where identification of a new compound has been made, in some cases, with less than 100 µg of material available. As late as 1930, one felt a sense of achievement in being able to assay milligram quantities of the substance sought. Today analysis of microgram quantities is a routine procedure in all clinical laboratories. For some substances such as iodine, thiamine, riboflavin, catechol amines and histamine, nanogram quantities are determined on a routine basis.

The reasons for this development are in the very nature of clinical chemistry. In this discipline one is limited to a biological material derived from a human. The sample is therefore always limited in size, especially since some humans (prematures) may weigh less than 1 kg. The total volume of plasma per kilogram is of the order of 50 ml. A 5 ml sample would represent 10 per cent of the total plasma in a premature.

In order to understand the disease process one must evaluate the functioning of the different organs of the body. This requires a battery of tests, especially during the time when the diagnosis is uncertain. As many as twenty components may need to be assayed on a single specimen. 5 ml
of blood usually represents 2–3 ml of plasma. It is obvious that, if the patient is not to be exsanguinated, microtechniques must be resorted to.

At the turn of the century, 100 ml of blood was required to evaluate its glucose content by a "micromethod". In the 1920's the "micromethod" for sugar or urea required 2 ml of blood. In the 1930's the "micromethod" for glucose required 0.1 ml of blood. Today the use of 0.1 ml of blood is considered as the "macromethod", and a method using a volume of the order of 0.01–0.02 ml is regarded as a micromethod. Using a single sample, comprising 0.1 ml of blood from the finger-tip, many laboratories are performing sugar, urea, sodium, potassium, chloride, CO₂ and pH tests on a routine basis.

The newer instrumentation is designed for microsamples. For example, instrumentation for sodium and potassium done simultaneously with the flame photometer requires 25 μl diluted to 5 ml as the manufacturers' recommendation.

A modern clinical laboratory is equipped to assay approximately 40 elements. Levels of myriads of organic compounds in biological fluids, including the enzymes, are also being determined. This includes those that occur naturally and drugs which are administered. In the author's laboratory last year, tests were made for over 160 different substances.

As distinct from the problems of that in industry or research, the routine laboratory must have these results within a certain time in order that they may be useful in the treatment of the patient. Thus speed is essential in any procedure.

The clinical laboratory also has another problem unique to itself. Many of these tests must be available on a 24 hour basis, 7 days a week. As an awareness of the value of these tests becomes more apparent to the physician, the numbers of tests performed daily approaches vast proportions. It is not uncommon for the larger hospitals to perform as many as 1000–2000 tests per day. Some of the giant medical centres perform even greater numbers. It must also be stressed that these are some of the most difficult and delicate techniques which have ever challenged an analytical chemist.

The above discussion has been presented as an explanation of the course that analytical chemistry has taken in the clinical laboratory.

**METHODOLOGY**

Generally speaking, the clinical laboratory relies a great deal on colorimetry or spectrophotometry for wet chemical analysis. The reason for this is apparent when one considers the speed possible with this method. Automation is most readily adapted to this technique with the variety of flow colorimeters available. The use of cuvettes with longer light paths permits an appreciable increase in sensitivity. Catalytic procedures such as enzyme assays, determination of iodine with the ceric arsenite system and others usually entail a colorimetric reading at the end.

However, almost all available analytical techniques find application. Total lipids are still assayed gravimetrically. Microtitrimetry may involve acid measurement of gastric fluid, chloride assay and calcium determination with EDTA. Oxidimetry is still used as a routine method in many laboratories for assay of calcium precipitated as the oxalate. The diverse instru
mentation discussed below shows that the clinical laboratories have gone far afield to adapt every available technique to their needs.

Separation methods include not only the conventional ones of extraction, filtration distillation and others, but also techniques developed relatively recently, e.g., column, gas, paper and thin-layer chromatography and electrophoresis on gels, starch, paper and cellulose acetate. These are used daily in many laboratories. For example, the identification of the source of a particular enzyme is of prime importance to the clinician who wants to know whether an increased lactic dehydrogenase in the serum originates from the heart or liver. The two types of dehydrogenase are referred to as iso-enzymes, since they act on the same substrate at the same optimum pH. They can be readily differentiated by starch gel or acrylamide gel electrophoresis. Starch block electrophoresis is often used for the separation of the different haemoglobins. Many of these techniques originated in the biochemical laboratory.

