THE METABOLIC ASSESSMENT OF PATIENTS WITH DIABETES MELLITUS.

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MacRury SM, Kilpatrick ES, Paterson KR, Dominiczak MH. Serum fructosamine/HbA1 ratio predicts the future changes in haemoglobin A1 in type II (non-insulin dependent) diabetic patients. *Clinica Chimica Acta* 1991; **199:** 43-51

Kilpatrick ES, Rumley AG, Small M, Dominiczak MH. Increased fetal haemoglobin in insulin-treated diabetes mellitus contributes to the imprecision of glycohaemoglobin measurements. *Clinical Chemistry* 1993; **39:** 833-835

Kilpatrick ES, Rumley AG, Myint H, Dominiczak MH, Small M. The effect of variations in haematocrit, mean cell volume and red blood cell count on reagent strip tests for glucose. *Annals of Clinical Biochemistry* 1993; **30:** 485-487

Rumley AG, Kilpatrick ES, Dominiczak MH, Small M. Evaluation of glycaemic control limits using the Ames DCA 2000 HbA_{1c} analyser. *Diabetic Medicine* 1993; **10:** 976-979

Kilpatrick ES, MacLeod MJ, Rumley AG, Small M. A ward comparison between the One Touch II and Glucometer II blood glucose meters. *Diabetic Medicine* 1994; **11:** 214-217

Kilpatrick ES, Rumley AG, Dominiczak MH, Small M. Glycated haemoglobin values: problems in the assessment of blood glucose control in diabetes mellitus. *British Medical Journal* 1994; **309:** 983-986

Kilpatrick ES, Rumley AG, Smith EA. Variations in sample pH and pO_2 affect Exactech meter glucose measurements. *Diabetic Medicine* 1994; **11:** 506-509

Smith EA, Kilpatrick ES. Intraoperative blood glucose measurements. The effect of haematocrit on glucose test strips. *Anaesthesia* 1994; **49:** 129-132

Kilpatrick ES, Rumley AG, Rumley CN. The effect of haemolysis on blood glucose meter measurements. *Diabetic Medicine* 1995; **12:** 341-343

Kilpatrick ES, Dominiczak MH, Small M. The effects of ageing on glycation and the interpretation of glycaemic control in Type 2 diabetes. *Quarterly Journal of Medicine* 1996; **89:** 307-312

Summary

This thesis critically evaluates two developments which have both brought about major advances in the assessment of glycaemic control in diabetic patients. First is the measurement of glycated proteins in the form of glycated haemoglobin and serum fructosamine and the second is the use of portable test strips and meters for the self-monitoring of blood glucose. Both methods of assessment have gained widespread acceptance but there remains a number of clinical and methodological problems associated with their use. This thesis reviews the literature relating to the use of these tests and describes a number of studies demonstrating new benefits and difficulties which exist.

The majority of clinicians now use glycated haemoglobin measurement as their principal objective indicator of glycaemic control in diabetic patients. We have performed several detailed studies elucidating clinically relevant aspects of glycated haemoglobin measurement which can affect the assessment of glycaemia in these patients.

The setting of target values for glycated haemoglobin measurement is severely hindered by the lack of standardisation in methodology. In an attempt to account for this, European guidelines define categories of glycaemic control as an HbA_1 or HbA_{1c} concentration so many standard deviations from a particular method's non-diabetic population mean. Using these guidelines, we found that HbA_1 could classify the same diabetic patients differently to HbA_{1c}, even when using the same instrument and reference range individuals. This new finding led, in part, to a change in the European guidelines for glycated haemoglobin targets.

Many commonly used glycated haemoglobin methods include both glycated and non-glycated fetal haemoglobin (HbF) in their result. This means patients with elevated HbF concentrations (≥0.5%) can have spuriously high glycated haemoglobin values. We presented the first evidence that insulin

treated adult patients had a significantly greater prevalence of elevated HbF concentrations than either non-insulin treated or non-diabetic controls, thereby compounding the problem in this group of patients. In addition, we found that HbF increased the apparent imprecision of these assays.

Discrepancies exist when comparing glycated haemoglobin and serum fructosamine as indicators of glycaemic control. We found that in newly diagnosed Type II diabetes, fructosamine only showed a correlation with HbA_1 after glucose control had stabilised and not during the period of changing glycaemia. However, because changes in fructosamine preceded those of HbA_1, the ratio of fructosamine/ HbA_1 was able to predict the change in HbA_1 in the forthcoming month. Thus, parallel measurement of fructosamine with HbA_1 provided additional information on the future trend of a HbA_1 concentration.

A further reason for disparity between fructosamine and glycated haemoglobin was established when investigating the effects of ageing on glycation in non-diabetic subjects. Mean HbA_{1c} values rose with increasing subject age while fructosamine and fasting plasma glucose values did not. Consequently, when Type II diabetic patient samples were classified according to European HbA_{1c} guidelines, significantly fewer patients were in good control and more in poor control when a young reference population was used compared to an age matched one. Thus, age related reference intervals may be required for glycated haemoglobin measurement.

Using test strips and meters to measure blood glucose has become widespread amongst diabetic patients in the community and in the monitoring of acutely ill hospital patients. We have documented studies which describe the effect on glucose measurement of variations in several physiological parameters. We also describe how new technology is improving glucose meter measurement.

In vitro variations of sample haematocrit have been shown to be a source of error in several glucose meter systems. Red cell count and mean cell volume are intimately related to haematocrit, so it makes it impossible to distinguish if the test strip error is due to haematocrit variations *per se* or to changes in the number of red cells. We found that in groups of individuals with different mean cell volumes, meter error was still related to the haematocrit of the sample rather than its red cell count.

Having established this, 10 patients undergoing cardiopulmonary bypass (whose haematocrits routinely fall to 20% intraoperatively) were investigated to determine the effect of *in vivo* variations in haematocrit. Changes in test strip glucose accuracy were found to be consistent with those shown previously *in vitro*.

While the use of whole blood to measure glucose is convenient, it is not possible, unlike plasma or serum, to tell if a sample is in any way haemolysed. We showed that extreme sample haemolysis can affect several glucose meter systems, while even modest degrees of red cell lysis were found to give clinically inaccurate results when using the Accutrend instrument.

Most glucose meter systems use the enzyme glucose oxidase as their basis of measurement. Like all enzymes, it may be affected by changes in ambient pH and substrate concentrations. We demonstrated that variations in sample pH and pO_2 can cause clinically important errors, especially when using biosensors such as the ExacTech glucose meter. Therefore, use of such a meter in acidotic or hypoxic patients may be inappropriate.

New blood glucose meter systems offer many potential advantages over previous systems. One such meter, the One Touch II, showed impressive performance when used in a laboratory environment. In a six week ward evaluation, the One Touch II demonstrated better meter accuracy, fewer blood glucoses too high to be measured and a greater user preference compared to the existing Glucometer II system. When used by out-patients, average values in

the glucose meter's memory were found to relate more closely to HbA_{1c} values than random clinic glucose measurements.

The ability to accurately assess present and prior glucose control by the means described has undoubtedly benefited the treatment of patients with diabetes mellitus. This thesis has demonstrated that although technological advances have led to improvements, interpretation of these indices cannot yet be used as a sole substitute for clinical judgement.

Chapter 1

Glycaemic Control Assessment: A Historical Background and Review of Current Methodologies.

1.1 Introduction

The non-enzymatic and irreversible binding of glucose to the amino acids of proteins has long been known to food chemists as the 'browning reaction'.[1] While this glycation process may alter the structure and function of proteins and thus be relevant to the small vessel complications of patients with diabetes mellitus,[2,3] glycation of haemoglobin and serum proteins (in the form of fructosamine) has become an important means of objectively assessing glycaemic control in diabetic patients.

The measurement of blood glucose itself remains central to the diagnosis of diabetes mellitus as well as being an important adjunct to glycated proteins in the monitoring of patients. Recent technological advances involving glucose test strips and meters has moved much of the monitoring of blood glucose from the laboratory into the hands of diabetic patients and health care staff.

The scope of this thesis relates to the clinical uses and difficulties which exist in using glycated haemoglobin, serum fructosamine and glucose test strips in the assessment of metabolic control in diabetes. Although other indicators of glycaemia (such as glycated albumin[4] and 1,5-anhydroglucitol[5]) have been proposed only these three are routinely used in clinical practice.

This chapter describes the historical development and uses of these tests, their underlying mechanisms of formation, and the current methods used for their measurement.

1.2 Historical Background

1.2.1 Glycated Haemoglobin

In 1962, Huisman and Dozy reported an increase in some of the minor fractions of haemoglobin in four diabetic patients who had been treated with tolbutamide.[6] Using column chromatography, they called these fractions 'fast' haemoglobins since they eluted before the main component, HbA_0. Their

increase was attributed to a reaction of tolbutamide to haemoglobin. However, attempts to reproduce this phenomenon *in vitro* proved unsuccessful.[6]

In 1967, an abnormal negatively charged haemoglobin band was observed by cellulose acetate electrophoresis in two patients being screened for abnormal haemoglobins at Tehran University hospitals. Both patients were suffering from diabetes mellitus. Further investigation by Rahbar found another 47 cases of the abnormal band, all occurring in patients with poorly controlled diabetes. The finding of a 'diabetic haemoglobin component' was thus reported in 1968.[7]

Soon it was demonstrated that the diabetic component had a chromatographic characteristic similar to that of HbA_{1c}, a minor Hb component described by Schnek and Schroeder in 1961 and found in non-diabetic adults in a proportion of 1-4%.[8] Structural studies later established that the diabetic haemoglobin was indeed identical with HbA_{1c}.[9]

Nearly a decade passed following Rahbar's discovery before a flurry of clinical studies were published which showed that the increased proportions of HbA_{1c} in diabetic patients could be used as a reliable index of glycaemic control over the preceding 6-8 weeks. The studies demonstrated correlation of glycated haemoglobin with other known indicators of diabetic control such as 24-hour urinary glucose excretions,[10] plasma 'glucose brackets',[11] daily mean plasma glucose[12] and the area under the curve of the glucose tolerance test.[13] Following these studies, and despite considerable methodological difficulties in measurement (see Chapter 2.2), acceptance of the use of glycated haemoglobin by diabetologists and other health care workers was rapid. The main reason for its attraction to clinicians was clear: for the first time they had an apparently simple tool which could give a completely objective assessment of a patient's glucose control.[14] Thus treatment regimes could now be changed without relying solely on a patient's description of symptoms or home urine/blood test results.

As well as its use in monitoring diabetic patients, it was hoped that measurement of glycated haemoglobin could also be used as an alternative to the laborious and inconvenient glucose tolerance test (GTT) in diagnosis. First studies to assess this were hindered by the non-uniformity of GTT criteria, resulting in discrepant results depending on which glucose cut-offs were used.[15,16] Even following the widespread recognition of WHO criteria for the GTT, and the undertaking of numerous studies, there continues to be some debate as to the usefulness of glycated haemoglobin in diagnosis and screening. A recent review of 25 studies confirmed that in a subject with a mildly elevated glycated haemoglobin level the test was not specific or sensitive enough to reliably distinguish between normality, impaired glucose tolerance or diabetes.[17] However, it is possible this is more a reflection on the poor reproducibility of the GTT[18] than any shortcoming of the glycated haemoglobin assay. In addition, some evidence suggests that the test may be more dependable when measured in younger (30-59yrs) rather than elderly subjects.[19]

Soon after the first clinical studies it was realised that glycated haemoglobin measurement could prove to be useful in answering one of the fundamental questions in diabetes, namely, are long-term microvascular complications of the disease related to the degree of control of hyperglycaemia?[20] Although early studies had suggested such a link,[21,22] the technical advances of glycated haemoglobin, capillary blood glucose measurement and intensified insulin regimes now made it possible to conduct more meaningful trials.[23] Indeed, once available, glycated haemoglobin measurement became the cornerstone of treatment evaluation in all such studies.[24] In particular, the largest and most comprehensive study, the Diabetes Control and Complications Trial (DCCT),[25] showed conclusively that glycaemic control as assessed by glycated haemoglobin can predict the risk of developing microvascular diabetic complications in Type I patients. As a

consequence, the usefulness of the assay was vindicated and its increased use recommended. [23,24,26,27] Moreover, it was suggested that the cost of using the test in routine clinical practice was likely to be offset by much larger savings due to the decreased need for procedures such as laser photocoagulation therapy, renal dialysis and renal transplantation.[23] The continued use of glycated haemoglobin measurement in diabetes assessment would thus seem assured.

1.2.2 Serum Fructosamine

By contrast with glycated haemoglobin, serum fructosamine measurement was established as a useful indicator of glycaemic control (over the previous 1-3 weeks) after a relatively short gestation. New Zealanders, Johnson, Metcalf and Baker were the first investigators to propose the use of a simple colorimetric assay for the assessment 'serum glycosylprotein' concentrations in 1982.[28] They based their assay on the ability of serum protein ketoamine linkages to glucose being able to act as reducing agents in alkaline solution. These protein ketoamines were generically termed 'fructosamines', not because of the involvement of fructose but because the resulting compound had structural similarities to this sugar.[29] Since all serum proteins can form ketoamines through glycation, fructosamine was found to give a similar indication of glycaemic control to that found when measuring total glycated protein and glycated albumin.[30-33] However, in contrast to assaying the latter, fructosamine could be measured by spectrophotometer instruments found in most clinical biochemistry laboratories. Therefore, the assay initially gave the promise of being a less expensive and quicker alternative to the majority of glycated haemoglobin analyses methods used at the time.[34,35] Indeed, early cross sectional studies seemed to show good correlations between fructosamine and glycated haemoglobin, [34-38] but subsequent studies have since shown that marked discrepancies can exist between the two measures,[39-43] not least

because they measure glycaemia over different time-scales. Nevertheless, the shorter time period measured by fructosamine has especially leant itself to use in pregnant diabetic patients[44] where frequent objective monitoring is essential for the health of both mother and fetus.[45,46]

Fructosamine, like glycated haemoglobin, has also been evaluated as a tool for the diagnosis of diabetes. After initial optimism[47,48] studies have since found it to lack the sensitivity and specificity required of a screening test.[49-53] Moreover, as a substitute for the GTT, it seems to be inferior to measuring glycated haemoglobin.[52,53]

1.2.3 Monitoring of Blood Glucose

Until the early 1980's, self monitoring of glycaemic control for most diabetic patients was limited to the measurement of glucose concentrations in urine samples. Many patients found this method to be insensitive, particularly due to the problems associated with the renal threshold for glucose. Indeed, at the time, adjustment of insulin dosages according to urine tests was likened to 'driving a car on roads with a 30mph limit with a speedometer that only begins to work at greater than 70mph'.[54] The measurement by patients of blood rather than urine glucose was perceived as a means of surmounting these problems.

Although for many years the accurate and precise measurement of blood glucose had been achievable in the laboratory setting,[55] the technology for portable instrumentation did not become available until the early 1970's. The first advance was in the development of 'dry' chemistry techniques which obviated the need for liquid reagents in glucose measurement. With this approach, the blood sample itself was the solvent in which the chemical reaction took place. The earliest demonstration of such technology for glucose measurement occurred over 30 years ago using glucose oxidase 'Dextrostix' strips from Ames which were similar in principle to those still in use today.[56,57] By the early 1970's, both Ames (now Bayer) and Boehringer Mannheim (BM)

had developed disposable test strip systems using this technique. Before reading, removal of blood from the strip was required either by blotting (BM) or with water (Ames). In these first strips visual assessment of the colour change (and therefore the blood glucose measurement) was at best semiquantitative, allowing only an estimate of low, medium or high values. Reflectance meters were then introduced by both companies which gave the promise of more accurate and consistent measurement.[58] However, these were bulky, expensive and not easy to use. For example, the Ames 'Eyetone' meter cost around £200, weighed 1.7kg, measured 18x 11x 5cm, required mains operation, a 30 minute warm-up period, calibration before each sample and employed a needle scale for reading.[59]

Despite these disadvantages, a number of papers in 1978 demonstrated the potential usefulness of the devices when used at home by diabetic patients.[54,60,61] It was hoped that the ability to measure near physiological blood glucose concentrations would allow more diabetic patients to achieve this degree of control.[54] It also meant that glucose concentrations could be assessed accurately without recourse to frequent hospital admission, especially in pregnant diabetic patients.[60,62]

Nevertheless, it was recognised that the potential market for meters would only be realised if they became less expensive and genuinely portable. In 1978, the Glucoscan (Lifescan Inc) was introduced which was relatively small (10x 6.4x 1.3cm), light (0.2kg), used rechargeable batteries, had a light emitting diode readout, factory calibration and could use Ames test strips.[59] Other companies followed suit and there then followed a period of steady improvement in the products of all manufacturers in terms of ease of use and cost. However, a significant advance occurred in 1987 with the introduction of 'non-wipe' systems by Lifescan Inc and Medisense Ltd which allowed glucose measurement without the need for blotting (or the now obsolete washing) of sample from the test strip. One of these meters, the ExacTech from Medisense,

was particularly elegant. It used a pen sized meter which, instead of detecting a colour change in a reagent strip, employed a disposable biosensor for the electrochemical detection of blood glucose.[63] Most new glucose meters are now 'non-wipe' and several also include features such as automatic timing of the test, time and date stamping of results, and the recording of these results in the meter memory.

The next major advance in blood glucose monitoring is likely to be in the form of 'non-jab' measurement using near infra-red techniques[64] but as yet no commercial system has been developed.

1.3 Glycated Haemoglobin and Fructosamines: Structure and Formation

1.3.1 Glycated Haemoglobin

Human haemoglobin demonstrates marked heterogeneity, mainly as a consequence of post-translational changes due to the non-enzymatic binding of various carbohydrates in a process known as glycation. Glycation occurs via a carbohydrate such as glucose reacting in its free aldehyde form with a haemoglobin molecule to form the Schiff-base compound, aldimine (Figure 1.1).[65] The formed aldimine can then meet one of two further fates: it can either dissociate back to its component carbohydrate and haemoglobin or, in a process that is 60-fold less rapid, it can undergo an intermolecular transformation known as the Amadori rearrangement to form a stable glycated ketoamine product (Figure 1.1).

Haemoglobin glycation occurs at several sites, namely, the amino terminal of both its α and β-chains, as well as at certain ε-amino groups.[66] However, it is only the modification at the N-terminal valine amino acid of the β-chain which imparts enough negative charge to the haemoglobin molecule to allow separation of the respective haemoglobins by charge-dependent techniques. In contrast, glycation at sites other than the β-chain amino terminus results in a compound with a charge not dissimilar to non-glycated haemoglobin and so is indistinguishable by these methods. However, this portion, which accounts for about half of all haemoglobin glycation, can be detected if glycation-specific 'total glycated haemoglobin' methods such as affinity chromatography are used (see Chapter 1.4.1).

The charge-separated haemoglobins of normal adult HbA_0 are jointly known as haemoglobin A_1 (HbA_1). Improved techniques have allowed further separation of HbA_1 into its constituent parts, HbA_{1a1}, HbA_{1a2}, HbA_{1b}, and HbA_{1c}. Glucose is the carbohydrate in the major fraction, HbA_{1c}, whilst other

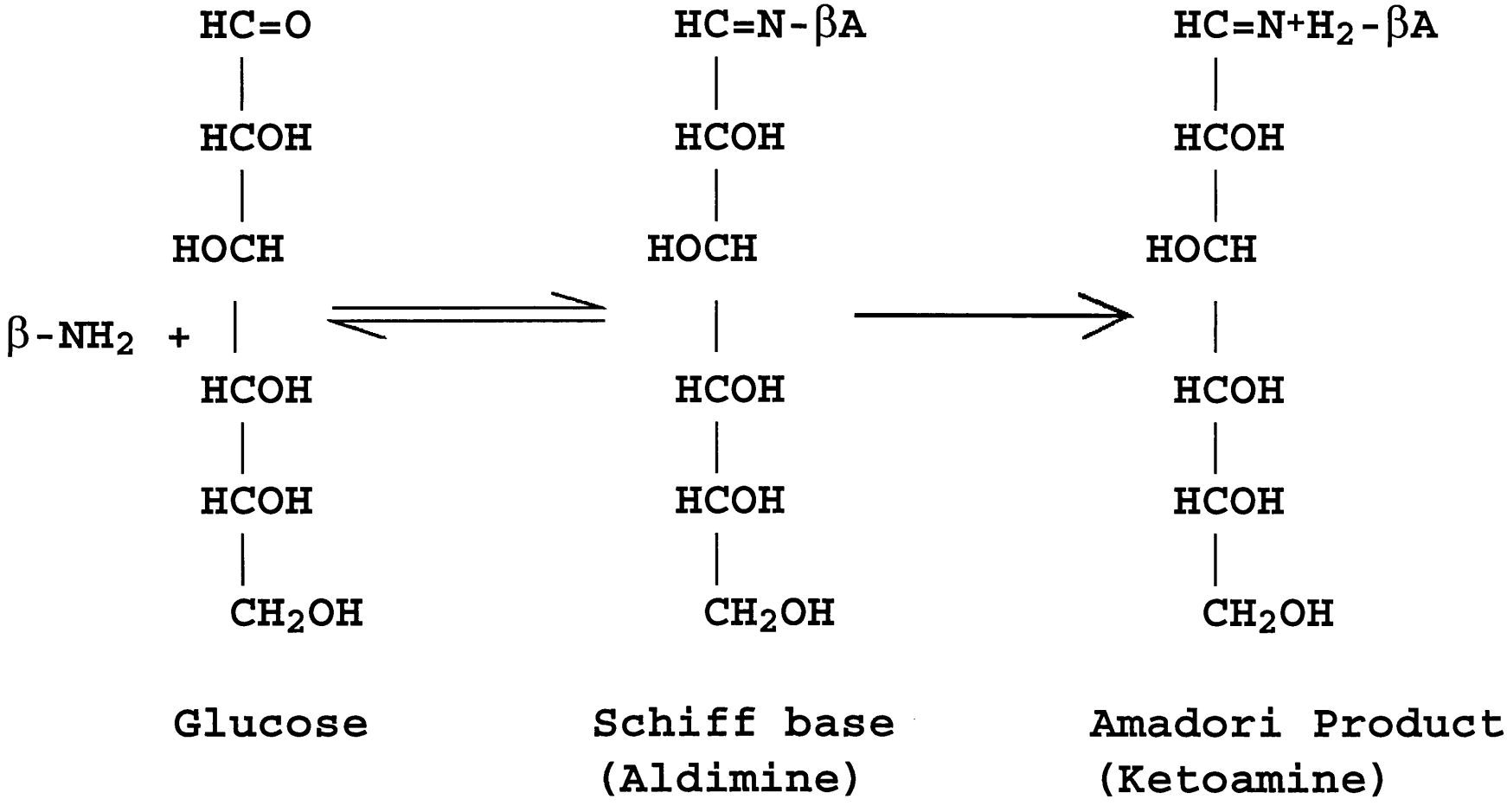


Figure 1.1: The formation of glycated haemoglobin from the reaction of glucose with the N-terminal valine of the haemoglobin β-chain.

carbohydrates, some of which have still to be established with certainty, constitute the other fractions (Table 1.1).[67]

Glycation of haemoglobin occurs continuously throughout the 120 day lifetime of the red cell,[68] so the eldest cells will be most glycated and the youngest least.[69] However, all ages of cells will have been exposed to recent levels of glycaemia while only the eldest cells will also have been exposed to glucose levels from 4 months previously. Therefore, the more recent the period of glycaemia, the larger its influence will be on the glycated haemoglobin value. Consequently, it has been suggested that half of a HbA_{1c} value is attributable to changes in glycaemia over the preceding month, a further quarter is due to the month prior to that, with the remaining quarter a reflection of months 3 and 4.[70]

1.3.2 Fructosamines

As mentioned in Chapter 1.2.2, serum fructosamine measurement is a measure of total glycated serum proteins. These glycated proteins are formed via a Schiff base and Amadori rearrangement in the same way as glycated haemoglobin. The fructosamine assay measures glycation at all sites on a protein molecule and so is not dependant on changes in molecular charge caused by glycation.

The major serum protein, albumin, has a half life substantially shorter than haemoglobin at around 20 days. Serum fructosamine therefore appears to reflect time-integrated glycaemia over a period of only 1-3 weeks.[71]

Haemoglobin		Modification	Abundance (%)
	A_o		95
HbA_1	HbA_{1a1}	fructose-1,6-diphosphate	0.2
	HbA_{1a2}	glucose-6-phosphate	0.2
	HbA_{1b}	carbohydrate (?)	0.5
	HbA_{1c}	glucose	4

Table 1.1: Table showing carbohydrates involved in the formation of charge-separated haemoglobins together with their approximate abundance in non-diabetic individuals.

1.4 Glycaemic Control Assessment Methodologies

1.4.1 Glycated Haemoglobin Methods

Methods in routine use for the measurement of glycated haemoglobin separate the molecule on the basis of either its charge, structure or antigenic properties. Table 1.2 gives the methods and instruments that are in routine use as described by the United Kingdom National External Quality Assessment Scheme (UK NEQAS).

The most popular means of measurement rely on the increased negative charge found in the glycated haemoglobin molecule to distinguish it from its non-glycated form. These assays include electrophoresis and ion-exchange chromatography.[72] The latter method is becoming increasingly popular with the development of rapid (as short as 4 minutes between samples) dedicated high performance liquid chromatography (HPLC) instruments which are much less labour intensive than electrophoresis. These new analysers have also made feasible the measurement of glycated haemoglobin 'on-site' at diabetic out-patient clinics.[73] New high-resolution HPLC instruments are now emerging which appear to show the relative lack of specificity that currently used HPLC 'reference' systems have for measuring HbA_{1c}.[74]

Boronate affinity chromatography separates glycated haemoglobin on the basis of its structure rather than charge. In this assay, separation occurs by the carbohydrate moieties present on glycated haemoglobin binding by condensation to the affinity reagent, di-hydroxyboronate.[75] This method is specific for all glycated haemoglobins irrespective of molecular charge or the site of glycation on the haemoglobin molecule. In addition, it is also able to detect the glycated portion of haemoglobin in patients with haemoglobin variants such as HbS, HbC or HbF. Thus, the term 'total glycated haemoglobin' has been used in describing this type of assay. Nevertheless, despite some reservations,[74] these glycated haemoglobin values are usually

HbA_1

Agar Electrophoresis
- Corning
- Helena Rep

Ion Exchange Chromatography
- Corning Glycomat
- Biomen HA 8121

HbA_{1c}

Affinity Chromatography
- Abbott Vision
- Abbott IMX

Affinity Electrophoresis
- Beckman Diatrac

Ion Exchange Chromatography
- Biomen HA 8121
- Corning Glycomat
- Shimadzu LC 6A

Immunoassay
- Dako Novoclone

Table 1.2: Table showing glycated haemoglobin methods routinely in laboratory use as measured by involvement in the United Kingdom National External Quality Assessment Scheme.

expressed as 'HbA_{1c} equivalents' by comparison with those obtained using an HPLC instrument. Like HPLC, some affinity chromatography methods have now been automated with 'on-site' use of instruments possible.[76]

More recently, immunoassays have been developed for the measurement of HbA_{1c}. The Dako Novoclone is an enzyme immunoassay performed on microtitre plates employing a monoclonal antibody raised against a haemoglobin molecule with glucose attached to the N-terminal valine.[77] Such a system is only suited to batch analysis in a laboratory but a portable instrument, the DCA 2000 analyser from Bayer Diagnostics, has been used successfully in our diabetic clinic.[78] It's assay is based on latex immuno-agglutination inhibition methodology and uses a disposable cartridge to measure each sample individually. In a situation similar to the affinity chromatography assays, both these immunoassay methods are calibrated with material to which HbA_{1c} values have been assigned using HPLC.

1.4.2 Fructosamine Methods

The fructosamine assay relies on the ability of serum Amadori compounds to directly reduce nitroblue tetrazolium (NBT) to the tetrazinolyl radical NBT^+, which disassociates to yield a highly coloured formazan dye in an alkaline environment.[79] The reaction rate is followed by measuring the increase in dye absorbance between 10 and 15 minutes incubation at 530nm wavelength. While NBT is reduced, the oxidation degeneration product of the Amadori compound would appear to be D-glucosone.[80]

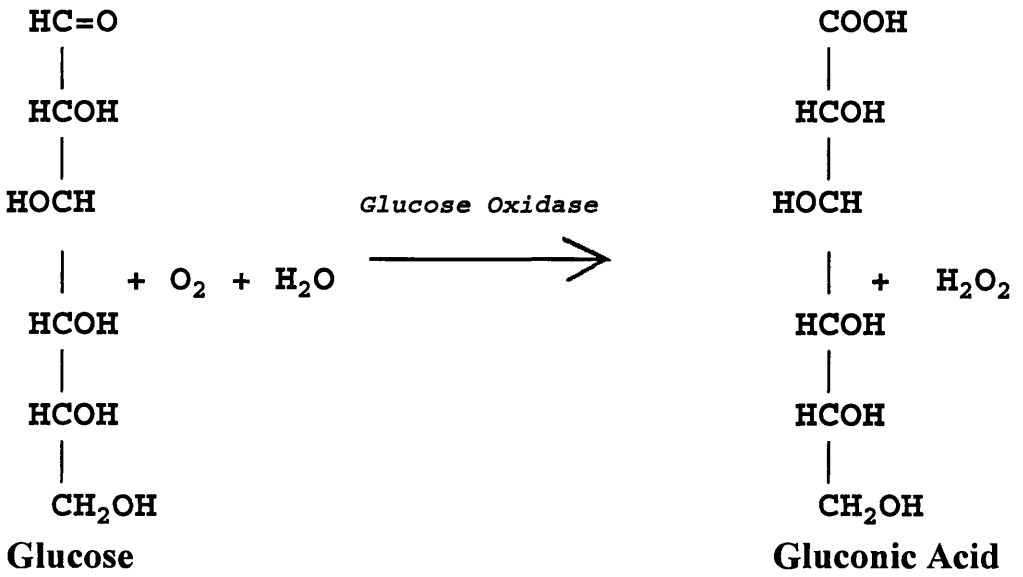
Since originally introduced, the commercial assays from Boehringer Mannheim and Roche underwent modifications in 1991 by including a detergent and uricase in the reagent, and by increasing the concentrations of both the carbonate buffer (from 0.1M to 0.2M) and the dye (from 0.25mmol/L to 0.48mmol/L).[81] This has helped reduce the effect of lipaemic and hyperuricaemic samples as well as diminishing protein matrix effects (see

Chapter 2.3.3).[82] At the same time, the detection wavelength of absorption changed from 530 to 550nm. Also, glycated polylysine replaced deoxymorpholinofructose (DMF) as the assay calibrant (see Chapter 2.3.1).[83] To accompany these wholesale changes, the units for fructosamine measurement changed from mmol/L to μmol/L with the results from the two assays not being directly comparable.[84]

1.4.3 Glucose Test Strip Methods

Nearly all glucose test strips employ the enzyme glucose oxidase as their basis of measurement. The strips are impregnated with glucose oxidase, peroxidase and a chromogenic dye. Glucose in the blood sample reacts with the enzyme system to bring about a colour change in the chromogen which is proportional to the concentration of glucose in the original sample (Figure 1.2a). The colour change can either be read visually and compared to a colour chart, or read with the aid of a reflectance meter and a digital readout. The glucose oxidase enzyme system has also been adapted for measuring blood glucose electro-chemically.[63] This method uses ferrocene as the electron mediator of the enzyme rather than molecular oxygen thus allowing the rate of reaction to be followed by a change in electrical current (i.e. amperometrically) rather than by a change in colour. More recently, Bayer has broken with tradition and introduced the Glucometer 4 meter which uses the enzyme hexokinase rather than glucose oxidase (Figure 1.2b). Hexokinase is already used by laboratories as a reference method for the measurement of plasma glucose. This enzyme also has the advantage of being less susceptible to compounds which can interfere with glucose oxidase measurement (see Chapter 2.4.3).

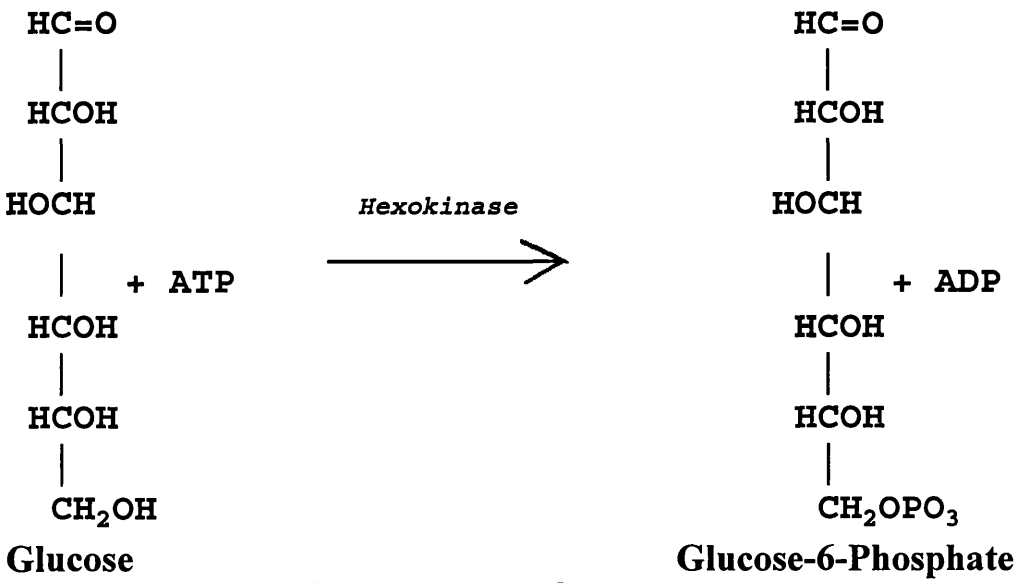
(a) Glucose Oxidase



Chromogen + H_2O_2 → Oxidised chromogen + H_2O

(Colourless) (Coloured)

(b) Hexokinase



Glucose-6-P Dehydrogenase

Glucose-6-P + NAD^+ → 6-Phosphogluconate + NADH + H^+

Diaphorase

NADH + Chromogen → NAD^+ + Oxidised chromogen

(Colourless) (Coloured)

Fig 1.2 (a) Test strip glucose oxidase enzyme system.
(b) Test strip hexokinase enzyme system.

