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COMPARATIVE EVALUATION OF ULTRAMICRO - AND MACRO-CHEMO ENZYME BASED ASSAYS OF GLUCOSE, CHOLESTEROL AND TRIGLYCERIDES

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ABSTRACT

A comparison of the absorbance, enzyme / substrate concentration, reaction efficiency and sensitivity has been made for enzyme-based clinical chemistry assays, using a conventional colorimeter versus a strip-microwell reader, in order to establish the value of ultra-microchemical procedure, with reaction volume 87 μl (light path length = 0.25 cm). By utilizing commercial kits available for the quantitation of serum glucose, cholesterol and triglycerides, it has been established that the micro method is highly cost effective (9-30 fold), reproducible and sensitive. Comparison of blood drawn by a finger prick (capillary) and venipuncture for normal and pathological specimens show reproducibility between different laboratory technologists and in reference with the values reported by an accredited reference laboratory. Since the micro method uses very little serum, it is most suitable for analyses of small samples, from large population-based field trials. However, the assay range has to be titrated for each commercial kit to establish the enzyme/substrate equivalence.

KEY WORDS : Micro-method, Blood Chemistry, Sensitive Assay, Capillary-venous Blood, Colorimetry.

INTRODUCTION

Chemo-enzyme based estimation of various biochemicals in plasma or serum is widely used for its selectivity and sensitivity. However, in the Indian context, these chemo-enzyme based assays are expensive and hence many clinical chemistry laboratories still employ cost-effective colorimetric assays like O-toluidene test for glucose, Zak's method for cholesterol, that are not very selective. Semi-automatic and automatic chemo-enzyme assay readers also employ expensive instrumentation, and the reagents or methods employed make many of these approaches close-ended, and restricted by the guidelines of the manufacturer.

Rylatt and Parish were the first to develop an ultra microchemical assay (1) for protein determination using the Coomassie brilliant blue dye-binding assay of Bradford (2). The assay of Rylatt and Parish was widely used by researchers and the concept of using a microwell reader, as an ultra-micro colorimeter proved successful for many biochemical estimations. However, the use of the microwell reader for general colorimetry never became popular with clinicians. With the advent of ELISA based immunoassays for hepatitis, AIDS and other tests, stripwell micro-readers became available, and offered a great degree of sensitivity and flexibility to clinical

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laboratories. Many of the manufacturers of ELISA microwell readers offered a wide range of filters differing by 10 nm in band width, covering the entire range of visible spectrum, thus improving the versatility of the instrument's use in colorimetric studies.

The use of capillary blood samples for diagnostic clinical chemistry has been advocated in the case of pediatric laboratory diagnosis (3), and where rapid results or constant monitoring is desired. Although, number of such reports are available for various analytes (4-6), no systematic paired study is available for common parameters like glucose and lipid profile in adults. Our present report provides a comparative study of capillary versus venous samples from the same patient under standardized conditions. We have also attempted to include the methodological approach, technical basis, superiority of instrument features, cost comparisons, precautions and sources of error, in order to enable the wide spread application of this microwell reader based microassay technology amongst clinicians. We also report the detailed comparative findings on the estimation of glucose, cholesterol and triglycerides using macro-colorimetric method and ultra-micro chemo-enzyme based assays, using a microwell strip reader.

MATERIALS AND METHODS

Materials : Plain vacutainer tubes without anticoagulant of Beckton and Dickinson, USA were purchased from Alfa & Omega Diagnostics, India. Heparin coated capillaries were obtained from Top Syringe Co. Mumbai, India. Chemo-enzyme assay kits of Minarini, Italy for glucose (GOD/POD) and cholesterol (CHOD/POD) and for triglycerides (GPO/POD) of Raichem, USA were supplied by CPC-Pharmaceuticals, Chennai, India. Uncoated spectrophotometer quality 8-well strips of Nunc Corporation, Denmark were purchased from Genetix, New Delhi, India.

Specimen Collection and Processing : Blood was collected from ten healthy volunteers and six patients through a reputed commercial clinical laboratory in Chennai. The same reference laboratory also provided the assay values for glucose, cholesterol and triglyceride estimated using the Boehringer Mannheim 4010 semi-auto analyzer with a quality control from EQAS of CMC-Vellore. Capillary blood was drawn from the same healthy volunteers and patients (random samples of diabetic and lipidemic). Immediately, 3-5 mm of a plug of molding type plasticizer putty was used to seal one end of the capillary tube. The capillaries containing samples (4 per batch) were placed in a 120x80 mm semi-micro test tube and centrifuged at 1000 x g for three minutes. The capillary tube was broken immediately, a few mm above the hematocrit and the plasma was transferred to 0.5 ml polypropylene Eppendorf tubes and kept frozen at -20^{0}C pending analysis. Similarly, venous blood was drawn from the same patients or volunteers using the vacutainer, and the serum was saved for studies. A comparative macroanalyses of glucose were done on venous drawn blood from thirty six volunteers in order to establish the relative value for the microassay.

