# Open Research Online

The Open University's repository of research publications
and other research outputs

## Automation of Gycohaemoglobin Measurement and its Application to Renal Patients.

## Thesis

For guidance on citations see FAQs.

Version: Version of Record

oro.open.ac.uk

# Automation of Glycohaemoglobin Measurement
# and its Application to Renal Patients

Naseem Akhter Goraya (BSc Hons)

A thesis submitted in fulfilment for the degree of Master of Philosophy,
Department of Biology, The Open University.

Dept. of Clinical Pathology,
Royal Sussex County Hospital,
Eastern Road,
Brighton,
East Sussex.
BN2 5BE.

DATE OF SUBMISSION: 5 AUGUST 1999
DATE OF AWARD: 28 JANUARY 2000

August 1999

*I begin with the name of Allah, the most merciful and compassionate.*

## Acknowledgements

I would like to take this opportunity to thank Dr. Furth (internal supervisor) and Dr. Bernard Rocks (external supervisor) for their help, support, and encouragement with this project.

I would like to thank Brighton Health Care NHS trust for their sponsorship both financially and allowing day-release from full-time work in order to complete this project.

I would also like to thank Dr. Sandhu (renal registrar) and nurse Osorio (renal sister) for their help in gathering clinical information on the renal patients on continuous ambulatory peritoneal dialysis (CAPD) treatment.

Finally I would like to thank my parents and sisters for their endless encouragement and support.

# Abstract

The work for this project was carried out in two parts. The first part of this project describes, evaluates, and compares a new pre-production automated analyser with the manual Pierce affinity method for the measurement of glycohaemoglobin (GHb). The Pierce affinity method has proven reliable for the measurement of GHb, but is very labour intensive requiring several hours for the completion of the test. The alternative new GHb analyser is simple and rapid to use.

The automated method is also more precise (CV < 5%) and is less prone to error than the manual assay. There was a good correlation between the two methods ( n = 50, y = 1.018x -0.1034, r = 0.9806 where y = Drew  x = Pierce, p = < 0.0001 using the assigned calibrant values of BR1 = 3.8%, BR2 = 10.0%. As a result of these investigations, we have now replaced our manual method with the automated analyser. Towards the end of this project we came under pressure from our endocrinologist to report our GHb results as 'DCCT aligned'. To this end we used the Primus DCCT aligned calibrants (intended for their own analyser) with values of Primus 1 = 5.8%, Primus 2 = 13.3%. The results of the correlation study of Biorad versus Primus calibrants was n = 139, y = 0.7585x + 2.0154, r = 0.9981 where y = Primus (GHb%)  x = Biorad GHb (%), p = < 0.001.

The second part of this project was an evaluation of the effect of continuous ambulatory peritoneal dialysis (CAPD) treatment on GHb results. The peritoneal dialysis solutions used in CAPD contain from 13.6 g to 38.6 g/l of glucose as an osmotic agent. During CAPD 100-200 g of glucose is absorbed from the glucose-containing dialysate fluids each day. Icodextrin is a starch-derived glucose polymer which also acts as an osmotic agent when administered intraperitoneally for CAPD, but unlike glucose-containing CAPD fluids does not contribute to large amounts of glucose absorption. The GHb measurement for this part of the evaluation was carried out using the Drew Scientific GHb-100 analyser after the first part of the project was successfully completed.

Contrary to what was expected CAPD did not cause a statistically significant difference in the concentration of GHb of non-diabetic patients compared to a non-diabetic group of patients who were not subjected to CAPD.

The research conformed to the ethical standards set out by the Royal Sussex County Hospital for research.

# List of Abbreviations

| | |
|---|---|
| **AGE** | Advanced glycation end products |
| **ATPase** | Adenosine triphosphatase |
| **CAPD** | Continuous ambulatory peritoneal dialysis |
| **Da** | Daltons |
| **DCCT** | Diabetes control and complications trial |
| **DIDMOAD** | Acronym for diabetes insipidus, diabetes mellitus, optic atrophy and deafness |
| **DMF** | Deoxymorpholinofructose |
| **DNA** | Deoxyribonucleic acid |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ELISA** | Enzyme-linked immunosorbentassay |
| **Hb** | Haemoglobin |
| **$HbA_1$** | Haemoglobin $A_1$ |
| **$HbA_{1a}$** | Haemoglobin $A_{1a}$ |
| **$HbA_{1b}$** | Haemoglobin $A_{1b}$ |
| **$HbA_{1c}$** | Haemoglobin $A_{1c}$ |
| **HbF** | Foetal haemoglobin |
| **HbS** | Sickle cell haemoglobin |
| **HDLs** | High-density lipoproteins |
| **HLA** | Human leukocyte antigen |
| **5-HMF** | 5-Hydroxymethyl furfural |
| **HPLC** | High performance liquid chromatography |
| **IAPP** | Islet amyloid polypeptide |
| **IDDM** | Insulin-dependent diabetes mellitus |
| **IGT** | Impaired glucose tolerance |
| **IUPAC** | International union of pure and applied chemists |
| **JCBN** | Joint commission on biological nomenclature |
| **LDLs** | Low-density lipoproteins |
| **MIDAS** | Multicentre investigation of icodextrin in ambulatory peritoneal dialysis |
| **MODY** | Maturity onset diabetes mellitus in the young |
| **MW** | Molecular weight |
| **NADH** | Nicotinamide adenine dinuceotide (reduced) |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate (reduced) |
| **NEQAS** | National External Quality Assurance Scheme |
| **NIDDM** | Non-insulin-dependent diabetes mellitus |
| **NTB** | Nitroblue tetrazolium |
| **PCV** | Packed cell volume |
| **pH** | - Log $_{10}$ of the hydrogen ion concentration |
| **PI** | Iso-electric point |
| **TBA** | Thiobarbituric acid |
| **TNFα** | Tissue necrosis factor alpha |
| **WHO** | World Health Organisation |

# Table of Contents

# 3.0 EXPERIMENTAL - METHODS AND MATERIALS

3.1 **Subjects**
3.2 **Sample Preparation**
3.3 **Equipment**
3.4 **Chemicals**
3.5 **Data Analysis**
3.6 **Glycohaemaglobin Measurement by the Pierce Affinity Chromatography Method**
    3.6.1 Principle
    3.6.2 Reagents and Columns
    3.6.3 Quality Control
    3.6.4 Assay Procedure
        3.6.4.1 Calculation
        3.6.4.2 Result Range and Interpretation
3.7 **Glycohaemoglobin Measurement by the Drew Scientific GHb-100 Affinity LPLC Automated Analyser**
    3.7.1 Principle
    3.7.2 Reagents and Columns
    3.7.3 Quality Control
    3.7.4 Primus Calibrant
    3.7.5 Assay Procedure
        3.7.5.1 Calculation

# 4.0 EVALUATION AND RESULTS OF THE GHb-100 ANALYSER FROM DREW SCIENTIFIC

4.1 **Effect of Haemoglobin Concentration on GHb Results**
4.2 **Effect of Using 1% Triton X-100 as a Haemolysing Reagent**
4.3 **Packed Cells versus Whole Blood**
4.4 **Precision-run Study**
4.5 **Between-batch Precision Study**
4.6 **Correlation Study**
    4.6.1 Correlation Study of Assigned Calibrant Values of
        BR1 = 4.3% BR2 = 11.3%
    4.6.2 Correlation Study of Assigned Calibrant Values of
        BR1 = 3.8%  BR2 = 10.0%
    4.6.3 Biorad versus Primus Calibrants
        4.6.3.1 The Effect of Re-standardisation using Primus Calibrants on the Existing Reference Range For GHb
4.7 **Column Life of the GHb-100 Analyser**
4.8 **Problems Encountered with the GHb-100 Analyser when in Routine Use**

# 5.0 EVALUATION AND RESULTS OF THE RENAL PATIENTS ON CAPD TREATMENT

# 6.0 DISCUSSION

# 7.0 CONCLUSION

# 8.0 REFERENCES

# List of Tables and Figures

## Tables:

## Figures:

# 1.0  INTRODUCTION

Glycohaemoglobin measurement is a test which is valuable in the monitoring and treatment of hyperglycaemic patients. Hyperglycaemia occurs in patients with diabetes mellitus and is reported to occur in non-diabetic renal patients on continuous ambulatory peritoneal dialysis (CAPD) who are dialysed with a high glucose content fluid (1). Both these conditions are discussed in detail.

## 1.1  Diabetes Mellitus

Diabetes Mellitus is a group of metabolic disorders characterised by chronic hyperglycaemia due to relative insulin deficiency, resistance or both. It is common and effects 30 million people world wide. Diabetes is usually irreversible, and although patients have a reasonably normal life-style, its late complications result in a reduced life-expectancy and considerable uptake of health resources. Macrovascular disease leads to an increased prevalence of coronary artery disease, peripheral vascular disease and stroke, while microvascular damage results in diabetic retinopathy and contributes to nephropathy (2).

### 1.1.1  Types of Diabetes Mellitus

Diabetes may be primary or secondary. Primary diabetes include:- type I or insulin - dependent diabetes mellitus (IDDM); type II or non-insulin-dependent diabetes mellitus (NIDDM); and the much rarer types of diabetes usually associated with other hereditary disorders. Secondary diabetes include diabetes due to destruction of the pancreas by drugs, disease or surgery and disease due to hormonal imbalance (3).

### 1) Type I Diabetes (Insulin-Dependent)

Insulin-dependent diabetes mellitus (IDDM) identifies patients who cannot survive without insulin treatment. The commonest cause is autoimmune destruction of the beta-cells of the islets of Langerhans. Insulin deficiency is so profound that ketoacidosis - the hallmark

of IDDM - will develop unless insulin replacement is given (3). The prevalence of IDDM shows a marked geographical variation with a 35-fold difference between Finland and Japan, the countries with the highest and lowest frequencies. In the UK the prevalence is about 0.25% and it accounts for about 20% of diabetes (2). Most patients present young and the peak age of onset is 12 years. At least 10% of diabetic patients aged over 65 years are insulin-dependent (3).

Type I diabetes is due to an interaction between environmental factors and a genetic predisposition to the disease. Inheritance is polygenic; major susceptibility loci include the human leucocyte antigen (HLA) class II genes (chromosome 6p) and the insulin gene (chromosome 11p). Possible environmental triggers for IDDM include various viruses (e.g. *coxsackie*) and neonatal exposure to bovine serum albumin in cows milk (2). Putative beta-cell autoantigens that might incite immune damage include glutamic acid decarboxylase (GAD) and insulin. Circulating antibodies against these and other antigens are detectable in most newly presenting IDDM patients, and also in some subjects with apparently typical NIDDM (2). The development of IDDM is the culmination of beta-cell destruction, often over many years. During the asymptomatic pre-diabetic phase, various beta-cell autoantibodies are present and subtle abnormalities of insulin secretion and glucose tolerance can be detected (2).

IDDM usually presents acutely with osmotic symptoms due to hyperglycaemia (thirst, polyuria, polydipsia), tiredness and weight loss. Minor symptoms include cramps, blurred vision and superficial infections. Nausea, vomiting and drowsiness usually denote impending ketoacidosis which nowadays only affects about 5-10% of newly diagnosed patients (3). Correction of hyperglycaemia (this impairs remaining beta-cell function) may induce a remission (honeymoon period) during which insulin requirements are low. Remission ends, sometimes after many months, when remaining beta-cells are destroyed by continuing autoimmune activity (2).

2

40-50% of patients with long-standing IDDM are at risk of significant microvascular complications (retinopathy, neuropathy, nephropathy), which are specific for diabetes, or macrovascular disease (atheroma). Evidence shows unequivocally that good glycaemic control prevents the specific diabetes-related complications (3). Overall mortality in IDDM is increased 4-7 fold compared with matched non-diabetic population, principally because of premature death from renal failure and coronary heart disease (3).

More accurate methods are being developed to predict which at-risk individuals will develop IDDM. Agents such as nicotinamide which protect against beta-cell damage, may be able to induce prolonged remission, or even prevent the development of IDDM (2).

**2) Type II Diabetes (Non-Insulin-Dependent)**

Non-insulin-dependent diabetes mellitus (NIDDM) identifies patients who do not need insulin treatment to survive, though they may need insulin in order to remain healthy (about 20% are insulin treated) (3). It is characterised by variable combinations of insulin resistance and insulin deficiency; the latter is less severe than in IDDM, and blood levels remain high enough to prevent excessive lipolysis and spontaneous ketoacidosis. Patients with NIDDM are therefore C-peptide positive (2).

NIDDM accounts for up to 85% of the diabetic population in most countries, affecting 5-7% of Western populations and 10% of people over the age of 70 years. The prevalence is often low in developing countries (<1%) and very high (40-50%) in certain groups which have undergone rapid Westernisation (e.g. Pima Indians in North America). It is becoming steadily more common in many countries. The peak age of onset is 60 years; most subjects are diagnosed after the age of forty years (3).

Genetic factors determine most of the susceptibility to the common type of NIDDM. 'Thrifty susceptibility genes', postulated to favour energy storage, might favour survival during harsh conditions but could lead to obesity, insulin resistance and NIDDM when food

3

is freely available. 'Thrifty genes' could act by inducing insulin resistance in the liver and muscle but not fat; their identity remains unknown (2).

Environmental factors contributing to the development of NIDDM include excessive energy intake and physical inactivity, which predispose to obesity and perhaps malnutrition *in utero* and infancy, which may impair beta-cell development. Diabetogenic drugs, pregnancy and excess counter-regulatory hormones can also induce NIDDM (2).

Obesity is present in over 50% of male and 70% of female patients and strongly predisposes to the disease. Truncal obesity, with visceral fat deposition, is particularly important in inducing insulin resistance and is associated with glucose intolerance, hypertension and dyslipidaemia (syndrome 'X'; Raven's Syndrome). Obesity may induce insulin restance through increased secretion of tissue necrosis factor alpha (TNFα)

Initially, insulin secretion rises and overcomes the effect of insulin resistance but ultimately fails and allows blood glucose levels to increase through a phase of impaired glucose tolerance (IGT) to overt NIDDM. The cause of beta-cell failure is unknown. The beta-cell product, islet amyloid polypeptide (IAPP) or its amyloid product that is deposited in the islets, have been implicated (2).

NIDDM diagnosed in adolescence 'Maturity Onset Diabetes Mellitus in the Young' (MODY) has in some cases been associated with mutations in the gene coding for glucokinase which is important in regulating insulin secretion in response to ambient glucose levels. Mutations in mitochondrial DNA are responsible for rare maternally inherited syndromes of diabetes with various neuromuscular syndromes (3).

About 50% of NIDDM patients present with classical osmotic symptoms due to hyperglycaemia, but many are diagnosed fortuitously or later suffering from diabetes-related complications. NIDDM is insidious and often present for several years before diagnosis. Up to 50% of all cases may be as yet undiagnosed (3). NIDDM cause microvascular diabetic complications and macrovascular disease; the major cause of death is coronary heart disease

(60% of cases). Microvascular disease, especially retinopathy, may be established at the time of diagnosis. Overall 5-year mortality is increased two-to-three fold and life expectancy reduced by 5-10 years (3).

Management commences with diet and life style, with a low-fat, high complex carbohydrate diet (and a caloric restriction for obese patients) and increased physical activity. These measures are usually inadequate and a sulphonylurea (insulin secretagogue; more useful in non-obese patients) or the insulin-sensitising biguanide, metformin, is added. Many patients ultimately require both oral agents and subsequently insulin (3).

Adjunctive treatments include the alpha-glucosidase inhibitor, acarbose, and anti-obesity drugs or very low calorie diets. Novel drugs include the thiazolidinedionic derivatives, e.g. troglitazone, which are insulin-resistance modulators (3).

### 1.1.1.1 Summary of Primary Diabetes (IDDM and NIDDM)

**Table 1** Summary of Primary Diabetes (IDDM and NIDDM)

|  | Type I (IDDM) | Type II (NIDDM) |
|---|---|---|
| Common age of onset: | Any, predominantly young | Any, predominantly middle and older ages |
| Family history of diabetes: | Uncommon | Common |
| Seasonal incidence: | More in winter months | No seasonal preponderance |
| Phenotype: | Thin | Obese |
| Haplotypes: | HLA B8, B15, B18, DR3, DR4, DQB, etc. | No preponderance |
| Onset: | Rapid | Slow |
| Symptoms: | Severe | Mild or absent |
| Urine: | Glucose and acetone | Glucose |
| Keto-acidosis: | Prone | Resistant |
| Serum insulin: | Low or absent | Usually depressed but may be raised |
| Islet cell antibodies: | Present at onset | Absent |
| Treatment: | Insulin | Diet |

**Diabetes Associated with Other Genetic Disorders**

Diabetes is sometimes a part of somewhat rare syndromes occurring in childhood and involving the neuro-endocrine and nervous system. Perhaps the least uncommon is the DIDMOAD syndrome. DIDMOAD is an acronym for diabetes insipidus, diabetes mellitus, optic atrophy and deafness. (Friedreich's ataxia, polyneuritis, retinitis pigmentosa, hypogonadism and obesity are other features sometimes involved). The genetic inheritance of these disorders can hardly be in question because they often occur in siblings or when the parents are consanguineous. The most likely explanation is that the syndrome is due to an inherited defect of embryonic neuroectoderm, from which primitive endocrine cells migrate to the gut and pancreas. There are many other associations with genetic disorders (3).

**1.1.1.2 Secondary Diabetes**

Secondary Diabetes results from destruction of the islets by surgery, disease or drugs. **Table 2** shows a list of causes of secondary diabetes.

**Table 2** Causes of secondary diabetes

| Cause | Example |
| --- | --- |
| *Liver Disease* | Cirrhosis. |
| *Pancreatic Disease* | Cystic fibrosis, Chronic pancreatitis, Pancreatectomy, Haemochromatosis, Carcinoma of the pancreas. |
| *Endocrine Disease* | Cushings's syndrome, Acromegaly, Thyrotoxicosis, Pheochromocytoma, Glucogonoma. |
| *Drug-Induced Disease* | Thiazide diuretics, Corticosteriod therapy. |

## 1.2    Renal Patients on CAPD Treatment

Chronic uraemia is associated with decreased glucose tolerance and other abnormalities reflecting impaired glucose metabolism (4-6). Fasting blood glucose levels are often normal or only moderately elevated but about 50% of the patients show an abnormal oral or intravenous glucose tolerance (4). Peripheral insulin resistance appears to be the most important pathogenetic factor for glucose intolerance in uraemia although decreased insulin secretion is observed in some patients (4-6). In addition, the basal circulating levels of many hormones including insulin and glucagon are often elevated, mainly due to their impaired degradation by the diseased kidneys (5). Other factors associated with end stage renal failure that are capable of interfering with glucose homeostasis are, accumulation of uraemic toxins, diminished physical activity, metabolic acidosis, hyperosmolality and medication (7).

During continuous ambulatory peritoneal dialysis (CAPD), which is an increasingly used form of chronic dialysis therapy, 2 litres of dialysis fluid remains in the patients abdomen at all times and are replaced with fresh fluid four to five times daily. CAPD as currently used only replaces about 5-10% of the function of two normal kidneys: four exchanges of 2 litres each in a 24 hr period equals 8 litres/day, or 5-6 ml/min; ultrafiltration using hyperosmolar fluid to remove excess salt and water may raise the clearance to 7 ml/min. CAPD provides round-the-clock function in contrast to the intermittent function provided by haemodialysis, and the latter, averaged over a period of a week, gives a comparatively low clearance. With the use of glucose containing CAPD fluids 100-200 g of glucose is absorbed each day from the dialysis fluid (8). In analogy with intermittent peritoneal dialysis (9) it is conceivable that such a continuous glucose load could result in further impairment of glucose tolerance by exhausting insulin secreting capacity.

After a glucose challenge, concentrations of glycated plasma proteins continue to

7

rise even after return to normoglycaemia. Amadori products continued to form as glucose concentrations fall (10). Similar changes might take place in dialysis, therefore CAPD patients were considered a suitable group for study.

### 1.2.1 Glucose and Other Omotic Agents in CAPD

In the peritoneal dialysis system, hydrostatic pressure in the blood compartment cannot be readily manipulated. Therefore, traditionally a solute (osmotic agent) is added to the peritoneal dialysis solution to create an osmotic driving force. During the process of ultrafiltration, the rate of ultrafiltration decreases with time due to dilution by the ultrafiltrate and absorption of the osmotic agent. Thus ultrafiltration will eventually cease after the dialysis solution is infused. The bigger the molecule of the osmotic agent, the longer the ultrafiltration lasts because solute absorption through the peritoneal membrane is slower. Thus, to achieve sustained ultrafiltration, an osmotic agent with a bigger, non-toxic, biocompatable molecule would be more advantageous than the smaller one at comparable osmotic gradients.

The osmotic driving force is dependent on the osmolality, which is a measure of the total number of osmotic agent molecules in the solution. It is directly related to the molal concentration of a solute, i.e., the number of moles per kilogram of solvent. In clinical practice, it is customary to measure the solute concentration as a percentage, i.e. actual weight per litre of solution. Large molecular weight solutes must be dissolved in higher percentage concentrations to obtain the same osmolality. The properties of charged molecules are different as the osmotic driving force does not depend on the polymer molecules themselves, but on the concentration of ionised electrolytes.

Glucose, which is the only osmotic agent in common use for CAPD, readily permeates through the peritoneal membrane. Using an intraperitoneal volume marker, Pyle et al. characterised the ultrafiltration profile of glucose based solutions (11). They demonstrated a rapid exponential decline in the ultra filtration rate with time in response to

a fall in osmotic gradient, due to a combination of glucose absorption and intraperitoneal dilution. Once equilibration of osmotic forces occurs, ultrafiltration ceased and reabsorption began. Since the equilibration times were between 2-3 hours with glucose solutions, the prolongation of the dwell time beyond this, commonly led to reabsorption of fluid that exceeded the ultrafiltrate (negative ultrafiltration); this was particularly noticeable in overnight exchanges.