**INSTRUMENTATION**

In order to cope with the problems discussed above, the clinical chemist has used available instrumentation and developed new models as the need required. Some of these instruments have been mentioned above. Only instruments used for microassays will be discussed below.

**Flame photometry**

The trend in the clinical laboratory is to use direct read-out instruments which determine both sodium and potassium simultaneously with digital read-out. One such instrument is made by the Instrumentation Laboratory Company of Boston, Massachusetts. The sample size of human serum (140 m equiv./l. for sodium and 5 m equiv./l. for potassium) is practical when 10 µl is diluted to 2 ml with a diluting fluid containing a lithium salt as the internal standard.

**Atomic absorption**

Atomic absorption equipment is practical and is used for the assay of calcium and magnesium in the routine laboratory. It cannot compete in speed or sensitivity with the flame photometer for sodium and potassium at present. Serum specimens diluted with a lanthanum chloride or strontium chloride solution can be assayed practically using 100 µl of sample diluted to 5–10 ml with diluting fluid. For other elements such as lead, sample preparation requires digestion or extraction of the sample and once this is done the instrument has no practical advantage over the dithizone method. The sensitivity is also lower. A practical instrument for the determination of calcium and magnesium in serum is made by the Perkin Elmer Company of Norwalk, Connecticut.

**Spectrofluorometry**

The spectrofluorometer is replacing the filter fluorometer in many laboratories because of its greater specificity for the substances analysed and, in many cases, greater sensitivity. For example, when riboflavin is assayed in urine or blood one can obtain the excitation and emission
spectra and ascertain that it is riboflavin and not an interfering substance that is being assayed. This problem of lack of specificity has markedly hindered the exploitation of this highly sensitive technique. A major problem with most spectrofluorometers is that one is charting the changing sensitivity of the detector in the different spectral regions. This has only recently been partly corrected for in the Amino-Bowman spectrofluorometer. Catechol amines, porphyrins, histamine and thiamine also find routine application with this instrument. Generally, quantities of the order of 10–100 ng (μg) are being assayed.

Electrophoresis

The Spinco instrument for paper electrophoresis is widely used in the Americas. 50–100 μl of sample is generally used for a determination. With the newer microzone attachment for this instrument amounts of the order of 2–5 μl of serum are fractionated on cellulose acetate for their protein distribution. Acrylamide gel electrophoresis with the instrument developed by Raymond is used routinely in many laboratories for haemoglobin identification. It has the advantage over the starch gel procedure in that it does not require a cold room.

Gasometry

The volatile gases present in the blood are carbon dioxide, oxygen and occasionally nitrogen. To these should be added the nitrous oxide and cyclopropane used in anaesthesia.

For carbon dioxide, microdetermination is usually made with a microgasometer. Serum volumes of the order of 10–30 μl are usually used. Recently the introduction of the pO₂ and pCO₂ electrodes has encouraged many laboratories to use this technique.

The gas chromatograph has been applied for this same purpose but suffers from the fact that only one sample can be fractionated at any one time. Since a chromatogram takes several minutes to develop, the application of this instrument is seriously limited where large numbers of determinations are to be performed daily. However, this procedure is most suitable for nitrogen assay. For the anaesthesia gases, and for special problems where only a few determinations need to be performed on any one day, the method can be applied.

In alcohol intoxication, the clinician is interested in knowing whether the patient has imbibed ethanol from ordinary beverages, methanol from denatured alcohol or isopropanol from rubbing alcohol. For this purpose the gas chromatograph is the instrument of choice. Only 10–100 μl of blood, gastric washing or saliva are required for testing. The water is removed from the sample with anhydrous copper sulfate and the vapour is injected into the gas chromatograph. An instrument designed for this purpose is available from the Perkin–Elmer Company of Norwalk, Connecticut.

Potentiometry

The requirements of the clinical laboratory have stimulated several companies to design stable equipment for estimating the pH of blood on samples of 10–50 μl using Sanz-type electrodes. Such instruments are made
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by Radiometer, Metraohm, Beckman and Instrumentation Laboratories. Beckman uses an aluminium heating block to maintain body temperature, namely, 37°C. The others circulate fluid from a constant temperature bath. These instruments may be supplied with Severinghaus type electrodes for estimating pO₂ and pCO₂ directly. These instruments use the fact that thin layers of certain plastics such as Teflon and polyethylene will pass CO₂ or oxygen but not significant amounts of water or salts. A potential developed at the membrane is recorded on the potentiometer of the pH meter due to interaction of the CO₂ or oxygen with the solution contained in the electrode. These electrodes require a careful operator, since equilibrium is reached more slowly than with pH measurement. Nevertheless this technique is finding increasing application in the clinical laboratory.