Macro-Chemo-Enzyme Assay : Ten microliters of undiluted sample made up to 220 µl with distilled water was taken in a semi-micro test tube and 780 µl of enzyme assay kit reagent was added. The assay volume (1 ml=path length of one cm.) was incubated to develop the color at 37^{0}C for 10 min. and the absorbance was read at 540 nm in an AIMIL-C160-MKII spectrophotometer. For standard values

the colour was developed with a range of standards as follows : (i) glucose (0-25 μg); (ii) cholesterol (0-20 μg); (iii) triglyceride (0-15 μg glycerol). Under these standard conditions with a light path length of one cm., one ml of total assay volume was found to be the minimum volume requirement for the macroassay.

Micro-Chemo-Enzyme Assay : Samples were diluted 1:1 with normal saline and 2-4 μl of sample was pipetted using capillary microtips into a microwell strip and to each well, 65 μl of reagent and 18-20 μl of makeup volume of water was added. Appropriate standards of glucose (0-25 μg), cholesterol (0-35 μg) and triglyceride (0-15 μg glycerol) were used for the ultra-microassays. The microwell strips were incubated at 37°C for 10 min. and the color was read at 540 nm in a stripwell reader model EL301, manufactured by BIO-TEK, USA. The total assay volume described above, was 87 μl and corresponded to a path length of 0.25 cm. In order to analyse the contribution of path length, the assay volumes were doubled (175 μl; path length=0.5 cm) or quadrupled (350 μl; path length=1 cm) by diluting with distilled water. Increase in microassay volume with constant reagent volume of 65 μl also provides a way to test the enzyme/substrate relationship and reaction stoichiometry.

In a separate experiment, the enzyme volume was increased to 130 μl and 260 μl for 175 μl and 350 μl total assay volume respectively. Proportionality of sample to enzyme ratio was maintained constant in this experiment.

Statistical Analysis : Analysis of Variance (ANOVA) was performed to determine the significance of values obtained from capillary samples compared to venous samples from the same patients/volunteers. Coefficient of variation was calculated for intra-assay variations. Correlation coefficients of assays were calculated with the regression equation. All statistical calculations were done using EPISTAT software.

RESULTS

Comparison of Macro-and Micro-Assays: An overall comparison of the standard optical density values obtained for glucose, cholesterol, and triglycerides (Figure-1a, b, c) using the macro assay (one ml) read in a colorimeter was two to three fold-less sensitive (less color for the concentration) as compared to the strip-well reader. Precision and accuracy of the micro method was acceptable(CV=0.18) when measuring glucose standards for the clinically relevant range and was good (CV=0.05) for cholesterol and triglyceride standards. A high correlation was observed between the values of macro and micro methods (r=0.987). The advantage of microassay over macroassay is demonstrated for one cm. (assay volume=350 μl) path length as well for, when the path length was limited in the microwell to 0.5 cm. (assay volume=175 μl) and 0.25 cm. (assay volume=87 μl). The data presented also demonstrates that when the enzyme volume is limited to 65 μl in the wells (path length=0.25 cm.), a non-linearity in the O.D. (Fig.-2a, 2b; for glucose and cholesterol) was observed, suggesting that there may be an enzyme deficiency or reaction inefficiency.

Effect of Path Length in the Microassay: The microwell assay was performed in three different volume regimen to provide different path lengths, namely 87 μl (0.25 cm.), 175 μl (0.5 cm.), and 350 μl (1 cm.). Developing the assay with 65 μl, 130 μl and 260 μl of reagent and standard makeup and specimen volume, we measured the influence of the path length on absorbance values. When the O.D. was measured for the three assays (glucose,