Chapter 2

A Review of Factors Affecting the Interpretation of Glycaemic Control.

2.1 Introduction

The ability to accurately assess present and prior glucose control has undoubtedly benefited the treatment of patients with diabetes mellitus. Clinicians can now set goals of glycaemic control for their patients by using glycated haemoglobin or fructosamine values while patients can empower themselves to meet these aims through the self-monitoring of blood glucose. However, errors in any of these measurements may either lead to the setting of unattainable goals or conversely the acceptance of spuriously good glucose control.

As these measurements of glucose control become further established it is easy to assume that any faults which may have existed must now have been removed. This chapter reviews the problems which can confound the assessment of glycaemia when using these tests.

2.2 Factors Influencing Glycated Haemoglobin Measurement

2.2.1 Lack of standardisation

A wide range of instruments and methods are available for glycated haemoglobin measurement (see Chapter 1.4.1). It is perhaps not surprising that instruments which measure different species (such as either HbA_1, HbA_{1c} or total glycated haemoglobin) tend to produce results which are not directly comparable. However, due to a lack of standardisation, even methods which purport to measure the same analyte can have widely differing reference intervals and give varying results for patient samples. Indeed, quality control returns to UK NEQAS showed poor between-laboratory agreement for glycated haemoglobin with an overall coefficient of variation of 20%.[85] As such, this lack of standardisation is regarded as one of the major drawbacks

that limits the clinical utility of glycated haemoglobin measurements[86] and both the British Diabetic Association[87] and the National Diabetes Data Group[88] see solving the problem as a major priority.

A number of different approaches have been used to address this issue. European guidelines have suggested that glycaemic control be classified according to how many standard deviations (SD's) a patient's HbA_1 or HbA_{1c} lies from the non-diabetic mean value for the particular assay.[89] Within 3SD of the non-diabetic mean is defined as good control, between 3 and 5SD is borderline and outwith 5SD is poor. Other groups have proposed alternatives such as comparing local laboratory values with those obtained when the same samples are measured by the DCCT central HbA_{1c} laboratory.[90] Another approach, used with a degree of success in the Netherlands, has been to use lyophilised calibrators to calibrate local instruments to produce similar values.[91,92] However, all these suggestions have not been without problems. For example, lyophilised specimens were found to be incompatible with total glycated haemoglobin assays and delay in the transport of samples between laboratories produced variations in results. In addition, all the proposed schemes, with the exception of the European guidelines, have the disadvantage of being time-consuming and expensive to maintain.

Full standardisation of glycated haemoglobin measurements is not expected until at least the end of the decade.[86] In the meantime, proposals such as the European guidelines are likely to continue to be the only means of comparing results obtained from different laboratories using differing methods.

2.2.2 Haemoglobinopathies

Normal adult haemoglobin is assembled from α-, β-, γ- and δ chains to form HbA_0 ($\alpha_2\beta_2$), fetal haemoglobin, HbF ($\alpha_2\gamma_2$) and HbA_2 ($\alpha_2\delta_2$).[68] As described above in Chapter 1.3.1, glycation of normal adult haemoglobin (HbA_0) leads to the formation of HbA_1 and HbA_{1c}. However, point mutations in the primary

structure of the β-chain can give rise to structural variants of haemoglobin, such as those found in HbS ($\beta^{6\ glu \rightarrow val}$), HbC ($\beta^{6\ glu \rightarrow lys}$) and HbE ($\beta^{26\ glu \rightarrow lys}$). Also, impaired synthesis of β-chains lead to conditions such as β-thalassaemia and hereditary persistent HbF. Patients with any of these haemoglobinopathies are likely to form glycated products such as HbS_{1c}, HbC_{1c} or HbE_{1c} either in addition to or instead of HbA_{1c}.[93]

The influence of abnormal haemoglobins on glycated haemoglobin measurement is variable depending on the method of analysis used.[94] In general, specific measurements of HbA_1 or HbA_{1c} in homozygous haemoglobinopathies will fail to detect any glycated haemoglobin and will necessitate measurements of derivatives such as HbS_{1c} or HbC_{1c}. In heterozygous haemoglobinopathies (e.g. HbAS, HbAC) or haemoglobin synthesis variants (e.g. β-thalassaemia), the same specific assays are likely to underestimate the degree of haemoglobin glycation by an amount dependent on the ratio of normal:abnormal haemoglobin. Examples of these specific methods include the electrophoretic, HPLC and the previously mentioned immunoassay methods. In some of these systems the problem is easily identified because the abnormal haemoglobins separate to form additional peaks, but in other HPLC and immunoassay techniques these abnormal haemoglobins may be indistinguishable from HbA_0.[95] The only assays able to give meaningful measurements in these conditions are those based on affinity chromatography because of their ability to measure glycation at any site of any haemoglobin molecule. However, even measurement of this 'total glycated haemoglobin' appears to be problematic since it would seem haemoglobins S and C glycate faster than native HbA_0, thus leading to overestimation of the glycated haemoglobin value in the heterozygous (e.g. HbAC) state.[96]

2.2.3 Fetal Haemoglobin

Fetal haemoglobin (HbF) has relevance to the measurement of glycated haemoglobin beyond that of abnormal haemoglobins. It is present physiologically as 75-100% of total haemoglobin in newborn children then declines during childhood to less than 1% in most adults.[97] However, for reasons unknown, this decline can be variable and incomplete, even in patients known not to have hereditary persistent HbF. The finding would be of no consequence was it not for the fact that HbF, whether glycated or not, co-migrates or co-elutes with the glycated haemoglobin peak of many commonly used electrophoretic and HPLC methods.[98,99] Thus, any detectable percentage of HbF in a patient sample is likely to be included in the HbA_1 or HbA_{1c} result of these methods leading to falsely elevated results.[98,100,101] Moreover, in paediatric diabetic patients, increased concentrations of HbF have been found compared to non-diabetic controls, thereby exacerbating the problem in these patients.[102]

2.2.4 Haemoglobin Derivatives

Haemoglobin derivatives arise from post-translational modification of haemoglobin. Examples are those resulting from reactions with glucose (to form glycated haemoglobin), urea-derived isocyanate (to form carbamylated haemoglobin) and acetylsalicylic acid (to form acetylated haemoglobin).[103] As is the case for abnormal haemoglobins, glycated haemoglobin methods are affected differently by the latter two derivatives. Both electrophoretic and HPLC methods show increases in glycated haemoglobin values with increasing degrees of uraemia as a consequence of rising concentrations of carbamylated haemoglobin.[104-106] For approximately every 15mmol/L of urea, glycated haemoglobin measurements increase by 1%.[106] In contrast, affinity chromatography and enzyme immunoassay methods seem free from interference. [106,107] Acetylated haemoglobin affects the same methods as the

carbamylated form, although in patients on chronic low doses of aspirin (200-300mg/day), the effect would appear negligible.[106] However, in rheumatoid patients receiving 4g/day, rises in glycated haemoglobin of 1.5% have been found when using HPLC methods.[108]

2.2.5 Labile Haemoglobin

Many original electrophoretic and liquid chromatography methods for measuring glycated haemoglobin showed marked fluctuations in results with acute changes in blood glucose.[109,110] This was due to these methods being unable to distinguish between HbA_1/HbA_{1c} and the intermediate aldimine or Schiff base.[111] This latter compound, also known as the labile haemoglobin fraction or pre-HbA_{1c}, has for over a decade been removed chemically[112] and should now only be of historical interest.

2.2.6 Decreased Red Cell Survival

Processes which lessen mean red cell life to shorter than 120 days will reduce the availability of haemoglobin for glycation. As a consequence, lower glycated haemoglobin values have been recorded in patients with chronic renal failure[113], immune haemolytic anaemia[114] and homozygous haemoglobinopathies.[115] This phenomenon is independent of the method of analysis used and cannot be easily corrected, so often the only recourse is to use an alternative method of glycaemic control assessment such as the measurement of serum fructosamine.[115]

2.3 Factors Influencing Serum Fructosamine Measurement

2.3.1 Lack of Standardisation

Calibration of the fructosamine assay has proved to be a major problem because of the lack of a trustworthy standard or calibrator. The obvious candidate, glycated serum albumin, has proven to be extremely difficult to manufacture to a known and reproducible amount.[116] Even if feasible, storage in a matrix suitably similar to human serum would pose further difficulties. As an alternative, the original fructosamine assay used the less desirable deoxymorpholinofructose (DMF). Although similarities between the reaction of NBT with DMF and with human serum existed, the reaction kinetics and absorption spectra were markedly different. The situation has since improved with the adoption of synthetically created glycated polylysine as a calibrator in new fructosamine kits. Reaction of this compound with NBT produces an absorption spectrum more akin to human serum than that found with DMF. It is hoped that this improvement may now have resolved most fructosamine calibration problems.[29]

2.3.2 Serum Proteins

Debate continues as to whether fructosamine concentration should be corrected for serum protein concentration. Empirically, it would not seem surprising that a measurement of glycated proteins is apt to be influenced by serum protein concentrations. Opponents of this view argue that, theoretically, glucose concentration is the rate-limiting step in the glycation reaction because available protein lysine residues will always be vastly in excess of reactive open-chain (carbonyl) glucose molecules.[117] However, much evidence has accumulated to suggest this is unlikely to be the case. An *in vitro* study has actually found that the amount of fructosamine formed is in first-order relation

to albumin concentration and not glucose.[118] The authors of the study postulated that as carbonyl glucoses were removed by glycation, more glucose molecules isomerised to the open-chain form to maintain equilibrium.

Clinical evidence would also support the fact that, even with improvements in the assay, pathological changes in serum proteins can affect fructosamine.[119] For example, patients with cirrhosis of the liver, the nephrotic syndrome[120,121] and hyperthyroidism (associated with increased protein turn-over)[122] all demonstrate reduced values. Even normoalbuminaemic Type I diabetic patients have shown diurnal variation in fructosamine levels closely related with changes in their serum protein concentration.[123] However, there has been consensus agreement that in patients with a serum albumin concentration greater than 30g/L, the effect will be negligible.[119]

It is generally assumed that albumin makes the largest contribution to total glycated protein concentration, but it has also been suggested that certain proteins such as IgA may have a greater influence on fructosamine values than others such as albumin, presumably because they glycate at different rates. This disproportionate contribution of IgA, either in well subjects[124] or in patients with paraproteinaemias[125], may thus partly explain why the involvement of serum proteins in fructosamine measurement remains controversial.

2.3.3 Interfering Substances

The original fructosamine assay was subject to interference from reducing substances other than ketoamine linkages, especially uric acid and ascorbic acid. Common photometric interferents such as bilirubin, haemolysis and lipaemia also interfered. Bilirubin and lipaemia caused increased fructosamine values while haemolysed samples produced falsely low ones.[29] The introduction of new fructosamine kits not only changed the calibrators, but also addressed hyperuricaemic samples by including a uricase, and lipaemic samples by including surfactant detergents. Increasing the concentration of

NBT in the reaction mixture also reduced the error due to interfering reducing substances other than uric acid.[116] Thus the new assay is significantly more robust than previously.

2.3.4 Body Mass Index

An unusual but consistent finding has been observed whereby obese diabetic patients and non-diabetic individuals have been found to have lower fructosamine values than lean ones.[126-128] Reductions of up to 25% have been noted in subjects with a body mass index (BMI) >30 kg/m^2 despite having identical HbA_{1c} and fasting glucose values.[126] Serum from these patients has also been found to glycate serum proteins at a slower rate *in vitro* than serum from less obese individuals.[128] However, the precise mechanism for this phenomenon remains unknown.

2.4 Factors Influencing Blood Glucose Test Strip Measurement

2.4.1 Lack of Standardisation

Whilst the use of whole blood in a clinical setting has the advantage of being convenient, whole blood glucose measurement, unlike plasma glucose, lacks a defined standard against which other methods can be calibrated.[129] However, the Yellow Springs Instrument (YSI) glucose analyser has become a *de facto* standard used by most glucose meter manufacturers to calibrate their instruments. The choice of the YSI owes more to it being one of the first widely available whole blood glucose instruments than to any analytical superiority. Indeed, because the YSI measures blood glucose indirectly (i.e. after dilution of the sample), results are liable to be affected by deviations in plasma protein concentrations, high lipid levels and the haematocrit of the blood specimens.[129] Thus, the lack of an agreed standard, and the limitations of

the adopted YSI instrument means that whole blood glucose measurements cannot be as universally concordant as that of plasma.

2.4.2 User Errors

Although newer glucose meters have helped address specific problems, many errors in test strip glucose measurement are still attributable to the user. These include errors due to the size and placement of the blood sample, the timing of the test and wiping of blood from the test strip.[130] Any other conceivable mistake in measurement such as the use of glucose-contaminated hands or inappropriate storage of test strips is sure to become apparent when any group of diabetic patients or health care staff are allowed use of the analysis.

Visual reading of test strips adds another source of error which can considerably reduce the accuracy of measurements in general use.[130] Patients tend to cluster their results around the standard colours on the container, despite instructions to interpolate.[131] Colour blindness and impaired colour discrimination, either inherited or acquired as a result of diabetes,[132] can also lead to difficulty in strip interpretation.[133,134]

Another important source of user 'error' is the falsification by patients of results obtained by glucose self-monitoring. In the first study to highlight the problem, 19 diabetic patients were given meters which had (unknown to them) memory chips built in. It was found that in 30% of glucose record diaries the patient had fabricated results, in 8% results had been omitted and in 26% there was disagreement between the meter result and that written in the diary.[135] The authors concluded that about a third of patients could not be relied on to keep unambiguous monitoring diaries, a figure confirmed in subsequent studies.[136]

2.4.3 Analytical Errors

Analytical compromises are unavoidable when using a glucose meter instrument costing £30-50 and test strips priced at £0.25 each. Indeed, it is

perhaps surprising how accurate and precise these meters can be considering such cost restraints. Nevertheless, some of the compromises present can lead to clinically important errors in glucose measurement.

Variations in blood sample haematocrit have been shown to be a source of inaccuracy in many blood glucose systems.[137-140] In general, blood samples adjusted to have a low haematocrits can give spuriously high glucose results and *vice versa*. This effect can be well in excess of that found when using instruments such as the YSI analyser. For example, for every 10% change in sample haematocrit the glucose concentration measured by Bayer Glucostix can vary by approximately 20%.[137]

When the glucose oxidase enzyme is used for glucose measurement, reducing agents found in the blood sample can interfere by a competitive effect on the redox detection system.[141] Hence, exogenously given substances such as ascorbic acid, paracetamol and salicylate as well as large amounts of endogenous uric acid, urea and creatinine can lead to falsely low results with many systems.[141]

Meter measurement of glucose test strips has been replacing visual reading mainly because of its ease of use and potential for more accurate results. However, case reports have documented examples where extremely high glucose values have given considerably lower results on the Boehringer Mannheim Reflolux S meter while manual reading of the strip has confirmed the gross hyperglycaemia.[142,143] With more meters being introduced which do not have any form of visual backup, there may be more scope for such errors to pass unnoticed.

Lastly, because glucose meters measure glucose concentration indirectly, they may (like the YSI instrument) underestimate glucose concentrations in extremely hyperproteinaemic or hyperlipidaemic patients.[144]

Chapter 3

Thesis Methodologies.

3.1 Introduction

This chapter presents a description of the methods of glycaemic control assessment used in common by the studies in this thesis. Thus, the laboratory assessment of glycated haemoglobin, serum fructosamine and blood/plasma glucose are included. The other methods of analysis peculiar to each study have been included in their relevant chapters.

3.2 Blood sampling

3.2.1 Glycated Haemoglobin Samples

All analyses of glycated haemoglobin were collected in containers with potassium ethylenediaminetetraacetic acid (K_2EDTA) anticoagulant. All samples were stored at 4°C and analysed within 3 days of collection.

3.2.2 Serum Fructosamine Samples

Fructosamine samples were collected in containers without anticoagulant. Serum was separated after centrifugation at 2500xg for 7 minutes. Serum was stored at 4°C and analysed within 1 week of collection.

3.2.3 Plasma Glucose Samples

Plasma glucose samples were collected in blood tubes containing potassium oxalate and sodium fluoride as an anticoagulant and an inhibitor of glycolysis respectively. Plasma was separated as above and glucose analysis performed within four hours of sampling.

3.2.4 Whole Blood Glucose Samples

Whole blood glucose samples were collected into either heparinised glass capillary tubes or, in the case of the Analox instrument, into capillary tubes containing sodium fluoride/potassium oxalate and sodium nitrite. All samples were analysed within 15 minutes of collection.

3.3 Glycated Haemoglobin

3.3.1 HbA_1 by Electrophoresis

Electrophoretic measurement of HbA_1 used the Corning Glytrac system (Ciba Corning Diagnostics Ltd, Halstead, Essex, UK). The whole blood sample (50μL) is incubated at 37°C with 150μL of labile removing haemodialysing reagent. One microlitre of haemolysate is dispensed onto an agar film which undergoes electroendosmosis in a citrate buffer. The film is then dried and scanned by a Corning 720 densitometer at 420nm which displays the value of HbA_1 as a percentage of the total haemoglobin. Between-batch imprecision (coefficient of variation, CV) was 4.33% at a mean HbA_1 concentration of 8.22%.

3.3.2 HbA_1 by High Performance Liquid Chromatography

Measurement of HbA_1 by high performance liquid chromatography (HPLC) used a Hi-AutoA1c, model 8121 instrument (Kyoto Daiichi Kagakiu Co. Ltd., Japan, distributors Biomen Ltd, Finchampstead, Berkshire, UK). Three microlitres of whole blood is added to 450μL of haemolysing solution which contains a labile removing reagent (tetrapolyphosphate at 48°C for 2 minutes at pH6). An HPLC ion exchange chromatographic column with a separation time of 4 minutes is used. Elutions are detected by a dual wavelength photometer (415 and 500nm) and measurements of HbA_1, HbA_{1c}

and HbF obtained by the integration of their corresponding peaks. Each measurement is expressed as a percentage of the total haemoglobin. Between-batch CV was 4.1% at a mean HbA_1 concentration of 12.1%.

3.3.3 HbA_{1c} by High Performance Liquid Chromatography

Haemoglobin A_{1c} measurement by HPLC used the same instrument as that described in Chapter 3.3.2. Both HbA_1 and HbA_{1c} measurements are obtained by the one sample injection. Between-batch CV was 2.2% at a mean HbA_{1c} concentration of 9.2%.

3.3.4 HbA_{1c} by DCA 2000 Immunoassay

The Ames/Bayer DCA 2000 (Bayer Diagnostics, Basingstoke, UK) portable immunoassay instrument was also used for HbA_{1c} measurement. As described in Chapter 1.4.1, monoclonal antibody to HbA_{1c} is used in a latex-agglutination immunoassay.

In this assay, total haemoglobin is measured first by reaction with potassium ferricyanide and then thiocyanate to form thiocyan-methaemoglobin. The quantity of haemoglobin is proportional to the absorbance of this compound at 531nm wavelength. For the measurement of specific HbA_{1c}, an agglutinator (a synthetic polymer containing multiple copies of the immunoreactive portion of HbA_{1c}) causes agglutination of latex coated with HbA_{1c}-specific mouse monoclonal antibody. This agglutination reaction causes increased light scattering which is measured as an increase in absorbance at 531nm. Haemoglobin A_{1c} in the blood specimen competes for the limited number of antibody-latex binding sites causing an inhibition of agglutination, a decreased scattering of light, and therefore a reduced

absorbance at 531nm that can be quantified by the instrument using a pre-programmed calibration curve.

All the necessary reagents are contained in a single reagent cassette, and following the introduction of a 1μL whole blood sample into the cassette all the reagent mixing and the measurements are carried out by the instrument within 9 minutes. Again, HbA_{1c} is expressed as a percentage of total haemoglobin. Between-batch CV during the study period was 4.2% at a mean HbA_{1c} concentration of 5.4%

3.3.5 HbA_{1c} by Novoclone Immunoassay

The Dako Novoclone HbA_{1c} (previously Novo Nordisk Diagnostics Ltd, Cambridge, UK, now Dako Ltd, High Wycombe, UK) system employs more traditional immunoassay techniques. In this assay, 500 μL of red cells are first washed, lysed in water then oxidised with ferricyanide to remove the labile fraction. The haemolysate is diluted in buffer and then added to the wells of a microtitre plate and incubated for 30 minutes. The wells are emptied and then incubated with 100μL of biotinylated HbA_{1c} antibody for 60 minutes. A further 100μL of avidin-peroxidase conjugate is added followed by another 60 minute room temperature incubation. Following washing of the wells, remaining bound horse radish peroxidase reacts with added *o*-phenylenediamine to produce a colour whose absorbance is measured at 492 and 620 nm by a microtitre plate reader. The percentage HbA_{1c} is calculated from a standard curve using standards to which HbA_{1c} values have been assigned using HPLC.

The assay is novel in that the total haemoglobin concentration is not measured. Instead, it is assumed the same quantity of any haemoglobin will bind to the microtitre plate, a fact that explains why this assay produces low

results in patients with haemoglobinopathies (see Chapter 2.2.2). Between-batch CV for this assay was 5.0% at mean HbA_{1c} concentration of 6.4%.

3.4 Serum Fructosamine

As mentioned in Chapter 1.4.2, the fructosamine assay has undergone improvements since its inception. One study in this thesis (Chapter 8) used the original fructosamine kit (Roche Diagnostica, Welwyn Garden City, UK). Analyses were performed on a Cobas-Bio centrifugal analyser (Roche Diagnostica) using the protocol outlined in Chapter 1.4.2. This assay had a reference interval for non-diabetic individuals of 2.1-2.8 mmol/L. Between-assay CV was 3.6% at 4.1mmol/L.

Following the introduction of the improved Fructosamine Plus kit (Roche Diagnostica), the units for fructosamine changed. The reference interval was now 202-276 μmol/L, but the same Cobas Bio instrument was used for analysis. The between-batch CV for this kit was 1.9% at 304 μmol/L.

3.5 Blood Glucose Reference Instruments

3.5.1 Introduction

In the studies described in this thesis, laboratory glucose measurements were performed on both plasma and whole blood. In general, studies relating to glucose test strip measurement were performed on whole blood while those on the studies of glycated haemoglobin and fructosamine used plasma. A range of these instruments were used as described below.

3.5.2 The YSI Glucose Analyser

The Yellow Springs Instrument Model 23AM (YSI Ltd, Hampshire, UK) was used to measure whole blood glucose. A 25μL blood sample is injected into the instrument and diluted in buffer. A membrane embedded with the enzyme glucose oxidase metabolises glucose in the sample to form hydrogen peroxide as one of its products (see Figure 1.2a). A polarised platinum anode oxidises the hydrogen peroxide thus:

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-$$

The glucose concentration is proportional to the current produced at the anode. The instrument was calibrated using standard solutions before each batch of samples.

3.5.3 The Beckman II Glucose Analyser

The Beckman analyser (Beckman, Yellow Springs, Ohio, US) also uses the enzyme glucose oxidase as its basis for measurement. However, in contrast to the YSI instrument, it measures the consumption of oxygen in the sample rather than the formation of hydrogen peroxide. This oxygen consumption is measured using an oxygen electrode. The instrument is only suited to the measurement of plasma since the presence of oxyhaemoglobin in the erythrocytes of whole blood will interfere with measurements. Between-batch CV for this instrument was less than 2%.

3.5.4 The Analox GM9 Glucose Analyser

The Analox GM9 analyser (Analox Instrument Ltd, Hammersmith, London, UK) was used for the measurement of whole blood glucose. Like the Beckman instrument it measures the consumption of oxygen following reaction of

sample glucose with glucose oxidase. Unlike the Beckman it can measure both plasma and whole blood samples. This is achieved by collecting the blood sample (at least 5μL) in capillary tubes containing nitrite which converts haemoglobin to methaemoglobin and thus prevents oxyhaemoglobin affecting the measurement. As with the YSI, calibration of the instrument was performed before each batch of samples.

3.5.5 The Dimension Analyser

Plasma glucose was also measured on the routine laboratory 'Dimension' instrument using a hexokinase method (Du Pont, Herts, UK), and monitoring formation of NADPH by the increase in absorbance at 340nm wavelength (Figure 1.2b). Between-batch CV for this method was <2%.

3.6 Blood Glucose Test Strips and Meters

Studies in this thesis have used a wide variety of glucose test strips and meters. The meters (with their respective test strips) include: the One Touch II (Lifescan Ltd, High Wycombe, Bucks, UK); the Reflolux II and Accutrend (12 second glucose strip) (both Boehringer Mannheim UK, Lewes, East Sussex, UK); the Glucometer IIM, Glucometer Gx and Glucometer 4 (all Bayer UK (Ames Division), Basingstoke, UK); and the ExacTech and Companion 2 (both Medisense Ltd, Abingdon, Oxon, UK). These meters are shown in Figure 3.1.

Each meter was calibrated according to the manufacturers instructions and operated using test strips within their expiry date. All meters were checked for accuracy using manufacturers recommended solutions and, where applicable, test paddles.

All systems bar the Glucometer 4 employ the glucose oxidase enzyme system as their basis for measurement as described in Chapter 1.4.3. The

Figure 3.1 Test strip blood glucose meters.

Top picture (L to R): One Touch II, Glucometer 4, Accutrend
(Bottom): ExacTech
Bottom picture (L to R) Glucometer Gx, Reflolux II

Glucometer 4 is instead based on the hexokinase enzyme. Only the ExacTech and Companion 2 meters measure glucose amperometrically rather than colorimetrically. The Companion 2 test strip differs from the ExacTech by having an additional 'blanking' electrode as a means of reducing chemical interference in the measurement. The older test strips belonging to the Reflolux and Glucometer II/Gx meters require wiping of blood from the test strip while all other meters used 'non-wipe' strips.

Uses of the meters peculiar to a particular study are described in the methods sections of the relevant chapters.

3.7 Statistical Analysis

All statistical analyses were performed using 'Statgraphics' and 'Statgraphics Plus' software (Statistical Graphics System. Rockville MD: Statistical Graphics Corporation Inc. 1986, 1995). Statistical tests used are described in their respective thesis chapters.

Chapter 4

The Effects of Fetal Haemoglobin on the Interpretation of Glycated Haemoglobin Measurements.

4.1 Introduction

Fetal haemoglobin (HbF) can co-elute or co-migrate with the glycated haemoglobin peak of several HbA_1 and HbA_{1c} methods (see Chapter 2.2.3). Thus HbF, whether glycated or not, will be included in the glycated haemoglobin result of these assays. At the time of this study, the most popular method of measuring HbA_1 in the UK was by electrophoresis.[145,146] Even now, it continues to be used in many hospital laboratories. Unfortunately, this assay is one of those known to be influenced by fetal haemoglobin concentrations.[98]

A known example of the problem associated with HbF occurred in a 63 year old male Type II patient attending the out-patient clinic at our hospital. Insulin treatment had been considered because his HbA_1 by electrophoresis of 13% had consistently indicated poor glycaemic control. Discrepancies between these HbA_1 results and glucose monitoring prompted the measurement of HbF by a routine insensitive electrophoretic technique. This estimated the patient as having 5% HbF, meaning that his true HbA_1 was nearer to 8%, and that his glycaemic control was, in fact, good.

In 1990, a high performance liquid chromatography (HPLC) glycated haemoglobin instrument, the Biomen HA-8121, was introduced to the UK. As well as being able to measure HbA_1 and HbA_{1c}, it was also able to simultaneously separate and measure small quantities of HbF in whole blood. It thus had the potential to be a useful tool in accurately assessing the effect that patient fetal haemoglobin has on the methods which include HbF in their measurement.

At the same time, European consensus guidelines for glycated haemoglobin measurement were becoming generally accepted and applied in clinical situations. In their original form, the guidelines defined good glycaemic control as a HbA_1 or HbA_{1c} within 2 standard deviations (SD's) of the non-diabetic mean value i.e. within the reference range or interval. Borderline control was between 2 and 4 SD's and poor control outwith 4 SD.[147] Although the

recommendations were originally aimed at non-insulin-dependent patients, their scope was subsequently broadened to include insulin treated and Type I patients as well.[148]

No prior study had accurately determined the concentrations of HbF found in adult diabetic patients. The present study describes the use of the Biomen instrument in establishing the prevalence of HbF in insulin treated and non-insulin treated diabetic adults compared to a group of non-diabetic controls. In addition, the effect that HbF concentrations may have on the clinical interpretation of electrophoretic HbA_1 results are evaluated with particular regard to the use of the consensus guidelines.

4.2 Methods

4.2.1 Patients

107 diabetic patients were chosen randomly from the outpatient clinic at Gartnavel General Hospital, Glasgow. They comprised 50 insulin treated (27 men, 23 women, median age 40, range 13-77 years) and 57 non-insulin treated (31 men, 26 women, median age 64, range 36-94 years) patients. Fifty seven non-diabetic control subjects (34 men, 23 women, median age 59, range 12-90 years) were also included.

4.2.2 Study Design

Whole blood was collected for the measurement of HbA_1, HbA_{1c} and HbF in all 107 patients and serum was also separated for fructosamine analysis in 82 of these.

HbA_1 was measured by electrophoresis (HbA_{1E}) (Ciba Corning Diagnostics Ltd) and high performance liquid chromatography (HbA_{1HPLC}) (Hi-AutoA1c, model 8121, Biomen UK Ltd) methods. Haemoglobin A_{1c} and HbF were measured concurrently by the same HPLC instrument.

Plasma glucose was measured by a glucose oxidase method (Beckman Instruments Inc).

4.2.3 Statistical Analysis

Statistical analysis was performed using Kruskal-Wallis one-way analysis of variance, chi-squared tests with Yates correction and Gaussian tail area probabilities where appropriate. The correlation coefficients were calculated by the least squares method.

4.3 Results

4.3.1 HbF in Study Patients

Insulin treated diabetic patients had a mean HbF concentration of 0.40% (range 0-2.7%) and non-insulin treated diabetics 0.30% (range 0-4.7%) ($p=0.056$ using Kruskal Wallis). Control patients had a mean HbF concentration of 0.34% (range 0-3.1%) (NS compared to the diabetic group).

Patients are considered to have elevated concentrations of HbF when the value is ≥0.5%.[102] 23/50 (46%) of insulin treated patients had increased concentrations of HbF compared to 13/57 (23%) of controls ($\chi^2 = 5.42$, $p<0.02$). In non-insulin treated diabetic patients, 14/57 (25%) had increased concentrations (NS compared to controls).

HbF concentrations did not correlate with measures of glycaemic control as represented by HbA_{1E}, HbA_{1cHPLC} or fructosamine.

Age and sex made no significant difference to the prevalence of increased HbF concentrations in insulin treated, non-insulin treated or control groups.

4.3.2 HbF and Glycated Haemoglobin Method Comparison

HbA_{1E} correlated with HbA_1 by HPLC in insulin treated patients ($r=0.84$, $p<0.001$) and non-insulin treated patients ($r=0.93$, $p<0.001$). The correlations

in these two groups improved when HbA_{1E} (where HbF comigrates with HbA_1) was compared to the sum of HbF_{HPLC} and HbA_{1HPLC} (r=0.92 and r=0.95 respectively) (Figure 4.1).

4.3.3 HbF and Comparisons Between Fructosamine and Glycated Haemoglobin

Serum fructosamine correlated best with HbA_{1cHPLC} (r=0.76, $p<0.001$) followed by HbA_{1HPLC} (r=0.72, $p<0.001$) and $(HbA_1+HbF)_{HPLC}$ (r=0.68, $p<0.001$). Correlation was least with HbA_{1E} (r=0.64, $p<0.001$).

4.3.4 HbF and Glycated Haemoglobin Assay Imprecision

In calculating the between-batch imprecision of an assay, quality control (QC) material is analysed with each assay run of patient samples. The spread of QC results is often expressed as a coefficient of variation (CV) defined as:

$$\frac{\text{Standard Deviation of QC results}}{\text{Mean Value of QC results}} \times 100\%$$

To illustrate the possible effect of HbF on the imprecision of the electrophoretic assay, HbF-free quality control material was used. Using this preparation, the estimated between-batch imprecision (CV) during 107 assay runs was 4.3% at a mean HbA_1 concentration of 8.22% (Figure 4.2a). Unlike QC material, however, patient samples contain variable concentrations of HbF which are included in HbA_{1E} measurements. To simulate the effect that variable patient HbF may have on assay imprecision the concentrations of HbF_{HPLC} measured in 107 diabetic patients were randomly added to the previous 107 electrophoretic quality control results. The mean calculated HbA_1 concentration rose to 8.57% and the CV to 8.33%. (Figure 4.2b). Thus, if 107 patient samples with the same HbA_1 were analysed instead of QC material, both the spread of results (imprecision) and the mean value (bias or accuracy) would increase.