Several authors have studied glucose absorption from glucose containing CAPD fluids. Lindholm et al. (12) studied glucose absorption from the dialysate in 13 non-diabetic patients undergoing metabolic studies. All patients exchanged 2 litres of dialysate four times per day. An average of 121 +/- 40 g glucose per day was absorbed each day representing an uptake of 71 +/- 10% of the glucose instilled in the peritoneal cavity.

Grodsteine et al. (13) found that an average of 182 +/- 61 g of glucose was absorbed each day. Most of their patients used five exchanges of dialysate per day. The percentage of the instilled glucose which was absorbed was 72%.

Baeyer et al. (14) found that the mean daily absorption of glucose was 117 +/- 14 g/d (four exchanges per day), while Keusch et al. (15) have reported an uptake of 104 +/- 8 g/d, and Splendiani et al. (16) an average uptake of 156 g/d.

Finally, De Santo et al. (17) have reported an average uptake of 271 +/- 5 g of glucose per day (five exchanges per day). These data show that under normal conditions most patients on CAPD absorb between 100 and 200 g of glucose per day, depending on the number of exchanges per day, the number of hypertonic solutions and the rate of absorption in the individual patient.

In addition, the continuous daily absorption of 100-200 grams of glucose from the dialysate imposed a substantial carbohydrate load, aggravating such long term metabolic complications as hyperinsulinaemia, hyperlipidaemia and obesity (18).

Lindholm et al. (12) and Breckenridge et al. (19) found an increase in high-density lipoprotein (HDL) cholesterol levels in previously normolipidaemic patients, and Chan et al.

(20) have reported higher levels of HDL cholesterol in CAPD patients than in patients treated with haemodialysis.

Lindholm et al. (12) and Khanna et al. (21) reported that serum triglycerides as well as cholesterol in all lipoprotein fractions tended to increase during the first 4-6 months of CAPD treatment. After 1 year these levels decreased again.

Baeyer et al. (14) found increased cholesterol levels and unchanged triglyceride levels after more than 6 months treatment. Keusch et al. (15) found increases in both serum triglycerides, serum cholesterol and HDL cholesterol after 9 months on CAPD. Turgan et al. (22) found increases in serum cholesterol levels but unchanged serum triglycerides in patients with restrictions of oral carbohydrate intake and a limited use of hypertonic solutions.

More recently the prolonged exposure to hyperosmolality has been implicated in the insidious damage to the peritoneum and host defences (23). These considerations have stimulated many investigators to search for an alternative osmotic agent with emphasis on correcting the metabolic and ultrafiltration deficiencies as well as achieving a more physiological solution capable of functioning over long dwell periods.

Various osmotic agents have been tried differing predominantly in their molecular size. Early research workers concentrated on minimising the metabolic side effects of glucose rather than altering the ultrafiltration profile; hence solutes of molecular size similar to or smaller than glucose were utilised (**Table 3**). In the majority of cases the rate of transperitoneal absorption exceeded the "metabolic" capacity resulting in serious hyperosmolar syndromes (24-26). Whilst glycerol (27-29) and amino acids (30-31), have had some long-term use, none of these have been found suitable for general use. Large molecular weight substances have also been tried, to modify both the ultrafiltration profile and limit metabolic side effects. Dextran (32), polyanions and gelatin (33) have had limited human and animal experimentation with disappointing results related to relative

10

insolubility, viscosity, allergenicity and accumulation in the body.

The lack of success in finding a suitable alternative agent, led to a re-examination of the fundamental role of osmotic forces in peritoneal dialysis. In biological systems, where water and solute requirements are similar to those on CAPD, the osmotic effectiveness of albumin (MW 68,000 Da) is well recognised. This physiological approach is based on the biological model of the permeable capillary wall, where only impermient solutes exert an osmotic force. The osmotic flow across such membrane is determined by the concentration gradient of impermient solutes rather than the difference in the total number of solutes across it, i.e. osmolality.

Albumin (human) is slowly absorbed from the peritoneal cavity, yields sustained ultrafiltration, and is non-toxic, non-immunogenic and readily metabolised. The proportion of bound to freely diffusible electrolytes in albumin solutions is known. However, the prohibitive cost of albumin renders it impractical for routine clinical use.

### 1.2.2    Glucose Polymers in CAPD

Whilst none of the above substances met the needs of an ideal osmotic agent the long dwell exchange of CAPD would be best met by a large molecular weight substance. The large size would limit the rate and amount of absorption from the peritoneal cavity thus giving sustained ultrafiltration. With selection of the substance of an appropriate reflection coefficient it would be possible to achieve ultrafiltration with solutions iso-osmotic to uraemic plasma. Glucose polymers could fulfill this role.

Icodextrin is a starch-derived glucose polymer which acts as an osmotic agent when administered intraperitoneally for continuous ambulatory peritoneal dialysis. A 7.5% w/v icodextrin solution is approximately iso-osmolar to serum but produces sustained ultrafiltration over a period of up to 12 hours in CAPD.

A randomised multicentre investigation of icodextrin in ambulatory peritoneal

dialysis (MIDAS) study group (34) was set up to evaluate the safety of icodextrin as an alternative osmotic agent to glucose in CAPD. MIDAS was the largest study of CAPD to have been conducted and has provided a wealth of information not only about the efficacy and safety of icodextrin and glucose, but also on morbidity in CAPD patients which had not been available before. About 5% of the U.K. CAPD population from eight major cities were included in the study.

The main aim of the study was to document the safety and tolerability of icodextrin in CAPD patients for six months, but its efficacy was also compared with glucose during the study. It demonstrated that the overnight use of icodextrin was well tolerated and produced ultrafiltration up to 5.5 times that of 1.36% glucose and was marginally better, though not significantly so, than the 3.86% glucose solution. In addition, there was lower carbohydrate absorption compared to the 3.86% glucose solution. Almost no patients had fluid resorption ('negative ultrafiltration') overnight on icodextrin compared with about a quarter of the control patients on glucose containing CAPD fluids. There was no evidence of any tachyphylaxis to icodextrin.

Patients in both groups tolerated their treatment well; the icodextrin group reported fewer adverse events than the control group. Cardiovascular disease accounted for most of the non-CAPD related adverse events. This is consistent with the high cardiovascular morbidity recorded at entry to MIDAS and the overall high level of cardiovascular disease in the CAPD population. Although there was a higher cardiovascular morbidity in the icodextrin group in entry, there was no excess cardiovascular morbidity during MIDAS.

The MIDAS study demonstrated the efficacy of icodextrin in CAPD patients. It suggested that icodextrin could replace glucose for the long dwell, particularly because of the sustained ultrafiltration it offers with the little likelihood of resorption of fluid from the peritoneum. It is at least as effective as high strength glucose (3.86%) and has potential advantages because it is iso-osmolar with uraemic serum (34).

Table 4 shows the composition of a range of commercially available CAPD fluids from Baxter containing glucose (1.36%, 2.27%, 3.86%) and 7.5% icodextrin.

**Table 3** Osmotic agents studied in CAPD patients (adapted from reference 35).

| Agents | Molecular weight (Da) | Charge | Reported side-effects |
|---|---|---|---|
| *Low molecular weight* | | | |
| Glucose | 180 | Nil | Hyperlipidaemia, obesity, hyperinsulinaemia |
| Fructose | 180 | Nil | Hypertriglyceridaemia; hyperosomolality |
| Sorbitol | 180 | Nil | Hyperosmolality and retention |
| Xylitol | 152 | Nil | Lactic acidosis; hyperosmolality |
| Glycerol | 92 | Nil | Short-lived ultrafiltration; glycerol related hyperosmolality |
| Amino-acids | 100 - 200 | +/- | Manufacturing and cost problems; increase nitrogen load; no optimum profile |
| *High molecular weight* | | | |
| Polyanions | 40 000 - 90 000 | - ve | Toxic to peritoneum |
| Polycations | 40 000 - 90 000 | + ve | Cardiovascular instability (rats) |
| Neutral dextran | 60 000 - 200 000 | Nil | Absorbed, intraperitoneal bleeding in rats ultrafiltration |
| Gelatine | 20 000 - 350 000 | +/- | Prolonged half-life/ immunogenicity |
| Glucose polymers | 250 - 22 000 | Nil | Retention of maltose |
| *Ideal osmotic agent* | | | |
| Albumin | 68 000 | - ve | None - but is prohibitively expensive |

**Table 4** Comparison of the composition a range of commercially available CAPD fluids containing glucose (Dianeal from Baxter) and 7.5% w/v icodextrin (also from Baxter). (36,37)

| | Dianeal PD4 with Glucose 1.36% | Dianeal PD4 with Glucose 2.27% | Dianeal PD4 with Glucose 3.86% | Icodextrin 7.5% w/v |
|---|---|---|---|---|
| Each one litre contains: | | | | |
| Anhydrous Glucose | 13.6 g | 22.7 g | 38.6 g | ——— |
| Anhydrous Icodextrin | ——— | ——— | ——— | 75 g |
| Sodium Chloride | 5.4 g | 5.4 g | 5.4 g | 5.4 g |
| Sodium Lactate | 4.5 g | 4.5 g | 4.5 g | 4.5 g |
| Calcium Chloride | 184 mg | 184 mg | 184 mg | 257 mg |
| Magnesuim Chloride | 51 mg | 51 mg | 51 mg | 51 mg |
| mmol per litre (approx.); | | | | |
| Sodium | 132 | 132 | 132 | 133 |
| Calcium | 1.25 | 1.25 | 1.25 | 1.75 |
| Magnesium | 0.25 | 0.25 | 0.25 | 0.25 |
| Chloride | 95 | 95 | 95 | 96 |
| Lactate | 40 | 40 | 40 | 40 |
| mOsm per litre (approx.) | 344 | 395 | 483 | 284 |

## 1.3    Diagnosis of Diabetes

The diagnosis is usually straight forward. Blood glucose is so closely controlled by the body that even small deviations become important.

1. In symptomatic patients, a single elevated blood glucose, measured by a reliable method, indicates diabetes. According to the World Health Organisation (WHO), a fasting venous plasma glucose of 8 mmol/l (144 mg/dl) or more is diagnostic of diabetes mellitus. Alternatively, a random venous plasma glucose of 11.1 mmol/l (200 mg/dl) or more establishes the diagnosis (2).

2. In asymptomatic or mildly symptomatic patients, the diagnosis is made on:

(a) One, preferably two, fasting venous blood glucose levels above 6.7 mmol/l (120 mg/dl); the equivalent venous plasma level is 7.8 mmol/l (140 mg/dl), or

(b) One, preferably two, random values above 10 mmol/l (180 mg/dl) in venous whole blood or 11.1 mmol/l (200 mg/dl) in venous plasma.

3. A glucose tolerance test (GTT) is unnecessary when the criteria above are satisfied, and should be reserved for true borderline cases.

4. Glycosuria is measured using sensitive glucose-specific dipstick methods. Glycosuria is not diagnostic of diabetes but indicates the need for further investigation. About 1% of the population have renal glycosuria in which there is a low renal threshold for glucose (2).

## 1.4    Measuring Control

### 1.4.1    Urine Tests

#### 1.4.1.1 Urine Glucose Test

Until the advent of home blood glucose monitoring, urine glucose testing was the chief day-to-day method of assessing diabetic control, the rationale being that urine glucose reflects mean blood glucose levels over the time the urine was collected.

Glucose is freely filtered at the renal glomerulas and actively reabsorbed in the proximal tubule. The reabsorption is limited, the maximal limit being the renal glucose threshold, which is exceeded when the blood glucose concentration is, on average >10 mmol/l. Glycosuria thus occurs when the blood glucose level exceeds 10 mmol/l, and from this point urine glucose concentrations should be proportional, to the increasing hyperglycaemia. Non-specific Benedict's reagent tests (Clinitest) and specific glucose oxidase strips (e.g. Clinistix) are available for urine glucose estimation.

Although urine testing is painless and less expensive than blood glucose monitoring, it is undesirable and misleading for several reasons (38).

1) The renal threshold is high in some individuals, such as those with long-standing diabetes, so that marked hyperglycaemia may exist without glycosuria.

2) The threshold is low in some people, such as in pregnancy and in children, so that glycosuria may occur with a normal blood glucose concentration.

3) The renal threshold varies between patients (including the above groups) but also changes in the same individual over time.

4) Fluid intake and urine concentration affect urine results.

5) The result does not reflect the blood glucose level at the time of testing but the average over the time of urine accumulation in the bladder. Autonomic dysfunction in diabetes may also lead to a mixture of recently formed and stagnant urine.

6) A negative urine test cannot distinguish between hypoglycaemia, normoglycaemia and moderate hyperglycaemia (i.e. up to 10 mmol/l).

7) Urine testing is less accurate than with a blood glucose meter.

8) Some drugs may interfere with urine testing.

Many tests have shown that urine testing is a poor method of assessing glycaemic control, giving only the crudest notion of the level of control (39,40). Urine testing is still recommended for those who are unable or unwilling to perform blood glucose testing (38), on the basis that some testing is better than no testing. For more stable NIDDM patients treated by diet or oral agents, urine glucose testing, when supplemented by regular glycohaemoglobin measurements, and perhaps fasting blood glucose tests, remains a reasonable means of monitoring control.

### 1.4.1.2   Urine Ketone Test

Urine ketones provide an indication of insulin deficiency and warn against impending or reflect established ketoacidosis (38). Commercial ketone tests are based on nitroprusside, which produces a purple colour with acetoacetate (e.g. Ketostix, Bayer Diagnostics), and also with acetone if glycine is also present in the reagent (e.g. Acetest tablets, Bayer Diagnostics). None of the nitroprusside-based tests detect 3-hydroxybutyrate which is quantitatively the most important ketone body.

False-positive tests with nitroprusside reagents are reported to occur with sulphydryl drugs such as Captopril and exposure to air produces false negatives (41). It is recommended that urinary ketone testing is done during acute illness, during pregnancy and

when symptoms of ketoacidosis are present (e.g. nausea, vomiting, abdominal pain). Blood 3-hydroxybutyrate levels are not routinely measured in clinical practice.

### 1.4.2 Blood Glucose Testing

### 1.4.2.1 Single Measurement of Blood Glucose Concentration

In IDDM, blood glucose levels fluctuate widely throughout the day and are generally unpredictable from day-to-day. Fasting or random blood glucose measurements therefore give little or no indication of the overall level of glycaemic control, even on the day in which the sample is taken. Because of this, there is a poor relationship ($r = 0.24$) between random and postprandial blood glucose tests and glycohaemoglobin levels in IDDM (42,43), confirming that these samples give little information about overall control in the preceding few weeks. Nevertheless, random and fasting blood glucose measurements are traditionally performed on IDDM patients in most diabetic clinics but serve not much more than to alert the physician to hypoglycaemia, which must be corrected before consultation can begin.

In NIDDM patients, blood glucose levels are more stable from day to day and have a similar pattern to that in non-diabetic subjects, though at a higher level and with larger postprandial peaks (44). There is thus a very good correlation ($r = 0.68$) between fasting plasma glucose and the mean daily plasma glucose levels and glycohaemoglobin percentage in NIDDM (43), so that a single clinic blood glucose value, particularly when fasting, provides a good estimate of overall control in NIDDM (44).

### 1.4.2.2 Blood Glucose Profiles

The best assessment of the variation in blood glucose concentrations throughout the day is provided by serially timed measurements either in hospital or at home. Self monitoring of capillary blood glucose by patients at home has become an integral part of modern intensified treatment of IDDM patients (45).

Although it is desirable that all patients receiving insulin perform routine blood glucose self-monitoring (46), only about 40% of IDDM and 30% of NIDDM patients in the USA do so (47). Amongst the hindrances to the increased use of self-monitoring are the discomfort of fingerprick blood sampling, the inconvenience of testing in terms of time and complexity, the high cost in some countries where there is no, or only partial, reimbursement of health service provision of supplies, and poor education of patients and health care professionals about the benefits. Improved methods of patient monitoring including non-invasive techniques are a priority.

Some benefits of blood glucose self-monitoring are:-

1) More accurate and patient-acceptable than the urine tests for glucose; i) on a day-to-day basis by the patient, ii) in the long-term by the physician

2) Defines the level of glycaemic control achieved; i) for research, ii) for routine clinical assessment

3) Identifies hypoglycaemia; i) impossible with urine testing, ii) particularly valuable in patients with loss of hypoglycaemia awareness

4) Acts as an educational aid

5) Increases patient participation, motivation and interest

6) Reinforces the patients feeling of 'being in control', thus offering independence and self-confidence

7) Improves quality of life

8) Reduces hospital admission

### 1.4.3  Glycohaemoglobin

Nomenclature :- In 1978 the term glycosylated haemoglobin was introduced to refer to the product of the non-enzymatic reaction between glucose and the free amino acid groups of haemoglobin (48). In 1983 Roth, on behalf of the International Union of Pure and Applied Chemists - International Union of Biochemists (IUPAC-IUB) Joint Commission on

Biochemical Nomenclature (JCBN) pointed out in his journal (49) that the JCBN suggested that the term glycated haemoglobin for such amino-linked-1-deoxyfructose derivatives of haemoglobin. In 1986 the JCBN finally recommended the term glycohaemoglobin (50). Because glycohaemoglobin is the officially recommended nomenclature, I shall be using this term in this project. **Table 5** summarises glycohaemoglobin nomenclature.

**Table 5** Summarises GHb nomenclature, based on the recommendations by the National Institutes of Health, Diabetes Data Group ( adapted from reference 51)

**Table 5. Haemoglobin Nomenclature (51)**

| | |
|---|---|
| HbA | The major form of haemoglobin, a native, unmodified tetramer consisting of two alpha and two beta chains. |
| $HbA_0$ | The major component of HbA, identified by its chromatographic and electrophoretic properties. Posttranslational modifications, including glucosylation, do exist in this fraction but do not significantly affect the charge properties of the protein. |
| $HbA_1$ (sometimes known as "Fast" Haemoglobin) | Posttranslationally modified, more negatively charged forms of HbA, as detected by chromatographic and electrophoretic methods. The total $HbA_1$ fraction, which because of a more negative charge, migrates more rapidly toward the anode in electrophoresis and elutes earlier in cation-exchange chromatography than does $HbA_0$. The chromatographically distinct components of $HbA_1$ are:- $HbA_{1a1}$ (fructose 1,6, diphosphate-N-terminal valine) $HbA_{1a2}$ (glucose-6-phosphate-N-terminal valine) $HbA_{1b}$ (unknown carbohydrate-N-terminal valine) $HbA_{1c}$ (glucose-N-terminal valine) |
| $HbA_{1c}$ | Adduct of glucose attached to the beta chain terminal valine residue by a ketoamine linkage. |
| Pre-$HbA_{1c}$ or Schiff base | A labile form of glucosylated haemoglobin containing glucose attached by aldimine linkage at the beta chain terminal valine residue. |
| Glucosylated Haemoglobin(s) | Haemoglobin modified by glucose at alpha and beta chain terminal valine residues and epsilon amino groups of lysine residues. |
| Total glyco-haemoglobin (total GHb) | $HbA_{1c}$ + glucose -non-N-terminal lysine sites. |

$HbA_1$ is a series of glycated variants where various carbohydrates are attached to the N-terminal valine of the beta chain of haemoglobin - either fructose 1,6-diphosphate

($HbA_{1a1}$), glucose-6-phosphate ($HbA_{1a2}$), or glucose ($HbA_{1c}$) or an unknown sugar ($HbA_{1b}$). The largest component (60-80%) is $HbA_{1c}$ and is frequently measured on its own. This glycation causes an increase in negative charge so that $HbA_1$ components are sometimes called fast haemoglobins because they run fast in electrophoresis systems. Total glycohaemoglobin (GHb) represents glucose attained to both the N-terminus ($HbA_{1c}$) and to other sites such as the epsilon-amino group of lysine, which do not alter the charge. The reference range for $HbA_{1c}$ is about 4-6% and that for $HbA_1$ and total GHb higher at about 5-7.5%, but the normal ranges must be established by each laboratory and cannot be interchanged (48).

Glucose reacts spontaneously and non-enzymatically with free amino groups on proteins to form covalent glycated proteins. The extent of protein glycation depends on the average glucose concentration to which the protein is exposed and on the half-life of the protein. Thus, long-lived structural proteins (e.g. lens proteins) may be damaged as a result of the abnormal increase in protein glycation found in diabetics. Indeed, it has been suggested that glycation of structural proteins in arterial walls and elsewhere might be responsible for some of the long-term sequelae of diabetes. Shorter half-life proteins such as haemoglobin may also undergo excessive glycation in diabetics (48).

The mode of reaction of glucose with proteins is shown in **Figure 1**. The carbonyl group of acyclic glucose reacts with the free amino groups via nucleophilic addition, forming a Schiff base or aldimine. The labile Schiff base may be hydrolysed back to glucose and protein, or it may undergo an Amadori rearrangement to form a relatively stable ketoamine linkage. The open chain of the amino sugar, 1-amino-1-de-oxyfructose (fructosamine), undergoes cyclization to a ring structure, mainly the pyranoside form. As this ketoamine linkage is fairly stable under physiological conditions and since there is no degrading enzyme(s) known in the mammalian tissue, once bound glucose stays with the protein throughout its lifespan (52).