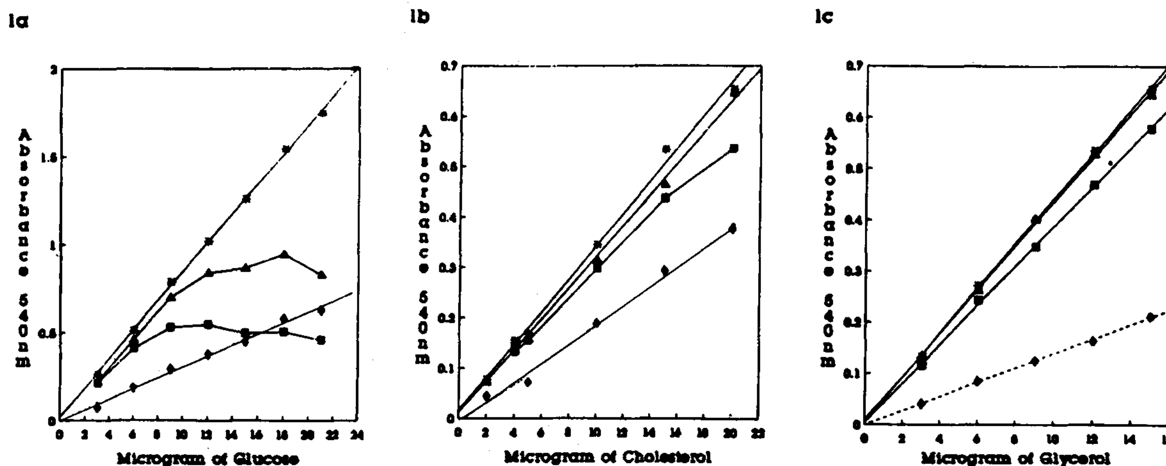

FIG. 1

a: Comparison of Macro- and Micro- assays for standard glucose. Data presented are single values for each concentration done on the same day by the same technologist.

—◆—◆— = Macro assay; 1000 μl; —✱—✱— = Microassay, 87 μl;

—▲—▲— = Microassay, 175 μl; —■—■— = Microassay, 350 μl.

Regression equation for 87 μl assay, y = 0.0169 + 0.0576 * X; r = 0.9969

b: Comparison of Macro- and Microassays for standard cholesterol. Data presented are single values for each concentration done on the same day by the same technologist.

—◆—◆— = Macro assay; 1000μl; —✱—✱— = Micro assay, 87 μl;

—▲—▲— = Micro assay, 175 μl; —■—■— = Micro assay, 350 μl.

Regression equation for 87 μl assay, y = 0.03254 + 0.0261 * X; r = 0.9970

c: Comparison of Macro- and Micro- assays for standard triglyceride. Data presented are single values for each concentration done on the same day by the same technologist.

—◆—◆— = Macro assay; 1000μl; —✱—✱— = Micro assay, 87 μl;

—▲—▲— = Micro assay, 175 μl; —■—■— = Micro assay, 350 μl.

Regression equation for 87 μl assay, y = 0.0479 + 0.0383 * X; r = 0.9961

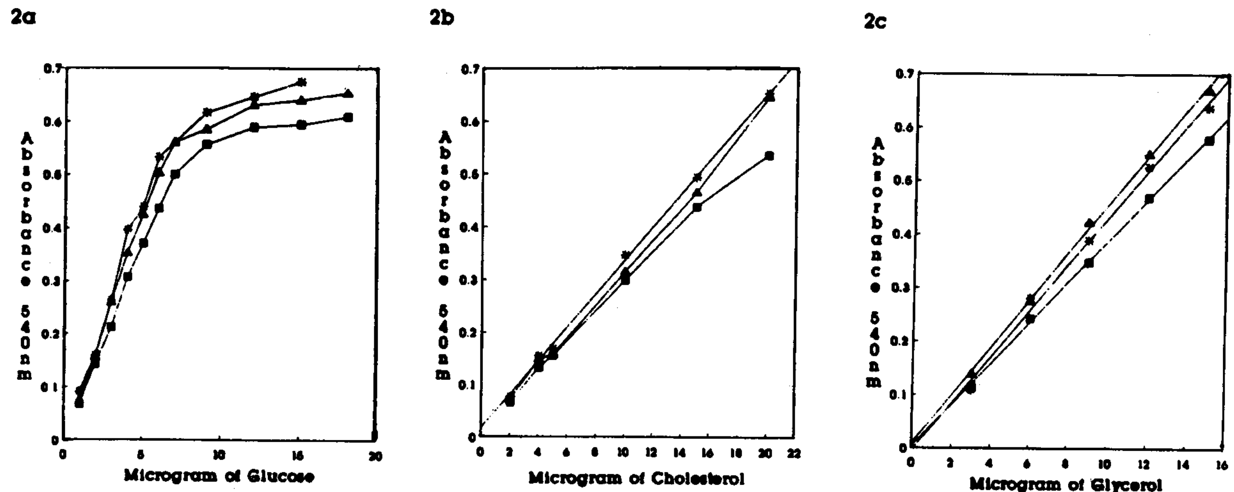

FIG. 2 : Comparison of the reaction volumes (path length) of micro assays for standard glucose (2a), cholesterol (2b) and triglycerides (2c). Data presented are single values for each concentration done on the same day by the same technologist.