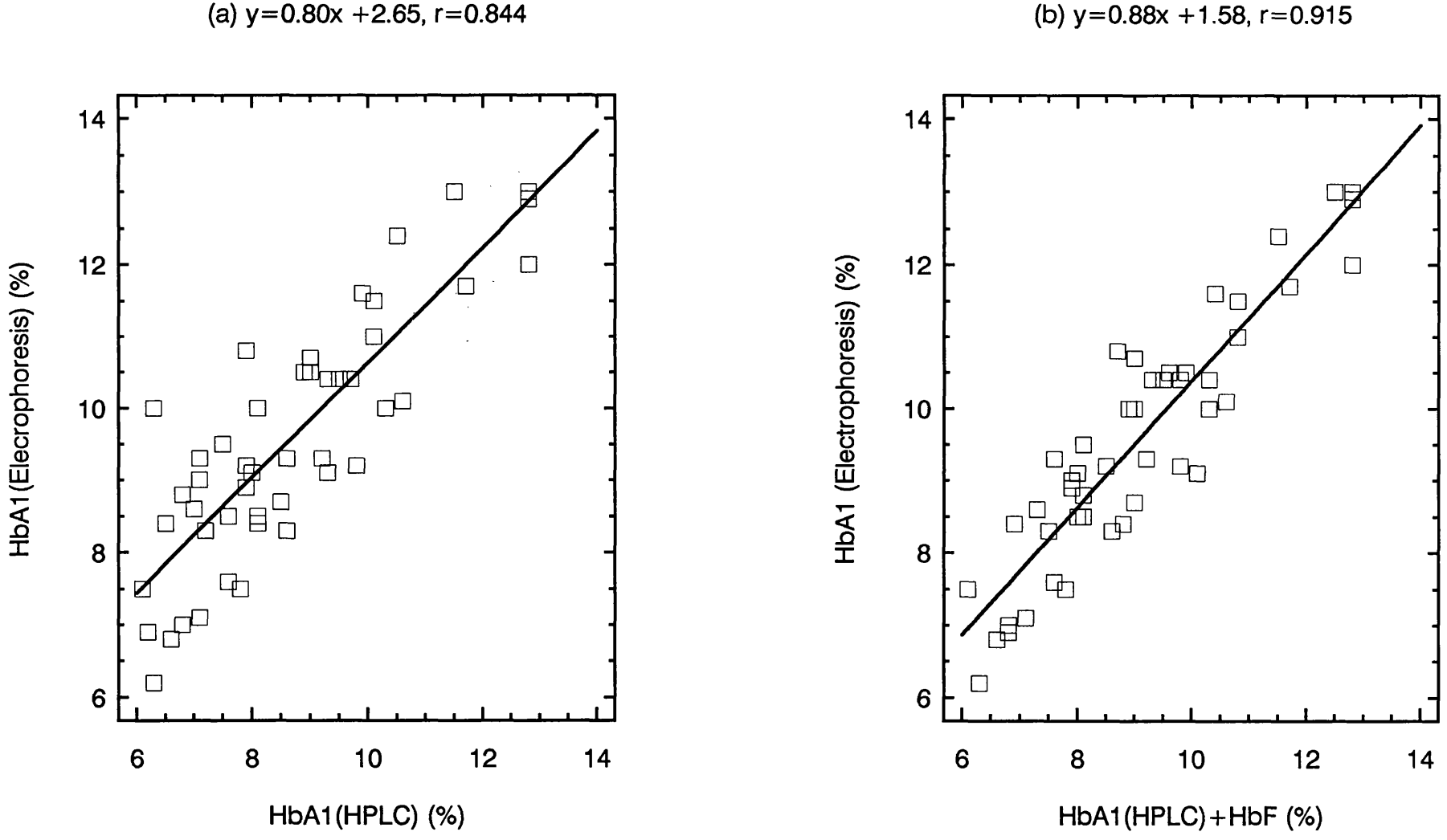


Figure 4.1 Relationship between electrophoretic and HPLC HbA_1 methods (a) without and (b) with the inclusion of HbF in the HPLC assay.

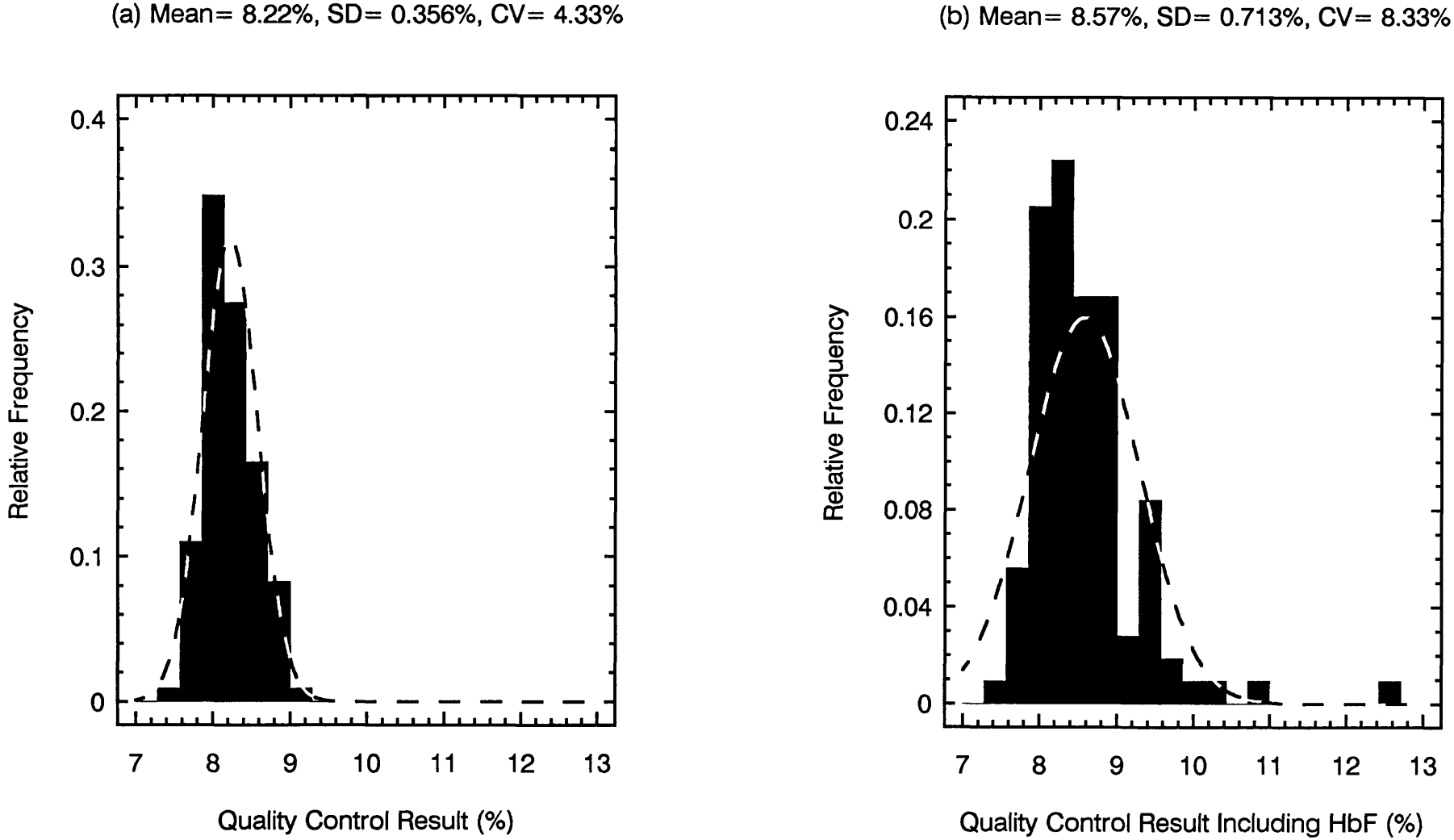


Figure 4.2 The increase in the imprecision of the electrophoretic HbA_1 assay (a) without and (b) with the inclusion of HbF.

4.4 Discussion

These results show that insulin treated patients have increased fetal haemoglobin concentrations compared to non-insulin treated and non-diabetic controls and that these values are not dependent on the age, sex or glycaemic control of the subject. In a paediatric population, HbF concentrations have previously been demonstrated to be higher in insulin-dependent diabetes than in non-diabetic controls but it was assumed that these raised values declined into adulthood.[102] Our observations would suggest that this may not be the case in some patients.

Elevated levels of HbF have been described in autoimmune diseases such as pernicious anaemia[149] and thyrotoxicosis.[150] Since Type I diabetes mellitus also has an autoimmune component,[151] it is perhaps not surprising that raised HbF concentrations may be present in this disease.

The reason for increased HbF concentrations remains speculative. Delayed transition from HbF to adult HbA has been described in the hyperinsulinaemic infants of diabetic mothers,[152] but with abnormal HbF concentrations appearing unrelated to age in this study, a reactivation of the HbF gene in insulin treated patients seems more probable.

This study has also shown that concentrations of HbF not only affect those patients with high HbF measurements (and therefore spuriously high HbA_1) but have a general effect on the imprecision and accuracy of the electrophoretic assay as a whole. Haemoglobin F added an average of 0.35% to the HbA_1 results thereby affecting assay accuracy. Since inter-patient HbF is variable, this added a random error which also increased the spread of results. The simulated experiment demonstrated the magnitude of this effect, showing that the between-batch CV (imprecision) for the assay was almost double that suggested by the use of quality control material alone.

When the true imprecision of the assay is so great it can markedly affect the classification of glycaemic control if European HbA_1 guidelines are used.

Applying these guidelines to the electrophoretic assay, where the local reference interval is 4.8-7.8%, good control (<2SD from non-diabetic mean) is <7.8%, borderline (2-4SD) is 7.8 to 9.3%, and poor control (>4SD) is >9.3%. If an acceptable assay run is defined as one which has a quality control sample result within two standard deviations from a quality control mean then, by our simulated experiment, a patient with a 'true' HbA_1 of 8.22% may have an electrophoretic measurement reported as being between 7.14% and 10.00% (Figure 4.2b). Using the above definition this corresponds to either good, acceptable or poor glycaemic control!

Looked at from a different perspective, fetal haemoglobin can also influence the use of this assay as a screening test for diabetes. If the sample with a HbA_1 of 8.22% was to be screened, 12% of acceptable assay runs would classify this patient as having a HbA_1 within the non-diabetic reference interval. More worryingly, it has recently been advocated that the diagnosis of diabetes in patients with borderline fasting plasma glucose values (6.4-7.8mmol/L) could usefully be made if their glycated haemoglobin measurement was >1% above the 'upper limit of normal'.[17] Not only may patients with high concentrations of HbF be wrongly diagnosed as diabetic, but the imprecision of the electrophoretic assay itself will increase 8% of acceptable assay runs by at least this 1%.

This study also demonstrated that comparisons between different glycated haemoglobin methods can be influenced by whether the assay includes HbF in results or not. When the electrophoretic HbA_1 measurement was compared with HPLC, the correlation between the 2 measurements in insulin treated patients was less (r=0.84) than when the measured HbF concentration was included (r=0.92). Thus, in laboratories employing the electrophoretic method of HbA_1 measurement, the potential effect of HbF concentrations must be taken into account when comparing different methods of analysis.

Comparisons between serum fructosamine and HbA_1 as different assessments of glycaemic control have shown wide variations in the degrees of correlation between the two measurements, varying from r=0.91[153] to a complete lack of correlation[40] (see also Chapter 1.2.2). The discrepancy in the time period of integrated glycaemia measured by the two assessments (1-3 weeks for fructosamine, 6-8 weeks for HbA_1) is likely to be partially responsible. However, an important new finding in the present study is that comparisons differ markedly with the method of glycated haemoglobin measurement and that HbF inclusion accounts for some of the discrepancies. Correlation between fructosamine and HbA_1 was least for the electrophoresis method (r=0.64). Using HPLC, the correlation was least when including measured HbF, $((HbA_1+HbF)_{HPLC})$, but improved when measuring just HbA_{1HPLC} (r=0.68 and r=0.72 respectively). Correlation with fructosamine was greatest with HbA_{1cHPLC} (r=0.76). Thus, future comparisons of the two assessments may benefit if precise HbA_{1c} assays are used instead of older, more imprecise glycated haemoglobin methods.

As new instruments for glycated haemoglobin analysis become available, it is tempting to assume that the problems associated with fetal haemoglobin will diminish as the electrophoretic method of analysis is gradually supplanted. However, it is probable that other glycated haemoglobin assays which include HbF will be affected in a similar way. Thus, the problems highlighted here will be applicable to other commonly used systems, such as the Corning 'Glycomat' HPLC analyser. At present, the Glycomat competes with the Biomen HPLC analyser for being the most commonly sited glycated haemoglobin instrument in UK laboratories.

In conclusion, the popular electrophoretic method of HbA_1 measurement may be more inaccurate and imprecise than quality control data suggests because of the presence of fetal haemoglobin in patient samples. This problem may be compounded in insulin treated patients because of their increased

prevalence of high HbF concentrations. As a consequence, there is reduced agreement between results from this assay and those obtained from other glycated haemoglobin methods as well as a poorer relationship with serum fructosamine concentrations. More importantly, the use of glycated haemoglobin as a means of assessing and diagnosing diabetes is prone to being less reliable when HbF-including methods are used.

In the years following the publication of this study,[154] two further studies have since confirmed the finding of raised HbF concentrations in adult insulin treated patients. The first found 23 of 60 adult Type I patients to have raised HbF concentrations compared to 9 of 60 control subjects.[155] More recently, a study involving 1,104 diabetic patients and 258 control subjects also found significantly higher concentrations of HbF in both insulin treated Type II and Type I diabetic patients.[156] Together, these results have prompted independent calls for the withdrawal of all glycated haemoglobin methods where fetal haemoglobin is a potential interferent in measurement[157,158] and hopefully has alerted clinicians and equipment manufacturers to the unacceptability of their continued use.

Chapter 5

An Evaluation of Glycaemic Control Limits Using the Ames DCA 2000 HbA1c Analyser.

5.1 Introduction

The introduction of the European consensus guidelines for glycated haemoglobin measurement[147] not only helped in classifying the glycaemic control of individual diabetic patients, but also provided an appealing means of comparing the results obtained from different centres when using a variety of instrumentation. Indeed, the use of such 'standard deviation scores' was heralded as 'a golden opportunity to compare accurately treatment and education regimens'.[159] However, we have demonstrated that methodological difficulties associated with some glycated haemoglobin methods can lead to the misclassification of diabetic patients when using these guidelines (see Chapter 4). Since glycated haemoglobin methods do not all suffer from the same limitations, we postulated that discrepancies might exist between assays when European recommendations are applied. Consequently, this study proposed to assess the agreement between different methods when classifying diabetic patients using standard deviation cut-offs.

In late 1991 when the study was conceived, a novel method for measuring HbA_{1c} was undergoing clinical trials before being officially introduced to the UK. The Ames (now Bayer) DCA 2000 analyser was a portable instrument employing all the reagents for a latex-agglutination immunoassay in a single disposable cartridge (see Chapter 3.3.4). It offered the promise of on-site measurement of glycated haemoglobin as a consequence of its small size, short testing time (9 minutes), and relatively inexpensive cost of the instrument (around £2000). The use of a monoclonal antibody to HbA_{1c} implied that the method was likely to show improved specificity for glycated haemoglobin compared to the routinely used Corning electrophoretic HbA_1 assay. In view of their markedly different methods of analysis, it seemed appropriate that these two assays should be chosen to assess whether results from different instruments could indeed be compared by using European recommendations. Therefore, this chapter describes a comparison of the DCA 2000 HbA_{1c}

analyser with other measures of glycated haemoglobin with particular regard to validating the consensus guidelines.

5.2 Methods

5.2.1 Patients

Reference interval individuals comprised 104 healthy non-diabetic individuals (median age 33.5 years, range 15-70) and a male/female ratio of 1:1.

A total of 152 insulin-treated or Type I diabetic patients (median age 43 years, range 15-84, 73 males, 79 females) attending the routine return diabetic, adolescent and pregnant diabetic clinics at Gartnavel General Hospital, Glasgow also participated in the study.

5.2.2 Study Design

One hundred and eight patient samples had HbA_1 measured using an electrophoretic method (Ciba Corning Diagnostics Ltd) and HbA_{1c} by the DCA 2000 analyser (Bayer Diagnostics Ltd). In 78 of these samples, blood for DCA 2000 analysis took the form of a 1μL capillary sample measured 'on-site' over a 6 week period at the clinics described above, while electrophoresis HbA_1 measurements were performed on a venous sample taken by the reviewing physician. The remaining 30 samples had both HbA_1 and HbA_{1c} measured on the same venous sample.

Venous results obtained from the DCA 2000 were also compared with those obtained from a laboratory based immunoassay HbA_{1c} system, the Novoclone (Novo Nordisk Diagnostics Ltd) in a further 44 patient samples.

During the same time period as patient sample measurement, a reference range (mean ± 2 standard deviations) for the DCA 2000 assay was established using the non-diabetic individuals. A local reference range for the electrophoresis HbA_1 assay had been determined previously.[35]

Diabetic patients samples were classified according to European guidelines using 2 and 4 SD cut-offs as described in Chapter 4.

5.2.3 Statistical Analysis

Statistical analysis used the McNemar test for paired results and the least squares method for correlations.

5.3 Results

5.3.1 Reference Individuals

The non-diabetic reference interval for HbA_{1c} on the DCA 2000 was found to be 4.38 to 5.70% (mean 5.04%, SD 0.33%), which was much tighter than that of 4.8% to 7.8% previously found with the electrophoretic HbA_1 method.

5.3.2 Method Comparisons

Haemoglobin A_{1c} values obtained by the DCA 2000 showed a significant correlation with the existing electrophoretic HbA_1 method ($r=0.894$, Figure 5.1). No differences were experienced between using capillary and venous blood samples. As expected, the relationship between the 2 HbA_{1c} assays (DCA 2000 and Novoclone) was superior ($r=0.954$, Figure 5.2).

5.3.3 Glycaemic Control Categories

Poor agreement existed between methods when using European guidelines to classify patients into good, acceptable and poor glycaemic control (Table 5.1). More patients were classified as being in poor control ($\chi^2= 30.1$, $p<0.00001$) and fewer in good control ($\chi^2= 10.3$, $p=0.0013$) when the HbA_{1c} method was used instead of HbA_1.

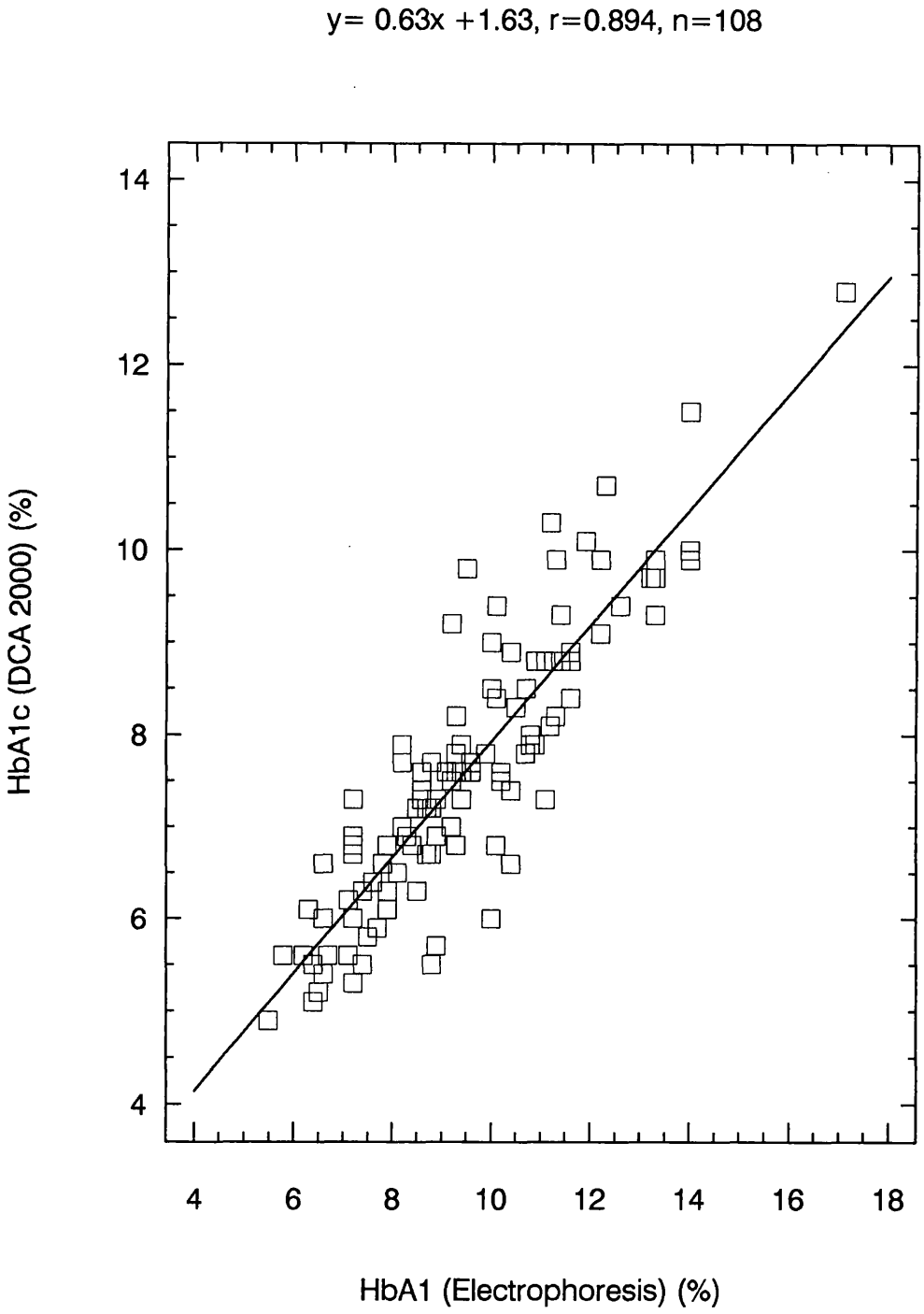


Figure 5.1 Relationship between DCA 2000 HbA_{1c} assay and the electrophoretic HbA_1 method.

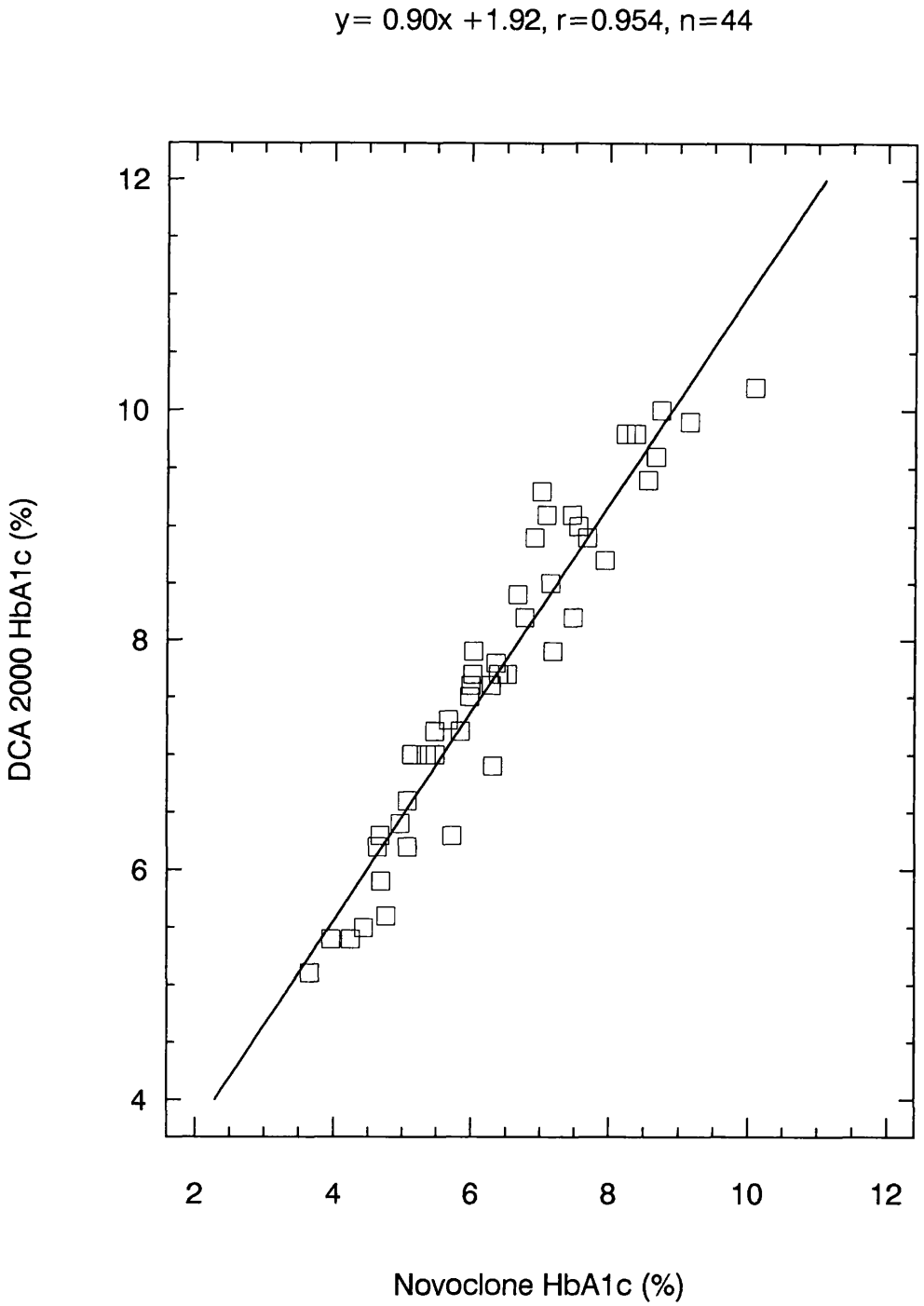


Figure 5.2 Relationship between DCA 2000 HbA_{1c} assay and the Novoclone HbA_{1c} method.

Assay	Glycaemic Control Category		
	Good (<2SD from non-diabetic mean)	Acceptable (2-4SD)	Poor (>4SD)
HbA_1 (Electrophoresis)	25 (23%)	33 (31%)	50 (46%)
HbA_{1c} (DCA 2000)	13* (12%)	13 (12%)	82** (76%)

*p=0.0013 and **p<0.00001 compared with HbA_1 by elecrophoresis.

Table 5.1 Glycaemic control categories for 108 diabetic patients classified using HbA_1 and HbA_{1c}.

5.4 Discussion

This study has shown that, when using European recommendations, HbA_{1c} measured by an immunoassay technique can classify diabetic patients differently to the electrophoretic HbA_1 assay. Sixty four percent more patients were classified as poorly controlled using HbA_{1c} compared with HbA_1. Likewise, 48% fewer patients were in good control with the HbA_{1c} assay.

Reasons for the discrepancy between the two methods centres on the fact that the non-diabetic SD for the DCA 2000 is much smaller than that for electrophoresis (0.33% vs. 0.75%), and so the >4SD definition of poor control is much tighter for this HbA_{1c} assay, perhaps as a result of its greater specificity. Whatever the reason, the 2 and 4SD categories for the electrophoretic assay equate to nearer 5 and 8SD when using this HbA_{1c} assay with the locally derived reference interval.

It seems unlikely that the findings in this study are peculiar to the indigenous West of Scotland non-diabetic population since the DCA 2000 manufacturers quote a reference range from 263 individuals which is almost identical (mean HbA_{1c} 5.1%, SD 0.3%). It also seems improbable that the new DCA 2000 is reporting inaccurate patient results since they largely agreed with those obtained by the Novoclone method. Indeed, the DCA 2000 instrument itself proved to be a useful tool for use at diabetic clinics, especially since it only required the minimum of technical skill to produce acceptable results. The only difficulty experienced was the simple capacity of the system. With a maximum throughput of six samples per hour it would be necessary to have more than one instrument in use if a large number of patients were to be tested.

In conclusion, we found that the use of different glycated haemoglobin methods can lead to a discrepant classification of glycaemic control in diabetic patients which may relate to the specificity of the assay used and the nature of the analyte being measured.

Primarily because of the presentation and publication of the findings in this study, the original European guidelines were changed so that the SD cut-offs were more attainable when using HbA_{1c} assays.[160] They now recommended that good glycaemic control be defined as a HbA_1 or HbA_{1c} less that 3SD's from the non-diabetic mean value, acceptable control (now renamed borderline) be between 3 and 5SD, and poor control be outwith 5SD.[89] These glycaemic control limits continued to be applicable to both Type I and Type II diabetic patients.

Chapter 6

The Classification of Glycaemic Control in Diabetes Mellitus using HbA_1 and HbA_{1c}.

6.1 Introduction

In September 1993, one of the most influential studies in the history of diabetes reported. The results of the Diabetes Control and Complications Trial (DCCT) showed an impressive reduction in microvascular complications in an intensively treated group of Type I patients compared to those who were treated conventionally.[25] In this study, the cornerstone of treatment evaluation was the measurement of glycated haemoglobin in the form of HbA_{1c}. Thus, the study results not only reinforced the need for good glycaemic control but also focused minds on the importance of an accurate and standardised means of assessing glucose control.

We previously found that the use of different glycated haemoglobin methods could lead to discrepancies in the classification of glycaemic control when using European consensus guidelines (see Chapter 5). However, some consequences of the study itself also raised further questions. Firstly, it was not possible to establish whether the discrepancies found were because two markedly different methods were used (electrophoresis and immunoassay) or if it was solely because one assay measured HbA_1 while the other measured HbA_{1c}. Secondly, although the study used locally derived reference intervals, they were not established using the same individuals at the same time which may have had a minor influence on our findings. Lastly, the change in guideline SD cut-offs in response to our findings (from 2 and 4 SD's to 3 and 5) meant the results of the original study may no longer have been applicable. During this period, the consequences of misclassifying a patient's glycaemic control were highlighted by the DCCT and helped cement the need for a further study to clarify the points raised. This chapter addresses these points.

The first aim of the study was to determine if the discrepant classification of diabetic patients remained when the new cut-offs of 3 and 5 SD were applied and the same non-diabetic individuals were used to construct reference intervals for the different assays. A second aim was to try and establish if,

when employing the new guidelines, HbA_1 fundamentally classified patients differently to HbA_{1c} even when the same high performance liquid chromatography instrument of analysis was used. If this proved to be the case then the interchangeable use of HbA_1 and HbA_{1c} for the measurement of glycaemic control would require reappraisal.

6.2 Methods

6.2.1 Patients

The reference range sample consisted of 106 non-diabetic individuals (42 male, 64 female, median age 36 years (range 16 to 82)) comprising hospital staff and families. Two hundred and eight consecutive patients (114 male, 94 female, 90 insulin treated, 118 non-insulin treated, median age 60 years (range 13 to 94)) attending the diabetic out-patient clinic at Gartnavel General Hospital, Glasgow also participated in the study.

6.2.2 Study Design

Two methods of glycated haemoglobin analysis were used: HbA_1 and HbA_{1c} were measured individually by high performance liquid chromatography (HPLC) (Hi-AutoA1c, model 8121, Biomen UK Ltd); HbA_1 was additionally measured by an electrophoretic method (Ciba Corning Diagnostics Ltd).

A locally derived non-diabetic reference range (mean ± 2 standard deviations) was established for the HPLC methods (HbA_1 and HbA_{1c}) and for the electrophoretic method. During the same time period, blood from the diabetic patients was collected for the same range of glycated haemoglobin analyses.

Diabetic patient samples were categorised according to European IDDM and NIDDM guidelines. Good glycaemic control was defined as a HbA_1 or HbA_{1c} value less than 3 standard deviations from a method's non-diabetic

population mean. Borderline control was between 3 and 5 standard deviations and poor control was above these limits.[89]

6.2.3 Statistical Analysis

Statistical analysis used the McNemar test for paired samples and the chi-squared test for unpaired proportions. The Gaussian distribution of the reference samples was verified using Kolmogorov-Smirnov one way analysis. Correlations used the least squares method.

6.3 Results

6.3.1 Reference Individuals

Table 6.1 shows the results of glycated haemoglobin measurements obtained from the reference population for each analysis with their respective good, borderline and poor control limits. The spread (standard deviation) of each assay's reference values are also expressed as a percentage of the method mean (sample coefficient of variation). Results for the electrophoretic HbA_1 method are consistent with those obtained previously in Chapter 5.

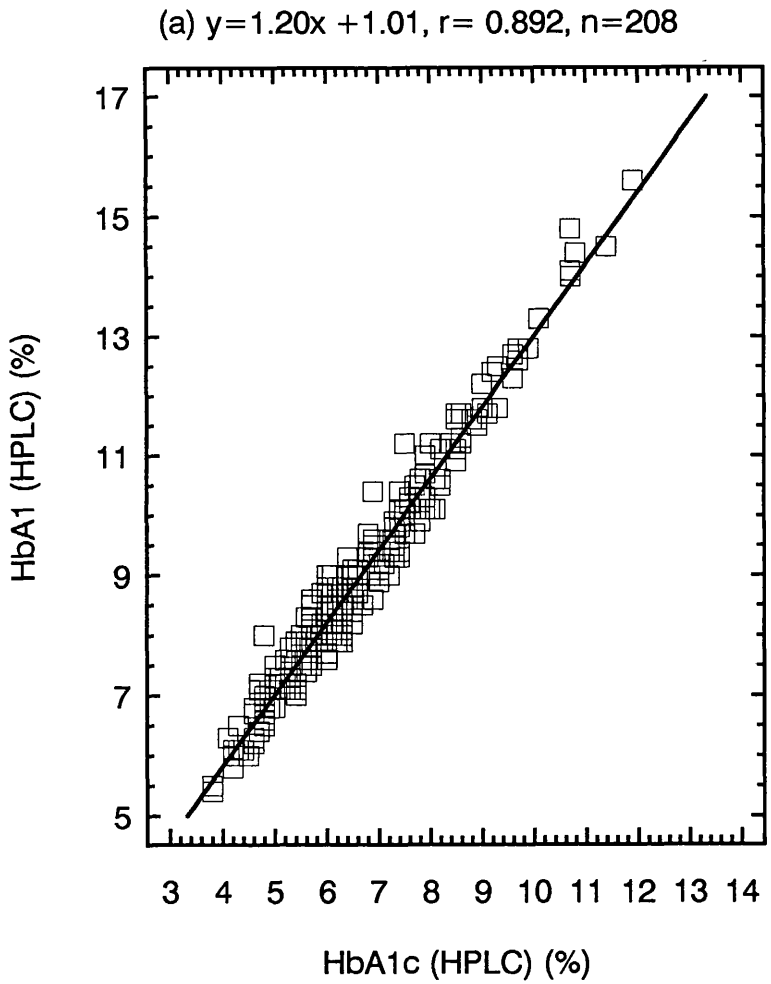
6.3.2 Diabetic Patients

Figure 6.1 demonstrates good correlation between the two HbA_1 assays and HbA_{1c}. HbA_1 by electrophoresis also correlated with HbA_1 by HPLC ($y=0.90x+1.61$, $r=0.888$).