20

## The Glycation Reaction
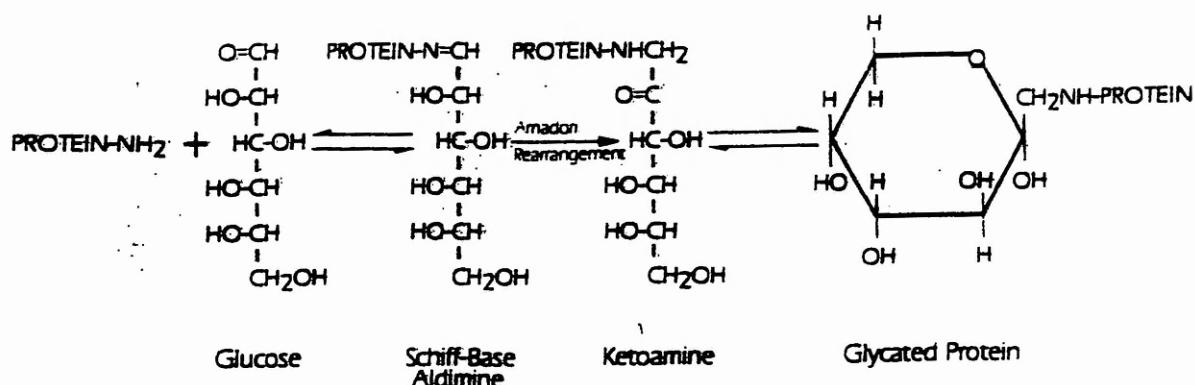


**Fig. 1** The glycation reaction (52).

Since the half-life of Hb is about 60 days, the $HbA_1$ value reflects the average level of blood glucose concentration over the previous 1-2 months. As a consequence, levels of $HbA_1$ tend to be higher in diabetics. The extent of elevation indicates the overall average degree of blood glucose control; in poorly controlled diabetics, it may rise as much as 25%. In type I diabetics, the percentage of total Hb present as $HbA_1$ provides doctors with a better out-patient index of diabetic control than blood or plasma glucose concentrations, since it is little affected by short-term fluctuations in blood glucose concentration (53).

In diabetic clinics, $HbA_1$ is mostly used to assess control of insulin-dependent diabetics and to complement home-monitoring of blood glucose. Studies have shown that the $HbA_1$ value correlates well with the mean blood glucose over the preceding 1-2 months and with clinical impressions of each patient's state of health. It provides an objective measurement of glycaemic control and can be used to define a treatment goal for both the patient and physician. Results of $HbA_1$ measurements have an important place in studying the relationship between diabetic control and the longer-term development of diabetic complications, and are valuable in the assessment of new treatment regimes. As a parameter

21

of diabetic control, glycohaemoglobin is measured in addition to the urinary and blood glucose.

It is unlikely that $HbA_1$ measurements will ever displace home-monitoring of blood glucose as the mainstay of day-to-day control of diabetes by patients themselves, their friends or relatives. Technically, $HbA_1$ measurements are more time-consuming and much more expensive than glucose measurements (53).

There are circumstances in which glycohaemoglobin measurements are very useful:-
1) They are of special value in the routine management of insulin-dependent diabetic children in poor metabolic control. Here, frequent determinations are necessary since in these patients the glucose profiles are prone to great variations, which may lead to changes in the glycohaemoglobin concentrations. In adults, the frequency of glycohaemoglobin measurements must depend on the stability of the diabetic control obtained; twice a year in stable diabetics and four to six times a year in unstable diabetics (54).

2) In home monitoring of blood glucose, glycohaemoglobin determination has gained acceptance as the best parameter to detect objectively the effect of this kind of therapy and to measure patient compliance. In particular, patients unsuitable for this kind of therapy are detected by frequent glycohaemoglobin measurement (54).

In the past clinicians have had to rely on historical information given by patients home urine or blood tests and clinic blood glucose measurements. All these may be unreliable or unrepresentative. Many patients feel perfectly well with relatively high blood glucose concentrations and the classical symptoms of polyuria and thirst may not occur until blood glucose concentrations are very high. The results of home monitoring may be incorrect either because patients are not proficient in carrying out the test or because they set out to mislead their doctors. Clinic blood glucose measurements provide no measure of control at other times. They may also not be representative because the patient behaves differently when a clinic visit is imminent or has an intercurrent illness which produces a

22

higher result than normal. The advent of glycohaemoglobin determinations has demonstrated the limitations of the traditional procedures for assessing diabetic control (55)

3) In monitoring diabetic pregnancy - strict conrol of pregnant subjects is necessary because of a high risk of foetal abnormality Leslie et al. (56) and Miller et al. (57) have demonstrated a positive relationship between high glycohaemoglobin levels during the first trimester of pregnancy and foetal anomalies. Their results revealed a significantly higher incidence of major congenital anomalies in the offspring of women with elevated glycohaemoglobin values. This was confirmed by Hinen et al. (58) who found that poor control of maternal diabetes and high glycohaemoglobin levels were associated with increased risk of perinatal death. Most foetal malformations occur before eight weeks of gestation, so problems of morbitity and mortality can be minimised only if patients with diabetes are maintained as euglycaemic, preferably before and during pregnancy (59). As a single glycohaemoglobin determination cannot satisfactorily predict the degree of regulation, both glycohaemoglobin and blood glucose should be determined regularly and frequently throughout the pregnancy (60).

4) The measurement of glycohaemoglobin is of the utmost importance in detecting the relationship between the degree of metabolic control and the occurrence of diabetic complications - see **section 1.5**. It must therefore be measured in all groups of patients subjected to different therapeutic regimens to establish long-range determinations of the degree of diabetic control obtained.

### 1.4.4 Fructosamine and Glycated Albumin

Analogous to the glycation of haemoglobin, serum proteins, the bulk of which is albumin, react with glucose and other sugars to form an aldimine linkage, which undergoes an Amadori rearrangement to a ketoamine. The concentration of glycated protein can be used as a measure of long-term glycaemic control over approximately the life-time of the

23

albumin molecule, i.e. about 2-3 weeks. This test is commonly known as the 'fructosamine' assay (61,62), although fructosamine is the generic name for the protein-ketoamine product, and should strictly refer to any glycated protein assay. Johnson et al. (63) were the first to introduce a simple colorimetric assay for fructosamine in 1982, based on the ability of fructosamines to act as a reducing agents in alkaline solutions, distinguishable from glucose and other reducing agents.

In the original assay (63), serum is added to carbonate buffer pH 10.8 (subsequently changed to 10.35) containing nitroblue tetrazolium (NTB), which is reduced to a formazan dye that absorbs at 550 nm. The original standard was a synthetic ketoamine, deoxymorpholinofructose (DMF), and gave a reference range of about 2.1-2.8 mmol/l. The assay was rapidly adapted for automated analysers. Numerous interferences were reported including lipaemia, bilirubin, ascorbate, haemolysis, uric acid and uraemia (61,62). The result is dependent on albumin concentration and there are arguments for and against correcting fructosamine values to the serum albumin value. While Johnson et al. (64) report that it is not necessary to correct for serum albumin concentration, others (65,66) have suggested otherwise.

Changes in the half-life of albumin may occur in catabolic and anabolic states (cachexia, thyroid disease) and in dysproteinaemias (paraproteinaemia, hypergammaglobulinaemia). The half-life of albumin is inversely related to its serum concentration. Results of fructosamine may also be invalid in the presence of cirrhosis of the liver, nephrotic syndrome, or after rapid changes in acute-phase reactants (67).

Recent improvements in the assay (68) include using a secondary standard of glycated albumin and incorporation of detergent into the reagent mixture to increase linearity and to eliminate protein-matrix effects and interference due to lipaemia. Added uricase eliminates interference from uric acid. The modified formulation and standard are commercially available (Boehringer Mannheim) and suitable for automated analysers.

Affinity chromatography and immunoassays can be used for the assay of both glycated total serum protein and albumin (69,70).

Fructosamine values correlate well with GHb in stable diabetes but less well when control has altered markedly in the past week or so (68). Because the test has good precision, low cost and is easily automated, it has been suggested as an alternative to GHb for monitoring control in diabetes. In situations where information is required about control over a shorter period than that measured by GHb, such as during diabetic pregnancy, fructosamine may be the method of choice (71). Other authors report that the correlation between the two tests is weak, reflecting the greater inter-individual variation in plasma proteins than in haemoglobin (66). Fructosamine assays are reported to be less reliable and GHb measurements are often preferred (66).

### 1.4.5 Other Glycated Plasma Proteins

Glycation of fibrinogen has been measured by affinity chromatography and reflects mean blood glucose over 2-3 days (72). Other products are discussed in **section 1.5 and** have been reviewed by Furth (73).

### 1.4.6 Glycation by Fructose

Fructose, a major dietary sugar, is a potent glycating agent forming advanced glycation end products (AGE) much faster than glucose. Although it reacts with lens protein and haemoglobin its study has been much neglected (74). The common assays for measuring glycation are unsuitable for measuring fructation and until a suitable, relatively simple assay is developed, its usefulness will go largely unexplored (75).

## 1.5 The Long-term Effects of Extensive Glycation in Diabetics

### 1.5.1 Potential Mechanisms in the Pathogenesis of Complications

Pathogenesis of the various diabetic complications may not be uniform. Distinct abnormalities might operate in nerve and kidney, for example, or several abnormalities might act in concert. Three possible mechanisms have received considerable attention: 1) the glycation of proteins; 2) the polyol pathway, and; 3) the haemodynamic hypothesis

#### 1) Can Extensive Glycation of Proteins Produce Disease?

The level of glycation of a protein *in vivo* is determined in part by its time of contact with a given level of hyperglycaemia. In addition, the turnover rate of the protein influences the extent of glycation. Proteins in plasma that turn over slowly, such as red-blood cell membranes (76), haemoglobin (77), albumin (78), low-density lipoproteins (LDLs) (79), high-density lipoproteins (HDLs) (80), and immunoglobulin G (81) become significantly glycated in diabetes. Outside the circulation, increased glycation has been found in lens (82), glomerular basement membrane, aorta, coronary arteries, and femoral nerve (83,84).

Interference with function of a protein by glycation requires either that the affected intrachain lysines be close to the active site(s) of the molecule or that stereochemical conformation of the protein be distorted. The function of some proteins is known to be altered by glycation, and in other cases the possibility is suspected but unproven. In the former category are haemoglobin, albumin, lens protein, fibrin, collagen, lipoproteins, the glycoprotein recognition system of hepatic endothelial cells (85), and antithrombin III (86). In the latter category are immunoglobulin G, red-blood cell membranes, circulating white-blood cells, myelin, and Von Willebrand factor.

Glycation of haemoglobin blocks the reaction of 2,3-diphosphoglycerate with positively charged residues on the beta chain, causing a slight but clinically significant increase in oxygen affinity. Glycated albumin inhibits the hepatic uptake of glycoproteins and is taken up into small blood vessels more rapidly than native albumin, but a report that

it binds to glomerular basement membranes (87) has not been confirmed (88). Glycated fibrin is less susceptible to digestion by plasmin (89), which might account for its extensive accumulation in diabetic tissues (90). Non-enzymatic glycation of the crystalline proteins of the lens may promote the formation of disulphide links between protein molecules (82); aggregates of crystalline protein with molecular weights in excess of $5 \times 10^{6}$ scatter light, i.e., they constitute a cataract.

It is not known whether glycation of collagen in glomerular basement membranes is related to their thickening in diabetes. There appears to be a generalised increase in basement membrane thickness (91). Glycated collagen is more insoluble and resistant to digestion because of increased intramolecular cross-linking (92), which may decrease its degradation. It has also been postulated that decreased proteoglycan synthesis causes increased permeability of basement membranes and the thickening is a compensatory response (93).

It has been reported that glycated collagen is antigenic in rats and that rats with streptozocin-induced diabetes form antibodies to glycation but not native collagen (94). If this is true, antibodies to glycated collagen could damage the glomerular basement membranes either directly or via immune complexes. Conceivably, the absence of severe microangiopathy in some poorly controlled diabetics could represent a reduced immunological response to glycated collagen.

Glycated skin collagen is resistant to digestion by collagenase (95). It is not clear whether glycation is responsible for connective tissue changes, such as tight waxy skin and limited joint mobility, that are said to indicate an increased risk of late complications (96).

Glycation of the red cell membrane could play a role in the 15% reduction in erythrocyte survival time (97) and perhaps in the loss of the normal red-blood cell deformability that occurs in poorly controlled diabetes (98). Normal red-blood cells pass easily through capillaries with luminal diameters smaller than their own because they are

deformable; loss of flexibility could cause sludging of blood and contribute to retinal and renal ischaemia (98). Glycation of myelin protein (99) may account in part for the functional changes in nerve conduction.

Theoretically, extensive glycation of insulin receptors could contribute to the reduced sensitivity to insulin that occurs in chronic hyperglycaemia (100) and is reversed by meticulous control (101). Membrane glycation in leukocytes conceivably might account for the reduced chemotaxis (102), phagocytosis, bacterial activity (103) and cell-mediated immunity (104) reported in diabetes, although this has not yet been explored. The defective response of T cells and B cells to mitogens can be restored by the normalisation of glucose (105). Extensive glycation of Von Willebrand factor could contribute to the increased platelet aggregation reported in poorly controlled diabetes (106). Advanced glycation end products have been proposed as a major factor in diabetic macrovascular disease (107). By cross-linking matrix and plasma proteins, they may accelerate development of atherosclerosis.

## 2) The Polyol Pathway

A second general mechanism possibly underlying diabetic complications is activation of the polyol pathway (108). In this pathway glucose is reduced to sorbitol under the influence of the enzyme aldose reductase (D-aldose: $NADP^+$ 1-oxidoreductase) with NADPH as cofactor. Sorbitol can then be oxidised to fructose with the production of NADH by the enzyme sorbitol dehydrogenase (L-iditol dehyrogenase). Aldose reductase is present in the retina, kidney papillae, lens, Schwann cells, and aorta, tissues that are frequently damaged in diabetes. Polyols have been implicated in the pathogenesis of cataracts (109), retinopathy (110), neuropathy (111) and aortic disease (112). In the lens, sorbitol, may cause osmotic swelling, which is initially reversible, but subsequently, $Na^+$, $K^+$-ATPase activity falls (109). How the latter interacts with the postulated role of

glycated lens proteins in the genesis of cataracts (82) is not known. In nerves polyols also inhibit $Na^+$, $K^+$-ATPase (113,114) a lesion that accompanies the characteristic myo-inositol deficiency found in experimental diabetic neuropathy (111). Although myo-inositol deficiency is routinely observed in nerves from diabetic rats and repaired by myo-inisitol feeding (115), biopsy specimens of sural nerve from humans with diabetic neuropathy failed to show diminished levels of myo-inisitol (115). Further studies will be required to understand the discrepancy. Experimentally, retinopathy (110), cataracts (109), nephropathy (108), and the metabolic abnormalities of peripheral nerve (111,114) can be prevented by inhibition of the polyol pathway. Sorbinil, an aldose reductase inhibitor tested in human trials, may relieve symptoms in painful diabetic neuropathy (116) and in long-term treatment appears to enhance nerve regeneration in diabetic humans (117). The use of these inhibitors as prophylaxis for complications has not been reported.

Non-enzymatic glycation of proteins and the polyol pathway may not be unrelated mechanisms. It is now known that fructose generated in the polyol sequence can non-enzymatically bind to protein (called fructation) and that fluorescence of collagen from diabetic animals is decreased by inhibitors of aldol reductase (118). It is thus possible that an active polyol pathway may contribute significantly to non-enzymatic glycation of proteins.

### 3) Haemodynamic Hypothesis

A third postulated general mechanism of tissue injury is based on the observation that blood flow is increased in patients studied shortly after the onset of IDDM (119). Because blood pressure is usually normal in such patients, it is likely that arteriolar resistance is decreased. The increased hydrostatic pressure in the capillary beds is thought to increase filtration of potentially damaging proteins and other macromolecules (including immune complexes) into the walls of blood vessels and mesangium, secondarily stimulating synthesis of mesangial and basement membrane components. The latter step is presumed to

29

enhance capillary 'leakiness', setting up a vicious circle. The haemodynamic hypothesis has received most attention in connection with diabetic renal disease (120). In view of the wide spectrum of dysfunction that characterises the diabetic state, the probability that a single abnormality like hyperfusion, by itself, could cause microangiopathy is low.

## 1.6    Methodology for Glycohaemoglobin Measurement

The major assay methods can be divided into categories, based on the manner in which glycated and non-glycated haemoglobin components are separated. Each is discussed in detail.

## 1.6.1  Methods Based on Charge Differences

### 1.6.1.1 Cation-exchange Chromatography

This is the most widely used method in clinical laboratories around the world. Procedures range from disposable microcolumns to expensive "high-performance" automated systems. The principle of the method is that many GHb species, including $HbA_{1c}$, are less positively charged at neutral pH than $HbA_0$, and bind less well to a negatively charged resin. Typically, the haemolysate is applied to a column filled with resin and the eluate is collected. The less-positively charged minor haemoglobin components, primarily $HbA_{1a}$, $A_{1b}$, and $A_{1c}$, elute before the main haemoglobin fraction, $HbA_0$, and their percentages of total haemoglobin can be easily determined with a spectrophotometer. Some procedures only separate $HbA_1$ ($HbA_{1a}+_b+_c$) from $HbA_0$; others separate the various minor haemoglobin components from one another, thereby allowing quantification of $HbA_{1c}$ (121).

Numerous commercial cation-exchange chromatography kits have been marketed for the measurement of glycohaemoglobin (122,123). Most involve haemolysis of anticoagulated whole blood, application of the haemolysate to a pre-filled disposable

column of cation-exchange resin, and finally, elution of $HbA_1$ with one buffer and of $HbA_0$ with a second buffer. Many variations of this technique have been reported, including a resin slurry method (124), and a rapid column-centrifugation method (125). A few cation-exchange methods have been described that separate the $HbA_{1c}$ from $HbA_{1a}^+{}_b$ (126-128). Typically, these methods also involve two buffers: the first to elute the $HbA_{1a}^+{}_b$ fraction, and the second to elute $HbA_{1c}$. $HbA_0$ is not eluted from the column, but is quantified indirectly by determining the absorbance of the haemolysate before application to the column.

Several high performance liquid chromatography (HPLC) systems have been marketed for the measurement of glycohaemoglobin by cation-exchange chromatography. The increasing numbers of glycohaemoglobin requests received by laboratories has resulted in the need for increased automation, turn-around time, and high sample throughput. Dedicated HPLC systems have become commercially available for the estimation of $HbA_1/HbA_{1c}$, although non-dedicated systems adapted to the analysis of glycohaemoglobin, are becoming more widely available. There is currently available one low pressure, and several high pressure cation exchange resin systems.

*Low pressure system:-* The Glycomat analyser and its successor the Hb Gold are manufactured by Drew Scientific, and distributed in the U.K. by Ciba Corning Diagnostics, and Biomen Ltd., respectively. This system uses two buffers and mini-columns, each capable of performing at least 150 separations before it has to be changed. The instrument will give results as $HbA_1$, $HbA_{1c}$, and may be adjusted for the estimation of HbF and HbS (129).

*High pressure system:-* There are several systems available:

a) The Diamat Analyser (used in the Diabetes Control and Complications trial) and its successor the variant Analyser are manufactured by Toya-Soda, Tokyo, Japan; distributed by Bio-Rad laboratories, Hemel Hempsted, U.K. These require sample pre-treatment before

31

loading onto the autosampler. The analyser utilises a tertiary gradient and produces $HbA_1$ and $HbA_{1c}$ results. The results are not obtained directly from the peak areas, but are calculated from comparison with calibrant chromatograms stored in the instrument; this is necessary to correct for peak broadening as the column ages.

With a different column and eluants the instrument can be used to quantify and detect a wide range of abnormal haemoglobins HbS, C, F, and E with the glycohaemoglobin protocol (130).

b) The Shimadzu HPLC haemoglobin System (Dyson instruments) also corrects the results obtained after comparison with calibration chromatograms; but unlike the Diamat system uses a binary elution gradient (130).

The greatest degree of automation is shown by the

c)   Daiichi Hi-Auto $A_{1c}$ HA 8121 manufactured by Kyoto Daiichi, Kagoto, Japan; distributed by Biomen Limited, Croyden, U.K. It is at present unique in its ability to dilute, haemolyse and remove the labile fraction of $HbA_{1c}$ within the instrument. This is achieved by incubation of the sample in a thermal-jacketed loop for 2 minutes at $48^{O}C$ in the presence of tetrapolyphosphate at pH 6. This instrument is dedicated to the measurement of $HbA_1$ and $HbA_{1c}$ (130).

In general, HPLC methods show excellent assay precision, and permit rapid separation of $HbA_{1c}$ from other minor haemoglobin components and $HbA_0$. Haemolysate is injected onto a small glass column filled with cation-exchange resin. For most, HPLC methods, a two-buffer system is used, similar to the disposable column methods. The first buffer, which has a higher pH and lower sodium ion concentration than the second, is pumped through the column under moderate pressure and at a rapid flow rate. After elution of the various minor haemoglobin fractions, the second buffer is pumped through the column to elute $HbA_0$. The absorbance of the eluate is monitored continuously, allowing quantification of each minor haemoglobin component. This method can be fully automated.

Despite its advantages, however, HPLC requires meticulous laboratory technique for achieving and maintaining optimal results. Equipment costs are high, but for laboratories that analyse many specimens per week, the cost per test is comparable with that of disposable microcolumn methods. As none of these analysers are capable of resolving the $HbA_{1c}$ peak from neighbouring peaks, experienced chromatographers question their accuracy.

### 1.6.1.2 Electrophoresis

### 1) Agar Gel Electroendosmosis

In 1980 Menard and co-workers (131) described a method using agar electroendosmosis. Haemoglobin $A_0$, being more positively charged than $HbA_1$, interacts more strongly with the fixed negative charge of the gel matrix, while the $HbA_1$ is swept towards the cathode by the electroendosmotic flow of buffer. A commercial kit (Corning Medical, Halstead, Essex. U.K.) incorporating electroendosmosis methodology compared favourably with a short column chromatographic method. Unlike ion-exchange chromatography, electroendosmosis was not significantly affected by fluctuations in temperature (132).

Puukka et al.(133) have shown that $HbA_1$ as measured by the electrophoretic method is acutely responsive to changes in glucose concentrations. The contribution of the labile fraction is eliminated by saline incubation of the erythrocytes or dialysis of the haemolysates before the assay. The removal of the unstable component before analysis of the electrophoretic method will minimise the effect of any acute changes in the blood glucose concentration on the level of $HbA_1$ and thus make it a more accurate indicator of long term glucose control in diabetic patients, but will complicate an otherwise simple and rapid procedure (133). Increased percentages of HbF are also known to interefere with glycohaemoglobin measured by electrophoresis based on the electroendosmotic separation, and carbamylated haemoglobin has been shown to interefere with methods based on charge

separation (132).