—■—■— = Micro assay, 87 μl; —▲—▲— = Micro assay, 175 μl; —✱—✱— = Micro assay, 350 μl.

cholesterol, triglyceride), we found that there was a slight improvement in absorbance (Fig. 1a, 1b, 1c) due to the increase in enzyme content.

The standards used for linear regression analysis in the case of all three analytes fall in the range of clinical relevance (Fig. 1 a-c). Considering a use of minimal sample volume of 1-2 μl in an 87μl assay format, we observed linearity in the measurement of 50-700 mg/dl for glucose; 70-1500 mg/dl for cholesterol; and 50-1200 mg/dl of triglycerides. This range covers clinically significant limits for glucose and cholesterol and a wider range in the case of triglycerides.

The microassay studies (87μl volume) demonstrated that there was plenty of reagent to handle even the highest concentration of substrate (Fig. 2b. and 2c). The data also shows some non-linearity in the glucose assay (Fig. 2a) at higher concentration (>7 μg glucose standard correlating to >700 μg/dl serum glucose if minimal volume i. e., one μl of the sample is used for the assay). By comparison, the micro assay for glucose was three times better, when compared to the macro assay using one-third of the reagent, and hence is approximately nine-fold more cost effective. In the case of cholesterol assay there was a 25% improvement in the micro assay (87 μl) when

compared to macro assay (one ml) and the savings on the reagent was sixteen-fold. Micro assay for triglycerides showed two to three-fold improvement in absorbance and an overall 25-30-fold reduction (based on reagent used and the corresponding color developed) in the reagent cost.

Comparison of Venous and Capillary Samples : We have compared representative normal and pathological blood samples collected by capillary and venipuncture methods and by different people handling the microassay technique. The results of this study are shown in table 1. There was no significant variation between analyte concentration measured by either of the specimen collection methods in clinically normal and patient samples (with CV % ranging from 0.5–3.7 %). Interestingly, even in diabetic specimen, the high blood glucose values are measurable with the available reagents, thereby proving the clinical relevance of the ultramicro assay format.

Precision, Accuracy and Recovery : Two individual technicians provided triplicate venous blood glucose measurements by both micro and macro assay formats on two different days for statistical analysis (Table 2). The values showed good agreement between those obtained from the macro assay performed in the reference lab and microassay values from our lab (ANOVA, $p<0.001$). Accuracy of the assay report was evaluated by a reference clinical laboratory based on their same day assay values and was found to be within a standard error of ±5.2 mg/dl for glucose (CV%=2.5). The intra and interassay imprecision studies are based on triplicate analyses done on sixteen patients' sera, a pooled control sera and standard substrates (100 mg/dl). The average intra and interassay imprecision (% CVs) as derived from triplicate analysis were ranging from 1.2–3.8%. Within day variations of values reported by micro assay format (87µl) were found to be minimal (ranging from 0.9–4.3 %).

Ten pooled sera of known concentration were supplemented with glucose 100mg/dl and assayed by the microassay (87 µl) format. The analytical recovery of the substrates added to the ten pooled sera were ranging from 97.4 – 98.2% with an average recovery of 97.8%.

Table 1 : Imprecision analysis of Micro-Chemo-Enzyme assays using venous and capillary blood

Samples	Glucose		Cholesterol		Triglycerides	
	Venous	Capillary	Venous	Capillary	Venous	Capillary
Normals *(n = 10)*	2.3	0.5	1.9	0.6	3.5	3.7
Diabetic & / Lipidemic *(n = 6)*	2.5	2.2	1.0	3.0	0.5	1.9

Table 2 : Comparison of Macro (1000 μl) and Micro (87μl) assay formats.