Despite the agreement between methods, Table 6.2 shows significantly fewer patients classified in good control and more as poorly controlled with HbA_{1c} (HPLC) compared to both HbA_1 assays (both $p<0.0006$). This was true for both insulin treated and non-insulin treated patients (no significant difference). The clinic patient median HbA_{1c} (HPLC) value was proportionately higher than HbA_1 (both by HPLC and electrophoresis) when

	HbA_{1c} (%) (HPLC)	HbA_1 (%) (HPLC)	HbA_1 (%) (Electrophoresis)
Reference Population Statistics			
Mean	4.02	5.88	6.30
Standard Deviation	0.28	0.46	0.75
Coefficient of variation	7.1%	7.8%	11.9%
Derived Glycaemic Control Categories			
Good (<3 standard deviations)	<4.87	<7.25	<8.55
Borderline (3-5 standard deviations)	4.87-5.44	7.25-8.17	8.55-10.05
Poor (>5 standard deviations)	>5.44	>8.17	>10.05

Table 6.1 Reference population statistics and derived glycaemic control categories (n=106)



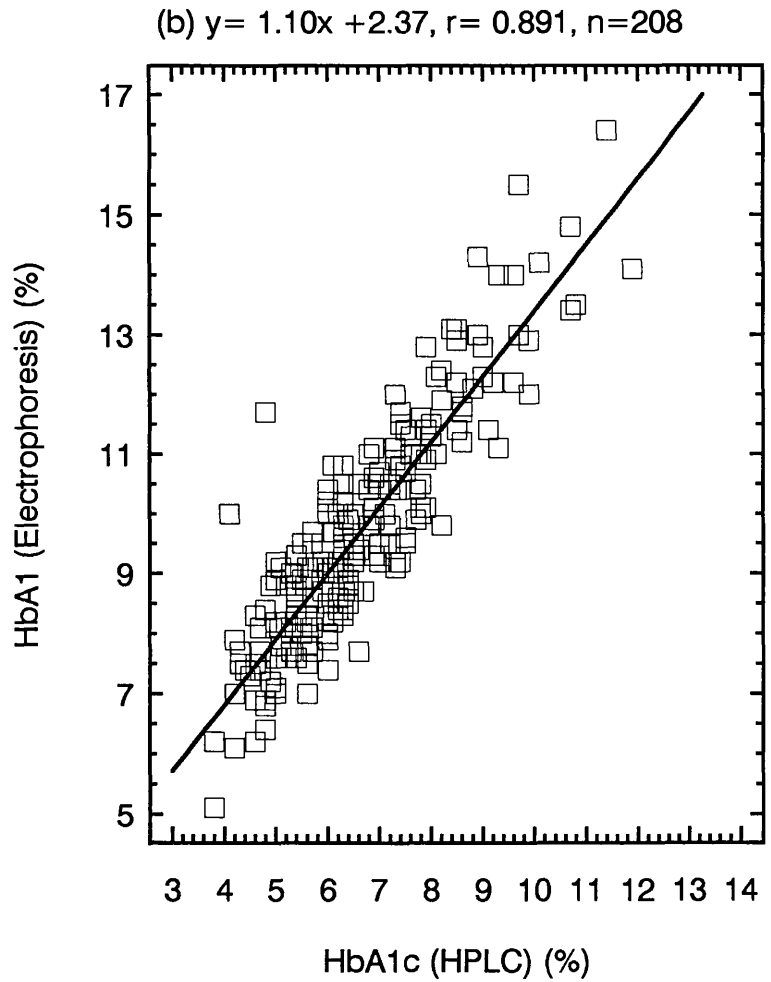


Figure 6.1 Relationship between (a) HPLC, and (b) electrophoretic HbA_1 methods, and HbA_{1c} measured by HPLC.

	HbA_{1c} (HPLC)	HbA_1 (HPLC)	HbA_1 (Electrophoresis)
Diabetic Patient Statistics			
Median value (%)	6.3	8.6	9.35
Standard Deviations from reference mean	8.0	5.9	4.1
% above reference mean value	56.7%	46.2%	48.4%
Glycaemic Control Category			
Good Control	25 (12%)	39* (18.8%)	63** (30.3%)
Borderline Control	26 (12.5%)	39* (18.8%)	71** (34.1%)
Poor Control	157 (75.5%)	130** (62.4%)	74** (35.6%)

* p= 0.0005 and **p<0.00001 compared with HbA_{1c} (HPLC)

Table 6.2 Diabetic patient statistics and glycaemic control according to European IDDM guidelines (n=208)

compared to the reference population. Constituents of HbA_1 other than HbA_{1c} i.e. HbA_{1a1}, HbA_{1a2} and HbA_{1b}, were estimated by subtracting the HbA_{1c} value from HbA_1. The median diabetic patient value for this was 2.3% which represented a 24% increase above the non-diabetic mean of 1.86%.
Figure 6.2 demonstrates why more patients were classified as poorly controlled using HbA_{1c} measurement. The distribution of diabetic patient samples using HbA_{1c} (HPLC), HbA_1 (HPLC) and HbA_1 (electrophoresis) is shown as a function of the standard deviations from their respective method means.

6.4 Discussion

The results of the Diabetes Control and Complications Trial have provided the best objective guide for desirable glycaemic control limits to prevent microvascular complications in Type I diabetes. In that study, the median HbA_{1c} of the intensively treated patients was 4 standard deviations from the mean non-diabetic value whilst the conventionally treated group was 8 standard deviations from the mean.[25] In the study described in this chapter, the median diabetic value was 4.1 standard deviations from the non-diabetic mean using electrophoretically measured HbA_1, 8.0 using HbA_{1c} (by HPLC) and 5.9 by HbA_1 using HPLC. Thus, depending on the method of glycated haemoglobin used, our same diabetic clinic patients could be described as equivalent to either the intensively treated group, the conventionally treated group or approximately midway between. Although our study included non-insulin dependent patients, it has been suggested that the results of the DCCT are likely to be equally applicable to this group with diabetes.[27]

In October 1993, submission of samples to the United Kingdom National External Quality Assessment Scheme (UK NEQAS) reported 4 different instruments for HbA_1 measurement (with the Corning electrophoresis being the commonest) together with 8 instruments for HbA_{1c} analysis (with HPLC

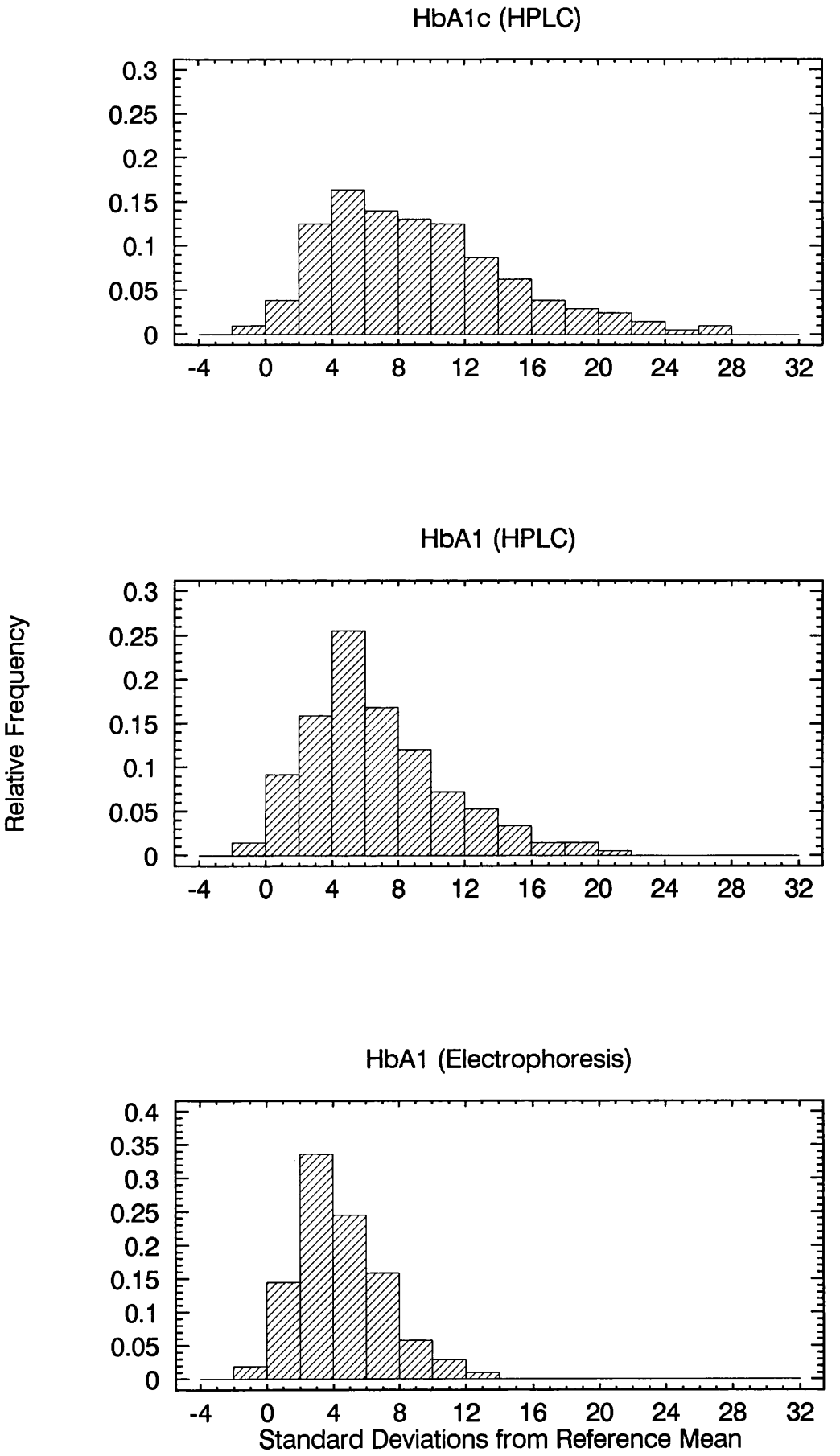


Figure 6.2 Distribution of diabetic patient samples as a function of standard deviations from respective method means.

the commonest method). This present study has clearly demonstrated that there is considerable discrepancy in the classification of glycaemic control when comparing the electophoretic HbA_1 method with HbA_{1c} HPLC analysis. Using revised European guidelines, 36% of our patients were poorly controlled (>5 SD's) using electrophoresis compared to 76% using HbA_{1c} measured by HPLC. This is consistent with our previous findings in Chapter 5 when comparing electrophoretically measured HbA_1 with HbA_{1c} measured by an agglutination inhibition immunoassay method.

Publication of the DCCT results emphasised the need for consistency between centres regarding the measurement of glycated haemoglobin.[26] One of the objectives of providing guidelines based on SD's was to allow diabetic units to compare data and perform useful audit. Since our routine assay at the time of this study was HbA_1 by electrophoresis (where more patients appear to have good control) we will always have erroneously appeared to have patients with better glycaemic control in comparison to a centre using a HbA_{1c} assay!

This discrepancy is not confined to only the electrophoretic HbA_1 assay. This study has also shown that a marked disparity between HbA_1 and HbA_{1c} categorisation remained even when patient specimens were measured by HPLC using the same instrument, time of analysis and reference range samples. Significantly more patients had poor control as assessed by HbA_{1c} than by HbA_1 (76% vs. 62%). The reasons for this appear twofold. Firstly, the spread (standard deviation) of the non-diabetic HbA_1 reference population results was relatively greater than HbA_{1c} (7.8% vs. 7.1% of the mean reference value). Thus more diabetic samples fell within 3 and 5 standard deviations by using HbA_1 rather than HbA_{1c}. Secondly, in comparison to non-diabetic values, patient HbA_{1c} values were relatively higher than HbA_1 (median value 57% vs. 46% greater than reference mean). The implication is that this is due to glycated analytes of HbA_1 other than HbA_{1c} (HbA_{1a1}, HbA_{1a2} and HbA_{1b}) rising less rapidly than HbA_{1c} itself.

There remained a significant difference in the classification of glucose control between the two HbA_1 methods: 62% of patients were poorly controlled using HPLC and 36% by electrophoresis. This was due to the electrophoretic assay exhibiting a relatively higher reference range standard deviation (11.9% of the mean vs. 7.8%). This disparity is likely to be due in part to the fact that, unlike the chosen HPLC method, both glycated and non-glycated fetal haemoglobin comigrates with HbA_1 in this electrophoretic assay and so is included in the HbA_1 result.[98] Chapter 4 showed that this may not only lead to spuriously raised HbA_1 values in some patients but an increase in the imprecision (and therefore reference range) of the assay as a whole.

In conclusion, this study has found marked differences in the classification of glycaemic control in diabetic patients when using HbA_1 measurement rather than HbA_{1c}. In relation to the Diabetes Control and Complications Trial, this inconsistency may have considerable consequences for the long term well-being of diabetic patients and may also influence the allocation of resources towards their treatment. Therefore, these findings reinforce the need for more standardisation in the methods used for the measurement of glycated haemoglobin. The adoption of a standardised HbA_{1c} would allow the development of clear guidelines for clinicians based on both recent and subsequent complications trials- a sentiment echoed by representatives from the British Diabetic Association in response to the publication of this study.[87]

Much of the correspondence which followed this study described alternative approaches which may be taken to address the guideline shortcomings. The author's suggestion,[161] which was also common to a number of communications,[162,163] was to express patient glycated haemoglobin results as multiples of the non-diabetic mean value (MoM) in a situation analogous to that already used in Down's syndrome and neural tube defect screening. This proposal was based on the fact that assay imprecision only affects the spread of results, it does not affect either the mean glycated

haemoglobin value of a reference interval population or the median value of a diabetic clinic population. Thus, by eschewing the use of standard deviations, the effect of variations in glycated haemoglobin assay imprecision would be minimised.

Applying this proposal to the study in this chapter, the median diabetic HbA_1 value using HPLC was 1.46MoM i.e. 46% greater than the non-diabetic mean value, which is similar to the value of 1.48MoM found when using electrophoresis. However, this method of comparison still leads to discrepancies when comparing HbA_1 with HbA_{1c}. Presumably because HbA_{1c} concentrations rise faster in diabetic patients than HbA_1, the median HbA_{1c} value implied poorer glycaemic control at 1.57MoM. As a guide, the intensively treated group in the Diabetes Control and Complications Trial had a median HbA_{1c} value of approximately 1.40MoM while that of the conventionally treated group was 1.80MoM.

Thus, whilst comparisons of either 2 HbA_1 or 2 HbA_{1c} methods by MoM's may be valid, the situation remains that comparing HbA_1 with HbA_{1c} can be problematic.

Chapter 7

The Effects of Ageing on Glycation and the Interpretation of Glycaemic Control in Type 2 Diabetes.

7.1 Introduction

We have shown some of the limitations that exist when attempting to classify glycaemic control in diabetic patients using glycated haemoglobin, primarily as a result of lack of standardisation in measurement (see Chapters 4 to 6). One of the proposed advantages of the serum fructosamine assay was that commercial quality control sera could be used to standardise this method of glycaemic control assessment and thus ensure comparable results from different laboratories.[47] The reasons as to why serum fructosamine has not therefore become generally accepted in preference to glycated haemoglobin are probably diverse but are likely to include its perceived lack of specificity for protein glycation[164] and, most tellingly, its lack of consistent correlation with glycated haemoglobin measurements.[40,42,43] Therefore glycated haemoglobin remains the most popular measure of prior glucose control, despite the inability to effectively compare results between centres.

The attempts to compare glycated haemoglobin results from different assays include the European guidelines and the 'multiples of the mean' proposal outlined in Chapter 6. Both approaches put great emphasis on the establishment of an accurate assay reference interval. Since locally derived reference intervals are traditionally determined using young, healthy hospital staff, any age variation in non-diabetic glycated haemoglobin may render comparison to older diabetic patients inappropriate. This study aimed to determine if non-diabetic glycated haemoglobin and fructosamine values vary with the age of the subjects chosen.

7.2 Methods

7.2.1 Patients

Two hundred and thirty two non-diabetic individuals (95 male, 137 female, median age 47, range 16-74, fasting plasma glucose < 6.4 mmol/L [165,166]) took

part in the study. These subjects comprised hospital staff and individuals attending a clinic for lipid assessment. The clinic patients were subsequently confirmed as having repeated fasting glucose values less than 6.4 mmol/L.

One hundred and twenty eight Type 2 diabetic patients (median age 63, range 50-75, median HbA_{1c} 6.4%) attending the Diabetic Clinic, Gartnavel General Hospital, Glasgow also participated.

7.2.2 Study Design

HbA_{1c} was measured by HPLC (Hi-AutoA1c, model 8121, Biomen UK Ltd) in all non-diabetic subjects. In a subgroup of 126 of these individuals (54 male, 72 female, median age 57 years, range 23-74), serum fructosamine (Fructosamine Plus, Roche Diagnostics), serum albumin and body mass index (BMI) were also measured.

The diabetic patients were classified according to amended European guidelines into good (HbA_{1c} value <3 SD from non-diabetic mean value), borderline (3-5SD) and poor (>5SD) glycaemic control using reference intervals derived from both an age matched (n=101, median age 63 years, range 50-75) and a younger population (n=108, median age 37 years, range 25-50).

Patients were also classified using 'multiples of the mean' (MoM) where the median clinic HbA_{1c} value is expressed as a proportion of the non-diabetic mean value (see Chapter 6.4). Both the mean non-diabetic values from the age matched subjects and younger subjects were used.

7.2.3 Statistical Analysis

Statistical analysis used the McNemar test for paired samples. Correlation coefficients were calculated by the least squares method. Regression slopes and intercepts were compared using t-tests.

7.3 Results

7.3.1 HbA_{1c}, Fructosamine and Age

A linear relationship between HbA_{1c} and age was obtained in the 232 non-diabetic subjects ($r=0.49$, $p<0.0001$). There was no significant difference between males and females in the regression line slope (0.0108 ± 0.00262 SEM vs. 0.0132 ± 0.00178 respectively) or intercept (3.631 ± 0.122 vs. 3.538 ± 0.090 respectively). Mean HbA_{1c} rose from 3.82% to 4.44% between the ages of 20 and 70 (Figure 7.1).

In the subgroup of 126 non-diabetic subjects, the rise in HbA_{1c} with age ($r=0.49$) was not reflected by similar increases in serum fructosamine concentrations (Figure 7.2) or fasting glucose measurements (Figure 7.3) ($r=0.07$, $r=0.009$ respectively, p=NS). Serum albumin concentrations (median 44g/L, range 35-52) and body mass indexes (median 25.5kg/m^2, range 18.2-35.2) showed no linear relationship to age in this sample ($p>0.05$). The fructosamine/albumin ratio was also unrelated to age ($p>0.05$).

7.3.2 Glycaemic Control Classification

As a consequence of greater HbA_{1c} values in elderly non-diabetic individuals, the reference interval derived from non-diabetic subjects age matched to the Type 2 patients was higher (mean HbA_{1c} 4.31%, SD 0.37%) than that of the younger age group (mean 4.03%, SD 0.30%). Therefore, according to European guidelines, fewer diabetic patients were in good control (14% vs. 25%) and more in poor control (73% vs. 53%) when the younger reference interval was used (both $p<0.05$ by McNemar tests, Table 7.1).

When using 'multiples of the mean', the same diabetic patients were 1.48MoM using the age matched reference interval and 1.59MoM using that derived from younger subjects.

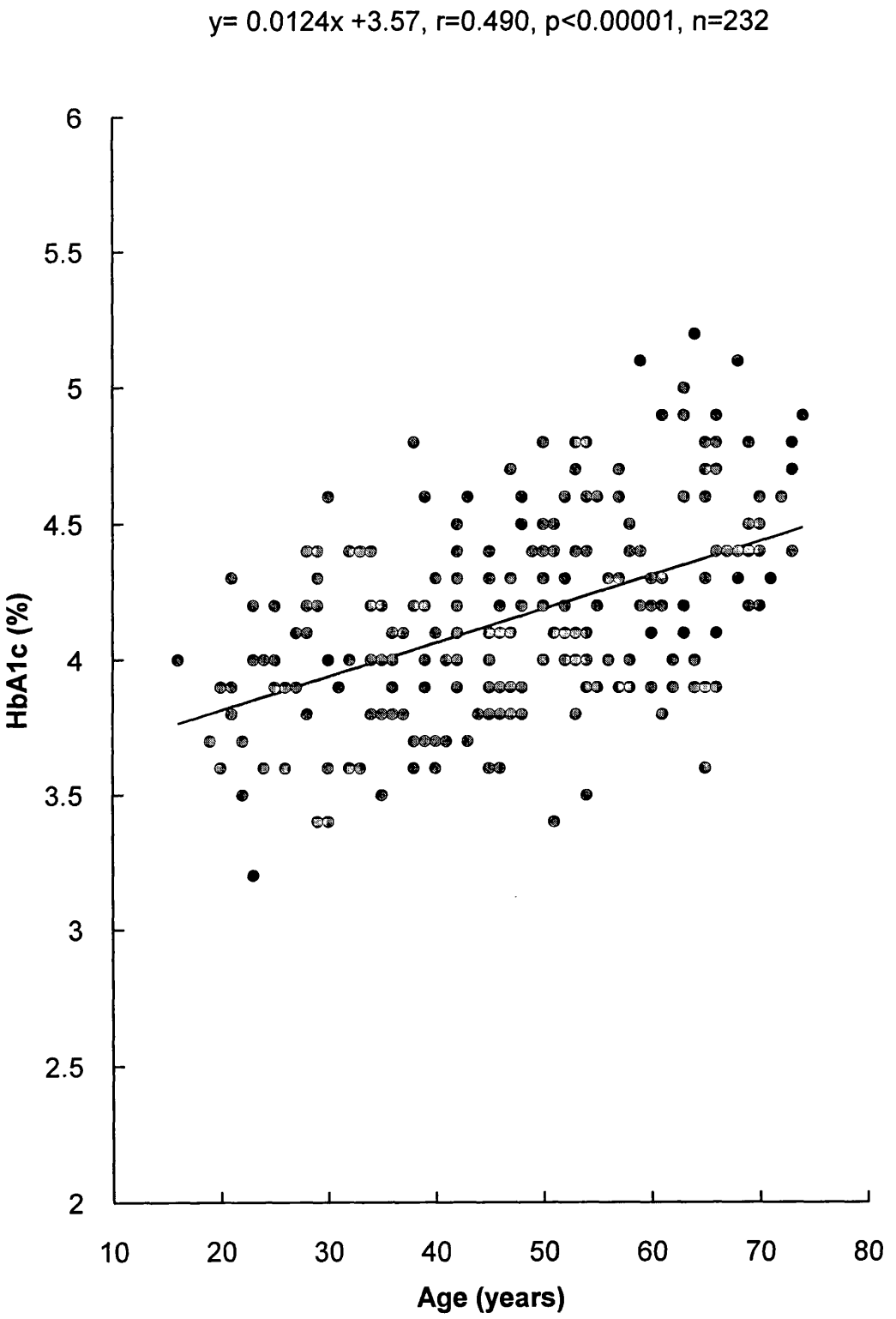


Figure 7.1 HbA_{1c} vs. age in non-diabetic subjects.

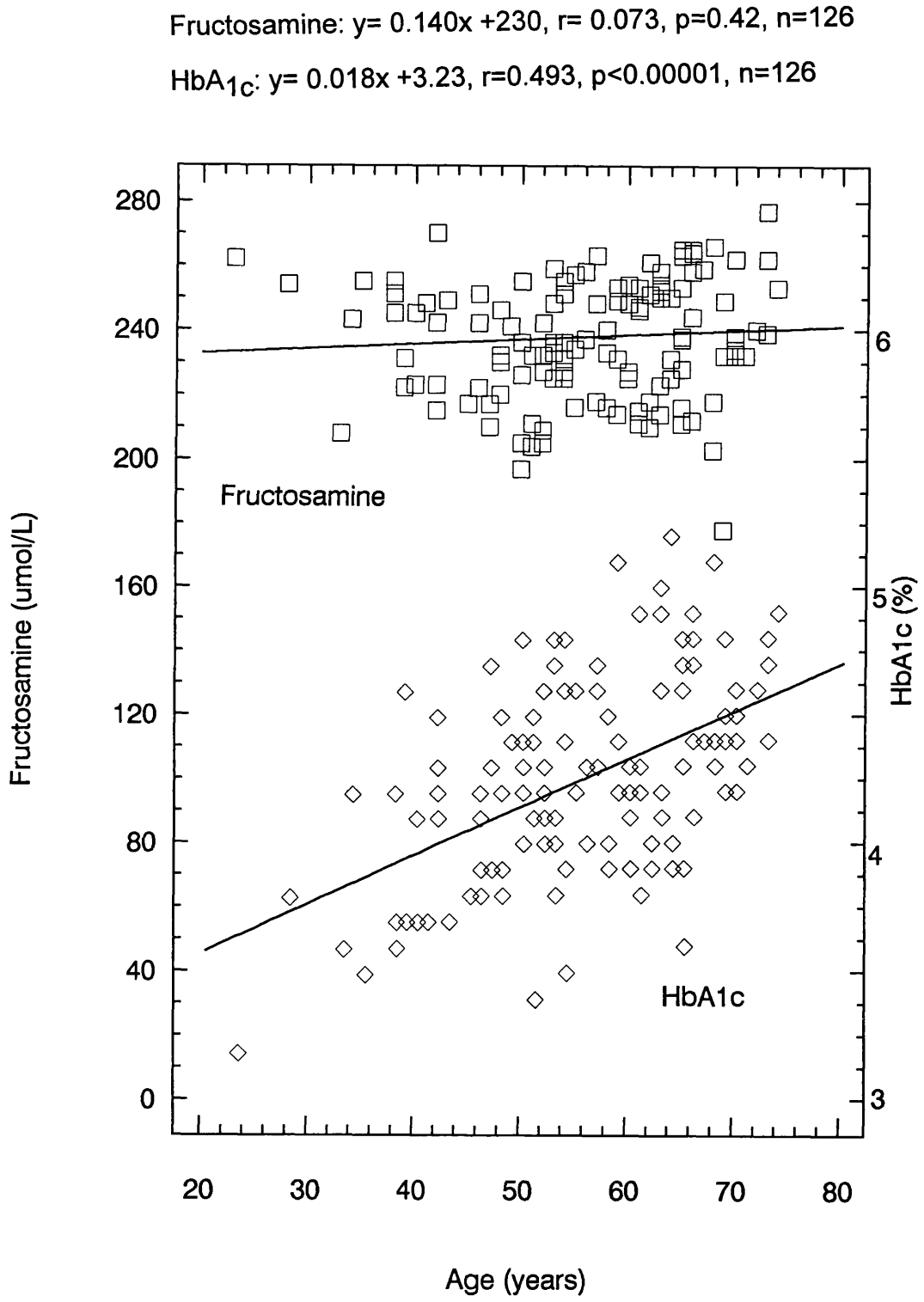


Figure 7.2 Serum fructosamine (□) and HbA_{1c} (◊) vs. age in non-diabetic subjects.

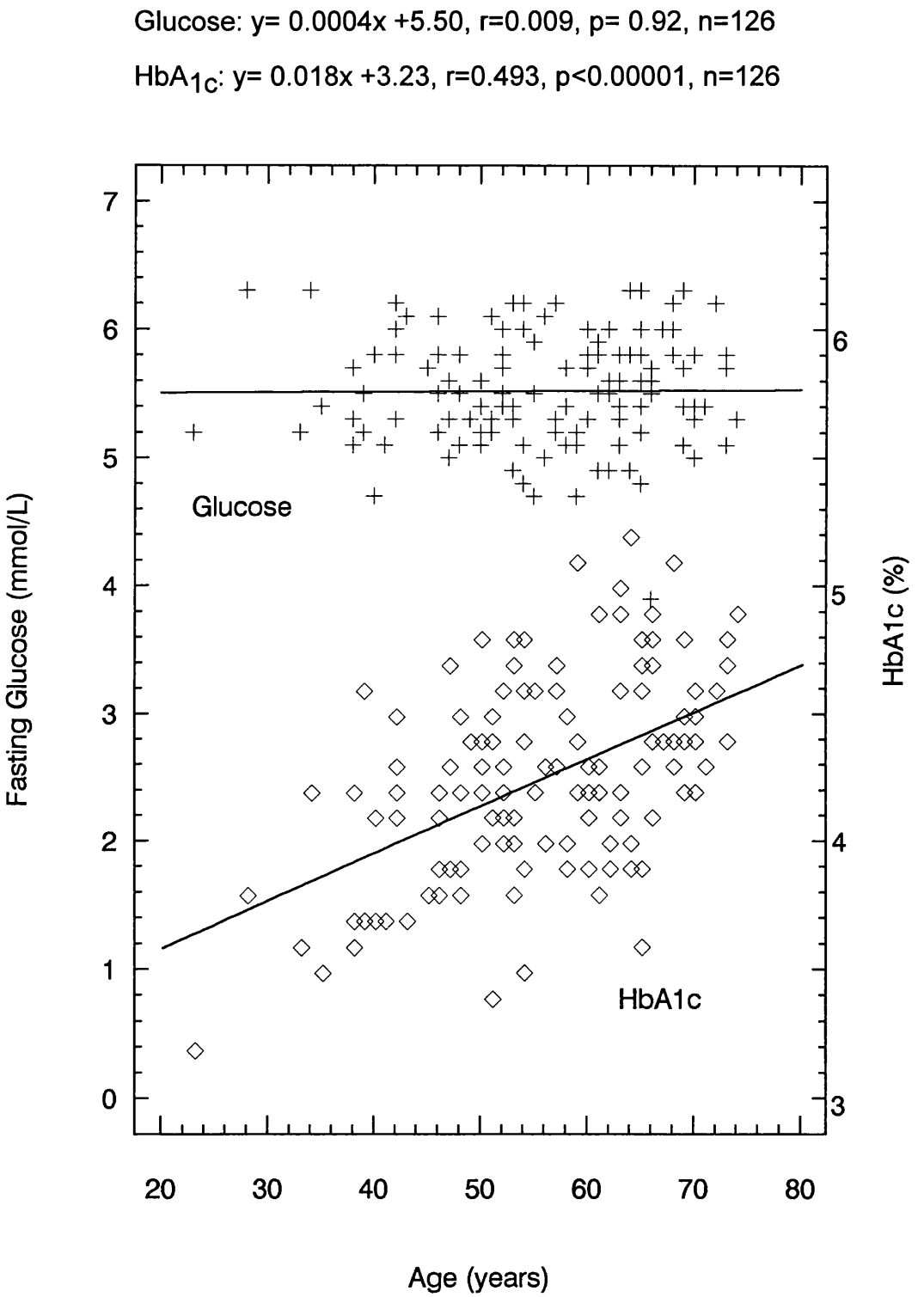


Figure 7.3 Fasting plasma glucose (+) and HbA_{1c} (◊) vs. age in non-diabetic subjects.

Non-Diabetic Controls	Glycaemic Control Category		
	Good (<3SD from non-diabetic mean)	Acceptable (3-5SD)	Poor (>5SD)
Age Matched (50-75yrs)	32 (25%)	28 (22%)	68 (53%)
Younger (25-50yrs)	18* (14%)	17 (13%)	93** (73%)

*$p<0.05$ and **$p<0.01$ vs. age matched controls.

Table 7.1 Classification of glycaemic control in 126 type 2 diabetic patients using age matched and younger controls.

7.4 Discussion

This study has shown that non-diabetic HbA_{1c} values increase with subject age which in turn may affect the assessment of glycaemic control in diabetic patients. By using a reference interval derived from young individuals, more Type 2 diabetic patients appeared in poorer glycaemic control when using European guidelines than if an age matched non-diabetic population was used (73% vs. 53%). The use of 'multiples of the mean' as a method of comparing glycated haemoglobin values from different assays was also affected. Again, the younger controls made the diabetic patients appear more poorly controlled. Indeed, the discrepancy (1.59 vs. 1.48MoM) was similar in magnitude to that found when comparing HbA_{1c} with HbA_1 in Chapter 6. Therefore, to take account of these problems, age related reference ranges may need to be used when interpreting HbA_{1c} results.

This report has also shown clear differences when comparing HbA_{1c} with fructosamine in non-diabetic subjects of varying ages. Some of this inconsistency can be explained by the fact that HbA_{1c} reflects glycaemic control over the preceding 6-8 weeks compared with 1-3 weeks for fructosamine.[14,71] Fructosamine measurements can also be influenced by other factors such as the serum albumin concentration and body mass index (BMI) of the patient (see Chapter 2.3). However, despite the subjects in this study having similar serum albumin concentrations / BMI's and presumably being in stable glucose control, HbA_{1c} values increased with subject age whereas fructosamine did not.

The reasons for this discrepancy remain speculative. Like other studies,[167,168] this investigation showed little change in fasting plasma glucose with increasing age which, at first glance, would appear to indicate that serum fructosamine is the more representative marker of glucose control. However, this deduction does not take account of the larger glycaemic excursions which are likely to occur post-prandially in elderly individuals.[167] As such, it remains

difficult to establish whether it is HbA_{1c} or fructosamine that is most accurately reflecting glycaemia in these subjects.

It has been suggested that degrees of glucose intolerance may explain only one third of the variance of glycated haemoglobin levels in non-diabetic subjects.[169,170] Thus, another possible explanation for our HbA_{1c} findings would be if a factor which changes with increasing subject age also affected HbA_{1c} values. For example, if red blood cell lifespan was longer in elderly subjects, this would allow greater glycation of their haemoglobin. In fact, it would seem that erythrocyte lifespan paradoxically shortens with increasing subject age.[171]

Our HbA_{1c} results are consistent with those of a previous more limited study of 48 non-diabetic individuals where values were found to be higher in elderly patients[172] although, curiously, this was not the case in another study where total glycated haemoglobin was measured instead of HbA_{1c}.[168] Our fructosamine data are also in agreement with a European fructosamine workshop report which concluded that, above 16 years, age had little effect on serum fructosamine concentrations.[119] However, no previous study has evaluated both fructosamine and HbA_{1c} in the same subjects. These age differences, if applicable to diabetic patients, may be an additional reason for the discrepancy found when comparing HbA_{1c} with fructosamine.