## 2) Agarose Electrophoresis

Recent years have seen improvements in electrophoretic methods. Agar systems have been replaced by agarose gels (Diatrac; Beckman) and electrophoretic methods have been automated (REP automated electrophoresis instrument; Helena Laboratories, Gateshead, UK). The Helena rapid electrophoresis (REP) system was the first automated electrophoresis instrument available commercially and can be used for a variety of clinical and research electrophoresis applications. The REP method is capable of quantitating abnormal haemoglobins e.g. HbS. Further work is under way to determine if a modified formulation of the gel can improve the resolution sufficiently to separate HbC (134).

## 1.6.1.3 Iso-electric Focusing

The greater specificity of $HbA_{1c}$ by iso-electric focusing makes an attractive alternative to chromatography, but some observers have commented that 'the separation is not, however, adequate for quantitation or isolation of $HbA_{1c}$, and that the 'technical difficulties involved in this method do not permit its routine clinical application' (135). Iso-electric focusing in polyacrylamide gels is a special type of electrophoresis that separates haemoglobins according to their iso-electric points (PIs) (136). The haemolysate is subjected to an electrical current in a gel that has been specially prepared to have a pH gradient. The pH gradient is established by means of specialised carrier ampholyte mixtures of polyamino and polycarboxylic acids with differing PIs. Each haemoglobin component "focuses" as a single band in a gel at its specific PI. $HbA_{1c}$ focuses well separated from $HbA_0$ and the other minor haemoglobin components. Haemoglobinopathies do not affect results, but pre-$A_{1c}$ does interfere. The necessary equipment is expensive and results correlate well with other methods for quantifying GHb, and the assay precision is comparable with that of other methods (136).

34

### 1.6.1.4 Factors Affecting Charged-based Methods

The various factors that affect the results of cation-exchange chromatography can be divided into two categories: those that are related to characteristics of the measurement technique (assay factors), and those that are related to some characteristic of the blood sample.

1) *Assay conditions*. Assay conditions that can affect test results include temperature, pH, ionic strength, column size and flow-rate (137,138). Failure to appreciate these important factors accounted for much of the variability in test results encountered shortly after the introduction of cation-exchange chromatographic procedures.

2) *Labile intermediates*. Shortly after the introduction of microcolumn techniques for measuring GHb, several studies suggested that formation of GHb was both rapid and reversible, raising important questions concerning the validity of GHb measurements as indicators of long-term glucose control (139-141). We now know that formation of GHb is a two stage process, with initial formation of an intermediate Schiff base from glucose and haemoglobin A. This labile component, called pre-$A_{1c}$ when glucose is attached to the $NH_2$-terminal valine of the beta chain, then either undergoes an irreversible molecular arrangement to form GHb (a ketoamine), or dissociate back to glucose and haemoglobin A (48). Unfortunately, the intermediate, which is acutely responsive to glucose concentrations, co-chromatographs with GHb and will falsely increase test results for $HbA_{1c}$. Thus, it is necessary to remove the intermediate before assay; otherwise, results will reflect a combination of short- and long-term glucose control. Among the methods available for removal of this labile component are incubating erythrocytes in saline solution and lysing blood samples in low-pH buffer (142,143). Fortunately, most manufacturers of cation-exchange microcolumns routinely include such lysing agents in their kits.

3) *Haemolytic anaemia's, phlebotomy, and pregnancy*. These situations all tend to decrease GHb results from expected values in proportion to the degree that erythrocyte

survival time is shortened (144,145).

4) *Haemoglobinopathies.* Several haemoglobin variants are associated with either falsely increased or falsely decreased GHb values, depending on the charge characteristics of the particular variant (146).

Blood from adults contains up to 0.5% of foetal haemoglobin (HbF). The HbF fraction is often significantly elevated in patients suffering from thalassaemias, in those with structural varients of haemoglobin (146), and rarely as a result of hereditary persistence of foetal haemoglobin (147). It is raised to a lesser extent in pregnancy and in a variety of other conditions. Levels of HbF have also been shown to be higher in a proportion of children and adolescents with type I diabetes than in controls and are often in excess of 0.5% (148). HbF is negatively charged and co-chromatographs with "fast haemoglobins" and will increase the apparent concentrations of $HbA_{1c}$ or $HbA_1$ quantitated by some ion-exchange methods (149). The Diamat, HA8121 and the Shimadzu systems all achieve acceptable resolution of $HbA_{1c}$ from HbF, but with the glycomat, $HbA_{1c}$ and HbF co-elute (130).

Positively charged haemoglobins e.g. sickle cell haemoglobin (HbS) may be retained by some column methods resulting in low haemoglobin fractions (146). In HbS, the charged glutamic acid residue in position six of the normal beta chain is replaced by an uncharged valine molecule.

The gene for HbS occurs especially in a wide area across Tropical Africa, in some parts of the countries bordering on the Northern shores of the Mediterranean, and in parts of the Middle East and Southern India. The prevalence of this gene in these areas varies from very low values to 40% of the population. In black Americans, the prevalence is 8%. The distribution of the HbS gene corresponds to areas in which falciparum malaria has been endemic and the persistence of this potentially lethal gene in high frequency in these areas results from the fact that heterozygotes die less frequently from severe falciparum malaria

during early childhood than children with only HbA (150).

The automated liquid chromatography systems (Diamat and Glycomat) identify Hb traits but not their glycated fractions. With the Shimadzu and Daiichi HA8121 abnormal haemoglobins are eluted with or shortly after $HbA_0$, and the only indication of an abnormality will be, in most cases, an unexpectedly low $HbA_{1c}$ (as a percentage of apparent $HbA_0$). The clinical implications of this is that a diabetic with a known haemoglobinopathy may well give a falsely low (apparently normal) glycohaemoglobin result in the presence of poor control. In most diabetic populations, these haemoglobin variants are relatively uncommon, but in clinical situations where these variants are found frequently, methods that are not based on charge differences might be preferable to cation-exchange chromatography (130).

5) *Interfering substances.* When haemolysates are prepared directly from whole blood, markedly above-normal concentrations of either triglycerides or bilirubin will falsely increase results for $HbA_1$ and $HbA_{1c}$ (137,151). Bilirubin migrates with the 'fast haemoglobin' and absorbs at the detecting wavelength. Lactescent plasma due to hyperlipidaemia can also cause elevation of $HbA_1$, since lactescence elutes in the first $HbA_1$ fraction and absorbs at 415 nm.

6)*Non-glucose adducts of haemoglobin.* Various substances other than sugars can form adducts with haemoglobin, thereby altering its charge characteristics. Unfortunately, in some cases, these adducts co-chromatograph with the minor haemoglobins, falsely increasing test results. Examples include individuals with opiate addiction (152), lead poisoning, uraemia, and alcoholism, as well as those receiving chronic treatment with large doses of aspirin. Aspirin modifies several sites, presumably lysines, on both the alpha and beta chains of HbA. Acetylation of lysine residues with aspirin confers a negative charge on the modified protein. The modified haemoglobin has altered electrophoretic and chromatographic (ion-exchange) properties, migrating ahead of $HbA_0$ like $HbA_1$. Patients

37

with long-term high-dose aspirin therapy may have two-fold increase in the modified haemoglobin (153).

Clinically, the most important of these interfering adducts occurs in uraemia. Fluckiger and co-workers, using a gas-liquid chromatography method, first demonstrated elevated levels of carbamylated haemoglobin in uraemic patients and showed that the elevated levels correlated well with the time-averaged urea concentrations in haemodialysis patients (154). In renal failure, significant numbers of patients have impaired glucose tolerance, and those on dialysis are usually dialysed against a fluid with a high glucose content. It is likely that some increase in $HbA_1$ will occur due to the presence of increased glucose concentration (although patients in chronic renal failure have a tendency to shortened red cell survival). However, it appears that most of the increase in uraemic patients is due to carbamylation of haemoglobin with cyanate or ammonia. Cyanate appears to react with protein amino groups, preferentially but not exclusively with alpha amino groups (154). Therefore, in uraemic patients $HbA_1$ results obtained using methods relying on charge separation must be interpreted with care. In most of the above situations, including uraemia, the $HbA_{1a}{+}_b$ fraction is more affected than $HbA_{1c}$. Thus, assays that quantify $HbA_{1c}$ specifically show only slight alterations (rarely greater than 1% GHb) from these interferences (149).

7) *Sample handling and storage conditions.* Numerous studies have been conducted on the effect of blood storage on test results. Stored at temperatures above $4^O$C, the $HbA_{1a}{+}_b$ fraction shows steady increases that are time and temperature dependent (137,155,156). $HbA_{1c}$ is only slightly affected. Thus, methods that determine $HbA_1$ will show falsely increased values if specimens are not handled properly before assay. Whole blood specimens are stable up to one week at $4^O$C, or for many months under ultra-cold storage conditions ($-70^O$C or colder). The clinician and laboratory personnel should co-ordinate efforts to ensure proper sample handling and storage procedures, to minimise any analytical

errors produced by inappropriate sample handling.

## 1.6.2  Immunoassay Methods

### 1.6.2.1 Enzyme Immunoassay

Novoclone HbA$_{1c}$ (Dako Diagnostics Ltd., Ely, U.K. - formerly known as Novo Nordisk) is an enzyme-linked immunosorbent assay (ELISA) kit based on a monoclonal antibody specific to the N-terminal end of the haemoglobin beta chain with glucose attached to it in its stable ketoamine form. Erythrocytes are washed, lysed and incubated for 10 minutes at 15$^{\circ}$C - 30$^{\circ}$C.

The resulting haemolysates are mixed with coating buffer and loaded immediately into microtitre plate wells where the haemoglobin species bind directly to the polystyrene surface. Unbound material is removed by washing and antibody conjugated to horseradish peroxide is added. Further washing removes excess conjugate and HbA$_{1c}$ is estimated by measuring the remaining peroxidase activity after timed reaction with *o*-phenylenediamine using a microtitre plate reader. Results are expressed as percentage HbA$_{1c}$ from a standard curve derived from the four calibrants analysed in duplicate on each plate. Partial automation of the procedure is now possible using the Tecan 5032 sample processor and washing system (Tecan U.K. Ltd., Reading U.K.).

The antibody used in this method recognises the first four amino acids on the beta chain of the haemoglobin, and therefore will not recognise HbS (beta 6 glu--- > val) or HbC (beta 6 glu--- > lys) where the amino acid change is on position six (130).

### 1.6.2.2 Immunoturbidimetry

A HbA$_{1c}$ method based on the homogeneous immunoassay (Tina-Quant) has been introduced by Boehringer Mannheim U.K. The assay is based on the photometric (570 nm) determination of total haemoglobin and the immunoturbidimetric determination of HbA$_{1c}$. An antibody, recognising the first four amino acids and glucose on the beta chain of

haemoglobin, reacts with $HbA_{1c}$ to give a soluble immune complex. Polyhaptens then bind to excess antibodies and the resulting agglutinated complexes are measured turbidimetrically at 340 nm. The agglutinated complex is inversely proportional to the analyte concentration in the sample. Due to the utilisation of high specificity antibodies, the method does not cross-react with $HbA_0$, $HbA_{1a}$, $HbA_{1b}$, acetylated haemoglobin, carbamylated haemoglobin or Schiff base. Additionally, as the antibody recognises the first four amino acids no interference should be seen with HbS or HbC.

A very similar immunoturbidometric method has recently been introduced by Roche Diagnostic Systems. This method includes pepsin in the lysing reagent, the proteolytic degradation making the beta-N-terminal structure more accessible for the $HbA_{1c}$ immunoassay. This method utilises an antibody which recognises the first three amino acids on the beta chain of the haemoglobin so, again, there should be no interference with HbS or HbC (130).

### 1.6.2.3 Immunoassay HbA1c Analyser (DCA 2000)

This analyser measures the concentration of $HbA_{1c}$ and the concentration of total haemoglobin; the ratio of the two is displayed as % $HbA_{1c}$. The analyser utilises a cartridge which contains all the necessary reagents. Haemoglobin $A_{1c}$ is estimated using a method based on inhibition of latex agglutination. A synthetic polymer containing multiple copies of the immunoreactive portion of $HbA_{1c}$ causes agglutination of latex particles coated with $HbA_{1c}$ specific mouse monoclonal antibody. This analyser has been designed specifically for use in the clinic or doctor's office laboratories. Finger prick samples are used. Specimens are analysed singly using a disposable cartridge and results are available in nine minutes (130).

High reagent costs and slow throughput make this analyser unsuitable for a hospital laboratory. In general the immunoassay methods are not as reproducible as the

40

chromatographic methods.

### 1.6.3 Colorimetric Methods

The first determination of glycohaemoglobin $HbA_{1c}$ was described by Fluckiger and Winterhalter (157) and refined by Fischer et al. (158). In their TBA assay 5-hydroxymethyl furfural (5-HMF) was liberated from $HbA_{1c}$ by oxalic acid and subsequently complexed with thiobarbituric acid (TBA) to yield a coloured complex which could be measured at 443 nm. A first evaluation of this technique was not successful. However, very good correlations with macro - or mini column methods have been described (135,159). Both Gabbay et al. (153) and Pecoraro et al. (159) have demonstrated that, in contrast to the chromatographic and electrophoretic techniques, the chemical assay detects glycosylation not only at the N-terminal amino group of the beta chains, but also the substantial glycation of the N-terminal amino groups of the alpha chain and of the epsilon amino groups of the lysine residues in both alpha and beta chains.

The TBA method can be automated very well and can be used for the determination of both glycohaemoglobins and glycated proteins (160). The various steps of the TBA method were evaluated (161,162), resulting in small changes in the original method. Only Dolholfer and Wieland (163) evaluated the assay critically. They stated that the TBA reaction yields erroneous results unless strictly standardised. Critical points are:-

a) The amount of protein must be kept identical in all samples

b) Dialysis for removal of glucose and $NaBH_4$ before hydrolysis is essential

c) The choice of acid, acid concentration, temperature and time of hydrolysis is critical

d) Appropriate $NaBH_4$ concentrations must be used for the reaction

Comparing the result of determining the glycation of total protein and albumin, 90% of glycated serum protein is accounted for by glycated albumin. HbF is not measured by the TBA-reaction and carbamylated haemoglobin has been shown not to interefere with the

41

specific thiobarbituric acid colorimetric method (163).

A completely different colorimetric method for the determination of glycohaemoglobins is based on the reaction of glycosyl residues with a phenol:sulfuric acid mixture (164). This reaction should yield a higher recovery of liberated glycosyl residues with an ever better correlation with the ion-exchange method than does the TBA method. Competition from the newer chromatographic methods have caused the colorimetric assays to decline in popularity.

## 1.6.4 Affinity Chromatography

Affinity separation of glycohaemoglobin was first discribed by Mallia and co-workers in 1981 (165). Affinity chromatography is a technique for separating large molecules on the basis of their chemical structure. The stationary phase, which is packed in a column, consists of an insoluble, inert matrix, such as agarose or cellulose, with an appropriate ligand attached. When the mobile phase containing the substances to be separated is passed through the column, the substances are separated by their different interactions with the ligand. The matrix is cross-linked agarose and the ligand is *m*-aminophenylboronic acid. Like the Amadori product of any protein, glycohaemoglobin contains *cis*-diol groups in the sugar portion of the adduct (adjacent hydroxy groups in the *cis* configuration) which form five-membered ring complexes with boronic acid (165).

The boronate has high affinity for 1,2-*cis*-diols, as in the stable ketoamine glycohaemoglobin complex, but not for the labile aldimines. The affinity column binds all ketoamine glycoproteins, including haemoglobins $A_{1c}$, $A_{1a}$, and $A_{1b}$. It may also be used for quantitating glycosylated plasma albumin, for which the turnover is more rapid than that of glycohaemoglobin.

Haemolysates may be prepared from whole blood or packed erythrocytes and when absorbed on to the solid phase washed with two buffers. The first buffer elutes the

42

non-glycated fraction, which does not have *cis*-diol groups and, therefore, does not bind to the boronic acid. The bound fraction, which should contain only GHb, is eluted with the second buffer which usually contains sorbitol, a sugar alcohol that displaces GHb from the column. **Figure 2** shows the interaction between glycohaemoglobin and the *m*-aminophenylboronic acid.



Immobilised boronic acid                    Binding of glycohaemoglobin
                                            to affinity matrix
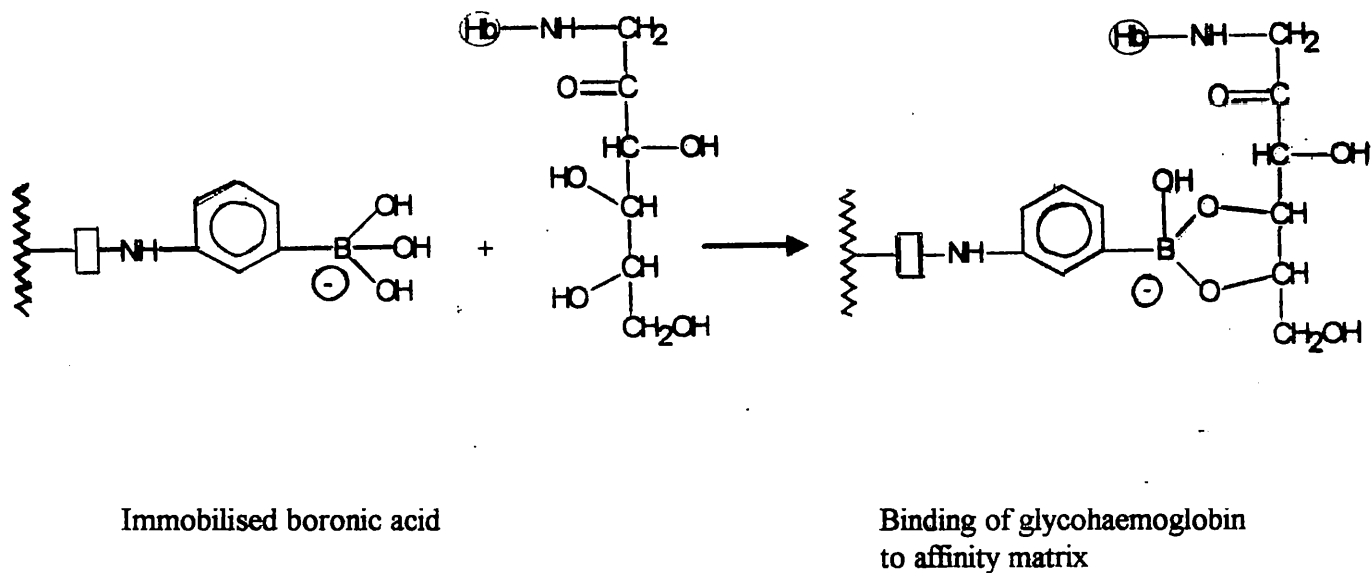
**Figure 2**    Interaction between glycohaemoglobin and the *m*-aminophenylboronic acid

The following affinity chromatography procedures were used in this project :-

### 1.6.4.1 Pierce Affinity Chromatography

Pierce affinity chromatography (Pierce and Warriner, Cambridge, U.K.) Glycotest II columns are filled with agarose gel bearing *m*-aminophenylboronate groups, which have affinity for *cis*-diol groups. The glycohaemoglobin containing the *cis*-diol groups are retained by the aminophenylboronate column while the non-glycohaemoglobin is eluted using a high concentration of the competing ligand, sorbitol. The absorbance of the separated glycohaemoglobin fractions are measured at 414 nm, and the percentage of

43

haemoglobin that is glycated is calculated (166). For the detailed methodology see **section 3.6** (167).

Attempts at automating the Pierce method by incorporating a mini-column containing gel in a flow based system were disappointing. With use the gel compacted giving slow throughput and drifting results (168).

### 1.6.4.2 Drew Scientific GHb-100 Analyser

Drew Scientific GHb-100 analyser (Drew Scientific Ltd., Sowersbywoods Industrial Estate, Burrow-in-Furness, Cumbria, U.K.). The GHb-100 analyser is a pre-production automated low pressure liquid affinity chromatography instrument for the assay of glycohaemoglobin. The GHb-100 analyser is designed to carry out tests on whole blood with a minimum of sample preparation and is provided with a 100 position autosampler for unattended automatic operation. The ratio of the glycohaemoglobin to non-glycohaemoglobin can be estimated in the presence of variants and without interference from foetal haemoglobin (HbF)

The GHb-100 analyser comprises of a pair of syringe pumps, three multiport rotary valves and a fixed wavelength flow-through spectrophotometer in conjunction with a computer control system to perform boronate affinity on haemoglobin samples. The rotary valves allow the syringe pumps to fill from the reagent bottles and then in reverse direction provide a flow of reagent to the chromatography column. Sample loading from the autosampler is achieved by using one of the rotary valves. The GHb-100 analyser uses low pressure liquid chromatography to separate glycohaemoglobin from non-glycohaemoglobin. The separated haemoglobin fractions are monitored by means of light absorption at 415 nm. The chromatogram obtained is stored by the internal computer. A software program performs the analysis of the chromatogram and generates a result report on both the cathode ray tube display and on the printer. For detailed instructions see **section 3.7** (169).

## 1.6.4.3 Advantages of the Affinity Method

Glycohaemoglobin measurement by affinity chromatography has many advantages over other methods. Affinity chromatography is not affected by the Pre-$HbA_{1c}$ as the boronate has a high affinity for 1,2 *cis*-diols, as in the stable ketoamine glycohaemoglobin complex, but not for the labile aldimines. The affinity column binds all ketoamine glycoproteins, including haemoglobins $A_{1c}$, $A_{1a}$, and $A_{1b}$. A possible explanation is that the Schiff base adducts are dissociated during chromatography and therefore do not have a strong interaction with *m*-aminophenylboronate. The affinity method, being totally insensitive to Schiff base, thus provides a more reliable measurement of stable GHb compared to the other methods, obviating the extra step of saline incubation (149).