Sample statistics	Micro Assay	Macro Assay
n	36	36
Range	75–400	75-400
Mean ± SD	180.9 ± 11.5	175.6 ± 23.1
CV %	1.2	3.8

DISCUSSION

A comparison of the available construction of electronic hardware and the handling of light path of the standard colorimeter versus the microwell reader shows four interesting differences. (i) Angle of light path, (ii) Path length, (iii) Presentation of light, (iv) Nature of light detector. In the standard colorimeter, the light path is horizontal and offers one cm. path in length and the source of light is continuous, from a tungsten bulb. Light detection in the colorimeter is done by a standard photovoltaic cell. By contrast, in the microwell reader, a fiber-optic source emits white light in flashes, along a vertical path that is picked up by a photodiode detector. The path length can be varied according to the volume of reactants in the microwell (87 μl=0.25 cm.; 175 μl=0.5 cm.; 350 μl=one cm.) Presumably, the optics present in the microwell reader and other features described above might offer greater sensitivity when compared to a colorimeter.

The following comments on the advantages, limitation and precautions to be taken in the microassay deserve careful review by the users of the microassay. By varying the reagent volume by two to four-fold with constant sample volume, we tried to determine the reproducibility and precision of the microstrip well reader. During this study, we found that most of the glucose oxidase - peroxidase (GOD - POD) assay kits for glucose estimation had been formatted for macro-format (one ml) and had poor adaptability to our microassay method. This could be due to either insufficient concentration of enzyme systems (GOD-POD-activity) which could not efficiently catalyze concentrated samples or the inappropriate ratios of enzyme / substrate / chromogen used. Wnereas, the other expensive kits like cholesterol (CHOD/POD) and triglycerides (GPO/POD) did not pose these problems.

According to Beer's and Lambert's laws (A=ECl, Wherein, A=absorbance, C= Concentration in moles/liter, and E=molar extinction coefficient, and l=path length), in the microassay, one would expect to see an increase in the absorbance with increase in path length (i.e. 350 μl vs 175 μl vs 87 μl). However, the apparent non-compliance to Beer Lambert's law in the case of absorbance values in Fig. 2a is due to the dilution of end chromogen rather than proportionally increasing reagent content. This anomoly was investigated by increasing the reagent content proportional to the path length and we found a strict conformity (Fig. 1a) to Beer-Lambert's law.

One of the limitations of microassay is the availability of active enzyme in the commercial reagents and there could be significant variation between batch to batch, due to the influence of poor refrigeration and storage conditions before its usage. Another precaution to exercise during the handling of high value pathological samples, is that we recommend a two-to four-fold dilution for diabetics and

hyperlipidemics. During micro-pipetting of samples, the use of capillary-type microtips is suggested for accuracy and wiping of the outside before transfer to microwells in order to avoid dripping and microdrop contribution to assay values.

Estimation of capillary blood glucose using filter paper spots, glucometers or dipsticks (4, 7-9) are already widely accepted and have proven to be suitable for monitoring the control of glucose levels of diabetic patients. Rapid and large population field trials, diagnosis in intensive care and want of minimum pain inflicting procedures in pediatric care dictated the wide spread use of capillary blood sample collection. In our present study, capillary blood collection by a finger-prick method was analyzed for its suitability to our ultra-micro chemo enzymatic assay protocols. A small amount of plasma/serum available from capillary collection, was insufficient to undertake replicate macro assay (one ml). Hence, we used reference values (venous) provided by the certified lab, to compare our results obtained by the ultra-micro assay procedure using both capillary and venous blood samples of the same patient (paired study).

The disadvantage of the capillary method is that in a few cases of hypovolemic individuals with high hematocrit, the values reported from the microassay tend to be exaggerated by 20-25%. In such instances, the results must be interpreted with caution. This haematocrit dependency of capillary blood glucose estimations was consistent with earlier reports about false diagnoses of hypo-glycemia in hemoconcentrated patients and of normoglycemia in anemic patients (10). It is therefore appropriate to conclude that significant differences between capillary and venous analyte levels, if any, are probably a result of haematocrit differences or errors in the collection technique, rather than due to the precision of the assay reported here.

In summary, we have shown that the intra-micro assay for three blood parameters, employing chemo-enzyme methodology, provides reliable results and is highly cost effective (nine to thirty-fold, depending on the assay) especially for assays using expensive cholesterol and triglyceride reagents. We suggest the use of a total assay volume of 87 µl volume (0.25 cm path length), which includes one to two µl of plasma/serum (if necessary, diluted 1:5) for the estimation of glucose, cholesterol and triglycerides. The assay is especially ideal, when blood samples have to be collected in pediatric wards, or in the field, using capillary method for large population studies at reduced cost, time and sample volume. The microassay technology requires little lab space, relatively inexpensive instruments and skilled technologists. Therefore, the ultra-micro assay described here is best suited for economically underprivileged laboratories.

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