These findings are of clinical relevance because, in contrast to fructosamine, HbA_{1c} reference intervals are likely to be influenced by the age of the subjects chosen and so may partly explain the diversity of locally derived reference intervals quoted when using the same glycated haemoglobin instrument.[173] In addition, if, as our data suggest, the glycated haemoglobin values from elderly subjects are closer to those of diabetic patients than younger individuals are, then it may also account for the finding that HbA_{1c} is a better screening test for diabetes in middle aged rather than elderly people.[19]

It would seem appropriate that clinicians should aim for their diabetic patients to have comparisons in glycated haemoglobin values made to those of their non-diabetic chronological peers. To this end, age related reference intervals may be required for glycated haemoglobin measurements to facilitate more accurate glycaemic control targets for patients and for better auditing of a clinic performance.

Chapter 8

Parallel Measurements of Fructosamine and Glycated Haemoglobin in Type 2 Diabetic Patients.

8.1 Introduction

As eluded to in Chapter 1.2.2, cross-sectional comparisons of serum fructosamine and glycated haemoglobin have revealed inconsistencies between the two measures. Some of the reasons for the discrepancies have already been described in Chapter 2.3, and include the effect of serum protein concentrations and obesity on the fructosamine assay. The studies in Chapters 4 and 7 have also demonstrated that comparisons may be influenced by both the choice of glycated haemoglobin analysis used and by age related changes in HbA_{1c} measurements.

Notwithstanding these causes of disagreement, it is likely that the major reason for disparity between the two assays is that fructosamine gives an indication of glycaemia over the preceding 1-3 weeks[71] while that of glycated haemoglobin is over a period of at least the prior 6-8 weeks.[14] In view of the different time periods they represent we postulated that the relationship between fructosamine and glycated haemoglobin may vary depending on whether glycaemic control was stable or undergoing change. In addition, if changes in serum fructosamine were to precede those of HbA_1, then the fructosamine/HbA_1 ratio could be useful as a prospective indicator of trends in HbA_1 in diabetic patients. Therefore, to resolve these issues, this study aimed to assess longitudinal changes in HbA_1 and fructosamine concentrations by observing them in a group of Type 2 diabetic patients immediately following treatment changes. Haemoglobin A_1 rather than HbA_{1c} was chosen as the form of glycated haemoglobin measurement because the study was performed at a time when this was the commonest means of measuring glycated haemoglobin before HbA_{1c} had gained widespread use in clinical practice.

8.2 Methods

8.2.1 Patients

Fourteen patients with Type 2 diabetes (9 males, 5 females, median age 56 years, range 36-72) were recruited from the Diabetic Outpatient Clinic. Six patients were newly diagnosed and eight were known diabetic patients with symptomatic hyperglycaemia over 4-6 weeks prior to attendance but with no evidence of intercurrent illness. All were free from diabetic complications and had normal renal function.

8.2.2 Study Design

In order to improve glycaemic control during the period of study, 2 patients were treated by dietary measures alone, 5 by diet plus and oral hypoglycaemic agents and a further 7 were commenced on insulin treatment, depending on previous diabetic status and treatment. Out of the 14 patients, 10 were followed for 16 weeks, 3 for 12 weeks and one patient terminated the study at week 8. Two patients had missing values for fasting blood glucose between weeks 2-4 of the study.

Whole blood was obtained for HbA_1 measurement by electrophoresis (Ciba Corning Ltd, UK) and the remainder centrifuged for the analysis of plasma glucose, serum fructosamine and serum albumin. Serum fructosamine was measured using the original fructosamine kit (Roche Diagnostica, UK) as described in Chapter 1.4.2. Fasting blood glucose was measured on a Beckman II glucose analyser (Beckman Ltd) and serum albumin (reference interval 35-45 g/L) on a SMAC II multi-channel analyser (Technicon Corporation, Basingstoke, UK).

8.2.3 Statistical Analysis

Statistical analysis was performed using the Student's t-test and Mann-Whitney U tests where appropriate. The correlation coefficients were calculated by the least squares method. Values were expressed as mean $\pm$ SD unless otherwise stated.

8.3 Results

8.3.1 Changes in Glycaemia

Figure 8.1 shows the changes in fructosamine, HbA_1 and fasting plasma glucose during the period of study. During the study there was no change in serum albumin concentration (41.6 $\pm$ 3.9 g/L initially and 43.1 $\pm$ 2.5 g/L at week 16). Mean fasting plasma glucose concentration fell significantly in each of the first 2 weeks and remained unchanged thereafter. There was a decrease in HbA_1 after 1 week and at 4 weeks and this continued to fall until 16 weeks. Fructosamine concentration decreased at 1, 2 and 4 weeks and remained unchanged thereafter.

8.3.2 Comparison of Fructosamine and HbA_1

The correlation between serum fructosamine and HbA_1 for all patients assessed in a cross-sectional manner at each of the eight visits over the 16 week period varied from $r= 0.13$ to $r= 0.87$ (Table 8.1) but only reached statistical significance ($p<0.05$) at week 12 ($p=0.018$) and week 16 ($p=0.001$). When the concentrations of fructosamine and HbA_1 were compared in individual patients over the 16 week period of study, significant correlations were found in 8 out of 14 patients (Table 8.2).

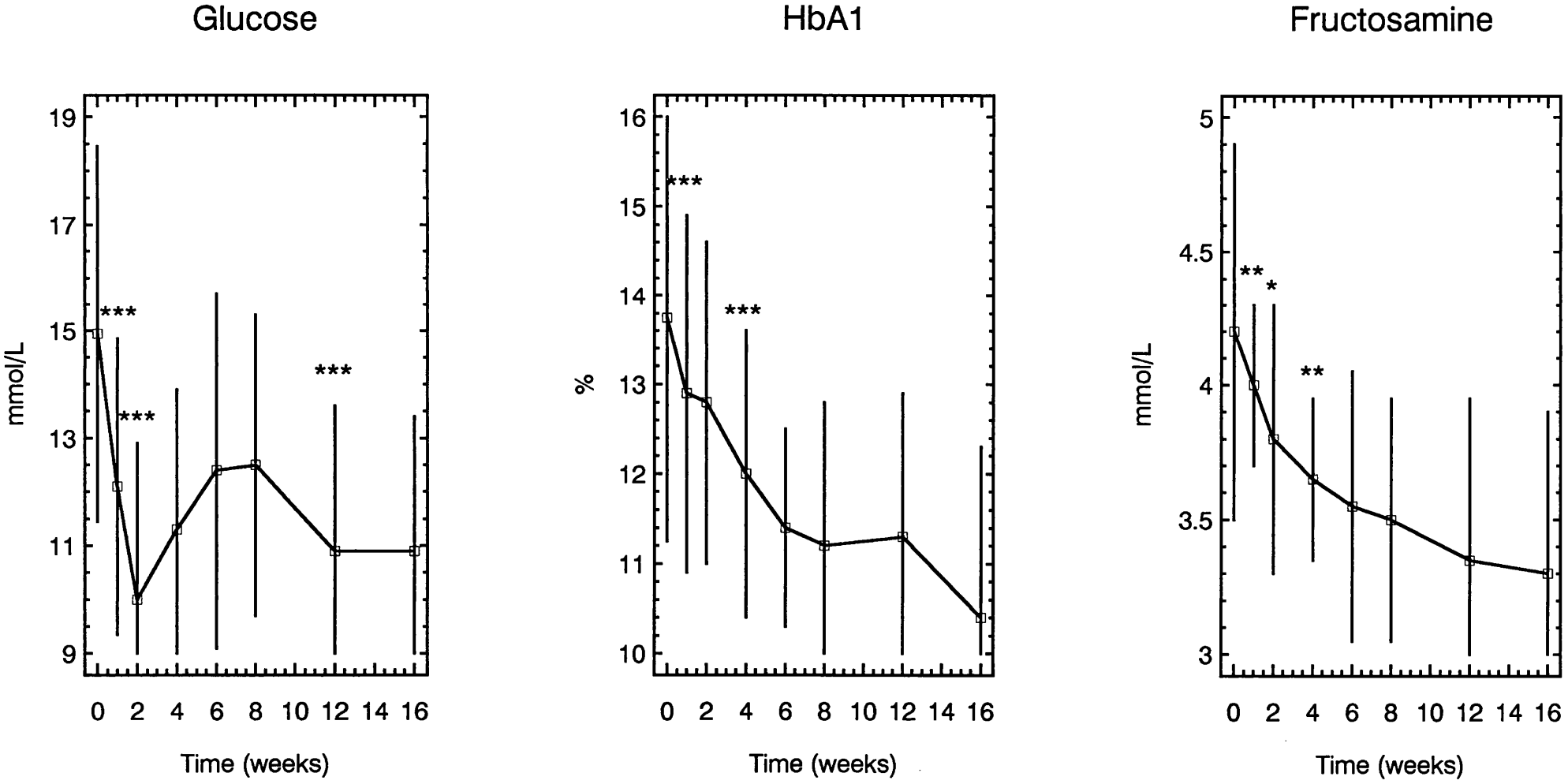


Figure 8.1 Plasma glucose, HbA_1, and serum fructosamine concentrations over 16 week monitoring period (mean±SD). * $P<0.05$, ** $p<0.02$, *** $p<0.01$ (each point compared to the analyte concentration at the previous visit).

Week	Correlation between Fructosamine and HbA_1	
	r	*P*
0	0.55	0.06
1	0.43	0.14
2	0.23	0.36
4	0.13	0.66
6	0.39	0.21
8	0.21	0.47
12	0.60	0.018
16	0.87	0.00092

Table 8.1 Correlation between HbA_1 and fructosamine at different time points in the group (n=14).

Patient Number	Correlation between Fructosamine and HbA_1	
	r	*P*
1	0.920	0.001
2	0.911	0.001
3	0.797	0.031
4	0.920	0.001
5	0.789	0.019
6	0.553	0.005
7	0.850	0.015
8	0.783	0.021
9	0.326	0.475
10	0.140	0.101
11	0.778	0.120
12	0.632	0.090
13	0.048	0.074
14	-0.121	0.818

Table 8.2 The relationship between serum fructosamine and HbA_1 in individual patients during 16 week monitoring period.

8.3.3 Fructosamine/ HbA_1 Ratio

The fructosamine/ HbA_1 ratio at a single time-point correlated with the change which occurred in the HbA_1 value the following month ($r=0.54$, $p<0.001$, Figure 8.2).

8.4 Discussion

There remains uncertainty as to the interpretation of the fructosamine concentrations in the diabetic clinic situation. The use of serum fructosamine as an indicator of glycaemia has been at least partly hindered by a lack of consistency found in cross-sectional comparisons with glycated haemoglobin.[34-43] Rather than dismiss fructosamine on this basis, this study has addressed the possible benefits that parallel measurement of fructosamine and HbA_1 may bring.

Few studies have evaluated longitudinal changes in serum fructosamine.[4,41] Those that have are either limited by sampling at clinic visits 5 months apart[41] or because detailed comparisons were not made with glycated haemoglobin.[4] Thus, the bulk of present data on the interrelationships between fructosamine and HbA_1 largely depend on cross-sectional analysis of groups of patients at a single time point.

The results presented in this chapter have shown that no one relationship exists between fructosamine and glycated haemoglobin: even amongst the same group of diabetic patients the correlation between fructosamine and HbA_1 values varied markedly (from $r= 0.87$ to $r= 0.13$) depending on whether the subjects were in stable or changing glycaemia. One of the best correlations occurred at week 0 when glycaemic control was poor but presumably stable, yet took 12 weeks following an acute change in glycaemia before becoming concordant again. This was because HbA_1 showed a sustained fall throughout the study period whereas fructosamine showed its most significant falls in the first month. Presumably this was a consequence of a long half-life protein

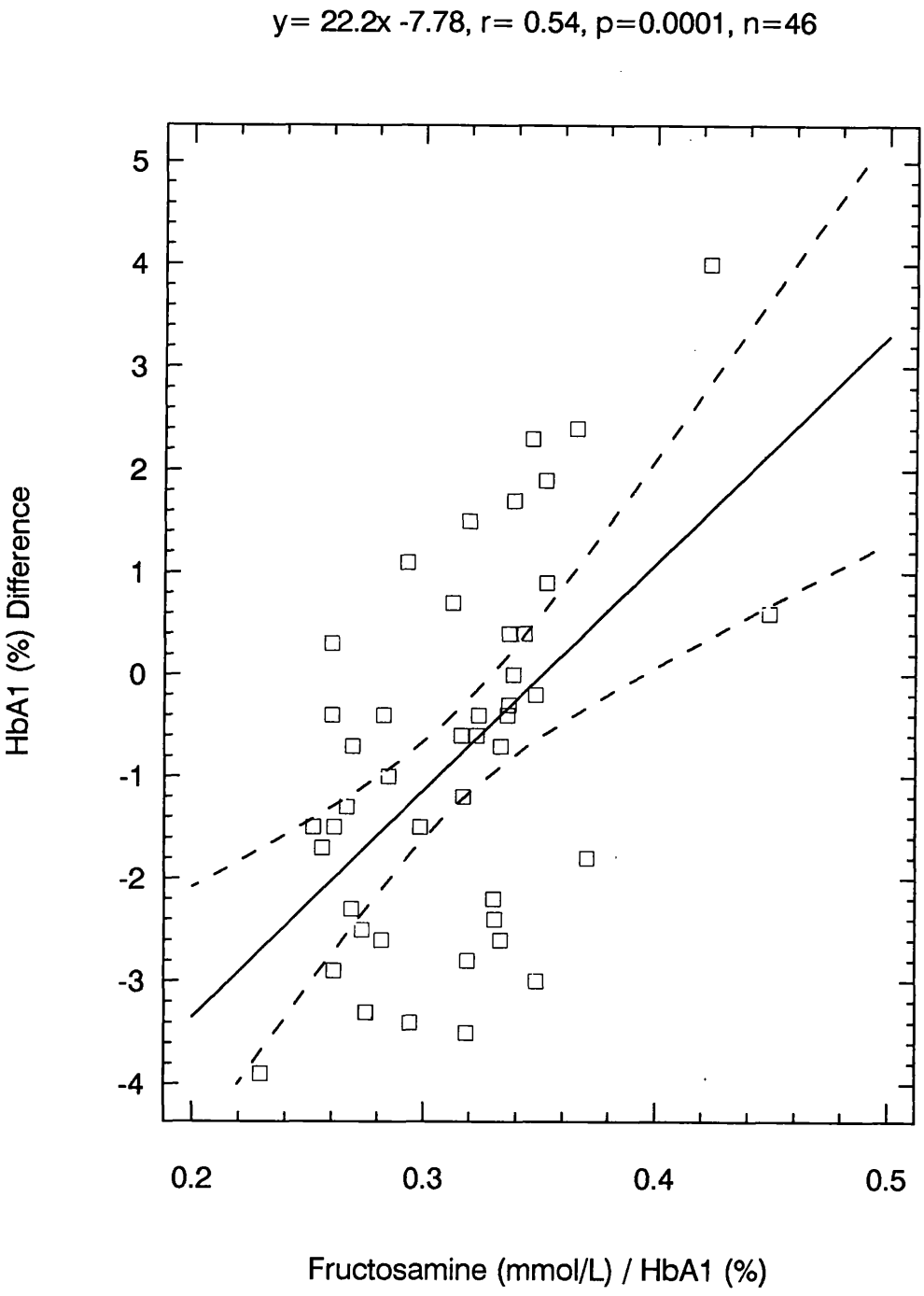


Figure 8.2 The correlation of the fructosamine/HbA1 ratio with change in HbA1 concentration over the following month.

(HbA_1) requiring a longer time to reach a new equilibrium than most proteins found in serum. These results are consistent with those of a previous study which showed fructosamine correlating with HbA_1 at admission to a diabetic day care unit (r=0.75), but not 2 weeks after discharge (r=0.29).[153]

It has previously been suggested that the parallel measurement of the two variables may provide a better insight into a patient's glycaemic control than the measurement of either HbA_1 or fructosamine alone.[40] The findings in this study have confirmed this by demonstrating that the fructosamine/ HbA_1 ratio can predict the changes in HbA_1 which occur at a patient's subsequent visit to the clinic. Thus, simultaneous measurement gives the clinician a patient's HbA_1 value together with an indication of whether it is improving or worsening. This may prove valuable if found to be applicable to pregnant diabetic patients or those on oral hypoglycaemics who are approaching the need for insulin treatment. From the findings in Chapter 4, it seems probable that measuring HbA_{1c} rather than HbA_1 will provide similar benefits.

In conclusion, this study has found that in Type 2 diabetic patients the different time period of glycaemia reflected by fructosamine and HbA_1 is a major contributor to discrepancies found when comparing the two methods. However, knowledge of this fact means that simultaneous measurement of fructosamine and HbA_1 can give valuable information on future trends in HbA_1 values.

Since the publication of this study, no further studies have reported on the longitudinal changes found when measuring fructosamine and glycated haemoglobin. The use of the fructosamine/ HbA_1 ratio has not become widespread, but at least one manufacturer of equipment and reagents (Boehringer Mannheim) advocates its use and have adapted their instruments for simultaneous measurement of the two analytes.

Chapter 9

The Effects of Variations in Haematocrit, Mean Cell Volume and Red Blood Cell Count on Reagent Strip Tests for Glucose.

9.1 Introduction

Throughout the 1980's measurement of blood glucose, other than that for diagnosis, showed a steady move away from laboratories into the hands of diabetic patients and health care workers. The use of glucose test strips and meters became commonplace not only amongst diabetic patients in the community but also for the monitoring of acutely ill hospital patients. The latter was illustrated by the fact that in 1994 over three ward glucose measurements were being performed in the author's hospital unit for every sample sent to the biochemistry laboratory.

Use of test strips in a hospital setting requires that they can give accurate results under a wide variety of physiological conditions. This and subsequent chapters in the thesis describe limitations in the performance of several popular glucose test strips and meters when a range of pathological specimens are used.

By 1992, *in vitro* variations in sample haematocrit had consistently been shown to be a source of error in several of the most commonly used reagent strip tests for glucose (see Chapter 2.4.3).[137-140] In general, blood glucose measurements by these methods usually varied inversely with increasing sample haematocrit. Thus, patients with abnormally low haematocrits had spuriously high test strip measurements and *vice versa.*

The author's recognition of this problem was stimulated by the case of a 72 year old man who had been admitted to the hospital's general medical wards with an exacerbation of chronic obstructive airways disease. The biochemistry laboratory was contacted because 'Glucostix' test strip and meter blood glucose measurements were consistently giving measurements of around 8 mmol/L while simultaneous venous samples sent to the laboratory were in excess of 20 mmol/L. Serum osmolality measurements indicated that the patient was likely to be truly hyperglycaemic. It transpired that the patient had a haematocrit value of 65% because of secondary polycythaemia and that this was causing spurious reductions in test strip glucose measurements.

Despite cases such as this, recognition of the influence of haematocrit amongst clinicians and nursing staff was not widely appreciated and many glucose strip manufacturers had not yet developed products which addressed this shortcoming. The study in this chapter was conducted around this time to assess the effect of other haematological indices on glucose test strip measurement.

The haematocrit of a sample is not a single entity, but is dependent on two other haematological parameters, namely, the red blood cell count (RBC) and the mean cell volume (MCV) of a sample. They are related as follows:

$$\text{Haematocrit} = \text{RBC} \times \text{MCV}$$

Consequently, patients with variable MCV may have the same haematocrit but widely differing red cell counts (Figure 9.1). The corollary of this is that studies which use normal subjects (presumably with similar MCVs) make it impossible to distinguish if the test strip error is due to haematocrit variations *per se* or actually due to changes in the number of red cells. Therefore, the study in this chapter aimed to ascertain how variations in haematocrit, RBC and MCV affect strip glucose measurement in 3 commonly used glucose meters.

9.2 Methods

9.2.1 Patients

Samples were obtained from 3 groups, each comprising of 3 non-diabetic males: The first group consisted of subjects with normal mean cell volumes (mean 88.9[range 88.2-89.7]fL), while 2 further groups comprised individuals with low (mean 70.4[range 67.5-72.5]fL) and high (mean 112.6[range 109.8-115.9]fL) MCVs.

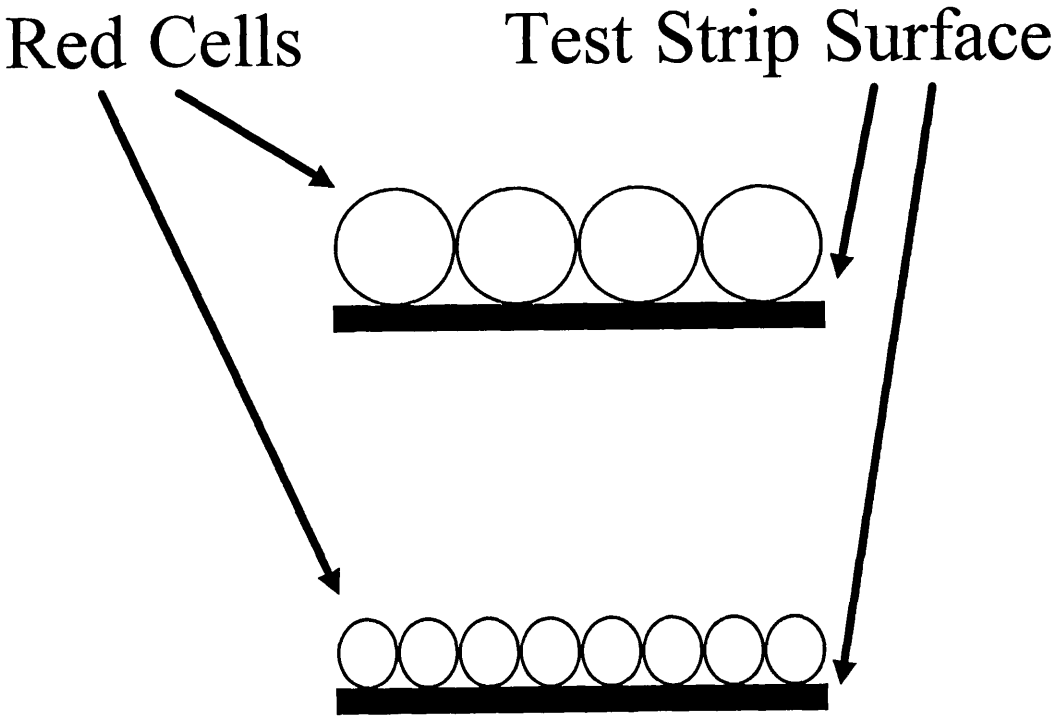


Figure 9.1 Stylised representation of 2 blood samples with the same haematocrit, but differing red cell counts.

9.2.2 Study Design

Blood was collected in K_2EDTA and had haematocrit, RBC and MCV measured using a Coulter S+4 analyser (Coulter Corp, Harpenden, Herfordshire, UK) before spiking with 10% glucose to produce samples with a mean plasma glucose of 12.1mmol/L. The reference interval for MCV using this analyser was 80-100fL.

The whole blood was centrifuged and homologous plasma either removed or added to produce samples with haematocrits from 20% to 60% in 10% increments.

Whole blood glucose for each sample was measured using the Glucometer Gx, the Reflolux IIM and the ExacTech credit card sensor. Plasma glucose was measured on a 'Dimension' analyser.

All whole blood and plasma samples for each subject were analysed within 30 minutes of one another and all measurements accounted for the dilutional effect of spiking.

9.2.3 Statistical Analysis

Simple and multiple regression was performed by the least squares method and comparisons of samples used the Mann-Whitney U test.

9.3 Results

9.3.1 Haematocrit and Glucose Meter Measurement

Glucose measurements were related to haematocrit in all three meters. Every 10% increase in haematocrit led to Reflolux glucose measurements falling by 6% ($r= -0.86$), ExacTech by 15% ($r= -0.87$) and Glucometer by 19% ($r= -0.91$).

9.3.2 Red Blood Cell Count and Glucose Meter Measurement

The variation of glucose strip accuracy (as a percentage of the plasma glucose measurement) with haematocrit was not significantly different between MCV groups in any of the meters.

Consequently, glucose strip accuracy correlated less well with red blood cell count than with haematocrit (r= -0.83 vs. r= -0.86 (Reflolux), r= -0.73 vs. -0.87 (ExacTech) and r= -0.74 vs. -0.91 (Glucometer)). Figure 9.2 demonstrates this for the Glucometer Gx. It shows the accuracy of this meter at a given red cell count differing according to MCV, but variations of accuracy with haematocrit to be independent of red cell size i.e. so long as the same area of a test strip is covered by red cells, identical glucose results will be obtained, regardless of whether this area is composed of a large number of small cells or a small number of large cells.

Multiple regression showed RBC measurement did not add significantly to the relationship between glucose strip error and haematocrit.

9.4 Discussion

Monitoring blood glucose in a hospital environment may commonly include patients with low or high haematocrits and low or high mean cell volumes. Low haematocrits can often be found in patients with blood loss or haematological disorders while high haematocrits may occur in polycythaemic and dehydrated subjects. Discrepancies between haematocrit and red cell count can occur in patients with anaemia because although all types exhibit a low haematocrit they may have either low, normal or high mean cell volumes depending on the aetiology. Neonates are a special case: even in health they have high haematocrits (55-65%) compared with adults but this is due more to having relatively high MCVs (109-128fL) than to any increase in red cell number.[174] For these reasons, it is important to the know whether a patient's

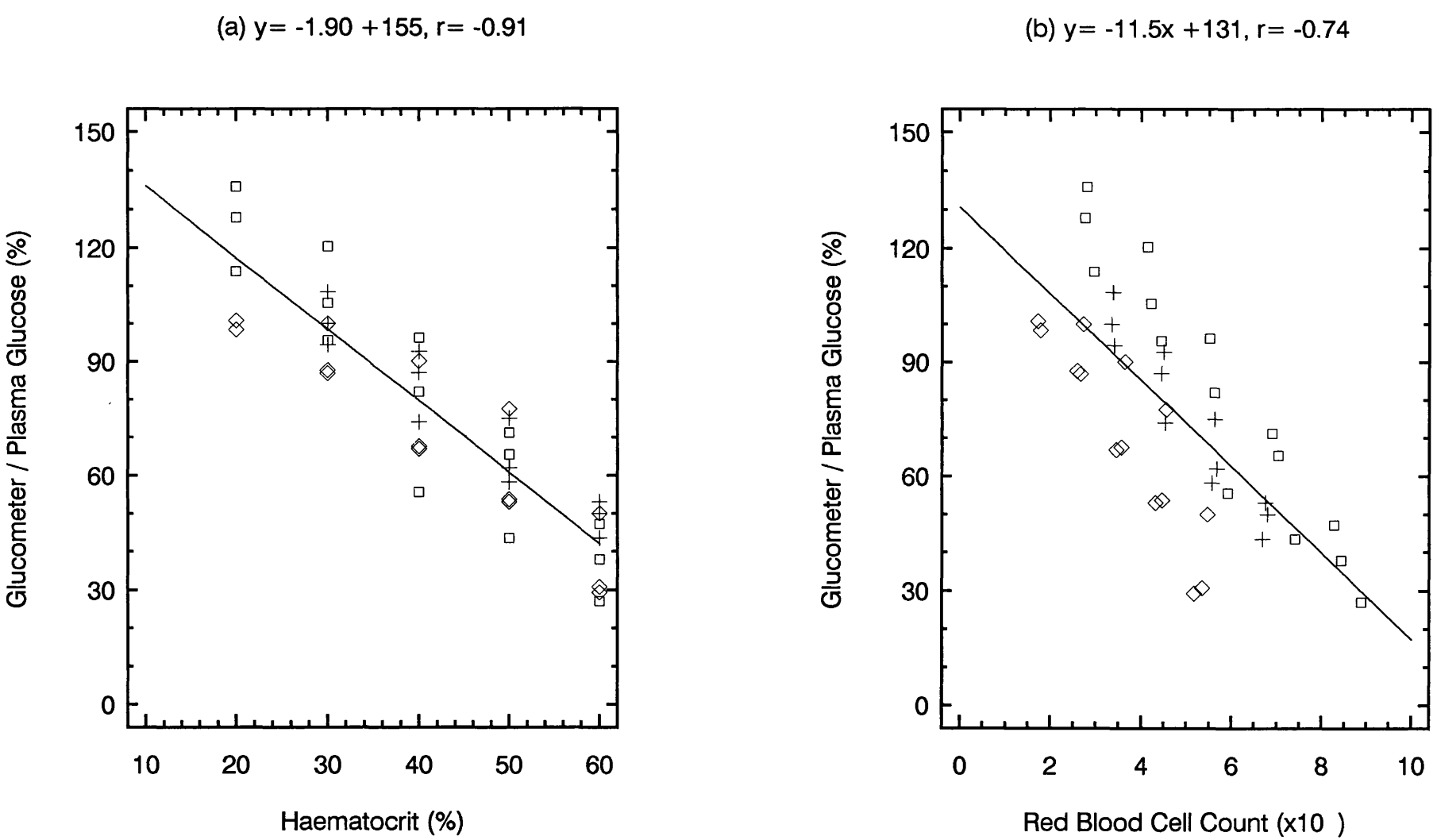


Figure 9.2 Effect of (a) sample haematocrit and (b) red blood cell count on Glucometer II blood glucose meter accuracy in subjects with mean MCV= 70.4 fL (□), 88.9fL (+) and 112.6fL (◊).

glucose meter result is likely to be affected either by their haematocrit or their red blood cell count *per se*.

This study has shown that three of the most commonly used meters in the United Kingdom exhibit changes in accuracy with sample haematocrit. In results consistent with a previous study,[138] the Reflolux IIM was found to be least affected, the ExacTech intermediate, and the Glucometer Gx worst.

One of the reasons postulated for high haematocrit samples affecting glucose strip accuracy is that the greater number of red cells causes a reduction in flow of plasma into the reagent pad, resulting in less glucose being available for reaction.[129] If red cells do indeed block 'holes' in the Glucometer and Reflolux strip matrices, then it might be expected that the main influence on meter measurement would be the red cell count rather than the haematocrit of a sample. But if meter accuracy is determined solely by red cell count then it should be independent of red cell size. The fact that it was not, and that haematocrit was, would suggest that the latter is the true source of test strip error.

Unlike the Glucometer and Reflolux, the ExacTech meter measures blood glucose via the electrical current produced by the oxidation of glucose through an electron mediator (see Chapter 1.4.3). Even with this system it also seems likely that variations in haematocrit, rather than red cell number, are responsible for the changes in this meter's accuracy.

The reasons for the differences in meter performance remain speculative. It may be partly explained by the Reflolux IIM meter allowing 2 minutes before measurement rather than the 50 seconds taken by the Glucometer Gx. The longer duration would allow more time for the passage of plasma to the reagent layer regardless of sample haematocrit. Although using a completely different measuring technique, variations in haematocrit presumably change the resistance of the ExacTech test strip to current flow.

In conclusion, these findings suggest that it is the simple proportion of a test strip covered by red cells, not their number, that is the main haematological determinant of glucose meter accuracy.

Chapter 10

The Effect of Haematocrit on Intraoperative Blood Glucose Measurement.

10.1 Introduction

Chapter 9 confirmed that *in vitro* changes in haematocrit are responsible for errors in test strip glucose measurement and that this effect is independent of the component red blood cell count and mean cell volume. However, at the time of that study *in vivo* confirmation of these experimental findings remained limited. Only a cross-sectional survey of renal diabetic patients had shown any evidence to support the *in vitro* studies.[139] No data existed on the possible effect of haematocrit variations within the same individual. This chapter presents the results of a study designed to investigate this.

Patients undergoing the procedure of cardiopulmonary bypass routinely show a marked reduction in their haematocrit, which potentially provides a good model for observing changes in glucose meter measurement. This study ascertained whether these intraoperative haematocrit changes could lead to spurious test strip glucose measurements *in vivo* when using three of the most commonly used contemporary blood glucose meters.

10.2 Methods

10.2.1 Patients

Ten patients (7 male, 3 female median age 63 years (range 33-79)) who were undergoing cardiopulmonary bypass at the Western Infirmary, Glasgow, were recruited into the study. None were known to have pre-existing diabetes. Six patients had coronary artery vein grafts, three aortic valve and one a mitral valve replacement.

10.2.2 Study Design

All patients were premedicated with either temazepam or lorazepam. In addition, seven patients were given omnopon and scopolamine intramuscularly. Anaesthesia was induced with a combination of midazolam,

etomidate and fentanyl. In two cases, only the latter anaesthetic agent was used. Muscle relaxation was achieved using an appropriate dose of pancuronium. Maintenance of anaesthesia involved a combination of fentanyl, nitrous oxide and enflurane in 8 patients and fentanyl/propofol in the remaining two. Six patients received intraoperative dopamine (2.5-3.5 μg/kg/min) and a further 2 required 3mg and 8mg of methoxamine.

Cardiopulmonary bypass was performed using a standard technique. This involved a pump prime of two litres of Hartmanns solution to which was added 8,000 units of heparin, 15 mmol of potassium chloride, 10g of mannitol, 50 mmol of sodium bicarbonate and 750mg of cefuroxime.

Blood samples were obtained before bypass and every 30 minutes following for the next two hours. Specimens were collected into a syringe with no anticoagulant via an arterial cannula. Each sample was analysed immediately by properly calibrated Glucometer II, One Touch II and Reflolux IIM meters. The blood samples were also decanted into fluoride oxalate vacutainers for the measurement of whole blood glucose by a reference method using a YSI Model 23 AM analyser. All reference samples were analysed within six hours of collection. Haematocrit was measured on a Corning 288 analyser (Ciba Corning Diagnostics, Halsted, Essex, UK).