With all methods, the importance of investigating unexpectedly low results and examination of individual chromatograms for unusual peaks cannot be overestimated. The best approach with patients with abnormal haemoglobins is to measure total GHb by affinity chromatography. Affinity chromatography is the only analytical system that is unaffected by the presence of abnormal haemoglobins. The affinity gel detects the glucose bound to haemoglobin, the type of haemoglobin (or indeed the type of protein) does not affect the separation. If there is complex mix of haemoglobin variants, then this assay must be the method of choice. The presence of carbamylated haemoglobin does not interfere with the specific phenylboronic gel affinity chromatography method, therefore this method can also be applied to renal patients with uraemia. (149).

Contrary to the statement of Mallia et al. (165), the affinity method has been reported to be susceptible to changes in pH and temperature (170); an increase in either was found to give a small increase of haemoglobin bound to the column. However, the affinity method was found to be considerably less sensitive to such changes than is the method using mini ion-exchange columns (170).

Ion-exchange and colorimetric results have both been shown to depend highly on

sample size variation (137,149). The application of a greater than recommended haemolysate concentration to commercially available ion-exchange mini-columns can shift the elution profile sufficiently to cause anomalous results because of poorer separation between the slow and fast fractions. Haemolysate concentration is even more critical in TBA colorimetry, because the results are expressed as nanomoles of hydroxymethyl furfural per 10 mg of haemoglobin. Any variation from the assumed quantity of haemoglobin per test will affect results proportionately. The affinity interaction, however, appears to be relatively independent from such errors since the final glycohaemoglobin concentrations are expressed in proportional and not absolute terms. Therefore, the affinity method is applicable to almost the entire range of concentrations encountered in normals and in diabetic patients (149,170).

### 1.6.4.4 Drew versus Pierce

The users of the Drew Scientific GHb-100 analyser can easily be trained in the use and maintenance of the analyser, as it is very simple and easy to use. Therefore, highly skilled personel are not required to analyse the glycohaemoglobin samples. Once loaded the samples can be left while the laboratory worker carries out other analyses, or may be left overnight. No reagent preparation is necessary as Drew Scientific provide all the necessary reagents pre-prepared. The GHb-100 analyser does not require poisonous reagents such as oxalic acid and thiobarbituric acid which are required for the colorimetric methods of measuring glycohaemoglobin. Once the glycohaemoglobin samples have been haemolysed and mixed thoroughly, they may be loaded on the GHb-100 analyser and assayed immediately.

The Special Chemistry section of Clinical Pathology at the Royal Sussex County hospital currently analyses approximately 900 patient samples per month for glycohaemoglobin. Until recently, glycohaemoglobins were assayed by the manual Pierce method, which is cheap in terms of consumables but is labour intensive. With the ever

increasing workloads, the continued use of the manual technique resulted in a backlog, increasing turn-around times (in excess of 5 working days), and contributed to stress, particularly for medical laboratory assistants and junior biomedical scientists.

There are cost pressures associated with this change in technique, but it is not viable to continue with the manual Pierce method if acceptable turn-around times are to be achieved. The consumables cost for the Pierce method has been kept low by 'regenerating' and re-using the columns which are sold as single-use, disposable items. In the past, this in-house treatment of columns has kept the cost per test low. It is, however, extremely labour intensive and current increases in workload cannot be sustained without increases in staffing. To use the Pierce columns correctly, as disposable items, would increase the consumables cost from £0.30 to £5.41 per test, equating to an increase in consumables cost of £58,428 per annum. Purchase of the GHb-100 analyser for this assay would increase the consumables cost to £0.59 per test which equates to an increase of £3,132 per annum. The capital cost of the GHb-100 analyser is £15,450 plus VAT if purchased before the end of March 1998. The revenue cost of consumables of the analyser, based on a workload of 10,800 per annum is £6,372 and the annual maintenance charge would be £1,810 after the one year warranty period. See **sections 3.6** and **3.7** for detailed methodologies.

### 1.6.5 Preferred Hospital Methods

For each laboratory the choice of method will depend on several factors, including set-up costs, the number of samples to be analysed, sample-handling details, running costs, and even the characteristics of the patient population (e.g. prevalence of haemoglobinopathies).

Because they are easy to automate, inspite of their many limitations, ion-exchange methods predominate. The "superior" affinity methods, until now, have proved difficult to automate and for this reason their use has declined. In addition to the Drew GHb-100

47

analyser, Primus, an American company have recently introduced an automated affinity method based on HPLC. If the machines prove satisfactory affinity methods may again become popular.

### 1.6.6    Problems of Standardisation with Glycohaemoglobin Methods

Each assay method measures glycohaemoglobin in a slightly different manner and assay conditions will differ between laboratories. In addition, there is no consensus on either a reference method or a single GHb standard. Thus, the actual numbers generated (including reference ranges) in one laboratory cannot be compared easily with numbers generated in another laboratory, even if the same basic assay is used. For example, a test value of 9% GHb in one laboratory might indicate that concentrations of blood glucose had been near the normal range, but in another laboratory might indicate high results. Under these less than ideal circumstances, each laboratory that performs GHb determinations must work closely with the medical practitioners who order the tests, to assure proper interpretation of results

A report published by the National Institute of Health Diabetes Data Group Expert Committee on glycosylated haemoglobin, suggests that intra-and interassay CVs of 5% are attainable, and should be achieved in any laboratory performing these measurements (171).

Different methods measure a range of different glycated fractions and the lack of a recognised standard has resulted in a large discrepancy in the results from the different systems. It is therefore at present impossible to define accuracy. Performance in external quality assessment can only be used to assess precision of a specific method within a laboratory (**Table 6**) or bias of one method from another.

**Table 6.** Within laboratory precision and between laboratory agreement for the major glycohaemoglobin methods. Data from the U.K. National External Quality Assurance Scheme (NEQAS) for Glycohaemoglobin (172).

| | Within Laboratory Precision (%CV) | | Between Laboratory Agreement (%CV) | |
| --- | --- | --- | --- | --- |
| | Within-batch | Between-batch | Normal result | High result |
| **HbA$_1$** Ion-exchange Chromatography | 3-10 | 4-12 | 6-14* | 6-14* |
| HPLC | 1-3 | 2-4 | 5-10 | 2-5 |
| Electrophoresis | 3-10 | 6-13 | 9-14 | 5-9 |
| **HbA$_{1c}$** Ion-exchange Chromatography | 2-8 | 3-4 | 9-12* | 9-12* |
| HPLC | 1-3 | 2-4 | 4-8 | 3-9 |
| Immunoassay | 2-8 | 2-10 | 10-12 | 7-8 |
| **GHb** (Manual) Affinity Chromatography | 3-5 | 3-8 | 12-16* | 12-16* |

\* No recent returns. Between laboratory agreement based on historical data.

One important result of assay imprecision is spurious widening of the reference internal, thereby limiting the usefulness of GHb to fine tune diabetic therapy.

There being no consensus on either a reference method or GHb standard, laboratories have a wide variety of quality control procedures. The materials are most often lyophilised haemolysates that are reconstituted in the local laboratory and used over several days or weeks. The stability of these materials has not, for the most part, been well documented. GHb standards called 'calibrators' are available from at least one manufacturer (Primus Corporation, Kansas City, USA).

Many laboratories monitor assay precision by using locally prepared controls - usually haemolysates of whole blood or packed erythrocytes from normal and diabetic samples (155,156). For laboratories that use ion-exchange chromatographic methods, controls must be stored -70$^{\circ}$C or colder to prevent gradual increases in the HbA$_{1a}$ $+$ $_b$ fraction (155,156). With proper storage conditions, controls will remain stable over many

months. Most laboratories include locally prepared controls in each assay, in addition to any commercial controls or standards. Manufacturers of commercial quality control materials face many of the same difficulties as local laboratories do in maintaining a high degree of long-term precision. One important facet of quality control is the establishment of non-diabetic reference interval in each laboratory that performs GHb determinations. In doing so, one must scrupulously avoid including samples from individuals with diabetes or even mildly impaired glucose tolerance, or with other factors such as haemoglobinopathies and anaemia's, that might affect glycohaemoglobin concentration.

# 2.0  AIMS OF THE PROJECT

The Clinical Pathology Department of the Royal Sussex County Hospital carries out about 10,800 glycohaemoglobin measurements every year. At present the manual Pierce affinity column method is used. This procedure is very labour intensive and consequently we have been considering ways of automating the test. Most of the established dedicated automatic analysers measure $HbA_{1c}$ by ion-exchange. As we choose to measure total glycohaemoglobin, it was our intention to develop an 'in-house' automated affinity method. During discussions with HPLC column suppliers we learned that Drew Scientific were developing an analyser based on the affinity procedure. Following our inquiries, Drew Scientific invited us to carry out the first clinical trials and evaluation for their pre-production analyser.

For the second part of my work I have studied GHb in long-term peritoneal dialysis patients. Patients undergoing peritoneal dialysis, for the treatment of chronic renal failure, are subject to high peritoneal concentrations of glucose (present in the dialysate) and it was thought that this could affect the patients GHb status. Because glycation products continue to form following a glucose challenge even after blood glucose has returned to normal, I hypothesised that I would find raised GHb concentrations in long-term CAPD patients.

The research conformed to the ethical standards set out by the Royal Sussex County Hospital for research.

# 3.0 EXPERIMENTAL-METHODS AND MATERIALS

The need was for an automated affinity method for the measurement of total glycohaemoglobin, that would cause minimal disruption to our established procedures. It was desirable to maintain our existing blood collection, sample preparation and reference ranges. Drew Scientific recommend dilution of whole blood in deionised water, while our existing method uses packed cells haemolysed in dilute Triton X-100. Drew Scientific also recommended calibrating their instrument using the diabetes control and complications trial (DCCT) (173) referenced $HbA_{1c}$ standards. We decided to find calibrants that, if possible, would give similar results to our existing method and hence avoid the considerable disturbance that would result from having to alter our existing reference value.

The glucose containing peritoneal dialysis solutions used in CAPD contain from 13.6 to 38.6 g/l of glucose. During CAPD large quantities of glucose are absorbed from the dialysate each day. This may provide a valuable source of energy to malnourished patients, but it may also cause further disturbances in the already abnormal uraemic metabolism of lipids, carbohydrates, proteins and amino acids. In this study I present results on the effects of CAPD in renal patients with special regard to the effects of glucose absorption from the peritoneal fluids on glycohaemoglobin concentrations.

## 3.1 Subjects

The total number of renal patients on CAPD studied = 67. The number of male CAPD patients = 42 ( 32 non-diabetic, 10 diabetic). The number of female CAPD patients = 25 ( 20 non-diabetic, 5 diabetic).

Routine blood samples for the measurement of glycohaemoglobin were used for the evaluation of the GHb-100 analyser from Drew Scientific. The samples were mainly obtained from the out-patients department at the Royal Sussex County Hospital, Brighton. Some glycohaemoglobin samples were also obtained from the hospital wards and general

practitioners.

Routine blood samples for the measurement of renal function tests (including urea and creatinine) on renal patients receiving CAPD treatment in the renal dialysis unit at the hospital mentioned above were intercepted after the routine test analysis had been completed. Glycohaemoglobin was then measured on these samples to obtain data for this project. Sixty routine biochemistry samples from non-diabetic, non-renal patients were selected after the requested tests were completed for use as controls. Number of male control patients = 40, number of female control patients = 20. Forty routine biochemistry samples from diabetic, non-renal patients were also used as controls when the requested tests were completed. Number of male control patients = 20, number of female controls = 20.

Random plasma glucose measurements were carried out routinely on the renal patients on CAPD by the renal physician. The results of urea, creatinine, and plasma glucose on renal patients on CAPD were obtained from the stored data in the laboratory computer.

Three to five 2 litre bags of commercially available dialysate solution were instilled each day into the peritoneal cavity of the renal patients on CAPD by the method of Oreopolous et al. (174). The number of exchanges containing either (1.36%, 2.27%, 3.86% glucose solutions and 7.5% w/v icodextrin was prescribed by the renal physician according to the need for fluid removal in each patient.

Patients on icodextrin used glucose containing CAPD fluids for the day-time dwells and 7.5% icodextrin in place of a bag of glucose solution for the overnight dwell, while patients on the glucose containing CAPD fluids used glucose solutions for the day-time and overnight dwells.

## 3.2  Sample Preparation

The blood samples for glycohaemoglobin measurement were taken into 7 ml lithium heparin tubes. The blood samples for renal function tests on renal patients receiving CAPD treatment and blood samples for routine biochemistry tests on non-diabetic, non-renal patients and diabetic, non-renal patients (controls) were also taken into 7 ml lithium heparin tubes. The blood samples for routine random plasma glucose measurements on the renal patients on CAPD were taken into 5 ml fluoride-oxalate containers.

On arrival at the laboratory the blood samples were centrifuged at 3000 rpm for 15 minutes. The routine samples for renal function tests and plasma glucose on CAPD patients and the control samples were analysed on the day of receipt. Samples for glycohaemoglobin measurement were stored at $4^{o}C$ and analysed within 3 days of receipt.

The renal function tests and plasma glucose measurements were analysed on the Vitros 950 analyser (Ortho-Clinical Diagnostics, Amersham, Buckinghamshire, HP7 OJS). The reaction sequence of the urea, creatinine, and glucose test methodologies are summarised below.

**Reaction sequence for urea on the Vitros 950 analyser**

$$H_2NCONH_2 + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{ammonia indicator} \longrightarrow \text{dye}$$
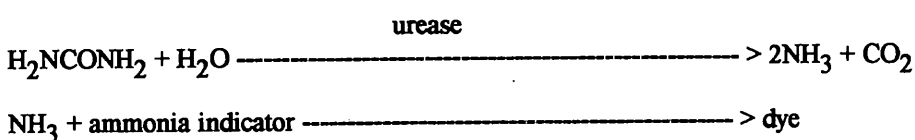
**Reaction sequence for creatinine on the Vitros 950 analyser**

$$\text{creatinine} + H_2O \xrightarrow{\text{creatinine amidohydrolase}} \text{creatine}$$

$$\text{creatine} + H_2O \xrightarrow{\text{creatine amidohydrolase}} \text{sarcosine} + \text{urea}$$

$$\text{sarcosine} + O_2 + H_2O \xrightarrow{\text{sarcosine oxidase}} \text{glycine} + \text{formaldehyde} + H_2O_2$$

$$H_2O_2 + \text{leuco dye} \xrightarrow{\text{peroxidase}} \text{dye}$$
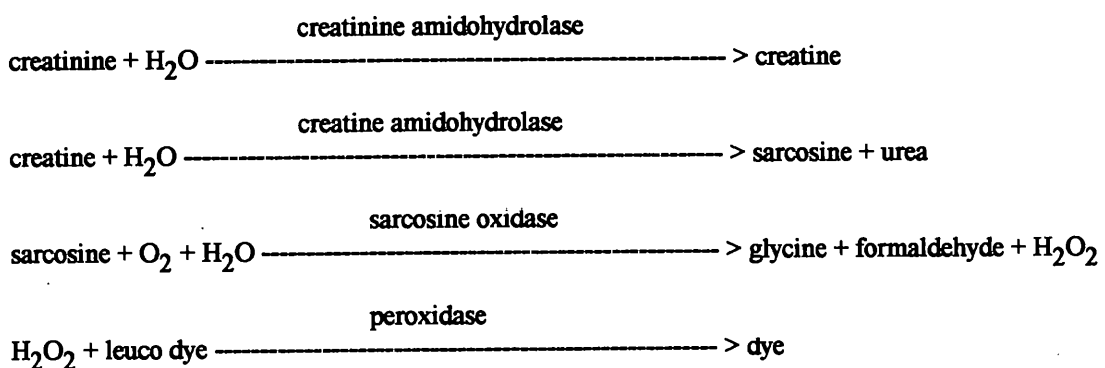
**Reaction sequence for glucose on the Vitros 950 analyser**

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\quad\text{glucose oxidase}\quad} \text{D-gluconic acid} + H_2O_2$$

$$2H_2O_2 + 4\text{-aminoantipyrine} + 1,7\text{-dihydroxynaphthalene} \xrightarrow{\quad\text{peroxidase}\quad} \text{red dye}$$
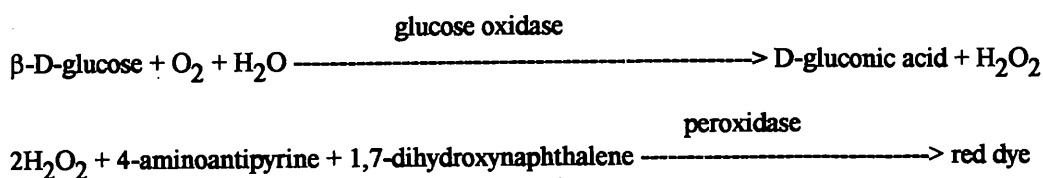
## 3.3    Equipment

1) Columns.

i) Pre-packed Glycotest II Analytical Columns from Pierce and Warriner, Cambridge, U.K.

ii) GHb-100 analyser columns from Drew Scientific, Ltd., Barrow-in-Furness, Cumbria LA14 4QR.

2) 7 ml lithium heparin blood collection tubes. Vacutainer. Brand sterile interior from Becton Dickinson Vacutainer Systems, France.

3) 5 ml lithium fluoride-oxalate blood collection tubes. Vacutainer. Brand sterile interior from Becton Dickinson Vacutainer Systems, France.

4) Absorbance spectrophotometer. Pharmacia Ultrospec III UV- visible spectrophotometer was obtained from Pharmacia Ltd. Pharmacia L.K.B., Milton Keynes, Buckinghamshire, MK9 3HP.

5) Centrifuge. Eppendorf Centrifuge 5416. Eppendorf Ltd., Cambridge, U.K.

6) Balance. Sartorious Handy Balance, resolution 0-1 mg. Anachem Ltd., Luton, Bedfordshire, LU2 06B.

7) L.K.B. diluter. Wallac, Finland.

8) Pipettes. Variable volume Gilsen automatic pipettes. Anachem Ltd., Luton, Bedfordshire, LU2 06B.

9) GHb-100 analyser. Drew Scientific Ltd., Barrow-in-Furness. Cumbria LA14 4QR.

## 3.4 Chemicals

1) Triton X-100. Aldrich Chemical Co. Ltd, Gillingham, Dorset, U.K.

2) All other chemicals were obtained from The British Drug Houses Ltd. BDH Laboratory Group, Pool, Dorset, U.K.

## 3.5 Data Analysis

Statistical analysis of the data was carried out using Minitab and Microsoft Excel linear and Demming's regression.

## 3.6 Glycohaemoglobin Measurement by the Pierce Affinity Chromatography Method.

### 3.6.1 Principle:- See Affinity Chromatography

### 3.6.2 Reagents and Columns

1) Columns. Pre-packed Glycotest II Analytical columns from Pierce and Warriner were used. They were stored at 4°C. After regeneration the columns could be re-used 16 times.

2) Haemolysing reagent. 0.1% Triton. 1 ml of Triton X-100 was dissolved in 1 litre of deionised water and stored at room temperature.

3) Wash buffer. 250 mmol/l ammonium acetate, 50 mmol/l magnesium chloride, pH 8.3.

35.5 g of anhydrous ammonium acetate and 20.3 g of magnesium chloride hexahydrate were dissolved in 1.5 litres of deionised, and adjusted to pH 8.3 using 1 mol/l of sodium hydroxide and made up to 2 litres. The wash buffer was stored at 4°C.

4) Elution buffer. 200 mmol/l Sorbitol, 50 mmol/l EDTA in 100 mmol/l Tris buffer, pH 8.3. 36.4 g of D-Sorbitol, 12.2 g of Tris(hydroxymethyl)methylamine and 18.6 g disodium EDTA dihydrate were dissolved in about 800 ml of deionised water. This was adjusted to pH 8.3 using 1 mol/l hydrochloric acid and made up to 1 litre and stored at 4°C.

5) Regeneration solution. 1% hydrochloric acid. 5 ml of concentrated hydrochloric acid were added to 500 ml of deionised water and stored at room temperature.

### 3.6.3 Quality Control

1) <u>In-house low control</u>:- From routine batches, 12 samples with glycohaemoglobin results of 7-10% were chosen. Haemolysates were prepared, pooled and frozen in 200 ml aliquots. Ranges of mean +/- 2 SD were established by assaying in duplicate on 20 batches, giving 40 results.

2) <u>In-house high control</u>:- From routine batches, 12 samples with glycohaemoglobin results of 17-20% were chosen. Haemolysates were prepared, pooled and frozen in 200 ml aliquots. Ranges of mean +/- 2 SD were established by assaying in duplicate on 20 batches, giving 40 results.

3) <u>Carry overs</u>. Two samples, one with around 5% glycohaemoglobin and one around 10% glycohaemoglobin were chosen from the previous batch. The carry overs were used as another internal quality control check.

### 3.6.4 Assay Procedure

1) A batch for glycohaemoglobin assay usually consisted of 44 samples plus low and high controls at each end and two samples carried over from the previous batch, i.e. 50 tests. The usual arrangement of the batch was:-

| Position | Control |
|----------|---------|
| 1 + 49 | Low control |
| 2 + 50 | High control |
| 17 | Low Carryover |
| 27 | High Carryover |

2) One set of 5 ml tubes were labelled 3-48 for the haemolysis step. Two sets of test tubes were each labelled from 1-50, to collect the fractions from the chromatography.

3) The low and high controls were already haemolysed. Using the LKB diluter, 100 microlitres of packed red cells from each patient and carryover sample were diluted with 2.0 ml of haemolysing reagent into its corresponding 5 ml tube. The sample was taken from the centre of the cells to avoid the buffy layer. The haemolysates were vortex mixed to ensure complete haemolysis.

4) The controls were allowed to thaw and were mixed well.

5) The wash and elution buffers were warmed to between $19^{O}C$ and $21^{O}C$. The reagent temperature was maintained at this temperature throughout the assay. The current set of columns were taken from the fridge and allowed to come to room temperature.

6) Then regeneration solution in which the columns were stored was discarded. Each column was primed with 2.5 ml pre-warmed wash buffer. When fully drained the columns were transferred to the first set of labelled test tubes and the priming solution discarded.