Sophisticated circuitry has resulted in remarkable stability with these instruments and reproducibility with expanded scales is of the order of ± 0.01 pH units. Electrodes are also available for calcium, potassium and sodium ion estimation. These electrodes lack the required specificity and are too insensitive at present to be used for microanalysis.

The polarograph may also be included in this class of instruments. It has found practical application in the routine clinical laboratory only for lead estimation. The very nature of this technique limits its use for large numbers of determinations because the samples have to be handled one at a time and the procedure is relatively slow. In medical research however, the instrument has found wide application.

Spectrophotometry

Infrared equipment (Perkin–Elmer, Beckman and Baird) is now found in many clinical laboratories. In the past, renal calculi were examined for the purpose of identification of its chemical composition by wet chemical analysis. The X-ray diffractometer has also been used for this purpose. However, the infrared technique seems to be favoured by the clinical chemist. Substances sought include apatite, calcium oxalate, magnesium ammonium phosphate, cystine, uric acid, xanthine and other substances each of which is an aid in the diagnosis of the underlying cause of the disease. Biliary calculi are also examined with this technique. In some laboratories identification of the nature of the barbiturate or drug in cases of intoxication is also determined by this procedure.

The recording u.v. and visible spectrophotometer is often used for drug identification and also for identification of methaemoglobin and sulphaemoglobin. A major use for this instrument is in estimating enzyme concentrations by appearance or disappearance of the substance sought. The reaction rate is measured by the slope of the curve on the recorder. The manually operated u.v. spectrophotometer is used for numerous routine determinations in clinical laboratories and today very few are without it.

The ultracentrifuge

The ultracentrifuge has found application in some clinical laboratories for investigation of the macroglobulins in serum and lipid fractionation. International is now competing with Spinco for this market which is still in the development stage.
Amperometric titration

Amperometric titrations are routine in the clinical laboratory for chloride estimation\textsuperscript{18}. The so-called "chloridometer", made by Aminco and Büchner, uses a silver anode to react with the chloride in solution at constant current. The time required for the sudden increase in current is measured. These instruments can be set at different current rates so that the same time, of the order of 50 seconds, can be used for 10 or 100 \( \mu l \) of serum. These instruments are remarkably stable and results are reproducible to \( \pm 1 \) per cent which is usually better than the precision of pipetting. Used in conjunction with the automatic dilutors, discussed below, this instrument is practical for routine work since the technician performs other tasks during the titration. Direct readouts are available so that results are given directly in m equiv./l.

While other applications, such as estimation of other elements and radicals, are practicable for this instrument, they have not found general use in the clinical laboratory for these purposes.

Radioisotope technique

Most hospitals have a separate radioisotope laboratory for the diagnosis and treatment of disease. Activities include such diagnostic procedures as the use of radioactive materials for locating tumours and measuring thyroid activity, blood volume red-cell turnover, vitamin B\textsubscript{12} deficiency and others. These laboratories will also treat patients with radioisotopes for control of certain types of neoplastic diseases.

The clinical laboratory also uses radioisotopes, but in some cases for quite a different purpose, which may be distinguished from the trend in the radioisotope laboratory by stating that it is purely chemical in nature. For example, in aldosterone assay the sample is chromatographed after a radioactive tracer of aldosterone has been added. The desired fraction is then located by the radioactivity and the aldosterone assayed by some chemical procedure. Similarly the presence of galactose transferase is detected in blood by the rate at which the blood releases radioactive CO\textsubscript{2} from radioactive galactose. Thus the radioisotope is used as a detector to aid in the separation and identification of the desired chemical component.

Radioactive iodine is used in some laboratories to measure the loss of iodine from a digestion procedure assaying for iodine. Thus the equipment used in the clinical laboratory is of a different nature than that used in the hospital radioisotope laboratory. In the radioisotope laboratory equipment is usually specialized for a particular purpose. In the clinical laboratory more versatile equipment is required. This includes such equipment as that for scanning a paper chromatogram for a spot, which may be eluted for colorimetric or fluorimetric estimation, and general counting equipment which can count a wide range of types of activity such as \( \gamma \)- and \( \beta \)-rays of widely variable energy ranges. This area of development is still relatively new in the clinical laboratory and is in a process of development.