10.2.3 Statistical Analysis

Meter accuracy at varying haematocrits was expressed as a percentage of the reference instrument values. Linear regression was by the least squares method. Method comparisons between all meter measurements and the reference instrument was by the Bland Altman residual plot method.[175] Bland Altman is a means of comparing a new method with that of an established or reference one. The differences between methods (the residuals) are plotted against the mean of the two results (for example, see Figure 13.1). Ninety five percent of samples are included by the mean residual value ± 1.96SD. If 95% of results

with the new method are deemed to be acceptably close to the existing method, then the new method can be adopted.

10.3 Results

10.3.1 Intraoperative Changes in Haematocrit

Table 10.1 shows the variation in haematocrit and reference blood glucose which occurred in the 10 patients during the cardiopulmonary bypass procedure.

10.3.2 Meter Accuracy and Changes in Haematocrit

Figures 10.1, 10.2 and 10.3 demonstrate the change in meter accuracy (as a percentage of the reference YSI glucose) with variations in haematocrit for the Glucometer II, One Touch II and Reflolux II meters respectively. Each figure is depicted in the same scale.

For every 10% fall in haematocrit, Glucometer II measurements rose by 22% and One Touch II measurements fell by 4%. The accuracy of the Reflolux II showed no significant relationship to changes in sample haematocrit.

10.3.3 Overall Meter Accuracy

The mean glucose during the study was 5.80 mmol/L. Using Bland Altman residual comparisons between the meters and the reference instrument, the One Touch II meter showed an overall mean bias of 0.3 mmol/L, with 95% of samples falling within +0.86 mmol/L and -0.26 mmol/L of the YSI analyser. The mean bias of the Reflolux II was 1.58 mmol/L (95% within +3.40 mmol/L and -0.24 mmol/L) and for the Glucometer II this was 3.25 mmol/L (95% within +6.18 mmol/L and +0.32 mmol/L).

The One Touch II also showed the best overall correlation with the reference analyser ($r=0.971$, $y=0.889x+0.94$). Although the Glucometer II

Sample Time (Minutes)	0	30	60	90	120
Median (Range)					
Haematocrit (%)	37 (28-42)	23.5 (18-27)	22.5 (18-31)	21.0 (19-31)	20.5 (17-30)
Glucose (mmol/L)	4.7 (4.2-6.5)	5.0 (4.3-6.2)	5.9 (4.4-7.1)	6.5 (4.7-8.9)	6.7 (5.6-8.6)

Table 10.1 Haematocrit and reference blood glucose concentrations at 30 minute intervals from commencement of cardiopulmonary bypass (n=10).

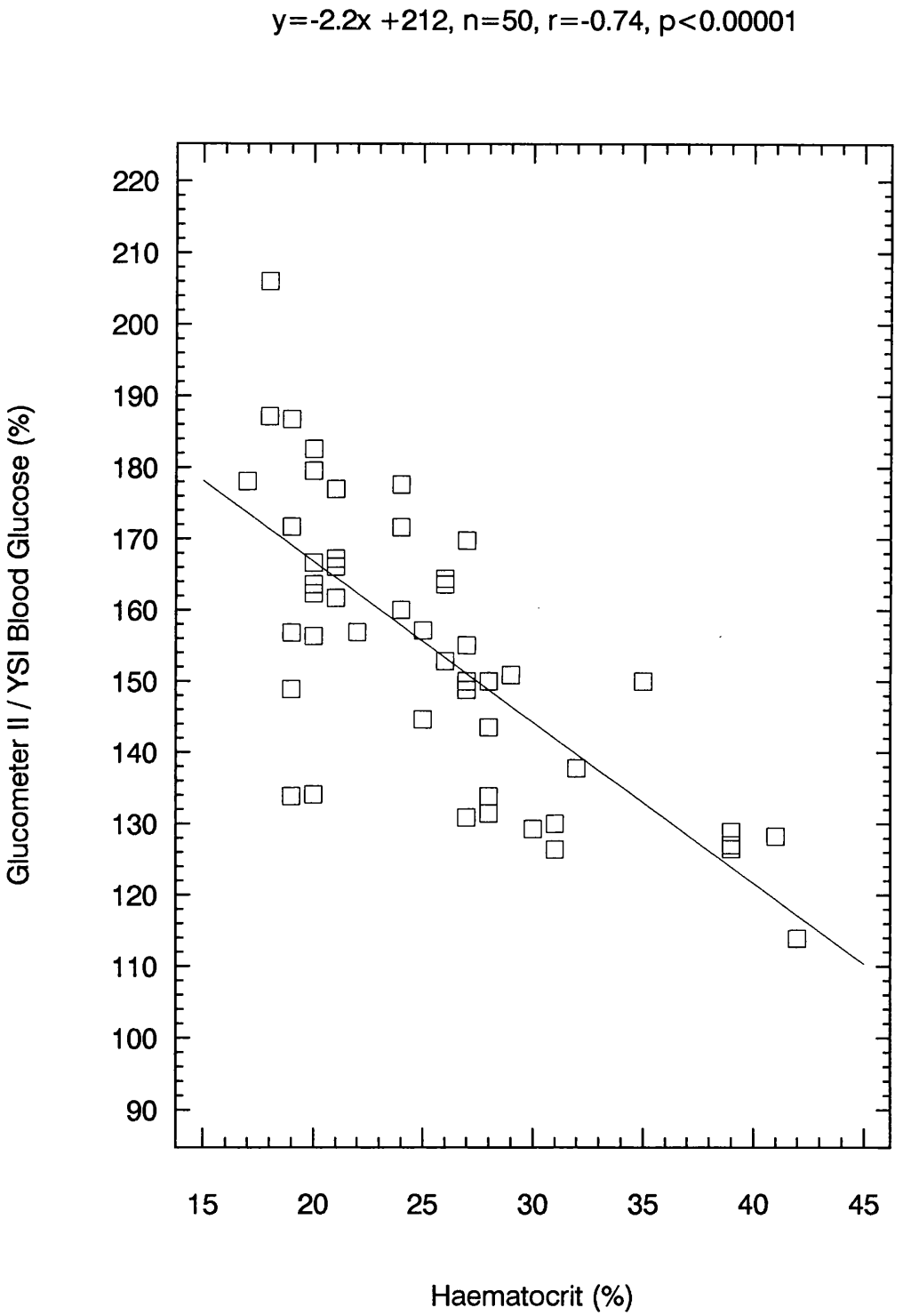


Figure 10.1 Effect of sample haematocrit on Glucometer II blood glucose meter accuracy.

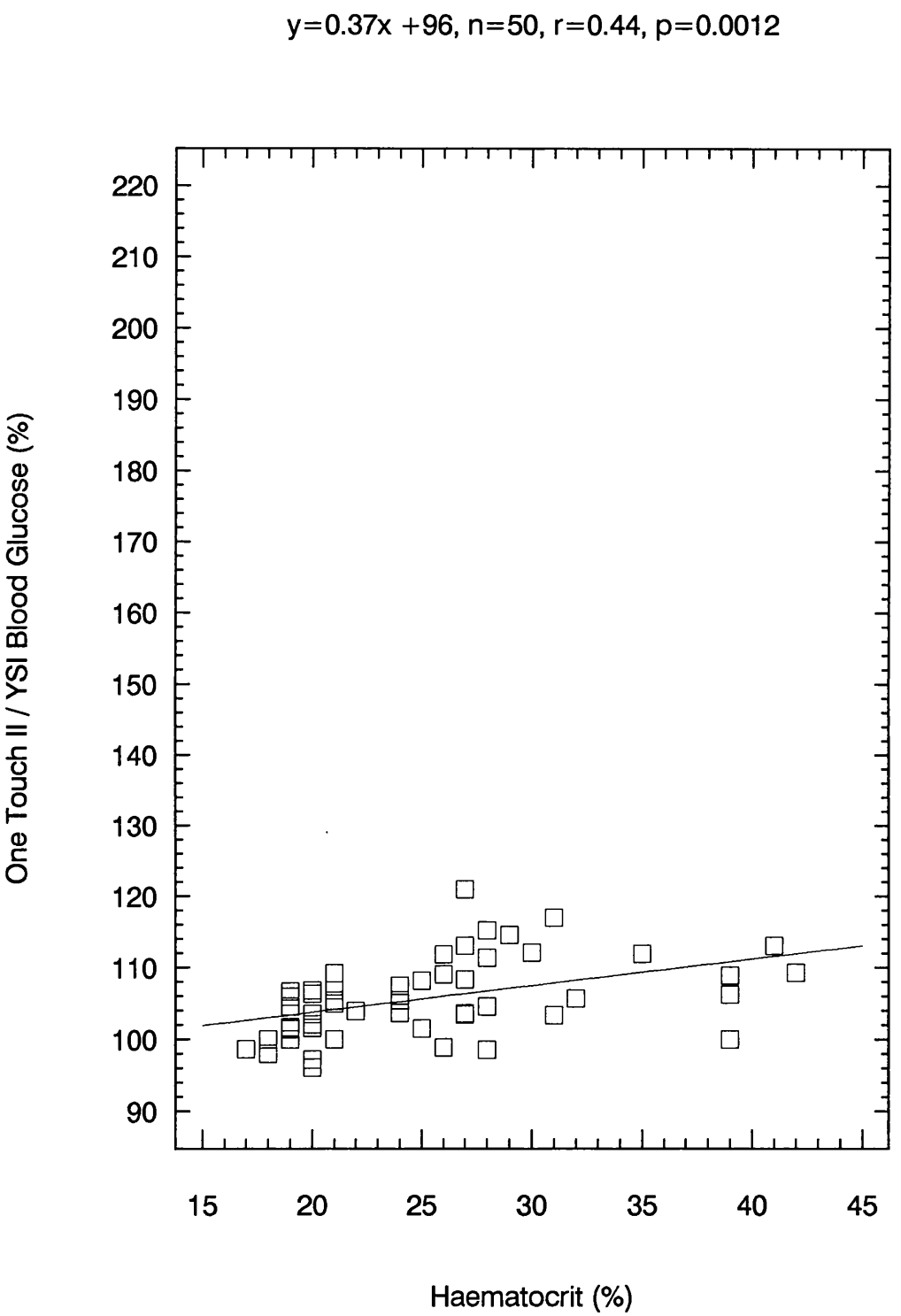


Figure 10.2 Effect of sample haematocrit on One Touch II blood glucose meter accuracy.

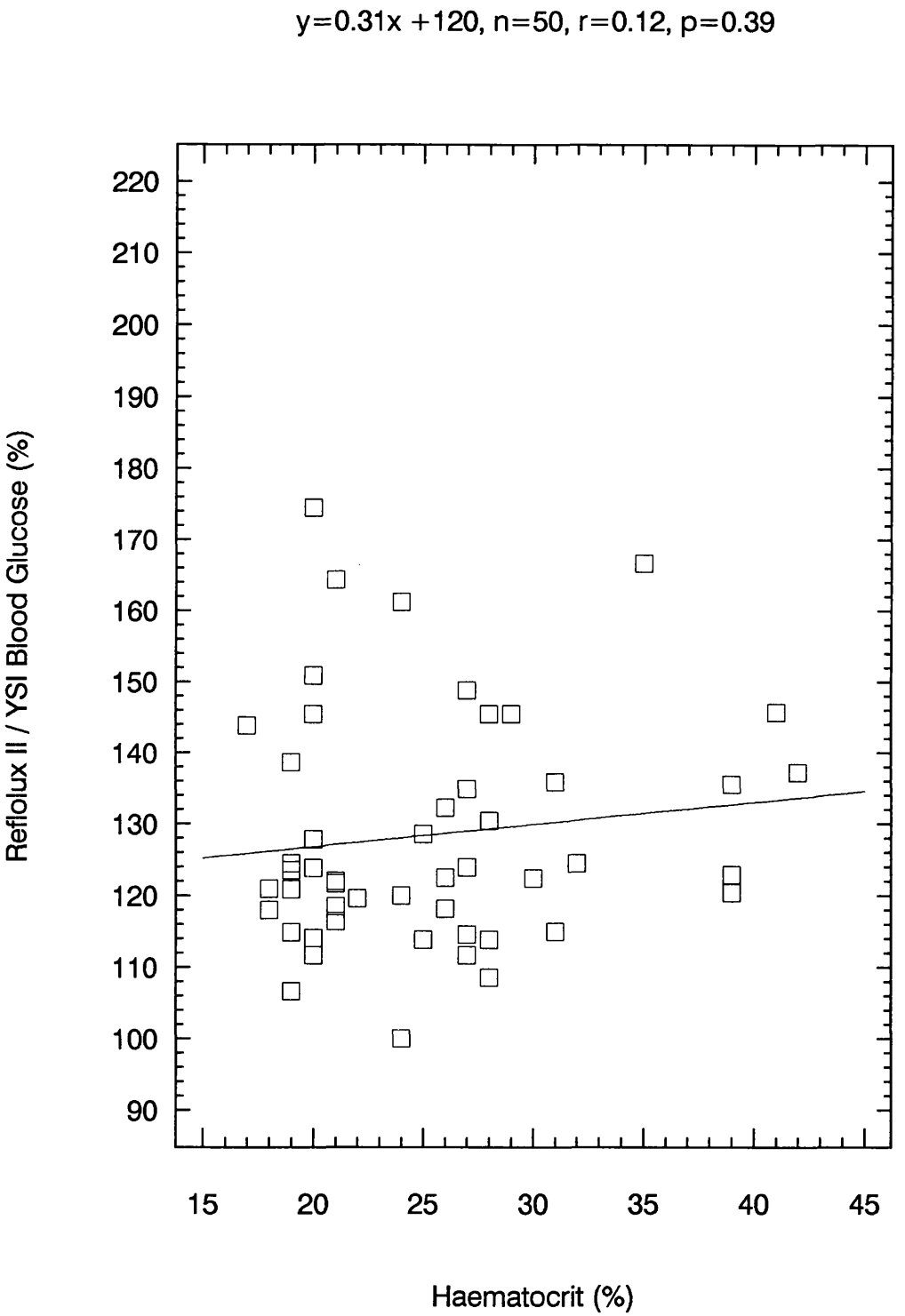


Figure 10.3 Effect of sample haematocrit on Reflolux II blood glucose meter accuracy.

correlated better than the Reflolux II (r=0.897, y=1.88x -1.86 vs. r=0.787, y= 1.01x +1.52 respectively), the effect of haematocrit on the Glucometer II is evident by the gradient and intercept of the regression line.

10.4 Discussion

Most published studies have varied haematocrits by either the addition or removal of homologous plasma from centrifuged whole blood samples. In contrast, Clark *et al* found that a group of patients with a low mean haematocrit due to renal failure gave different glucose test strip results compared to a group of controls.[139] In the present study we have varied haematocrit in the same individuals by a method which routinely occurs during cardiopulmonary bypass.

We have shown that Glucometer II glucose measurements vary inversely with a reduction in haematocrit. For every 10% fall in haematocrit, there was a 22% increase in measured glucose. This compares with our *in vitro* study (see Chapter 9) which showed a 19% increase for the same change in packed cell volume.

The same experimental investigation showed that a 10% haematocrit fall gave a 6% rise in glucose when measured by the Reflolux II. This change, however, was not apparent in our results, presumably because of the relatively poor relationship obtained between the Reflolux II measurements and the reference YSI instrument.

The One Touch II meter is peculiar to others in that both low and high haematocrit samples can give spuriously low glucose readings (see Chapter 13). Over the haematocrit range of this study, there was a modest 4% fall in glucose measurements with every 10% reduction in haematocrit which is consistent with the experimental effect of haematocrit found in Chapter 13, Figure 13.2. Overall, the One Touch II blood glucose meter performed closest to the reference YSI analyser during the period of the investigation.

It has been recommended that glucose meters should aim to have measurements which fall within 15% of the reference method.[130] Outwith this range errors are more likely to be clinically significant. We have demonstrated that the measurement of glucose using test strips can lead to this degree of inaccuracy during the procedure of cardiopulmonary bypass. For the Glucometer II, a change in haematocrit of less than 10% will confer such a difference.

Coronary heart disease is more prevalent in Type 2 (non-insulin-dependent) diabetic patients.[176,177] This means these individuals may comprise up to 20% of coronary artery bypass referrals.[178] It is thus especially relevant that cardiopulmonary bypass, in particular, should cause this problem in blood glucose measurement.

Glucose meter manufacturers have patient haematocrit limits outwith which measurements are not recommended (Glucometer II 35-50%, Reflolux II 35-70%, One Touch II 25-60%). However, these are not always widely appreciated, nor is it invariably possible to know the haematocrit of an individual at the time of sampling. An awareness of the effect is vital if the incorrect administration of intraoperative insulin is to be avoided.

In conclusion, we have demonstrated *in vivo* variations of test strip glucose measurements with the changes in haematocrit experienced during cardiopulmonary bypass. This data can presumably be extrapolated to any patient who has a low haematocrit and should be borne in mind by clinicians when dealing with intraoperative glucose measurement or in situations such as in Intensive Therapy Units.

Chapter 11

The Effect of Variations in Sample pH and pO_2 on Blood Glucose Meter Measurement.

11.1 Introduction

Most glucose test strips currently in use employ the enzyme glucose oxidase as the basis of their method of measurement (see Chapter 1.4.3). Glucose, molecular oxygen and water are the substrates of this enzyme. It therefore seems reasonable to suggest that changes in sample oxygen tension may result in discrepancies in glucose measurement. In addition glucose oxidase, like all enzymes, shows variations in activity with changes in pH. However, before the study described in this chapter, the sole effects of sample pH and pO_2 on glucose test strip measurement could not be accurately assessed because of the inability to isolate them from the multitude of other blood gas changes which are usually present in such samples.

This study describes a novel technique for producing blood samples with specified levels of pH, pO_2 and pCO_2 and applies it to establish if, and to what extent, five of the most commonly used glucose meters in the UK are influenced by independent changes in sample pH and pO_2.

11.2 Methods

11.2.1 Preparation of Blood Samples with Variable pH

On two occasions 21mL of venous blood (haematocrit 42%) was collected in lithium heparin sample tubes. One was spiked with a 10% glucose solution (10 μL in 1mL blood) to provide samples with an initial blood glucose concentration of 8.75 mmol/L and the other was left unaltered and had a concentration of 5.35 mmol/L. In total, five 2 mL samples had increasing concentrations of strong acid added (0 to 80μL of 1M HCl) whilst another five samples had increasing concentrations of additional strong alkali (0-80μL of 2M NaOH). Samples with less than 80μL added were made up to this volume with 0.9% NaCl. Each sample was stored in a 37°C water bath before being tonometered in turn for 20 minutes using an EQUILibrator Model 300

tonometer (RNA Medical Acton Ma, USA) at 37°C and a constant gas mixture of 9.8% O_2 and 5.6% CO_2. This instrument equilibrates the partial pressures of a blood sample with that of a connected gas cylinder. On completion, blood gases were immediately measured using a calibrated Instrumentation Laboratory Model 1302 blood gas analyser (Instrumentation Laboratory (UK) Ltd, Warrington, Cheshire, UK). This technique allowed the preparation of 10 blood samples with a very similar mean pO_2 (9.09 ± 0.096 (SEM) kPa) and pCO_2 (5.52 ± 0.024 kPa), but a pH range of 6.54 to 7.73. Throughout the procedure, a 2mL blood sample that had 80μL of 0.9%NaCl added (but had not been tonometered) was kept in a 37°C water bath.

11.2.2 Preparation of Blood Samples with Variable Oxygen Tension

Venous blood was collected and spiked as before to achieve an initial glucose concentration of 11.2 mmol/L. The sample was stored in a water bath set at 37°C. Two millilitres of sample was placed in an Instrumentation Laboratory Model 237 tonometer at a temperature of 37°C. The instrument was adapted to allow the gas input to come from a Boyles International anaesthetic machine (Omeda Ltd., Harrow, Essex, UK) which permitted a variable flow of nitrogen, oxygen and carbon dioxide. Blood was tonometered for 5 minutes and then immediately measured by a calibrated ABL Model 505 blood gas analyser (Radiometer Ltd., Crawley, West Sussex, UK). The flow of gases was set so as to achieve a minimal change to mean pH (7.28 ± 0.012) and pCO_2 (7.98 ± 0.25kPa) in 10 separate tonometered blood samples whilst allowing glucose measurements on samples with a pO_2 range of 2.0kPa to 33.6kPa.

11.2.3 Measurement of Blood Glucose

Immediately after blood gas analysis, duplicate blood glucose measurements were performed on the following five properly calibrated blood glucose meters:

the Accutrend, the ExacTech Companion, the Glucometer IIM, the One Touch II, and the Reflolux II. Within-assay coefficient of variations (n=10) using the Sugar Chex 'mid' solution (Streck Laboratories Inc, Omaha, NE, USA) were 2.9%, 5.2%, 4.9%, 6.6% and 1.9% respectively.

Duplicate reference whole blood glucose measurements were performed on an Analox Model GM9 glucose analyser concurrently with the measurements performed by the blood glucose meters. This instrument was chosen because it deoxygenates the blood sample before analysis and so is unlikely to be affected by variations in pO_2 (see Chapter 3.5.4). However, to prevent the possibility of the Analox analyser giving spurious measurements due to changes in pH the venous sample stored in a water bath was used as the reference measurement.

11.2.4 Statistical Analysis

Linear regression analysis was performed by the least squares method. Reference and meter blood glucose measurements were expressed as a mean of two values. Meter accuracy was expressed as a percentage of the reference measurement.

11.3 Results

11.3.1 Effect of Sample pH

Table 11.1 describes the relationship of all meters to changes in pH and pO_2. Only the ExacTech showed any significant ($p<0.05$) linear relationship to changes in pH (Figure 11.1). The Accutrend ($r=0.022$, $p=0.952$), Glucometer II ($r=-0.41$, $p=0.24$), One Touch II ($r=0.021$, $p=0.95$) and Reflolux II ($r=-0.53$, $p=0.12$) were unaffected. By extrapolation, compared to a sample of pH 7.40, the ExacTech meter underestimated a glucose measurement by greater than 15% below pH 6.95, and overestimated beyond 15% above pH 7.85.

Meter	pH		pO_2	
	Bias (mmol/L)[a]	Linear Regression[b]	Bias (mmol/L)[a]	Linear Regression[b]
Accutrend	+0.80 ± 0.83	y=0.91x +122, r=0.022	-0.69 ± 0.46	y=0.33x +97, r=-0.825
ExacTech	-0.18 ± 0.97	y=33x -141, r=-0.694	+0.62 ± 2.07	y=-1.63x +126, r=-0.794
Glucometer II	+0.10 ± 0.44	y=-8.0x +159, r=-0.414	+0.79 ± 0.66	y=0.227x +105, r=0.373
One Touch II	+0.84 ± 0.48	y=0.50x +111, r=0.021	+0.86 ± 0.54	y=0.35x +113, r=-0.667
Reflolux II	-0.31 ± 0.63	y=-12.7x+186, r=-0.531	+0.34 ± 0.83	y=0.267x +100, r=0.327

[a]Mean ± SD (Meter - Analox) and [b] Meter / Analox (%) vs. pO_2 (kPa)

Table 11.1 Meter bias and relationship to changes in sample pH and pO_2

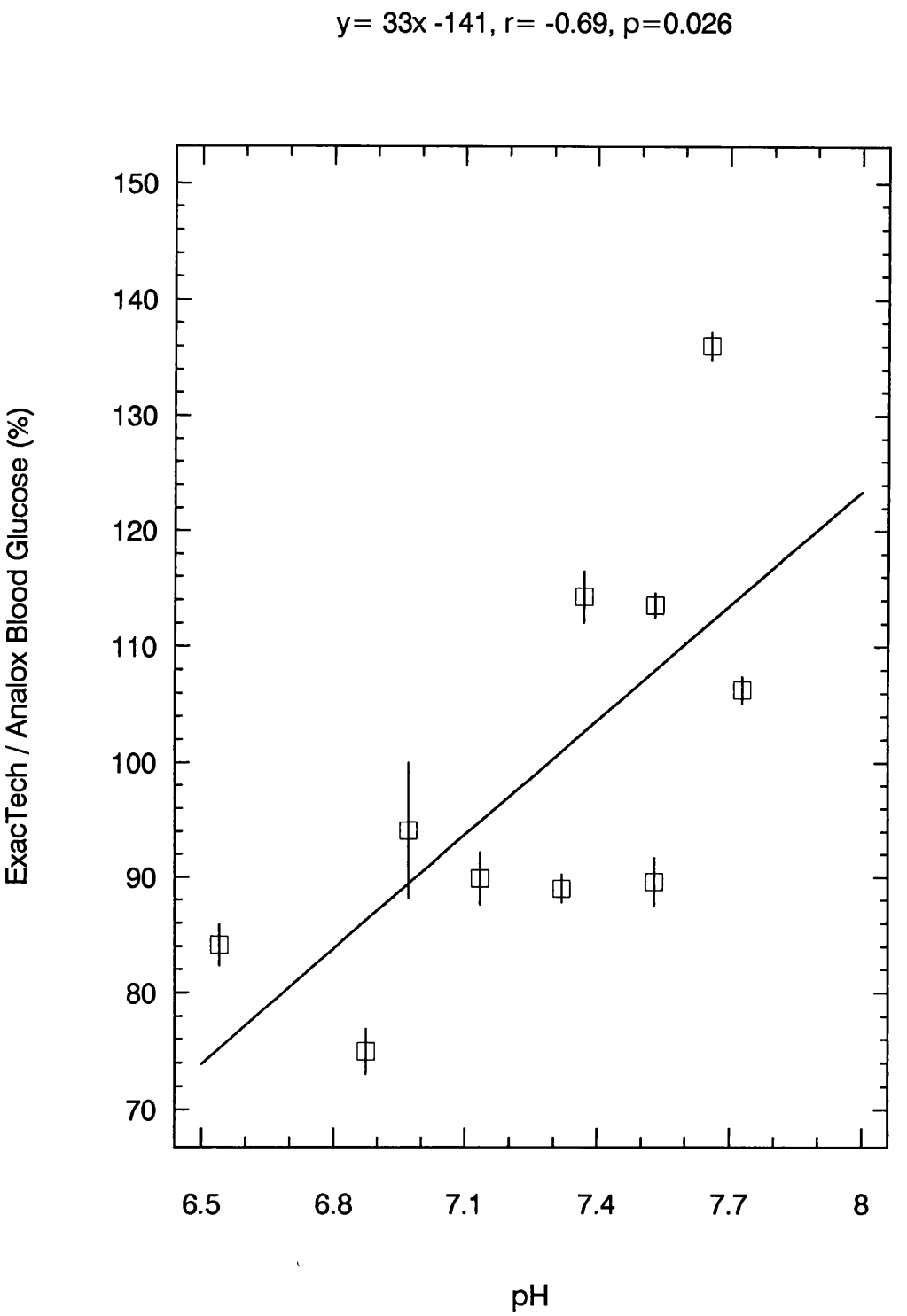


Figure 11.1 Effect of sample pH on ExacTech blood glucose meter accuracy (standard error bars included).

11.3.2 Effect of Sample pO_2

The meters which showed significant changes in glucose measurement with increasing oxygen tension were the ExacTech (r=-0.79, p=0.006) (Figure 11.2a), Accutrend (r=-0.82, p=0.003) and One Touch II (r=-0.67, p=0.035). The Glucometer II (r=0.37, p=0.29) and Reflolux II (r=0.33, p=0.36) had no association. The relationship of the ExacTech, unlike the other meters, was much improved when the oxygen tension was expressed logarithmically (r=-0.95, p=0.00002) (Figure 11.2b). Extrapolating from the log plot, the glucose measurement by the ExacTech was equal to that of the Analox at a pO_2 of 11.5kPa. The meter underestimated by greater than 15% at a pO_2 below 5.8kPa and overestimated to the same degree beyond 22.9kPa. A 15% deviation for the Accutrend and One Touch II represented a pO_2 change of 45kPa and 42kPa respectively.

11.3.3 Overall Accuracy of Meters at Varying pH and pO_2

During the study, all meters showed a mean bias within 1 mmol/L of the reference instrument, but as a result of the effect of pH and pO_2 on the ExacTech meter, it showed the greatest deviation from the Analox in both experiments (Table 11.1).

11.4 Discussion

As management decisions are taken according to the results obtained by ward based blood glucose meters,[179] it is particularly important that both clinicians and patients are aware of all the potential sources of error in the meter system they use.

Using the recommendations that glucose meters should aim to have measurements which fall within 15% of the reference method,[130] we have shown that the ExacTech meter can fall outwith these limits below a blood pH of 6.95 before any other possible sources of error are taken into account. Thus,

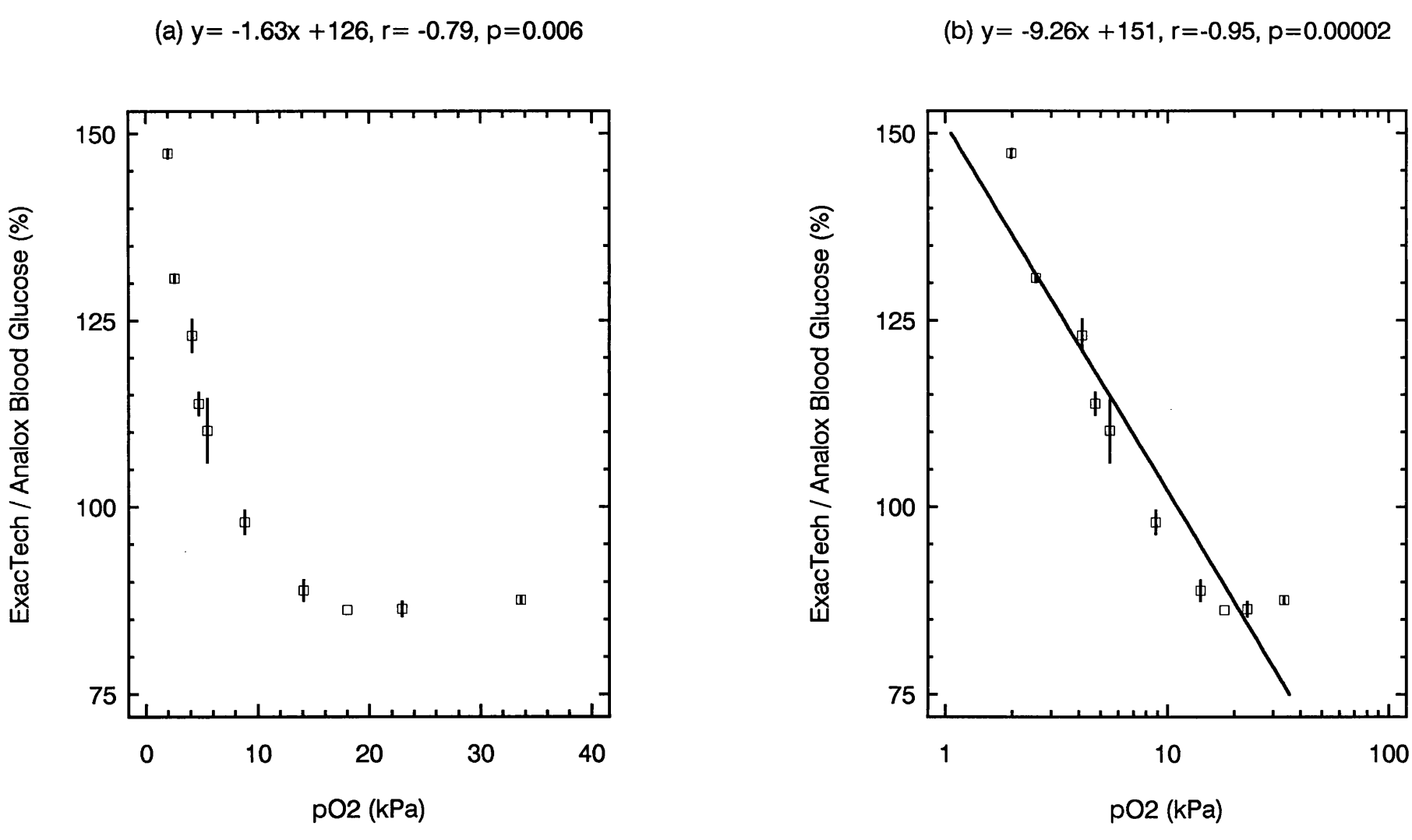


Figure 11.2 Effect of (a) pO_2 and (b) $\log_{10}pO_2$ on ExacTech blood glucose meter accuracy (standard error bars included).

a severely acidotic patient may obtain an inappropriately low result on using this meter. If the acidosis is due to diabetic ketoacidosis the patient is also likely to have a raised haematocrit due to dehydration. We have already shown that in the ExacTech meter a 10% increase in haematocrit leads to a 15% fall in measured glucose (see Chapter 9). This may therefore compound any underestimate in glucose measurement.

The ExacTech meter also showed the greatest variability in measurements taken at different blood oxygen tensions. At the sample pH we obtained, the meter was accurate at a pO_2 of 11.5 kPa but gave an error of greater than 15% at oxygen tensions below 5.8kPa and above 22.9kPa. The former oxygen tension may occur in hypoxic patients or if a venous sample is used instead of capillary. Indeed, Matthews *et al* found the ExacTech meter to have a 10% positive bias when using venous instead of capillary blood in 182 patients.[180]

Our oxygen tension findings are also consistent with those of Halloran[181] except his meter measurements, even at a normal capillary pO_2, showed an additional positive bias of around 30%. The bias is most probably ascribable to the fact that the equilibrating gas he used was a nitrogen/oxygen mixture which lacked carbon dioxide. All the samples were therefore likely to have been rendered severely alkalotic and thus subject to the pH changes described above.

Both the Accutrend and the One Touch II meters demonstrated reductions in meter measurements with increasing pO_2. However, a 15% error due to this effect alone would not occur in clinical practice.