7) Using the LKB diluter, 50 microlitres of each haemolysate were picked up and dispensed on to the top of the appropriate column with 0.5 ml wash buffer. This was allowed to soak in completely and was left for a further 15 minutes.

8) 2.5 ml of wash buffer were added to each column, followed by a further 2.5 ml when there was room. This was allowed to drain completely. The eluate was mixed thoroughly. This was the non-glycohaemoglobin (wash) fraction.

9) Keeping the columns in order, they were transferred to the second set of test tubes. 3.0 ml of pre-warmed elution buffer were added and allowed to drain completely. The eluate was mixed thoroughly. This was the glycohaemoglobin (elution) fraction.

10) The Ultrospec III spectrophotometer, printer, and computer were switched on. The computer (IBM, model P52) was pre-programmed to calculate the % GHb from the elution and wash fractions, and also to set the wavelength to 414 nm.

11) Samples were selected to act as carryovers on the next batch, and the carryover controls were placed with the next batch of samples.

12) After analysis samples were stored at $4^{O}C$ for one week before disposal in an autoclave bin.

13) After use the columns were transferred to their storage rack. Each column was washed with 2.5 ml of deionised water and the drainings discarded. Then 2 x 4.5 ml of the regeneration solution was applied to each column which was then stored wet at $4^{O}C$.

14) Columns were discarded at or before their 16$^{th}$ use (166).

### 3.6.4.1 Calculation

The % glycohaemoglobin was calculated using the following equation:-

$$\% \text{ GHb} = \frac{\text{A414 nm Elution}}{(\text{A414 nm Wash} \times 5.55/3) + \text{A 414 nm Elution}}$$

This calculation was done automatically by the ultrospec computer. A = absorbance.

### 3.6.4.2 Result Range and Interpretation

The result ranges for glycohaemoglobin and their interpretation using the Pierce affinity chromatography method are listed below.

| Result range | Interpretation |
|---|---|
| < 2.0% | Very low; hypoglycaemia very likely |
| 2.01-3.1% | Low; serious risk of hypoglycaemia in diabetic patients |
| 3.11-4.7% | Normal, but risk of hyopglycaemia in diabetic patients |
| 4.71-7.5% | Normal; excellent diabetic control |
| 7.51-8.5% | Just above normal, but good diabetic control |
| 8.51-9.7% | Above normal, but acceptable diabetic control |
| 9.71-13.0% | High, poor diabetic control |
| 13.01-17.0% | Very high; extremely poor diabetic control |
| > 17% | Very high; uncontrolled diabetes |

These ranges and interpretations were set on the basis of locally-determined normal range, and were agreed with Dr N Vaughan, Consultant Endocrinologist at the Royal Sussex County Hospital.

## 3.7 Glycohaemoglobin Measurement by the Drew Scientific GHb-100 Affinity LPLC Automated Analyser

### 3.7.1 Principle :- See Affinity Chromatography

### 3.7.2 Reagents and Columns

Column. GHb-100 analyser columns obtained from Drew Scientific.

Reagent A. GHb-100 analyser reagent (wash buffer) obtained from Drew Scientific.

Reagent B. GHb-100 analyser reagent (elution buffer) obtained from Drew Scientific.

Sample vials. GHb-100 sample vials obtained from Drew Scientific.

### 3.7.3 Quality Control

Biorad 1 (low) and Biorad 2 (high) controls were obtained from Biorad Laboratory Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempsted, Herts., HP2 7TD. The controls were provided in the lyophilized form for increased stability. On receipt the controls were reconstituted with 0.5 ml of deionised water, and allowed to stand for 10 minutes. Before sampling, the vial was gently swirled to ensure homogeneity. The controls were diluted using the LKB diluter set at 10 microlitres to pick up the control and 1 ml to pick up the deionised water. The controls were mixed for homogeneity and stored at -20$^{\circ}$C until required. The GHb-100 analyser was calibrated using assigned calibrant values of Biorad 1 = 3.8% Biorad 2 – 10.0%, unless otherwise stated.

1) <u>In-house low control</u>:- From routine batches, 12 samples with glycohaemoglobin results of 5.0-6.5% were chosen. Haemolysates were prepared, pooled and frozen in 2 ml aliquots.

2) <u>In-house high controls</u>:- From routine batches, 12 samples with glycohaemoglobin results of 9.5-10.5% were chosen. Haemolysates were prepared, pooled and stored in 2 ml aliquots.

3) <u>Carryovers</u>. Two samples, one with around 5% glycohaemoglobin and one around 10% glycohaemoglobin were chosen from the previous batch. The carryovers were used as an internal quality control check.

### 3.7.4 Primus Calibrant

Primus 1 (normal) and Primus 2 (high) calibrants were obtained from Primus Corporation, 7235 Central Avenue, Kansas City, USA. The calibrants were provided in the lyophilized form for increased stability. On receipt the calibrants were reconstituted with 400 microlitres of deionised water and allowed to stand for 10 minutes. Before sampling, the vial was gently swirled to ensure homogeneity. The calibrants were diluted using the LKB diluter set at 10 microlitres to pick up the calibrant and 1 ml to pick up deionised

water. The calibrants were mixed for homogeneity and stored at -20°C until required.

| | | HbA1c Component HPLC Affinity(175) | Total GHb HPLC Affinity(176) |
|---|---|---|---|
| Lot no. 1056 | Calibrater 1 | 5.8% | 7.0% |
| Lot no. 1057 | Calibrater 2 | 13.3% | 19.7% |

### 3.7.5 Assay Procedure

1) A batch of glycohaemoglobin samples normally consisted of 50 or 99 samples, according to the assay requirements. Depending on the circumstances, the GHb-100 analyser was flushed with new reagents and /or calibrated before analysing the samples. After each sample analysis the results were printed. When the batch was complete a summary of results was printed. Each GHb-100 glycohaemoglobin kit consisted of a column, reagents A and B and sample vials to provide 500 assays. Each sample took approximately 4 minutes to complete.

The usual arrangement for controls in a 50 sample batch was:-

| Position | Control |
|---|---|
| 1+49 | Biorad 1 (Low control) |
| 2+50 | Biorad 2 (High control) |
| 17 | Carryover (low) |
| 27 | Carryover (high) |

The usual arrangement for controls in a 99 sample batch was:-

| Position | Control |
|---|---|
| 1+98 | Biorad 1 (Low control) |
| 2+99 | Biorad 2 (High control) |
| 49 | Carryover (low) |
| 50 | Carryover (high) |

2) One set of 5 ml tubes were labelled from 3-48, or 3-97 depending on the assay requirements.

3) The Biorad 1 and Biorad 2 controls were already haemolysed. Using the LKB diluter, 10 microlitres of packed cells from each patient and carry over sample were diluted with 2 ml of deionised water into its corresponding 5 ml tube. The sample was taken from the centre of the cells to avoid the buffy layer. The haemolysates were vortex mixed to ensure complete haemolysis.

4) The heamolysates were then assayed immediately, but could be stored at 4°C for 3 days according to the manufacturer (Drew Scientific).

5) The controls (Biorad 1 and Biorad 2) were allowed to thaw and mixed well.

6) 1 ml of the haemolysate was pipetted into the appropriate sample vial placed on the autosampler tray.

7) The GHb-100 analyser was programmed to carry out the required number of tests, i.e. 50, or 99 samples.

8) The GHb-100 analyser was programmed to carry out a background check on deionised water and if necessary the GHb-100 analyser was programmed for calibration. The calibrant used was Biorad 1 ( assigned value GHb = 3.8%) and Biorad 2 ( assigned value GHb = 10.0%).

9) The background was checked and when necessary the calibration was checked before proceeding with the assay.

10) When the GHb-100 analyser completed the batch, a continual flow of reagent A was maintained at 50 microlitres per minute.

### 3.7.5.1 Calculation

A table of results were produced with the ratio of GHb to total haemoglobin.

# 4.0 EVALUATION AND RESULTS OF THE GHb-100 ANALYSER FROM DREW SCIENTIFIC

## 4.1    Effect of Haemoglobin concentration on GHb Results

The haemoglobin (Hb) concentration interferes with the ion-exchange and electrophoresis methods (134,137,149). Normally physiological variations in haemoglobin concentration will not give rise to large deviations, but careless handling during preparation of the haemolysate can enhance the effect. The application of greater than recommended haemolysate concentration to commercially available ion-exchange mini-columns can shift the elution profile sufficiently to cause anamolous results because of poorer separation between the slow and fast fractions.

Haemolysate concentration is even more critical in the TBA colorimetry, because results are expressed as nanomoles of hydroxymethyl furfural per 10 mg of haemoglobin. Any variation from the assumed quantity per test will affect the resulting values proportionately (149). The affinity interaction, however, appears to be relatively independent from such errors since the final glycohaemoglobin concentrations are expressed in proportional and not absolute terms (166).

A study of the effect of Hb concentration on GHb results was carried out on the GHb-100 analyser using a series of haemoglobin dilutions. 10 samples with GHb results of around 10% were collected (9.6%, 9.4%, 10.3%, 10.4%, 10.2%, 9.9%, 9.6%, 9.6%, 10.2%, 9.4%). The plasma from the 10 samples was aspirated and pooled together in a container. The remaining packed cells were pooled together in a separate container.

The haemoglobin dilutions were made using the LKB diluter as follows:-

| Packed Cell Volume (%) | Volume of Packed Cells (microlitres) | Volume of Plasma (microlitres) | Total Volume (microlitres) |
|---|---|---|---|
| 10 | 100 | 900 | 1000 |
| 20 | 200 | 800 | 1000 |
| 30 | 300 | 700 | 1000 |
| 40 | 400 | 600 | 1000 |
| 50 | 500 | 500 | 1000 |
| 60 | 600 | 400 | 1000 |
| 70 | 700 | 300 | 1000 |
| 80 | 800 | 200 | 1000 |
| 90 | 900 | 100 | 1000 |
| 100 | 1000 | 0 | 1000 |

The haemoglobin dilutions were then treated as whole blood samples. 10 microlitres of the haemoglobin dilutions were haemolysed with 1 ml of deionised water using the LKB diluter and mixed thoroughly. The haemoglobin dilutions were then placed in the sample rack of the GHb-100 analyser and the machine was programmed to carry out GHb assay on the dilutions.

The effect of Hb concentration on GHb results can be seen in **Table 7**. A packed cell volume (PCV) from 30% to 70% shows a very small increasing trend in GHb results (0.3% GHb).

**Table 7** Effect of Hb concentration (PCV%) on GHb%

| PCV% | GHb% |
|---|---|
| 10 | --- |
| 20 | --- |
| **30** | **8.7** |
| 40 | 8.8 |
| 50 | 9.0 |
| 60 | 9.1 |
| **70** | **9.0** |
| 80 | 9.2 |
| 90 | 9.5 |
| 100 | 9.8 |

— Error (low sample concentration)

## 4.2 Effect of Using 1% Triton X-100 as a Haemolysing Reagent

The Pierce affinity method requires 1% Triton X-100 as a Haemolysing reagent, while Drew Scientific recommend deionised water as a haemolysing reagent.

Triton X-100 is a detergent and a more powerful haemolysing reagent than deionised water. A study was carried out to determine the effect of using 1% Triton X-100 as a haemolysing reagent on the GHb-100 analyser. A sample with a known GHb result was used in the evaluation. 10 microlitres of whole blood was haemolysed with 1 ml of 1% Triton X-100 and deionised water respectively, using the LKB diluter. **Figure 3** shows a typical GHb chromatogram of the haemolysate analysed by the GHb-100 analyser using deionised water as a haemolysing reagent. **Figure 4** shows a GHb chromatogram of the haemolysate analysed by the GHb-100 analyser using Triton X-100 as a haemolysing reagent.

It can be seen from Figure 3 that when deionised water is used as a haemolysing reagent there is good separation of glycohaemoglobin (peak 2) from total haemoglobin (peak 1). It can be seen from Figure 4 that when Triton X-100 is used as a haemolysing reagent, the column became adversely affected and could not separate glycohaemoglobin from total haemoglobin. Once the column was damaged by the use of Triton X-100, the separation problem with the column could not be rectified. The damaged column was discarded and replaced with a new column. The use of Triton X-100 as a haemolysing reagent for the GHb-100 analyser was discontinued and deionised water - as recommended by Drew Scientific was used thereafter.

**Figure 3** A typical GHb chromatogram of the haemolysate analysed by the GHb-100 analyser using deionised water as the haemolysing reagent (key provided).
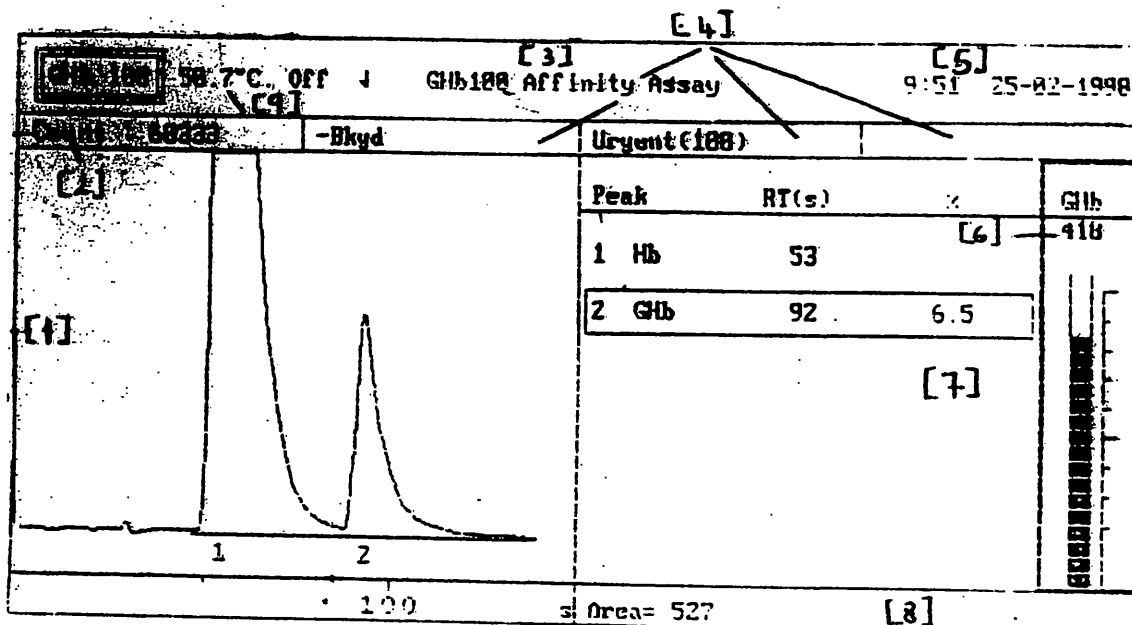


Key:-

[1] **Display Area**  This area of the screen is used to display traces and prompting messages when operator action is required.

[2] **Count**  This displays the assay count number.

[3] **Laboratory Name**  This displays the laboratory name that has been programmed into the parameter file.

[4] **Status Window**  Three boxes which display current activities of the system.

[5] **Date and Time**  The current date and time.

[6] **Kit Status**  This displays the number of tests remaining in numerical and visual form.

[7] **Results Window**  This is used to display the numerical results of the analysis and messages for the operator.

[8] **Batch Information**  Displays the progress through the batch. e.g. Batch : 12 - 23 B(C4) means that the current sample is 12, the last sample in the batch is 23. Background subtraction is in force. C4 is the background well. (Not shown here).

[9] **Temperature Display** Displays peltier temperature and current status of the peltier device.

**Figure 4** A GHb chromatogtam of the haemolysate analysed by the GHb-100 analyser using Triton X-100 as a haemolysing reagent
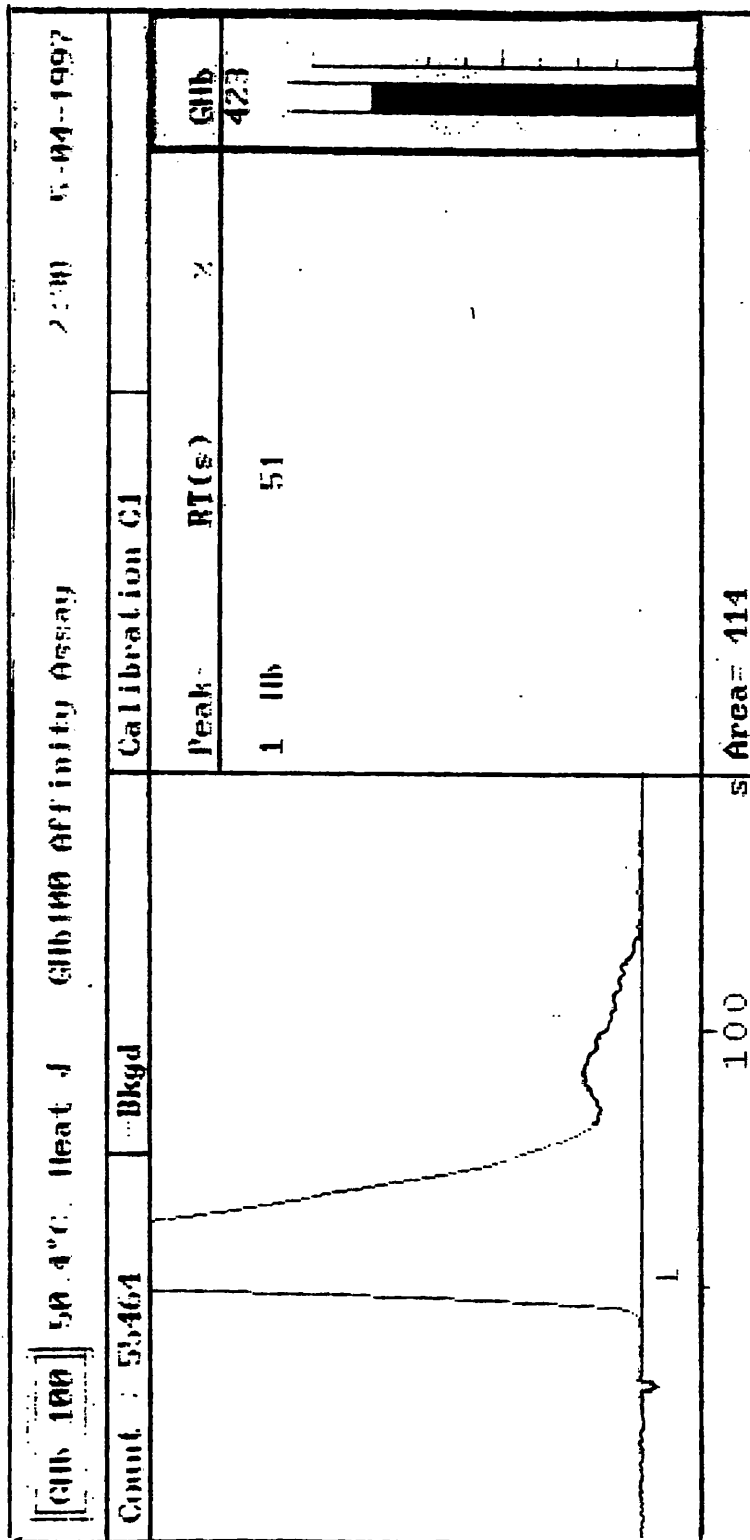
## 4.3    Packed Cells versus Whole blood

The Pierce method uses packed cells for the preparation of haemolysates, while Drew Scientific recommend whole blood for the preparation of haemolysates for use on the GHb-100 analyser. Packed cells are more convenient to use for the measurement of GHb in our laboratory as most of the whole blood samples are centrifuged on receipt to obtain serum or plasma, which is then used for the measurement of various other biochemical analytes. In addition, the use use of packed cells eliminates the need to mix the samples prior to haemolysing. The whole blood samples require thorough mixing prior to haemolysing to obtain a homogenous sample as cells tend to settle out on standing.

A study of packed cells versus whole blood was carried out on the GHb-100 analyser. Drew Scientific recommend 10 microlitres of whole blood to be haemolysed with 1 ml of deionised water. Forty samples with known GHb results were haemolysed in two sets using the LKB diluter. The whole blood haemolysates were prepared first. The whole blood samples were then centrifuged at 3500 rpm for 15 minutes to obtain the packed cells for the packed cell haemolysates.

a) The first set of haemolysates were made using 10 microlitres of whole blood with 1 ml of deionised water.

b) The second set of haemolysates were made using 10 microlitres of packed cells with 2 ml of deionised water.

Using linear regression, the results of packed cells versus whole blood was calculated. The results of the study of packed cells versus whole blood is shown in **Figure 5.** It can be seen from Figure 5 that there is a good correlation between the GHb results obtained from the packed cells and those from whole blood samples. n = 40, y = 0.9572x - 0.0987 where y = whole blood GHb (%)  x = packed cells GHb (%), r = 0.9904.