Other instrumental techniques

In some laboratories the X-ray spectrometer is being applied for calcium,
potassium, sulfur, phosphorus, iron and zinc assay in blood. Sample size is of the order of 10–50 \( \mu l \). While sodium can be assayed in serum, low counting rates preclude the use of the X-ray spectrometer for this purpose at the present time. When this problem is solved, it is most likely that it will supplant the flame photometer and atomic absorption technique for the elements because of its versatility, speed and use of a small sample. This technique also has the advantage of not requiring any reagents and is non-destructive. Thus 25 \( \mu l \) of serum dried on a piece of filter paper can be assayed for all the elements simultaneously. The paper can be stored and reassayed later.

In addition to the larger pieces of equipment listed above, numerous other instruments, which have been developed specifically for the purpose, are in daily use in the clinical laboratory. These include a variety of types of centrifuges, colorimeters, heating blocks, microtitrators, mixers, lyophilizing equipment, flash evaporators and others. The clinical laboratory is now a prime consumer of scientific equipment and numerous commercial organizations are directing their prime efforts towards satisfying this need.

**AUTOMATION**

It is only natural that the clinical chemist has been vitally concerned with the development of automated equipment in order to cope with the large numbers of determinations necessary daily.

The Technicon autoanalyzer has the limitation that the sample size is too large (of the order of 0.3–0.5 ml) for micro-assay. This is a limitation of the method of sampling with the peristaltic pump. However, it is practical to test for numerous components on this single sample. Where the initial sample is smaller than 0.5 ml it is practical, for some tests, to dilute the sample and still perform these determinations adequately.

Microanalysis in the clinical laboratory is most often concerned with liquid samples. It is therefore possible to use automatic dilutors. A microsyringe samples volumes of the order of 10–50 \( \mu l \) and a larger syringe ejects the diluting fluid or reagent through the same nozzle. This rinses the nozzle for the second sampling. The total time required is of the order of 5–10 sec. The laboratory is thus able to cope with the large numbers of samples to be assayed daily. Further reagents can be added with an automatic syringe. Where these procedures can be applied, greater speed is possible than with completely automatic procedures.

**PRESENT TRENDS**

At present, most components of blood can be determined on a 10 \( \mu l \) sample. Since many times this volume is obtained from a finger-prick of even the smallest infant, it is doubtful whether a significant further reduction in volume of sample will be attempted. Further development will rather take the direction of reducing the volume used for certain components for which, at present, larger samples are required. Creatinine is an example of one substance commonly required, for which a highly sensitive and specific procedure, which lends itself to assay of large numbers of specimens, has not been developed.

The tendency today is to measure the microsample and proceed from
there with larger volumes rather than adding microquantities of reagents. This latter technique requires specialized equipment which does not lend itself to convenient assay of large numbers of samples. Techniques of higher sensitivity and more sensitive instrumentation are therefore preferred.

Extensive automation of procedures has already taken place. This will continue and will be extended to microsamples as this field develops. It is in this field that clinical chemists have been most concerned in the last few years.

The application of the newer instrumentation such as atomic absorption techniques, infrared spectrophotometry, gas chromatography and X-ray spectrometry continues to be extended. Spectrofluorometry is also being applied more widely.

Screening procedures are being extended more generally as disease is better understood. For example a law has been passed recently, in Illinois, requiring infants to be tested for phenylketonuria routinely. For this purpose one method determines the phenylalanine in the blood. The is best done, at present, with a spectrofluorometer. Since a preferred procedure would be based on heel- or finger-prick to obtain the sample, it is apparent that a microtechnique is required. Pressure is being constantly applied by health organizations that other tests be done routinely. One such example would be the test for haemoglobin type by electrophoresis. It is apparent that these procedures will have to be automated if the population is to be covered adequately. Thus truly micro automatic techniques need to be further developed.

Certain substances, such as cobalt and manganese occur in blood at the pg/ml level; yet these substances are of great importance in metabolism. Neutron activation has been disappointing because of the massive equipment required and the extended time needed to make a result available to the physician. This problem exists both for inorganic and organic components of body fluids and tissue. Such substances as the protein hormones in blood, histamine and even the catechol amines in blood still present a problem in trace analysis. It is in this field that newer approaches have to be made, perhaps with equipment still not yet developed.

By performing millions of microdeterminations daily, clinical chemists all around the civilized world pay tribute to the pioneers of microchemistry who have made the advancements in this discipline possible.

References