The reasons remain speculative as to why the ExacTech electrochemical method for glucose measurement should show the largest error in both experiments. Experimental results from a prototype electrochemical method with non-physiological samples suggested the ExacTech meter would be unaffected by changes in pH.[182] However, it would appear the ExacTech implementation of this method, when using blood as samples, can be influenced, presumably by redox reaction interference from hydrogen ions. The

decreasing ExacTech meter measurements found when increasing sample oxygen tension may result from the use of the electron mediator ferrocene for glucose measurement: increasing competition between oxygen and ferrocene for the glucose oxidase enzyme is likely to result in the production of increasing amounts of hydrogen peroxide relative to the formation of the reduced form of ferrocene, ferricinium (see Figure 1.2). Hydrogen peroxide is less easily reoxidised than ferricinium which means test strip current flow is likely to be diminished and so any glucose concentration measured is apt to be reduced.

Our findings are of especial clinical relevance in the management of acutely unwell patients. As well as those individuals already mentioned with severe diabetic ketoacidosis, patients in Intensive Therapy Units commonly have acid-base disturbances and/or require ventilation for respiratory failure. Use of the ExacTech meter in these situations is more liable to lead to erroneous results.

In conclusion, changes in pH and pO_2 affect the most commonly used blood glucose meter systems variably. Whilst there is little question that the ExacTech Companion meter can perform accurately in well patients using capillary blood,[63] caution may need to be exercised when interpreting measurements in severely acidotic or hypoxic patients.

Since the publication of this study the other electrochemical meter marketed by Medisense, the Companion 2, has become widely available and the test strips have recently become obtainable on NHS prescription. Initial experience with this meter would suggest that although it is still affected by variations in sample haematocrit, the addition of a third electrode in the strip has minimised the effect that sample pO_2 has on meter accuracy.[183] This would help explain the finding that results from venous samples are now insignificantly different from capillary ones when using this test strip.[184] It would therefore seem appropriate from the evidence in this chapter that the

company continue to concentrate their efforts on the marketing of this product rather than the ExacTech.

Chapter 12

The Effect of Sample Haemolysis on Blood Glucose Meter Measurement.

12.1 Introduction

Extra-laboratory measurement of analytes such as blood glucose has led to an increase in the use of whole blood rather than serum or plasma as a specimen for biochemical measurement. Whilst the use of whole blood in a clinical setting has the advantage of being convenient, it is not possible, unlike plasma or serum, to tell if the sample being used is in any way haemolysed.

When a new method is introduced into a laboratory it is routine practice to assess the extent to which it is affected by sample haemolysis. However, before this study, the effect of haemolysis on glucose meter results had not been established, presumably because the samples usually used (serum and plasma spiked with haemoglobin) would be inappropriate for assessing glucose test strips which can be affected by sample haematocrit (see Chapters 9 and 10) as well as sample pH and pO_2 (see Chapter 11). A rapid means of obtaining samples which contained lysed red cells together with their red cell membranes was required. This study has adapted a method used for obtaining enzymes from white cells (sonication) to establish if, and to what extent, seven of the most commonly used glucose meters in the UK are influenced by varying degrees of sample haemolysis.

12.2 Methods

12.2.1 Preparation of Samples with Variable Haemolysis

Forty millilitres of venous blood (glucose concentration 7.35 mmol/L, haematocrit 40.0%) was collected without a tourniquet into lithium heparin sample tubes. Six millilitres was completely haemolysed by ten 1 second exposures to ultrasound using a MSE Soniprep 150 sonicator (MSE Ltd. Loughborough, UK). The sonicated blood (pO_2 4.0kPa), including the lysed cell membranes, was then immediately added to untreated blood (pO_2 5.0kPa) in increasing amounts (0 to 850μL) to yield 9 four millilitre samples with

increasing levels of haemolysis. The calculated plasma free haemoglobin concentrations (0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 g/dL) were confirmed at the end of the study by measurement in a Philips Model PU150 (Philips Scientific Ltd. Cambridge, UK) scanning spectrophotometer.[185] The free haemoglobin concentrations represented the proportion of intact red cells falling from 40% of blood volume to 31.5%.

12.2.2 Measurement of Blood Glucose

Each sample (in random order) was measured in duplicate on the following properly calibrated blood glucose meters using the August '94 generations of test strips: the Accutrend (12 second strip), the Companion 2, the ExacTech Companion, the Glucometer IIM, the Glucometer 4, the One Touch II and Reflolux II. Forty microlitre sample volumes were used throughout.
The reference glucose instrument used in the study was an Analox Model GM9 glucose analyser. This is able to measure glucose in plasma, in whole blood with intact red cells or in lysed whole blood.[186] Duplicate reference measurements were performed on each sample concurrently with the meter measurements.

12.2.3 Statistical Analysis

Linear regression analysis was performed by the least squares method. Reference and meter blood glucose measurements were expressed as a mean of two values. Meter accuracy was expressed as a percentage of the reference measurement.

12.3 Results

12.3.1 The Effect of Haemolysis on Blood Glucose Meter Measurement

Only the Accutrend showed any significant ($p<0.05$) linear relationship to the degree of haemolysis in the sample (Figure 12.1). Below a plasma haemoglobin concentration of 5g/dL, all other meters demonstrated results which were within 15% of the value obtained on the untreated sample.

12.3.2 The Effect of Extreme Haemolysis on Blood Glucose Meter Measurement

Using the completely haemolysed sonicated blood, extreme levels of haemolysis (14.2 g/dL) were found to affect the Accutrend meter (glucose value 108% greater than reference), the ExacTech (+98%), the Glucometer II (-32%) and the Companion 2 (-41%). The Reflolux gave an error code using completely haemolysed blood. The One Touch II and Glucometer 4 meters again gave results which were within 15% of the untreated sample.

12.4 Discussion

Manufacturers of glucose meters often recommend that their products be used exclusively with capillary blood samples. A degree of haemolysis in these samples is almost unavoidable.[187] Moreover, it has been estimated that, even when using careful techniques, around 5% of capillary specimens are likely to show visible red cell lysis.[188] Thus any effect that haemolysis may have on glucose meter measurements cannot be underestimated, especially since it is so difficult to detect.

In this study we have shown that, below 5g/dL haemolysis, the Accutrend meter shows an increasingly positive bias with increasing degrees of haemolysis. Using the recommendation that glucose meters should aim to have measurements which fall within 15% of the reference method,[130] and that

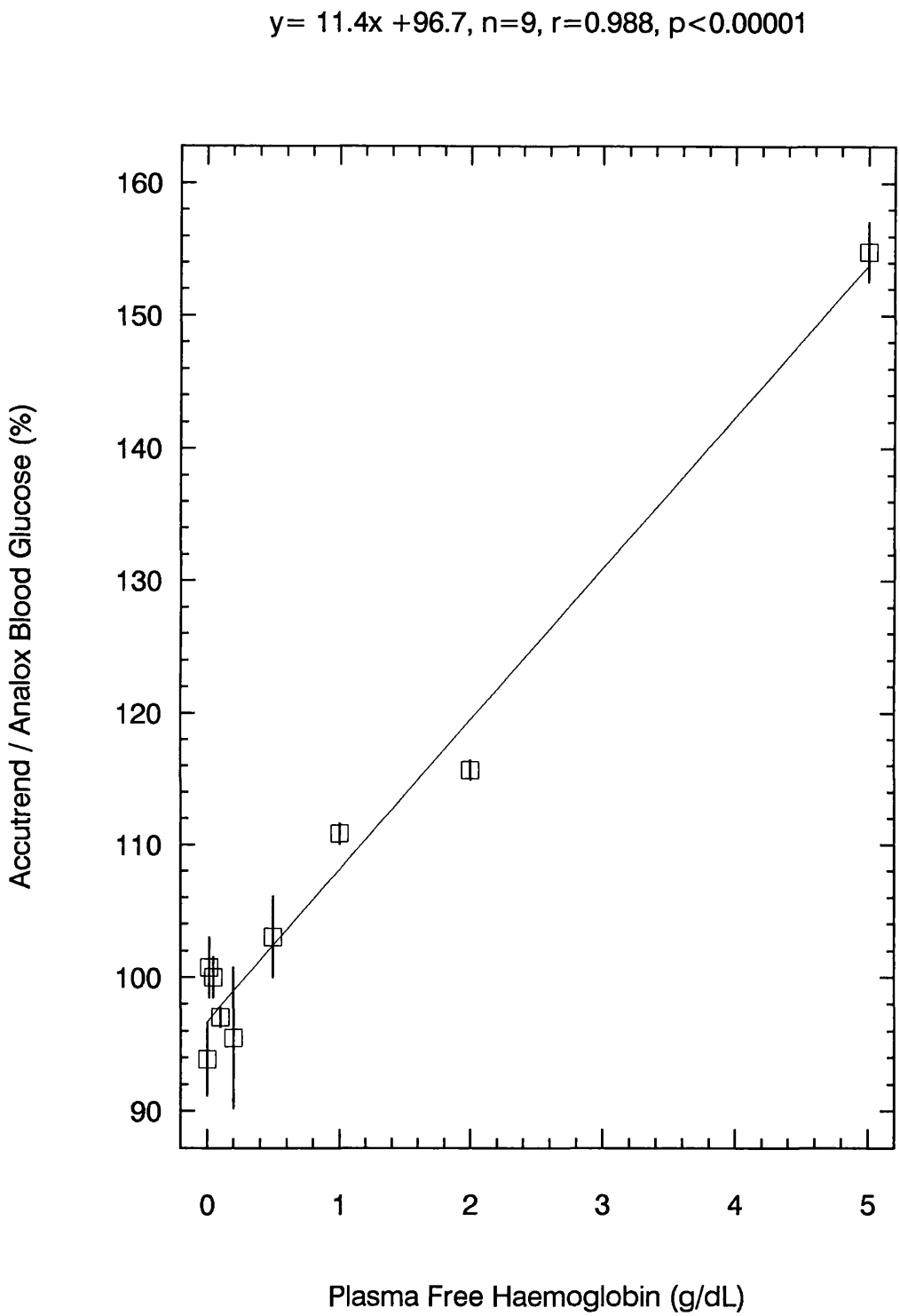


Figure 12.1 Effect of sample haemolysis on Accutrend blood glucose meter accuracy (standard error bars included).

outwith this range errors are more likely to be clinically significant, this study has shown that for every 7% of red cells lysed, such a 15% increase in the glucose value of the Accutrend meter is obtained.

Together with the Accutrend meter, the ExacTech, the Glucometer II and Companion 2 meters all showed large (>30%) fluctuations in glucose concentration when using an extremely haemolysed sample. While a similar sample would hopefully not be found often in clinical practice, it is interesting to speculate that this may be one of the causes of 'flier' results obtained by meter users.

The reason why the Accutrend meter should be most affected by haemolysis is unlikely to be due to any difference in the proportion of intact red cells to plasma since this meter is relatively unaffected by variations in haematocrit.[189] The Accutrend is also unaffected by changes in sample pO_2 (see Chapter 11) so the use of venous blood, rather than capillary, should not have affected our results. Presumably, free haemoglobin presents a darker colour at the colour field of the Accutrend test strip. This leads to a lower percentage reflectance and hence a higher blood glucose measurement. Since intact red cells are usually retarded by the glass fibre matrix of the test strip[189] then, unlike haemolysed specimens, haemoglobin in non-haemolysed samples would not normally have the opportunity to interfere.

It is of relevance that often the capillary samples most difficult to obtain (and therefore most likely to show haemolysis) are those found in situations where the patient has compromised peripheral circulation due to being acutely unwell. Therefore, use of the Accutrend meter in such situations may more commonly lead to spuriously raised blood glucose results.

In conclusion, haemolysis in a blood sample would appear to affect blood glucose meter measurement by varying degrees depending on the instrument used. Whilst a number of meters showed inaccuracies using extremely haemolysed samples, the Accutrend meter demonstrated a positive bias at more

modest degrees of red cell lysis. Thus, an unexpected result may need to be treated with caution, confirmed visually, and repeated by either the meter or, if relevant, in a laboratory.

Personal communications with Boehringer Mannheim since the publication of this study have confirmed that the company were aware of a problem with sample haemolysis when using their Reflotron analyser. Since the Accutrend meter is based on the same technology, they were not surprised we saw a similar effect, although they felt the problem was unlikely to be significant in most clinical situations.

Chapter 13

A Laboratory Evaluation of the One Touch II Blood Glucose Meter.

13.1 Introduction

In 1992 a new blood glucose meter, the One Touch II from Lifescan Ltd, was introduced into the UK after being successfully launched in the United States. It was unique in addressing the 4 principal clinical problems associated with extra-laboratory blood glucose analysis at that time, namely, errors due to the size and placement of the blood sample, the timing of the test and wiping of blood from the test strip.[130] It achieved this by flagging insufficient samples, automatically timing the test on correct sample placement, and requiring no wiping of the sample from the test strip. In the UK, the ExacTech meter from Medisense Ltd was the only other contemporary meter which offered close to this degree of sophistication. However, although the ExacTech also employed a 'non-wipe' technique, even this meter was unable to detect small samples consistently, start timing automatically, have an analytical range of 0-33.3mmol/L, keep a date and time stamped record of the last 255 meter readings, record quality control results separately, or download this information into a PC.

Before the potential advantages of this meter could be confirmed clinically, it was important to assess the adequacy of the meter when tested in a laboratory environment to see if the problems known to affect other meter strips were applicable to this instrument. This study describes such an evaluation.

13.2 Methods

13.2.1 Study Design

Accuracy of the meter was assessed in comparison with a YSI Model 23AM blood glucose analyser. Forty eight samples were collected in K_2EDTA anticoagulant. While the glucose values of some samples were allowed to fall by glycolysis, others were spiked with a 10% glucose solution to achieve

hyperglycaemic samples. The mean value of duplicate measurements was used in both instruments.

Imprecision of the meter was obtained using both EDTA whole blood and manufacturer's control solution samples. Samples designated as having low, mid and high glucose concentrations were analysed 20 times to provide a measurement of within-assay precision. Between-assay precision was assessed using the same control solutions over a 20 day period.

To assess the effect of haematocrit on meter measurements, EDTA samples from 2 non-diabetic individuals were spiked with 10% glucose to obtain YSI blood glucose concentrations of 10.0 and 10.3 mmol/L at 40% haematocrit. They were then adjusted to obtain haematocrit values ranging from 0-60% by the addition or removal of homologous plasma. Two glucose samples from each subject were used in both instruments.

Common interferents of the glucose oxidase enzyme system were added individually to whole blood samples to assess their effect on One Touch II strip measurement. Pathological concentrations of bilirubin (up to 317μmol/L), lipaemia (up to 1000mg/dL Intralipid), urate (up to 1.2mmol/L) and ascorbate (up to 50mg/L) were investigated. In addition, specimens with toxic and non-toxic concentrations of paracetamol (up to 300mg/L), salicylate (up to 350mg/L) and ethanol (up to 400mg/dL) were used. All results were adjusted for any specimen dilution incurred by spiking.

The effect of sample volume on the imprecision of the One Touch II instrument was assessed using sample volumes 5-40μL using an adjustable 100μL Gilson air displacement pipette. Measurements below 5μL used an Absoluter positive displacement pipette.

13.2.2 Statistical Analysis

Comparison of meter accuracy with the YSI instrument was by a Bland Altman residual plot.[175] Imprecision measurements were expressed as coefficients of

variation (CV's). In the haematocrit experiment, One Touch II glucose values were expressed as a percentage of the YSI result and the curve fit used a fifth order polynomial interpolation.

13.3 Results

13.3.1 Accuracy

Specimen values from 0.1 to 29.2 mmol/L were obtained. Figure 13.1 shows the residual plot comparison with the YSI instrument. The One Touch II showed a mean bias of +0.55 mmol/L (95% CI +0.22 to +0.89) with 95% of samples (±1.96 SD) between +2.83mmol/L (95% CI +2.25 to +3.42) and -1.72mmol/L (95% CI -2.31 to -1.14).

13.3.2 Imprecision

Table 13.1 shows the within-assay and between-assay imprecision for both control and whole blood solutions.

13.3.3 Haematocrit

Figure 13.2 shows a graphical representation of One Touch II meter accuracy at different haematocrit values. Results only deviated by greater than 15% from the YSI values at haematocrits above 50%.

13.3.4 Interference

Tables 13.2a and 13.2b describe the effect of the chosen interferents on glucose measurement taking into account specimen dilution. A change of greater than 15% was not observed in any specimen used.

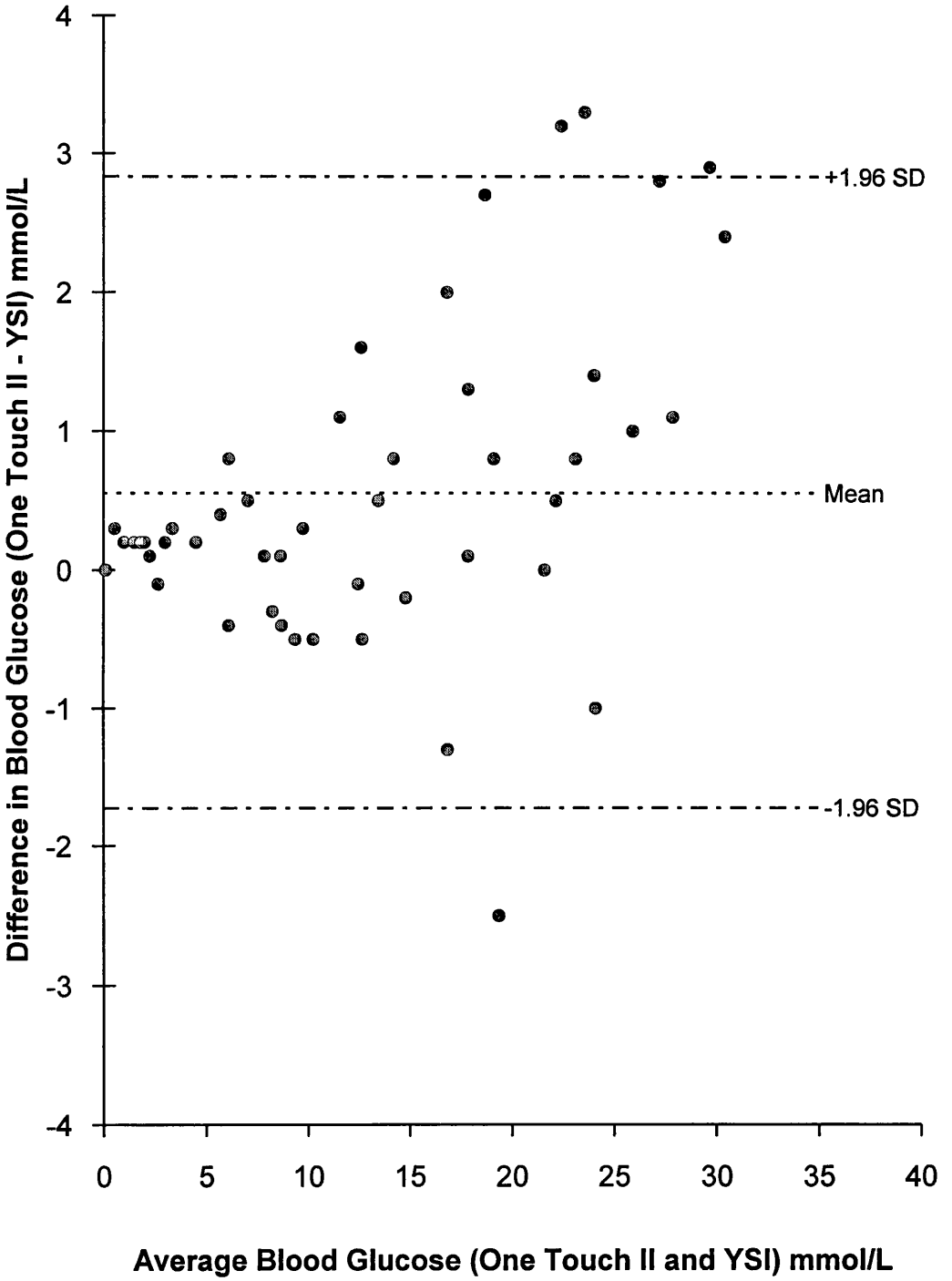


Figure 13.1 Bland Altman relationship between One Touch II blood glucose meter and YSI instrument.

		n	Mean	S.D.	C.V.
Within-Assay					
Control	low	20	3.07	0.065	2.14
	mid	20	6.20	0.141	2.28
	high	20	19.49	0.477	2.45
Blood	1	20	3.65	0.069	1.88
	2	20	7.32	0.146	2.00
	3	20	15.71	0.429	2.73
Between-Assay					
Control	low	12	3.08	0.062	2.02
	mid	16	6.13	0.189	3.08
	high	12	18.9	0.565	2.98

Table 13.1 Table showing within-assay and between-assay imprecision (coefficient of variation) of the One Touch II blood glucose meter.

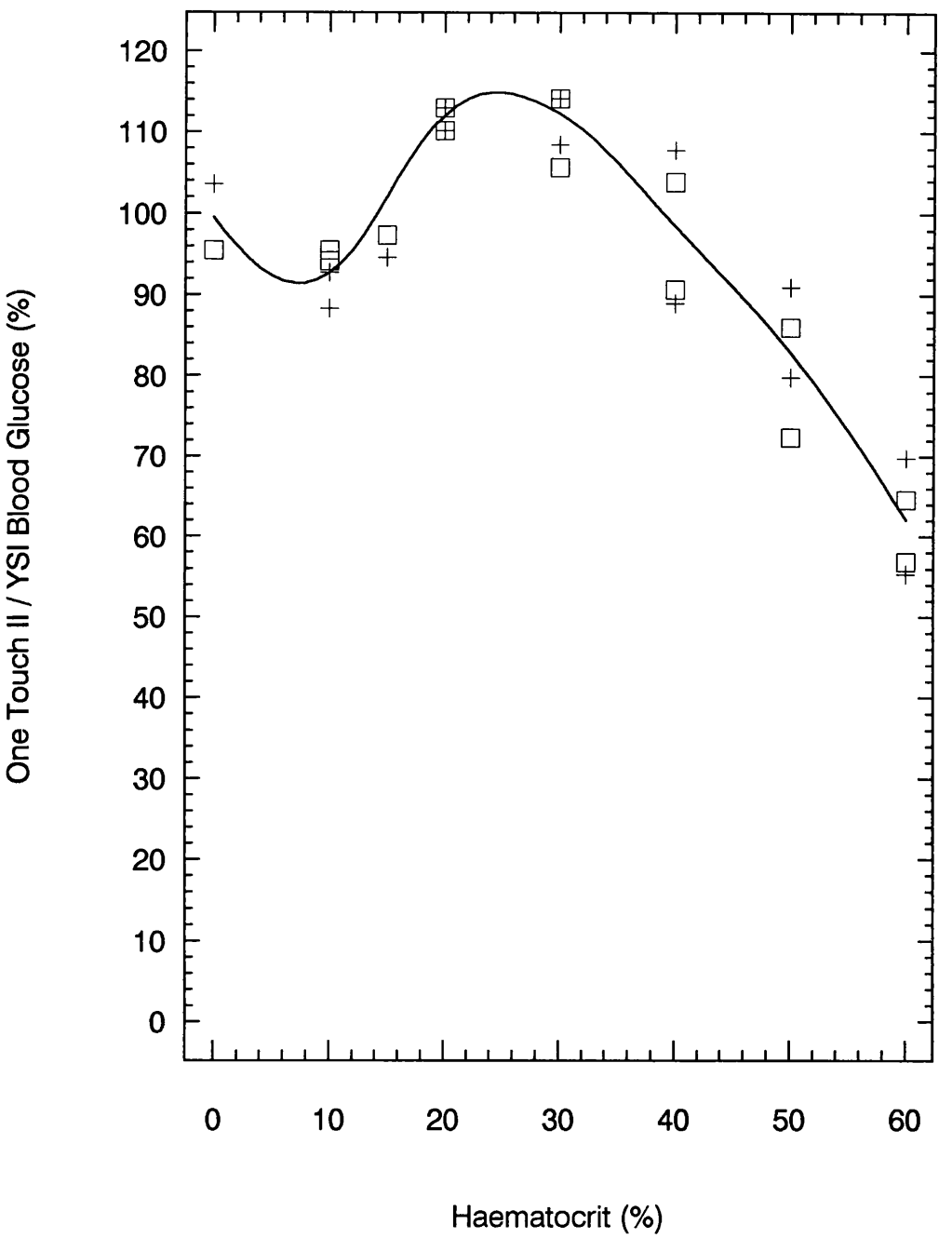


Figure 13.2 Effect of sample haematocrit on One Touch II blood glucose meter accuracy in 2 subjects (□ and +).

SALICYLATE	**0 mg/L**	**175 mg/L**	**350 mg/L**
Sample A	8.3	8.35	8.2
Sample B	7.85	7.875	8.08
URATE mmol/L	**0.3 mmol/L**	**0.8 mmol/L**	**1.2**
Sample A	7.8	7.7	7.5
Sample B	7.95	7.77	8.30
ETHANOL	**0 mg/dL**	**80 mg/dL**	**400 mg/dL**
Sample A	8.25	8.35	8.25
Sample B	7.8	8.0	8.0
ASCORBATE	**10mg/L**	**30mg/L**	**50mg/L**
Sample A	8.3	8.3	8.08
Sample B	7.8	7.7	7.42
PARACETAMOL	**0mg/L**	**30mg/L**	**300mg/L**
Sample A	8.55	8.54	9.18
Sample B	7.95	7.93	8.14

Table 13.2a Effect of increasing concentrations of potential interferents on the One Touch II glucose meter in 2 blood samples (A and B).

BILIRUBIN

Sample	Bilirubin (umol/L)	YSI (mmol/L)	One Touch II (mmol/L)
A	51	16.0	17.3
B	135	14.2	15.4
C	156	14.3	15.5
D	237	13.5	13.8
E	317	13.8	14.5

LIPAEMIA

mg Intralipid/dL	YSI	One Touch II
0	9.8	9.7
100	9.6	9.6
200	9.5	9.5
300	9.4	9.6
400	9.4	9.6
500	9.3	9.5
600	9.2	9.4
700	9.2	9.2
800	9.1	9.2
900	8.9	8.7
1000	8.8	9.4

Table 13.2b Effect of increasing concentrations of potential interferents on the One Touch II glucose meter.

13.3.5 Sample Volume

Measurements were flagged as insufficient with sample volumes of less than 3μL. Table 13.3 shows the effect on imprecision when using both air displacement and positive displacement pipettes. At sample volumes of less than 5μL, the meter commonly interpreted the sample as being that of a control solution.

13.4 Discussion

This study showed that for most samples likely to be encountered in clinical practice the One Touch II is a robust instrument for measuring blood glucose, at least in a laboratory setting.

When assessing the accuracy of the meter, all samples bar one (which had a glucose value of 0.4mmol/L) were within 15% of the reference YSI instrument. Therefore, in the laboratory, this meter did not report any results which could be described as clinically unacceptable.

Imprecision was similarly impressive. When using either control solutions or whole blood specimens, the within-batch coefficients of variation were all less than 3%. Surprisingly, the between-assay results were little different to those obtained within-assay.

The One Touch II showed a peculiar response to changes in blood sample haematocrit. Most meters show a linear response to increases in haematocrit, so that low haematocrit specimens give falsely high results and *vice versa* (see Chapter 9). However, while high haematocrit samples indeed gave spuriously low results, the One Touch II showed only minor fluctuations in glucose results at low haematocrits. In fact, results obtained with 0% haematocrit (i.e. plasma) samples were as accurate as those obtained when using specimens with normal packed cell volumes. Nevertheless, the effect shown at high haematocrits means that in patients with high packed cell volumes (e.g. in neonates,

Volume (μL)	n	Mean (mmol/L)	SD (mmol/L)	CV (%)
Using Adjustable 100μL Air Displacement Pipette:				
5	10	4.34	0.495	11.4
10	10	4.77	0.313	6.56
15	10	4.50	0.416	9.25
20	10	4.46	0.315	3.03
25	10	4.40	0.240	5.46
30	10	4.38	0.148	3.37
40	10	4.68	0.103	2.21
Using Positive Displacement Pipette:				
3	10	4.17	0.266	6.38
5	10	4.83	0.164	3.40

Table 13.3 Effect of sample volume on the within-assay imprecision of the One Touch II glucose meter.

dehydration or chronic obstructive airways disease) the results require to be interpreted with caution.

As explained in Chapter 2.4.3, interference from endogenous and exogenous reducing agents can affect the glucose oxidase enzyme system. In addition, colorimetric interference and the quenching of colour development can arise from icteric and lipaemic samples. This study showed that, even with extreme degrees of chemical pathology or drug toxicity, the One Touch II is unlikely to give unacceptable results.

The manufacturers of contemporary glucose meter 'wipe' systems usually recommended that sample volumes should be in excess of 15μL. In many cases this is not achieved, not only because of a poor skin puncture but also because users are given no guide from the meter as to whether samples are sufficient or not. This investigation showed that even sample volumes of only 3μL could give accurate and precise results with this meter, although on occasions this was recorded as a control sample in the meter memory. At volumes below 3μL it was not possible to cover the entire reagent pad of the strip with blood so, appropriately, the meter would flag that the placed sample was insufficient.

The effect of sample pH, pO_2 and haemolysis on the One Touch II are described in Chapters 11 and 12 and show that the meter is largely unaffected by any variations in these parameters.

In conclusion, with the caveat of high haematocrit samples, the One Touch II meter would appear to show a good laboratory performance, especially when dealing with potential interferents and low volume samples.

Chapter 14

A Ward Comparison Between the One Touch II and Glucometer II Blood Glucose Meters.

14.1 Introduction

We have shown that the One Touch II blood glucose meter could give accurate and precise measurements when used by laboratory personnel (see Chapter 13). However, while a number of other systems using reflectance photometers also worked well in both the laboratory setting[190,191] and in controlled studies[192], it was found they could give spuriously good results when compared to nursing staff using the meter under less than ideal conditions.[193,194]

To establish if the promising laboratory performance of the One Touch II conferred an improvement in ward glucose measurement, we performed a six week evaluation of the meter in a medical ward with a specialist interest in patients with diabetes, and compared it to the existing Glucometer II system in use.

14.2 Methods

14.2.1 Patients

Twenty seven acute admissions to Ward 7A, Gartnavel General Hospital, Glasgow participated in the study. This is a ward with a specialist interest in diabetes.

14.2.2 Study Design

All the nursing staff (comprising student nurses to ward sister) from Ward 7A were involved in the study. They had all been previously given official instruction on how to use the existing Glucometer II system. The One Touch II meter was demonstrated to them by one of the Diabetes Nurse Specialists in the ward.

Two extensively used One Touch II meters were used to complement the 2 Glucometer II meters already present on the ward. Each meter in use was checked daily using control solutions supplied by the manufacturers. The

Glucometer II meters also participated in a manufacturer's quality assurance scheme within the hospital and were given an accuracy rating of 'good', which is the highest grade achievable.

For a six week period, the 27 acute admission patients had 267 capillary blood glucose measurements taken by the nursing staff from a single capillary stab. The Glucometer II instrument was used first followed by the One Touch II. This order of sample application was used for three reasons. Firstly, since the Glucometer II requires a larger sample volume, it was likely that this meter would obtain the greatest amount of blood if always used first. Secondly, it meant that the nursing staff performing the Glucometer test would conduct it as close to the routine manner as possible. Lastly, the Glucometer II (unlike the One Touch II) does not indicate clearly if the blood volume is insufficient, so if used second with a small sample may have lead to erroneous results.

Blood has to be applied to the One Touch II test strip whilst in the meter. To prevent cross infection between patients the manufacturer's disposable transfer pipettes were used.

In 129 of the measurements the investigators obtained at least 25μL of heparinised capillary blood from either the same finger jab or from a second performed directly afterwards. These samples were measured on a YSI Model 23AM blood glucose analyser which was sited on the ward. This analyser was calibrated twice daily and all samples were measured within 15 minutes from collection.

Recent (within 1-3 days) haematocrit measurements were obtained on patients comprising 250 of the samples.

At the end of the study, the participating nursing staff were given a simple questionnaire to complete comparing different aspects of the two meters. They were asked to tick if they felt the One Touch II was the same, better, much better, worse or much worse than the Glucometer II in terms of (i) ease of use;

(ii) feel/robustness and (iii) confidence in results. Finally they were asked which meter they preferred overall.

14.2.3 Statistical Analysis

Method comparison between the two meters used Bland Altman residual plots.[175] Linear regression used the least squares method.

14.3 Results

14.3.1 Glucose Meter Accuracy Compared with Reference Instrument

Using Bland Altman 'limits of agreement' between the meters and reference the One Touch II showed a mean bias of +0.44 mmol/L (95% CI 0.20 to 0.67) with 95% of samples (±1.96SD) falling within +3.08 mmol/L (95% CI 2.68 to 3.49) and -2.26 mmol/L (95% CI -2.62 to -1.80) of the YSI analyser (Figure 14.1). The Glucometer II had a mean bias for all samples of +0.96 mmol/L (95% CI 0.54 to 1.38) with 95% of samples falling between +5.62 mmol/L (95% CI 4.89 to 6.34) and -3.71 mmol/L (95% CI -4.43 to -2.98) (Figure 14.2).

Results were within 15% of the reference in 107 out of 129 (82.9%) measurements using the One Touch II and 82 of the 124 (66.1%) measurements performed on the Glucometer II. Of the samples below 4 mmol/L with concurrent YSI measurements, 4 out of 11 Glucometer II results were within 15% and 7 out of 11 within 30% of the reference. The One Touch II had 6 from 11 within 15% and 11 from 11 within 30%.

The One Touch II was found to be as or more accurate than the Glucometer II in 106 out of 129 (82.2 %) samples.