To see individual results for the study of packed cells versus whole blood see **Table 1 in Appendix.**

68

**Figure 5** Correlation between GHb results determined from packed cells and from whole blood.
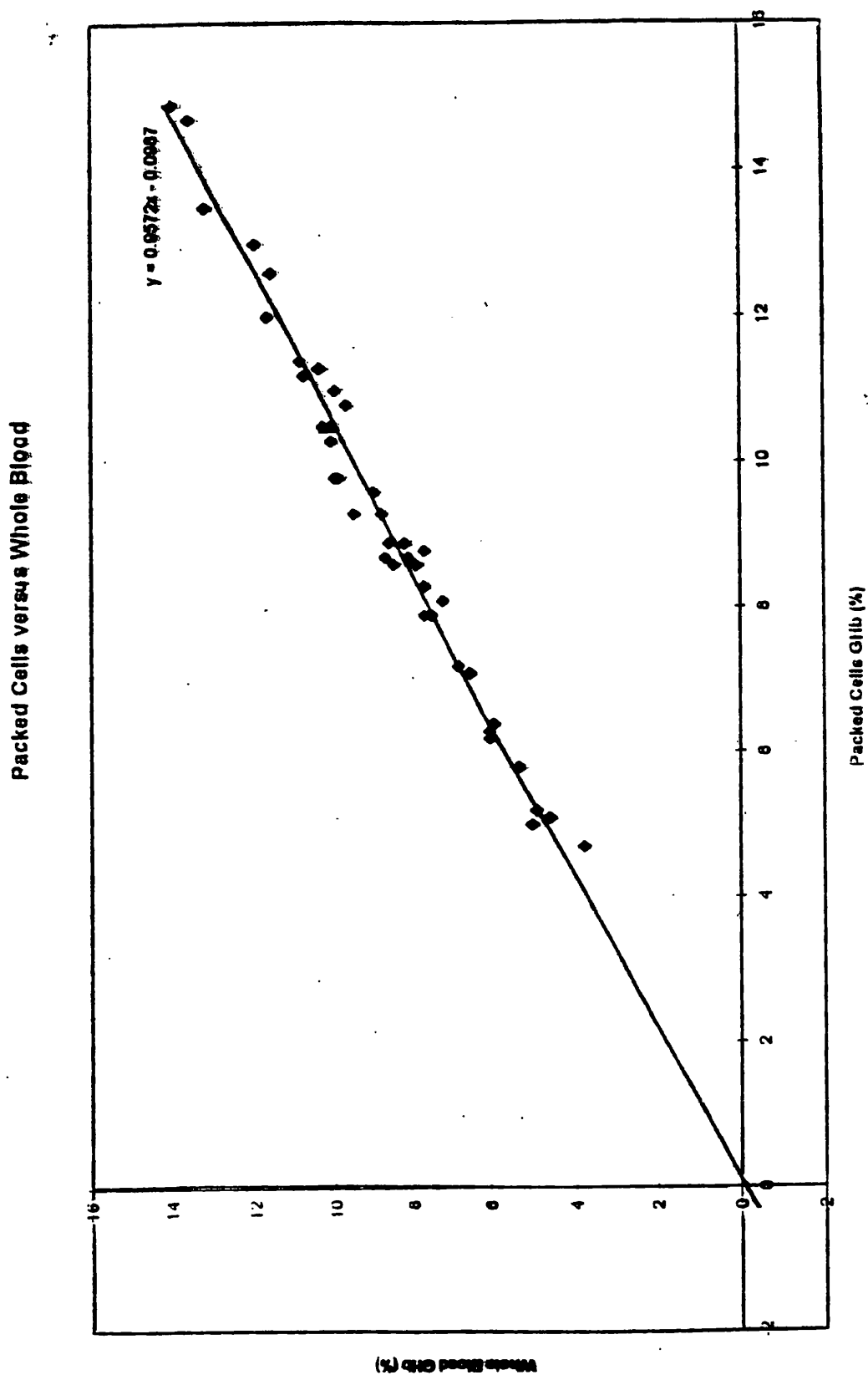
## 4.4    Precision-run Study

A report published by the National Institute of Health Diabetes Data Group Expert Committee on glycosylated haemoglobin, suggests that intra and interassay CVs of 5% are are attainable, and should be achieved in any laboratory performing these measurements (171).

A precision-run study was carried out on the GHb-100 analyser from Drew Scientific. Two samples with known GHb results were used in the precision study evaluation.

a) The first sample with a known low GHb value was analysed 40 times. 10 microlitres of packed cells were haemolysed with 2 ml of deionised water.

b) The second sample with a known high GHb value was analysed 53 times. 10 microlitres of packed cells were haemolysed with 2 ml of deionised water.

The results of the precision-run study are shown in **Tables 8** and **9**. It can be seen from Tables 8 and 9 that the GHb-100 analyser is a very precise instrument for measuring both low and high GHb ranges.

List of statistical abbreviations used:-

| | |
|---|---|
| N = | number |
| MEAN = | mean value |
| MEDIAN = | median value |
| TRMEAN = | truncated mean value |
| STDEV = | standard deviation |
| SEMEAN = | standard error of mean value |
| MIN = | minimum value |
| MAX = | maximum value |
| Q1 = | 1st quartile value |
| Q3 = | 3rd quartile value |
| % CV = | coefficient of variation (%) |

a) <u>Low GHb Precision-run</u>

**Table 8** The GHb results (%) obtained for the low GHb precision-run

| n | GHb % | n | GHb % | n | GHb % | n | GHb % |
|---|---|---|---|---|---|---|---|
| 1 | 5.5 | 11 | 5.6 | 21 | 5.6 | 31 | 5.8 |
| 2 | 5.6 | 12 | 5.7 | 22 | 5.7 | 32 | 5.7 |
| 3 | 5.5 | 13 | 5.6 | 23 | 5.8 | 33 | 5.7 |
| 4 | 5.7 | 14 | 5.7 | 24 | 5.8 | 34 | 5.8 |
| 5 | 5.8 | 15 | 5.7 | 25 | 5.8 | 35 | 5.8 |
| 6 | 5.6 | 16 | 5.8 | 26 | 5.7 | 36 | 5.6 |
| 7 | 5.6 | 17 | 5.6 | 27 | 5.7 | 37 | 5.8 |
| 8 | 5.6 | 18 | 5.7 | 28 | 5.8 | 38 | 5.8 |
| 9 | 5.7 | 19 | 5.8 | 29 | 5.8 | 39 | 5.9 |
| 10 | 5.7 | 20 | 5.7 | 30 | 5.8 | 40 | 5.9 |

n = 40  MEAN = 5.7125  MEDIAN – 5.7000  TRMEAN = 5.7139  STDEV = 0.0992

SEMEAN = 0.0157  MIN = 5.5000  MAX = 5.9000  Q1 = 5.6000  Q3 = 5.8000

The calculated % CV for the low GHb precision-run = 1.74%.

c) <u>High GHb Precision-run</u>

**Table 9** The GHb results (%) obtained for the high GHb precision-run.

| n | GHb % | n | GHb % | n | GHb % | n | GHb % |
|---|---|---|---|---|---|---|---|
| 1 | 10.6 | 15 | 10.7 | 29 | 10.8 | 43 | 10.9 |
| 2 | 11.0 | 16 | 10.7 | 30 | 11.0 | 44 | 11.0 |
| 3 | 11.1 | 17 | 10.6 | 31 | 11.0 | 45 | 11.0 |
| 4 | 10.9 | 18 | 10.7 | 32 | 11.0 | 46 | 11.0 |
| 5 | 11.1 | 19 | 10.7 | 33 | 10.8 | 47 | 11.0 |
| 6 | 11.1 | 20 | 10.4 | 34 | 10.7 | 48 | 11.0 |
| 7 | 11.2 | 21 | 10.6 | 35 | 10.8 | 49 | 11.1 |
| 8 | 11.1 | 22 | 10.8 | 36 | 11.1 | 50 | 11.0 |
| 9 | 11.2 | 23 | 10.8 | 37 | 11.1 | 51 | 10.9 |
| 10 | 11.1 | 24 | 10.7 | 38 | 10.9 | 52 | 11.0 |
| 11 | 10.7 | 25 | 10.9 | 39 | 11.0 | 53 | 10.7 |
| 12 | 10.8 | 26 | 10.8 | 40 | 11.1 | | |
| 13 | 10.6 | 27 | 11.0 | 41 | 11.1 | | |
| 14 | 10.7 | 28 | 10.8 | 42 | 10.9 | | |

n = 53  MEAN = 10.885  MEDIAN = 10.900  TRMEAN = 10.891  STDEV = 0.192

SEMEAN = 0.026  MIN = 10.400  MAX = 11.200  Q1 = 10.700  Q3 = 11.000

The calculated % CV for the high GHb precision-run = 1.76%

## 4.5    Between-batch Precision Study

A study of between-batch precision was carried out on the GHb-100 analyser. In-house low and high controls were used to assess the between-batch precision of the GHb-100 analyser during the evaluation period. To prepare the in-house controls - see **section 3.7.3**.

The GHb results of the between-batch precision on the in-house low and high controls are shown in **Table 10.**

The between-batch results of the in-house low and high controls are summerised below.

In-house low control

n = 89  MEAN = 6.3697  MEDIAN = 6.3000  TRMEAN = 6.3654  STDEV = 0.2986

SEMEAN = 0.0317  MIN = 5.8000  MAX = 7.0000  Q1 = 6.1000  Q3 = 6.6000

The calculated % CV = 4.69%.

In-house high control

n = 89  MEAN = 9.6831  MEDIAN = 9.7000  TRMEAN = 9.6728  STDEV = 0.3283

SEMEAN = 0.0348  MIN = 9.0000  MAX = 10.9000  Q1 = 9.4000  Q3 = 9.9000

The calculated % CV = 3.39%.

**Table 10.** Between-batch precision of in-house low and high controls

| Batch no. | Low GHb (%) | High GHb (%) | Batch no. | Low GHb (%) | High GHb (%) |
|---|---|---|---|---|---|
| 1 | 6.7 | 10.2 | 46 | 6.1 | 9.4 |
| 2 | 7.0 | 10.4 | 47 | 6.3 | 9.6 |
| 3 | 6.9 | 9.9 | 48 | 6.4 | 9.8 |
| 4 | 6.5 | 9.8 | 49 | 6.3 | 9.5 |
| 5 | 6.8 | 9.8 | 50 | 6.3 | 9.4 |
| 6 | 6.9 | 10.0 | 51 | 6.3 | 9.5 |
| 7 | 6.7 | 9.9 | 52 | 6.2 | 9.5 |
| 8 | 6.5 | 9.8 | 53 | 6.6 | 10.1 |
| 9 | 6.7 | 10.1 | 54 | 6.8 | 10.0 |
| 10 | 6.6 | 9.9 | 55 | 6.1 | 9.5 |
| 11 | 6.8 | 10.2 | 56 | 6.6 | 10.0 |
| 12 | 7.0 | 10.4 | 57 | 6.7 | 9.8 |
| 13 | 6.4 | 10.0 | 58 | 6.9 | 10.1 |
| 14 | 6.6 | 10.1 | 59 | 6.6 | 10.9 |
| 15 | 6.4 | 9.7 | 60 | 6.8 | 10.0 |
| 16 | 6.2 | 9.4 | 61 | 6.1 | 9.2 |
| 17 | 7.0 | 9.7 | 62 | 6.0 | 9.2 |
| 18 | 6.3 | 9.9 | 63 | 6.0 | 9.4 |
| 19 | 6.2 | 9.7 | 64 | 6.0 | 9.2 |
| 20 | 6.3 | 9.6 | 65 | 6.2 | 9.5 |
| 21 | 6.3 | 9.5 | 66 | 6.3 | 9.6 |
| 22 | 6.5 | 9.8 | 67 | 6.1 | 9.4 |
| 23 | 6.2 | 9.5 | 68 | 6.0 | 9.5 |
| 24 | 6.3 | 9.5 | 69 | 6.2 | 9.5 |
| 25 | 6.2 | 9.7 | 70 | 6.5 | 9.6 |
| 26 | 6.7 | 10.1 | 71 | 6.4 | 9.7 |
| 27 | 6.7 | 9.6 | 72 | 6.4 | 9.7 |
| 28 | 6.4 | 9.7 | 73 | 6.4 | 9.7 |
| 29 | 6.6 | 9.9 | 74 | 6.4 | 9.7 |
| 30 | 6.7 | 9.9 | 75 | 6.5 | 9.9 |
| 31 | 6.6 | 9.8 | 76 | 6.2 | 9.8 |
| 32 | 6.7 | 10.0 | 77 | 5.9 | 9.0 |
| 33 | 6.7 | 9.9 | 78 | 5.8 | 9.0 |
| 34 | 6.7 | 10.1 | 79 | 6.3 | 9.4 |
| 35 | 5.9 | 9.6 | 80 | 6.1 | 9.4 |
| 36 | 6.3 | 10.2 | 81 | 6.0 | 9.4 |
| 37 | 6.1 | 9.4 | 82 | 6.1 | 9.4 |
| 38 | 6.1 | 9.2 | 83 | 6.3 | 9.7 |
| 39 | 6.5 | 9.8 | 84 | 6.1 | 9.5 |
| 40 | 6.2 | 9.7 | 85 | 6.2 | 9.3 |
| 41 | 6.1 | 9.3 | 86 | 6.3 | 9.5 |
| 42 | 6.0 | 9.4 | 87 | 6.0 | 9.4 |
| 43 | 5.8 | 9.3 | 88 | 6.2 | 9.6 |
| 44 | 6.0 | 9.3 | 89 | 6.1 | 9.4 |
| 45 | 6.0 | 9.4 | | | |

## 4.6    Correlation Study

The Pierce affinity method of measuring GHb was used as a reference method for the correlation and calibration of the Drew Scientific GHb-100 analyser. A two-point calibration was carried out using Biorad 1 (BR1 lot no. 33531, expiry date November 1998) and Biorad 2 (BR2 lot no. 33532, expiry date November 1998) as GHb calibrants. Biorad have assigned mean total haemoglobin values for the Pierce method as Follows:-

BR1 mean = 4.3%    range (3.5 - 5.2%)

BR2 mean = 11.3%    range (9.0 - 13.5%)

The aim of the correlation study was to determine the assigned calibration value that would give the best correlation of GHb results between the Pierce and Drew method. A good correlation between the two methods mentioned would permit our laboratory to use the existing reference range set for the Pierce method to be used for the Drew method, i.e. the GHb result reporting will not be affected.

## 4.6.1 Correlation Study of Assigned Calibrant Values of BR1 = 4.3% BR2 = 11.3%

The GHb-100 analyser from Drew Scientific was calibrated using the mean values given for the Pierce affinity method by Biorad i.e, BR1 = 4.3%, BR2 = 11.3%.

The result of the correlation study using BR1 = 4.3% BR2 = 11.3% as the assigned calibrant values is shown in **Figure 6**. It can be seen from Figure 6 that the correlation is fairly good. n = 89, y = 1.0626x + 0.2464 where y = Drew GHb (%) x = Pierce GHb (%), r = 0.9834, p = < 0.0001. The Deming regression for the correlation studies was calculated using Pierce precision = 2.0% CV (166), Drew precision = 1.75% CV (mean of precision-run study 1.74, 1.76).

The percentage difference of GHb results between the Pierce and Drew methods is shown in **Figure 7**. It can be seen from Figure 7 that there is a positive bias of GHb results obtained for the Drew method when using the assigned calibrant values of BR1 = 4.3% BR2 = 11.3%. However, most of the result differences are within 0 to +15%.

The mean difference of GHb results (%) between the methods (Drew-Pierce) = 0.7719.

The mean percentage difference of GHb results (%) between the methods (Drew-Pierce /Pierce x 100) = 9.440.

To see individual results for the correlation study of assigned calibrant value BR1 = 4.3% BR2 = 11.3% see **Table 2 in Appendix.**

Correlation Study BR1 = 4.3%  BR2 = 11.3%

$y = 1.0826x + 0.2464$
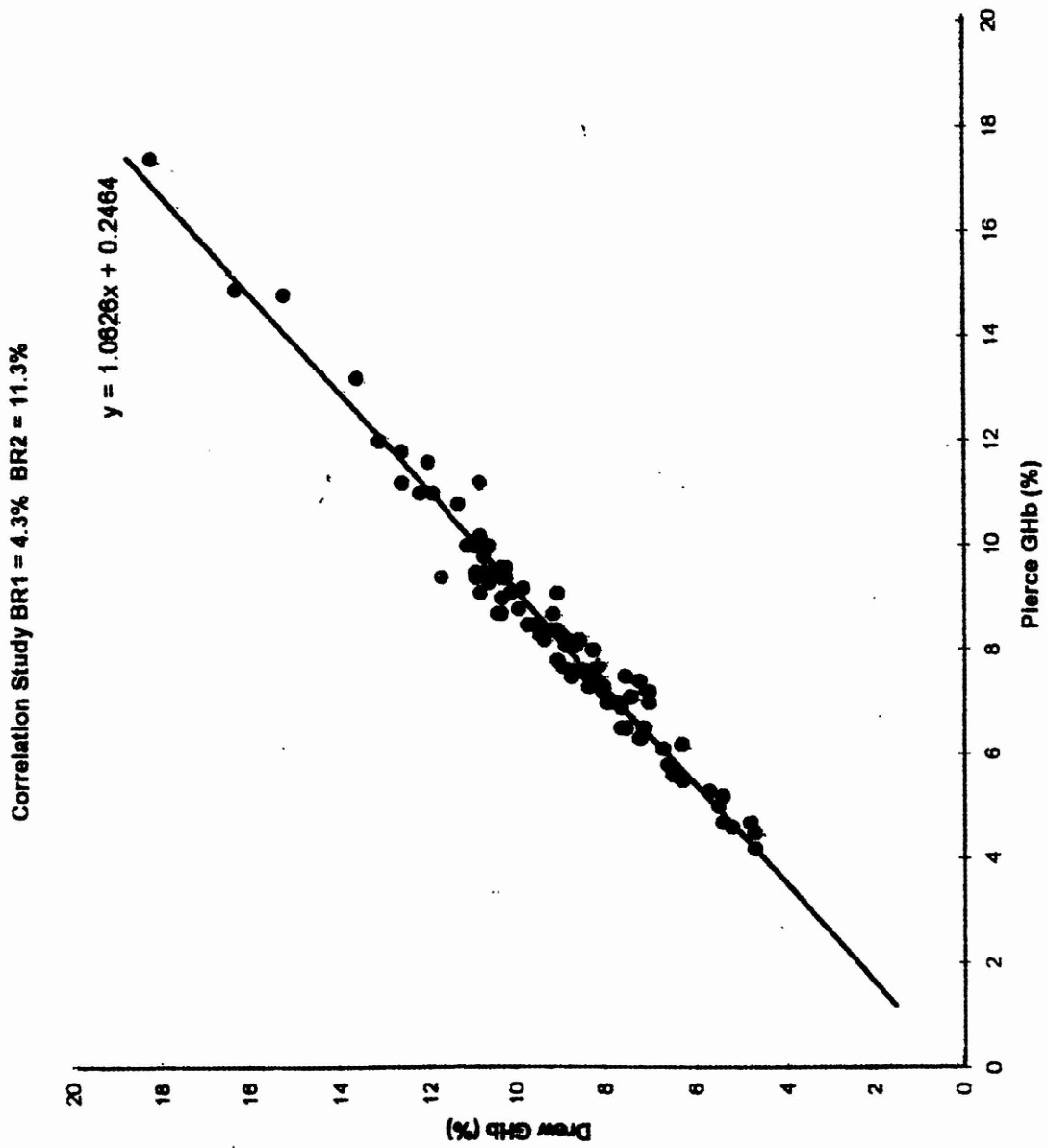
**Figure 7.** Percentage difference in GHb results between the Pierce and Drew methods for the assigned calibrant values of BR1 = 4.3%  BR2 = 11.3%.
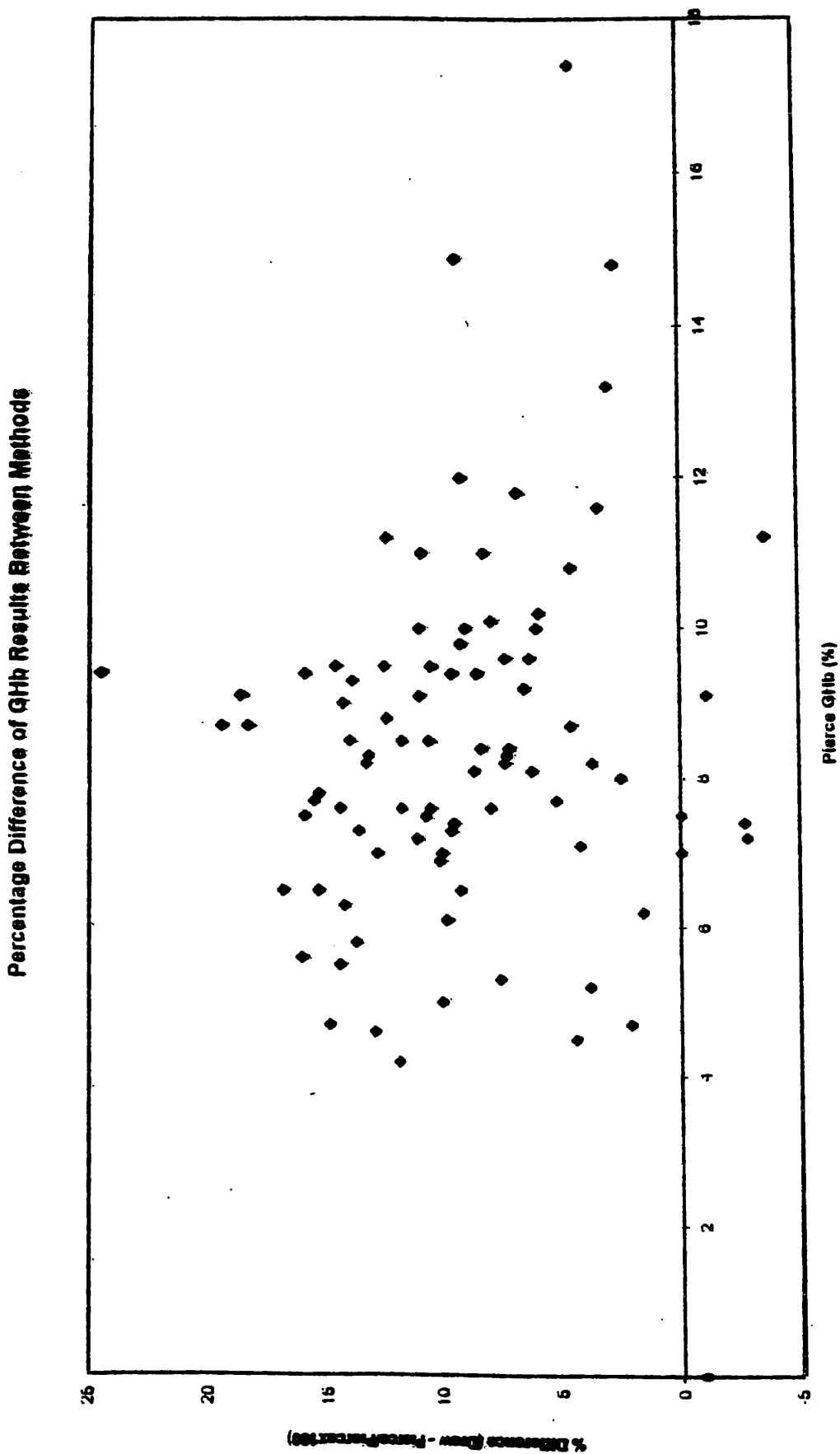
### 4.6.2  Correlation Study of Assigned Calibrant Values of BR1 = 3.8% BR2 = 10.0%

Various calibration values were assigned to BR1 and BR2 to obtain a much better correlation of GHb results between the GHb-100 analyser and the Pierce method. By trial and error, a very good correlation between the two methods mentioned was obtained (BR1 = 3.8%, BR2 = 10.0%).