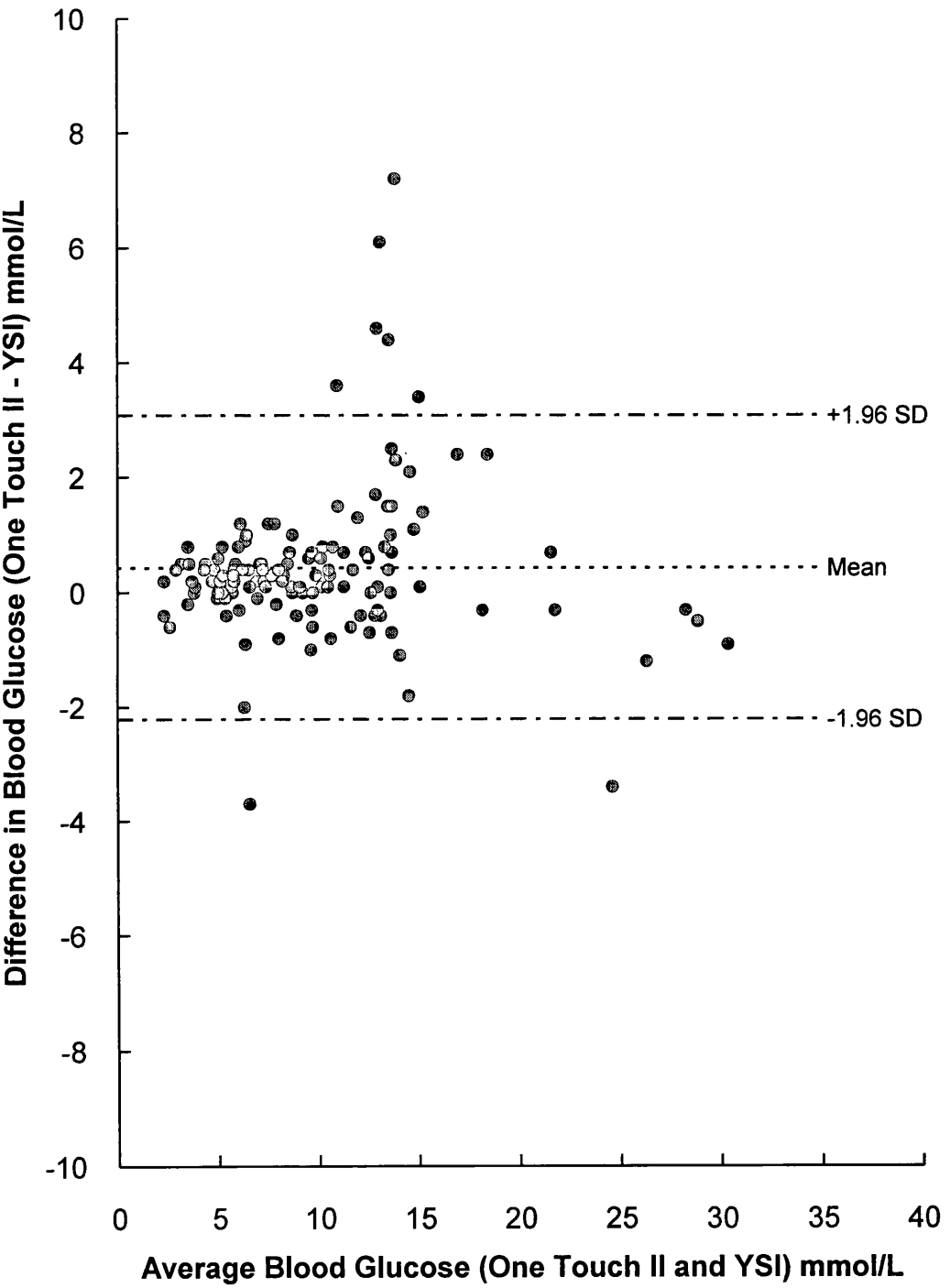


Figure 14.1 Bland Altman relationship between One Touch II blood glucose meter and YSI instrument.

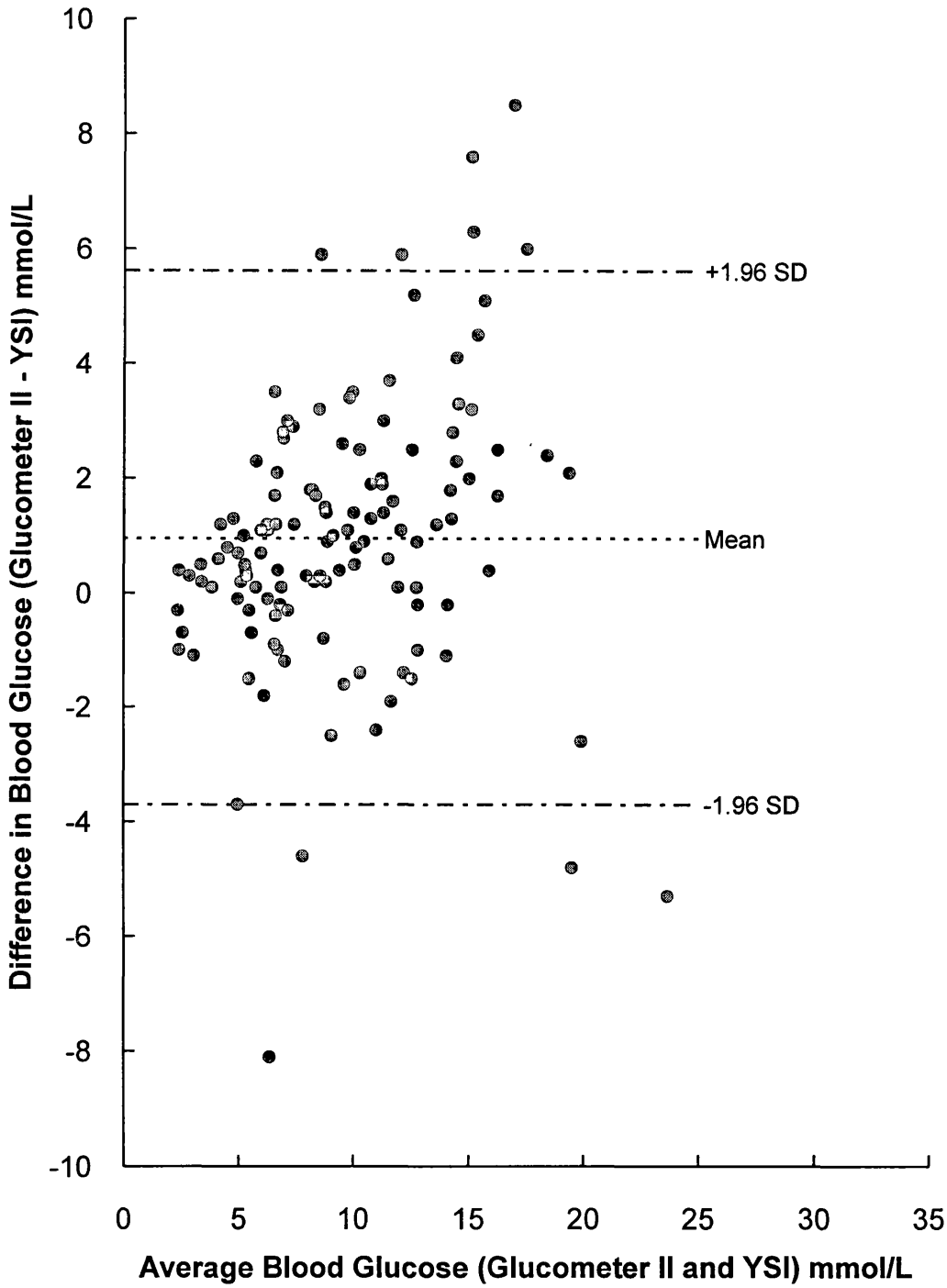


Figure 14.2 Bland Altman relationship between Glucometer II blood glucose meter and YSI instrument.

14.3.2 Glucose Meters Accuracy Over Same Analytical Range

Correlation of the One Touch II meter against the YSI for the samples over the entire meter range was as follows: $r=0.969$, $p<0.00001$, $y= 0.982x +0.617$. The correlation over the same analytical range as the Glucometer II (2.0-22.0 mmol /L) showed: $r=0.953$, $p<0.00001$, $y= 1.047x +0.08$. The same correlation for the Glucometer II meter demonstrated: $r=0.865$, $p<0.00001$, $y= 0.973x +1.20$.

14.3.3 Glucose Meter Accuracy Over Recommended Haematocrit Ranges

The manufacturers of the One Touch II meter recommend samples should have a haematocrit between 25-60%. For the Glucometer II, this is 35-50%. The respective correlations of these meters with the YSI were $r=0.974$ and $r=0.876$ within these haematocrit limits.

14.3.4 Agreement Between Meters

The correlations between the two meters were $r=0.896$ for all samples and $r=0.893$ for those with concurrent YSI measurements.

14.3.5 Measurements Outwith the Analytical Range of the Glucometer II

There were 17 occasions during the study period where the One Touch II meter was able to give a measurement which exceeded the upper analytical limit of the Glucometer II. On 7 of these instances the One Touch II recorded a glucose concentration greater than 25 mmol/L of which 3 were above 30 mmol/L. No samples were recorded as exceeding the analytical range of the One Touch II during the assessment.

No investigation specimens were measured by the Glucometer II as being below its lower measurement limit (2 mmol/L).

14.3.6 Questionnaire Results

Of the five nursing staff who responded to the questionnaire, four felt the One Touch II ease of use, feel/robustness and confidence in results were 'better' or 'much better' than that of the Glucometer II. The remaining nurse felt the former two attributes were the 'same' for both meters but had 'much better' confidence in the One Touch II results. All that replied preferred the One Touch II meter overall.

14.4 Discussion

In this study we attempted to give a comparison between two blood glucose monitoring systems which was more likely to reflect the measurements obtainable in a clinical setting rather than those achievable under laboratory conditions.

We found the One Touch II meter to perform closer to the reference YSI blood glucose analyser than the Glucometer II in both direct correlation and 'limits of agreement'. As a result, the One Touch II meter proved to equal or better the Glucometer II in 82% of measurements.

In this study, when using the recommendation that glucose meters should aim to have measurements which fall within 15% of the reference method,[130] 83% of One Touch II and 66% of Glucometer II results achieved this goal. Measurements at or near the hypoglycaemic range were of particular importance. At these concentrations the same deviation from the reference will yield a proportionately larger percentage error. At measurements of less than 4 mmol/L both meters had results which were greater than 15% from the YSI, but only the Glucometer II had ones which were beyond twice this desirable maximum error.

We have shown that variations in haematocrit are the sole haematological measurement responsible for discrepant glucose test strip results, with the Glucometer II showing particular susceptibility (see Chapter 9). However,

when only considering samples within both meter manufacturers recommended haematocrit limits the One Touch II still compared more favourably with the reference than the Glucometer II.

The 17 cases during the evaluation period where the One Touch II gave a measurement which was outwith the analytical range of the Glucometer II are likely to have prevented unnecessary venepuncture for additional emergency blood glucose measurements by the biochemistry laboratory. This only applies, of course, where the individual has had a high blood glucose concentration previously confirmed by the laboratory and whose condition is not worsening. In this situation, as well as being beneficial to the patient (by allowing a quicker therapeutic response) this also has cost implications to a diabetic unit.

The fact that nursing staff unanimously preferred using the One Touch II meter cannot be overlooked and may indicate the potential for greater compliance in self-monitoring of blood glucose.

In conclusion, the potential advantages of the One Touch II meter over the Glucometer II in the setting of a diabetic ward would appear to have resulted in better meter accuracy, fewer blood glucoses too high to be measured and a greater user preference.

Since the publication of this study it has been interesting to note that the two dominant glucose meter manufacturers in the UK (Boehringer Mannheim and Bayer) have responded to products such as the One Touch II by developing instruments which mimic many of the features found in this meter. For example, with the Accutrend Alpha from Boehringer Mannheim, blood is also applied to a strip already inserted into the meter and timing of the test starts automatically on sample application. In common with all new glucose meters, this instrument also features a 'non-wipe' system. In addition, meters such as the Accutrend and Companion 2 now demonstrate the ability to record date and time stamped results in the meter memory for future downloading into a PC, if necessary.

It is hoped that the meter improvements from these manufacturers will, like the One Touch II, improve their ease of use and their accuracy of results.

Chapter 15

A Patient Comparison of the One Touch II and Accutrend Blood Glucose Meters.

15.1 Introduction

The new generation of blood glucose meters can perform significantly better than older systems when used in a ward setting (see Chapter 14). However, the introduction of new blood glucose systems to the UK has left diabetic patients with a bewildering choice of at least 15 different meters. Often the newer meters also appear to have similar features to one another, thereby making a choice even more difficult. For example, the One Touch II and Accutrend meters both epitomise the recent advances that have occurred in non-wipe technology, analytical range, reduction in test time and recording of patient results in meter memories. To see if differences exist between the newer meters, part of this study has compared the relative accuracy and acceptability of the One Touch II and Accutrend blood glucose meters when used by diabetic patients.

Not every diabetic out-patient clinic has the resources to fund on-site glycated haemoglobin measurement, so in-clinic random glucose measurement is often used as an immediate assessment of glycaemic control. As an alternative, this study has also assessed the relative usefulness of using the mean value of the glucose results stored in the memories of the One Touch II and Accutrend meters.

15.2 Methods

15.2.1 Patients

Seventeen diabetic patients (10 male, 7 female, 15 Type I, 1 insulin treated Type II, median age 39 years, range 18-69, median duration of diabetes 8 years, range 0-27) attending the diabetic out-patient clinic, Gartnavel General Hospital, Glasgow participated in the study. Patients had either not used a meter previously or were using one and had expressed a preference to change. None had prior experience of the meters used in the study.

15.2.2 Study Design

A 16 week crossover study was performed. The diabetic patients were randomly assigned either a One Touch II or Accutrend blood glucose meter for eight weeks before crossing over to the other. They were educated in the use of both meters by the same Diabetes Nurse Specialist.

Patients were asked to perform 4 pre-prandial blood glucose measurements per day on 3 occasions per week. These were recorded using a diary and confirmed by the meter memories. In addition, to assess the meter accuracy, the patients were invited to attend weekly for a simultaneous measurement of capillary whole blood glucose by the meter (using their own technique) and by a reference Analox Model GM9 instrument. The sample used for the reference measurement was from either the same finger stab as that made by the patient or from one taken directly afterwards.

Haemoglobin A_{1c} was also measured in the patients using a Bayer DCA2000 instrument at the start of the study, at meter changeover and at study end. Random clinic glucose measurements using the reference instrument were also obtained at these times.

At the end of the study, patients were allowed to keep the meter they had preferred using.

15.2.3 Statistical Analysis

Linear regression used the least squares method. Bland Altman residual plots were used for glucose method comparisons. Comparison of glucose values with HbA_{1c} used the McNemar test.

15.3 Results

15.3.1 Meter Accuracy

The results of both meters correlated with those of the reference instrument (One Touch II, r= 0.966, n=132; Accutrend, r=0.885, n=120). The One Touch II showed a mean bias of -0.63 mmol/L (95% within +2.71 and -3.97 mmol/L) and the Accutrend a mean bias of -0.73 mmol/L (95% within +4.75 and-6.21 mmol/L)(Figures 15.1 and 15.2). Seventy five percent (99/132) of One Touch II and 74% (89/120) of Accutrend meter results were within 15% of the reference value (NS difference).

15.3.2 Meter Acceptability

At study completion, 15 out of the 17 patients chose the One Touch II as their preferred meter ($p<0.005$). The reasons for choosing the One Touch II were cited as either due to the improved ease of use of the One Touch II or the fact that the Accutrend did not operate reliably at temperatures below 15^0C. Of the 2 who chose the Accutrend, one preferred its shorter test time (12 seconds vs. 45 seconds) while the other, with impaired vision, favoured the larger results display.

15.3.3 Meter Memory Values and HbA_{1c}

Patients performed a total of 3,524 meter measurements. A mean of 112 One Touch II and 107 Accutrend measurements were recorded by each user. Haemoglobin A_{1c} values at study crossover and study end were obtained on 16 patients. For each of the eight week periods, the mean Accutrend and One Touch II glucose values correlated with the subsequent HbA_{1c} measurement ($p<0.00001$, Figure 15.3). This was not the case when using the random clinic value ($p=0.14$, Figure 15.4). This meant that the average meter glucose values predicted more HbA_{1c} results within 2 standard deviations of the assay (i.e.

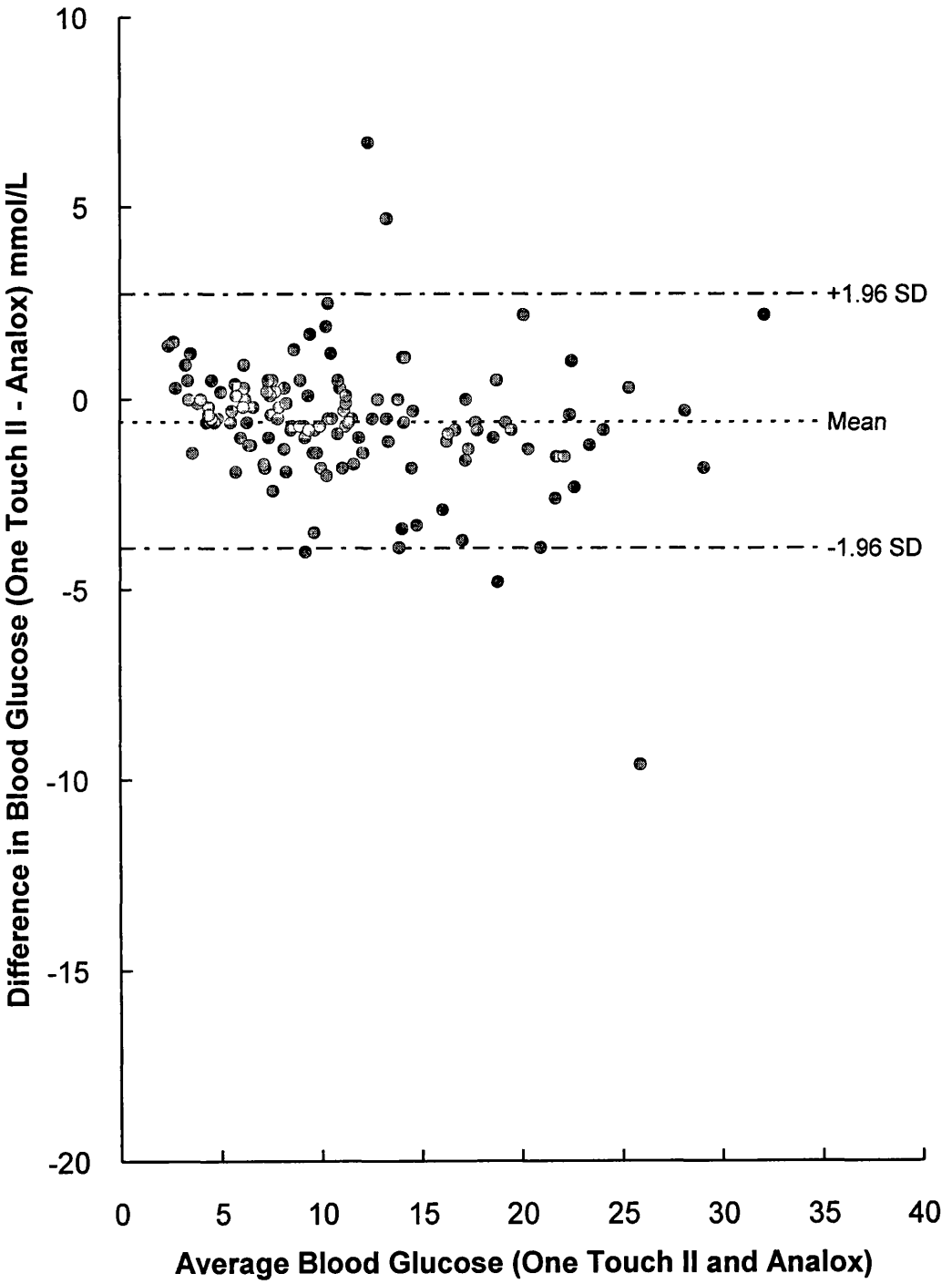


Figure 15.1 Bland Altman relationship between One Touch II blood glucose meter and Analox instrument.

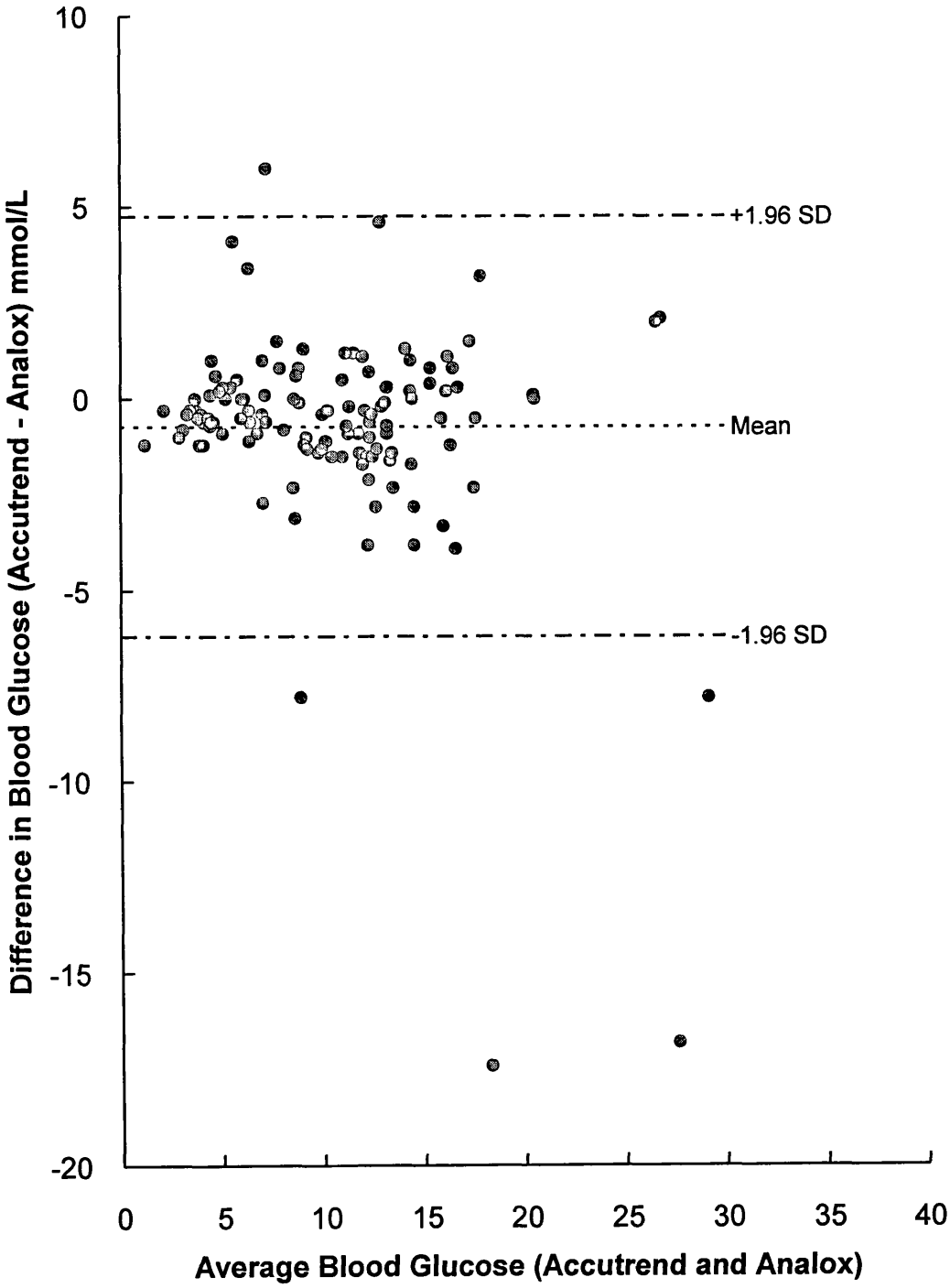


Figure 15.2 Bland Altman relationship between Accutrend blood glucose meter and Analox instrument.

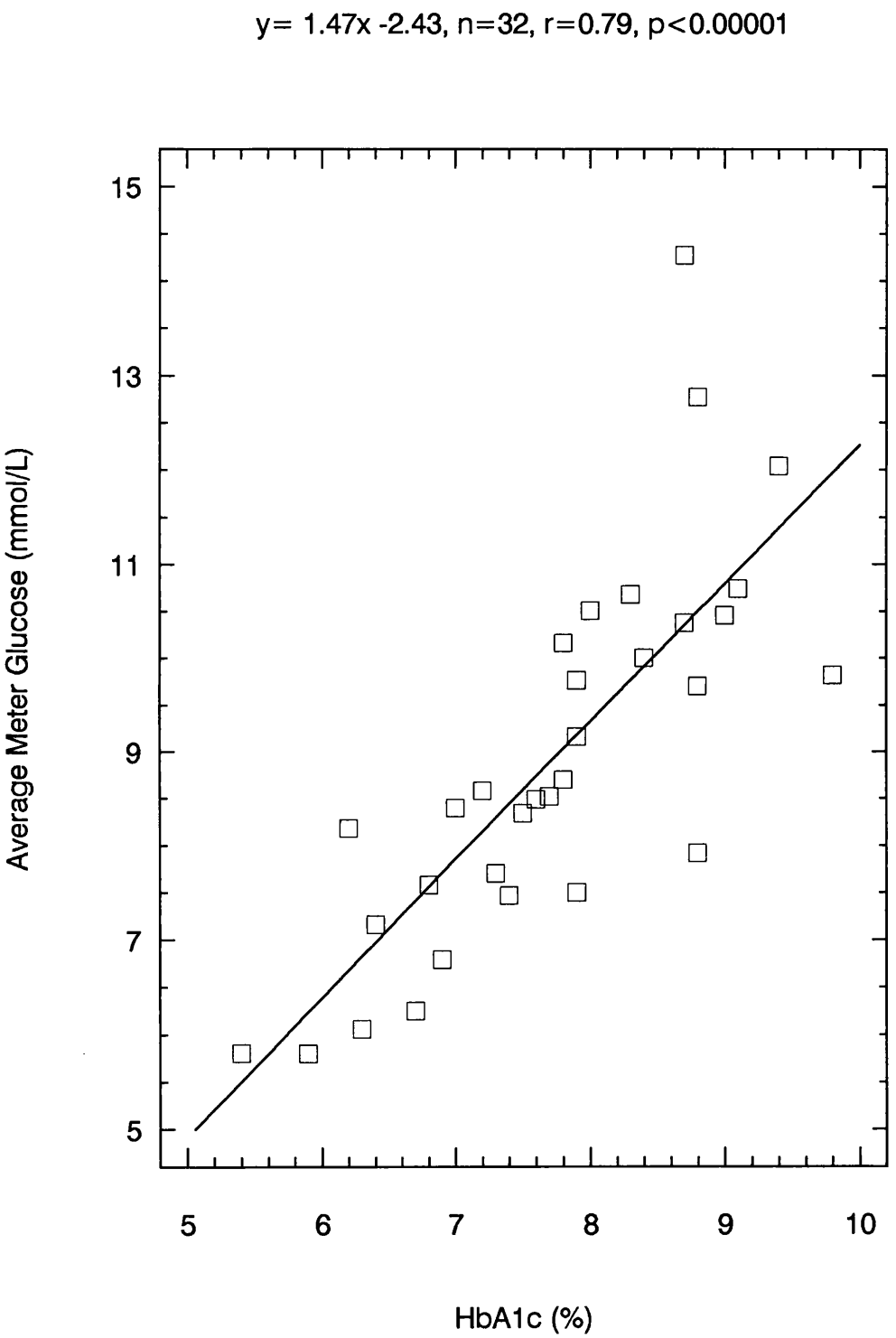


Figure 15.3 Relationship between average glucose meter value over the period of 8 weeks and subsequent HbA_{1c}.

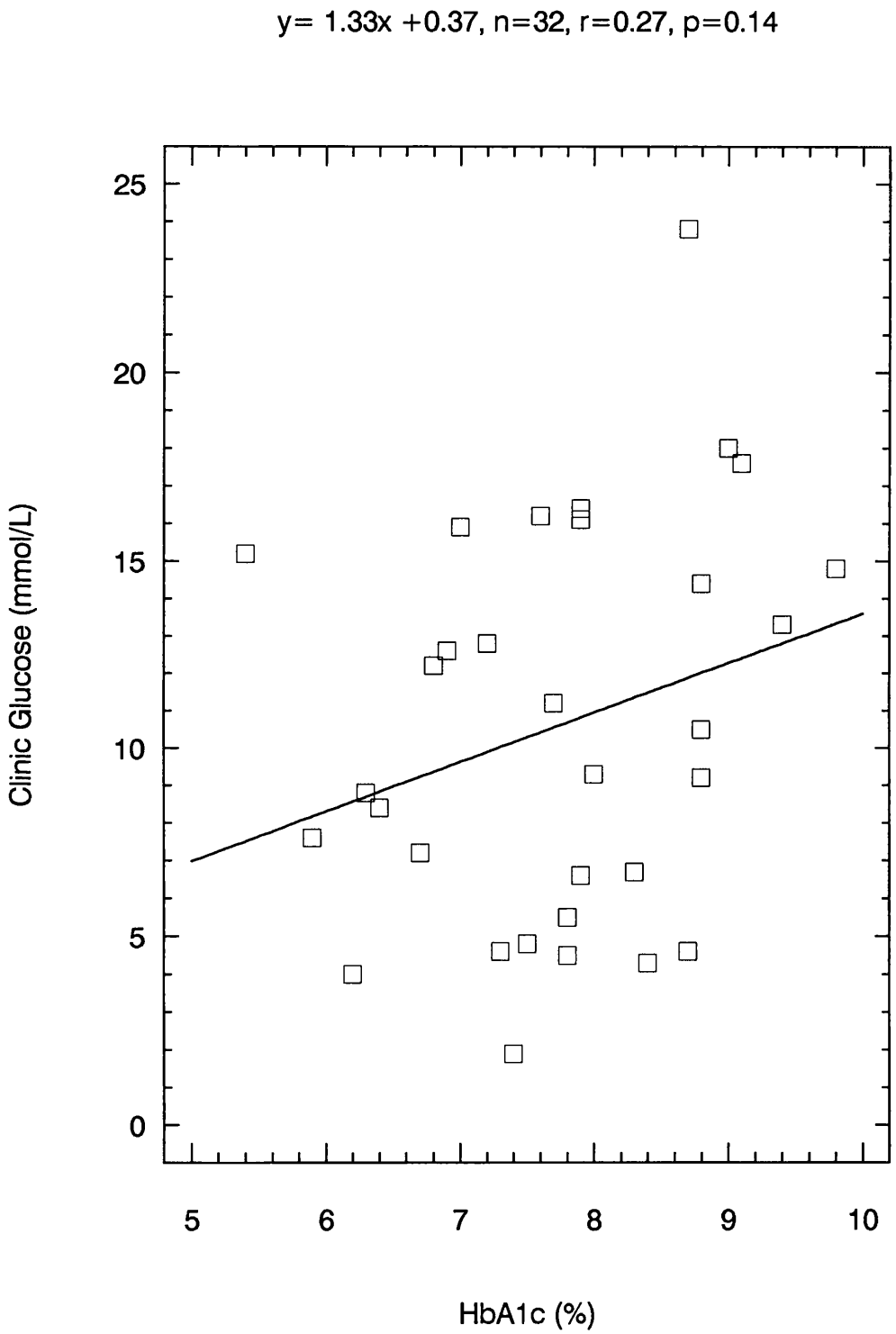


Figure 15.4 Relationship between clinic random blood glucose value and HbA_{1c}.

0.6% HbA_{1c}, see Chapter 5) than the random glucose measurement (23/32 vs. 11/32, p=0.003).

15.4 Discussion

Improvements in glucose meter technology are only of use if it can be demonstrated that patients are happy using these meters and can obtain acceptably accurate results. Although the meters used in this study have many similar features, they also differ in a number of respects. For example, the Accutrend meter can obtain results in 12 seconds compared to 45 seconds with the One Touch II while the Accutrend requires a larger sample volume than the One Touch II (11 μL vs. 3-5μL). Additionally, the One Touch II, unlike the Accutrend, displays written prompts for each process in the meter measurement but does not have the Accutrend's automatic bar-code calibration of test strips.

For all these differences, this study has shown that the One Touch II and Accutrend blood glucose meters show similar accuracy to one another when used by the same diabetic patients. Both meters had 74% of samples achieving the recommended goal of results within 15% of the reference instrument value.[130] This compares to the 83% of One Touch II samples which met this standard when the meter was used by trained nursing staff (see Chapter 14). In contrast, when using direct correlation and residual plots, the One Touch II showed greater overall agreement with the reference instrument than the Accutrend. This implies that the Accutrend readings which are not within 15% of the reference tend to be less accurate than the One Touch II.

The fact that 15 patients preferred to keep the One Touch II in preference to the Accutrend would appear to indicate that patients consider ease of use and reliability in meter measurement more important than simply the speed with which the test is performed.

It is of interest that the mean glucose value obtained by patients on their meter over the period of 8 weeks was so closely related to their subsequent HbA_{1c} value (Accutrend r= 0.79, One Touch II r= 0.81, together r=0.79). In a clinical situation, such a value recorded in the meter memory of insulin treated patients would appear to be a superior means of assessing glucose control than a random glucose measurement performed before an out-patient consultation (r=0.27, p=0.14). Predicting a HbA_{1c} value outwith 2 standard deviations of the true value may completely misclassify the glycaemic control of a patient when using European guidelines (see Chapters 4 to 7). In this study, the average meter glucose concentration predicted nearly three quarters of the patients to within 2SD, while with the random clinic concentration this was only one third. Therefore, for clinicians and patients attending clinics without the funding for on-site glycated haemoglobin measurement, the use of the average value in meter memories would appear to be a more reliable indicator of glucose control.

On a practical note, the Accutrend meter requires downloading of its recorded glucose values before an average value can be calculated, whereas the One Touch II has this facility as a function of the meter.

In conclusion, it would appear that the One Touch II and Accutrend blood glucose meters have comparable accuracy when used by diabetic patients. However, for the majority of patients the One Touch II meter is preferable because of its superior ease of use. This study has also shown that the glucose values stored in meter memories would seem to be more useful than in-clinic random glucose measurements when assessing the glycaemic control of insulin treated patients.

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