The result of the correlation study using BR1 = 3.8% BR2 = 10.0% as the assigned calibrant values is shown in **Figure 8**. It can be seen from Figure 8 that the correlation is very good. n = 50, y = 1.018x -0.1034 where y = Drew GHb (%)  x = Pierce GHb (%), r = 0.9806, p = <0.0001.

The percentage difference of GHb results between the Pierce and Drew methods is shown in **Figure 9**. It can be seen from Figure 9 that there is no positive or negative bias of GHb results obtained for the Drew method when using the assigned calibrant values of BR1 = 3.8% BR2 = 10.0%. Most of the result differences are within -5 to +10%.

The mean difference of GHb results (%) between the methods (Drew-Pierce) = 0.0440.

The mean pecentage difference of GHb results (%) between the methods (Drew-Pierce /Pierce x100) = 0.432.

To see individual results for the correlation study of assigned calibrant value BR1 = 3.8% BR2 = 10.0% see **Table 3 in Appendix.**

**Figure 8.** Correlation study of assigned calibrant values of BR1 = 3.8%   BR2 = 10.0%.

Correlation Study BR1 = 3.8%  BR2 = 10.0%

y = 1.018x - 0.1034

Pierce GHb (%)

Draw GHb (%)
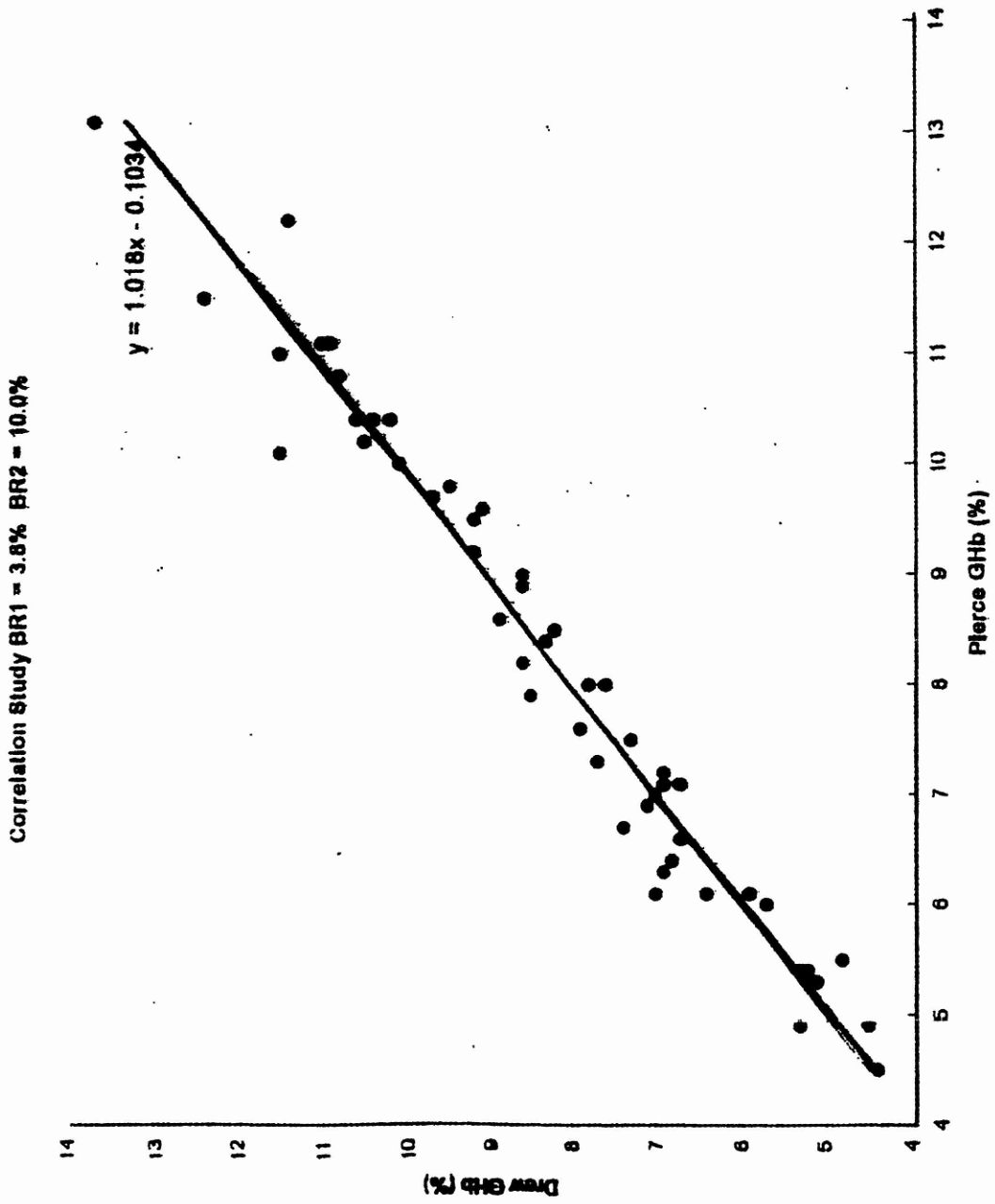
**Figure 9.** Percentage difference in GHb results between the Pierce and Drew methods for the assigned calibrant values of BR1 = 3.8%  BR2 = 10.0%.
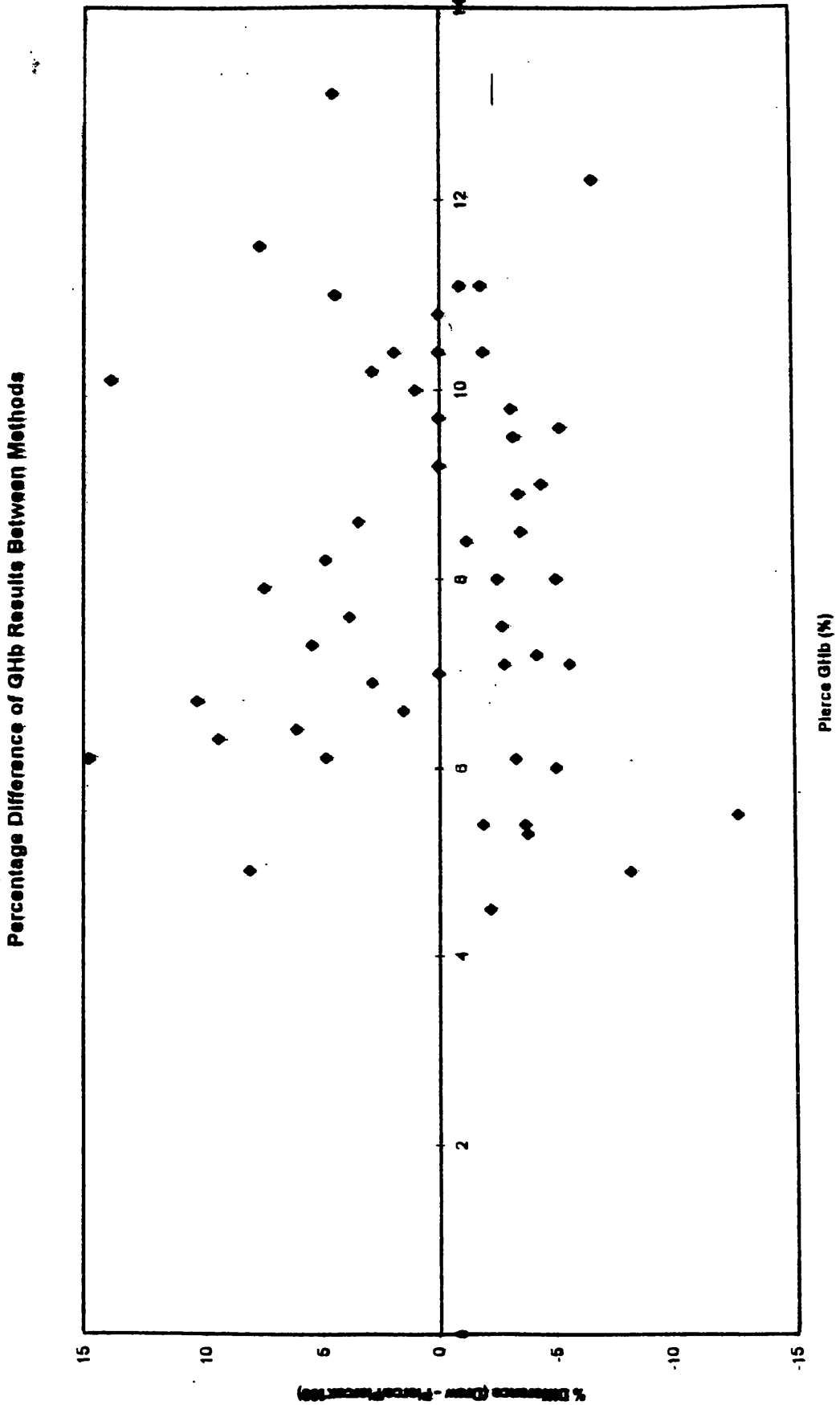
## 4.6.3 Biorad versus Primus Calibrants

Towards the end of this project we came under pressure from our endocrinologist to report our results as 'DCCT aligned'. To this end we used the Primus DCCT aligned calibrants (intended for their own affinity analyser) with the values of Primus 1 = 5.8%, Primus 2 = 13.3% (175).

The GHb-100 analyser was calibrated using the assigned calibrant values of BR 1 = 3.8% BR2 = 10.0%, and Primus 1 and Primus 2 calibrants were analysed as samples four times each.

The GHb results for Primus 1 were:  5.1%, 5.3%, 5.3%, 5.2%.     Mean result = 5.2%.

The GHb results for Primus 2 were:  14.7%, 14.7%, 14.8%, 15.0%Mean result = 14.8%

The mean GHb results obtained for Primus 1 and 2 were quite close to the DCCT GHb values assigned for $HbA_{1c}$ by HPLC affinity (175) (Primus 1 = 5.8%, Primus 2 = 13.3%). We therefore decided to correlate the GHb results obtained on the GHb-100 analyser using Biorad and Primus as calibrants in separate runs.

139 samples were haemolysed using 10 microlitres of packed cells with 2 ml of deionised water. The haemolysates were then analysed on the GHb-100 analyser using the assigned calibrant values of BR1 = 3.8%, BR2 = 10.0%. After completion of the analysis, the GHb-100 analyser was calibrated using Primus 1 = 5.8%, Primus 2 = 13.3%, and the measurements were repeated.

The results of the correlation study of Biorad versus Primus calibrants is shown in **Figure 10**. n = 139, y = 0.7585x + 2.0154 where y = Primus GHb (%)  x = Biorad GHb (%), r = 0.9981, p = < 0.0001.

The percentage difference of GHb results between Biorad and Primus calibrants is shown in **Figure 11**. It can be seen from Figure 11 that the Primus calibrants gave a positive bias for low GHb values and a negative bias for high GHb values. Most result differences are within -15 to +15%.

To see individual results for the correlation study of Biorad versus Primus calibrants
see **Table 4 in Appendix.**

**Figure 10.** Correlation study of Biorad versus Primus Calibrants
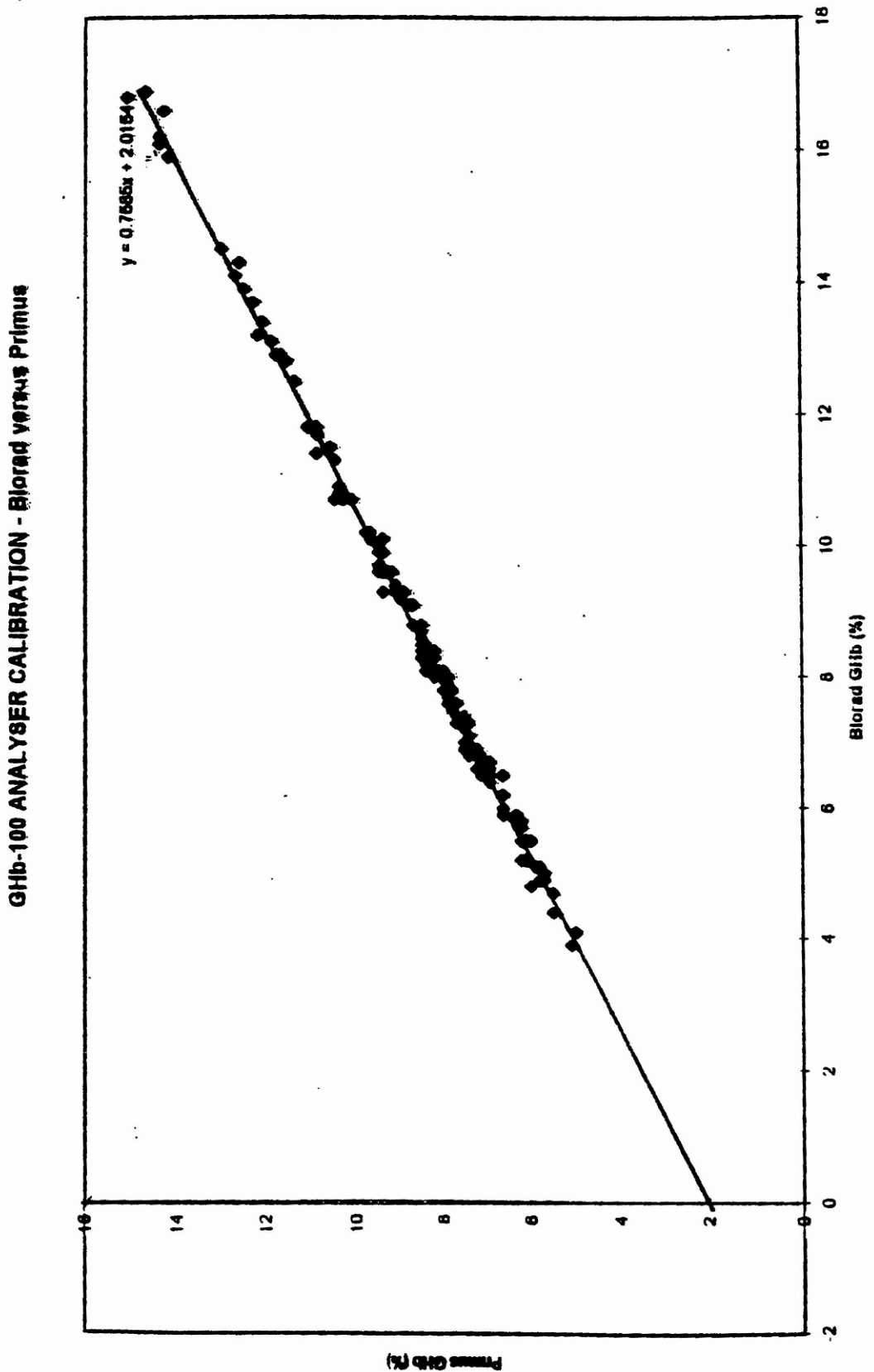
# Figure 11. Percentage difference in GHb results between Biorad and Primus Calibrants
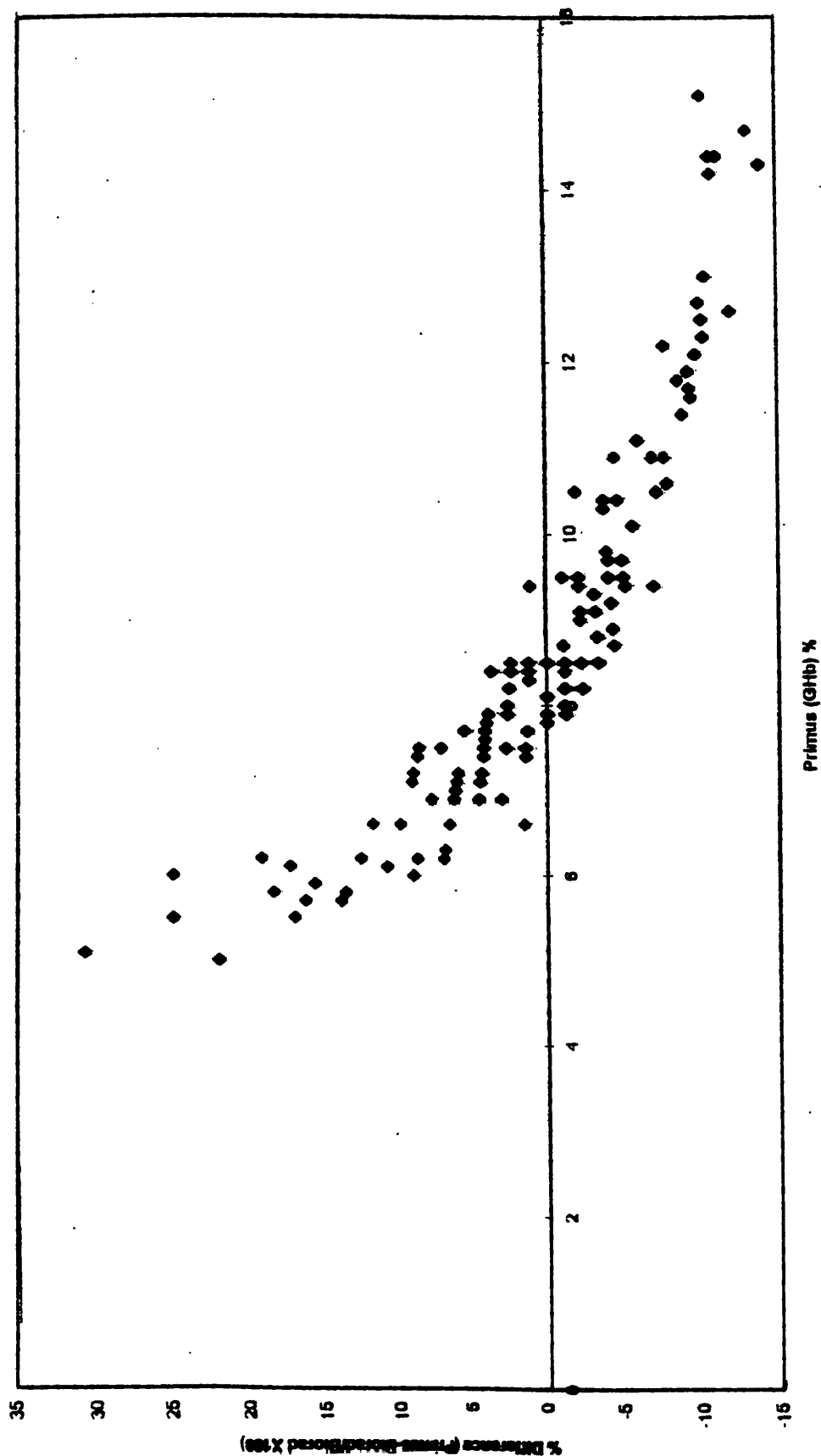
## 4.6.3.1 The Effect of Re-standardisation Using Primus Calibrants on the Existing reference Range for GHb

The large diabetes control and complications trial (DCCT) (173) has shown that it is desirable to reduce the glycohaemoglobin concentrations towards the upper end of normal. In order that the results from this laboratory are comparable with those elsewhere, and may also be clinically related with the outcomes in the DCCT, the Clinical Pathologist at the Royal Sussex County Hospital has now decided to realign our existing calibration (BR1 = 3.8%, BR2 = 10.0%) to be the same as that of the DCCT (Primus 1 = 5.8%, Primus 2 = 13.3%).

The hospital laboratory will continue to use the GHb-100 affinity chromatography analyser for the measurement of total glycohaemoglobin (GHb), but will be calibrated in terms of $HbA_{1c}$. The effect of this is summarised in **Table 11**. In the left hand column is shown the current ranges and associated comments which relate to the existing calibration (BR1 = 3.8%, BR2 = 10.0%). In the third column the effect of the re-standardisation is calculated so as to show how the numeric results would change for the same comments. As these do not take account of the advantages of more intense treatment demonstrated in the DCCT, the new comments will be aligned with the outcomes of the DCCT as shown in the right hand column. The new reports will state that the results are DCCT-aligned.

The DCCT result was calculated using the formula:

DCCT result = (0.76 X GHb result calibrated using BR1 = 3.8%, BR2 = 10.0%) + 2.02

**Table 11** The effect of re-standardisation using Primus calibrants on the existing reference range

| | Existing comment and results | Existing comment, DCCT-recalculated | New DCCT |
|---|---|---|---|
| < 2% | Very low, hypoglycaemia very likely. | < 3.54% | < 3.00% |
| 2.01 - 3.1% | Low, serious risk of hypoglycaemia in diabetic patients. | 3.54 - 4.37% | 3.00 - 3.99% |
| 3.11 - 4.7% | Normal, but risk of hypoglycaemia in diabetic patients. | 4.37 - 5.59% | 4.00 - 4.99% |
| 4.71 - 7.5% | Normal, excellent diabetic control. | 5.59 - 7.71% | 5.00 - 5.99% |
| 7.51 - 8.5% | Just above normal, but good diabetic control. | 7.71 - 8.47% | 6.00 - 6.99 |
| 8.51 - 9.7% | Above normal, but acceptable diabetic control. | 8.47 - 9.38% | 7.00 - 7.99% |
| | Diabetic control should be improved - at risk of complications. | | 8.00 - 9.49% |
| 9.71 - 13.0% | High, poor diabetic control. | 9.38 - 11.88% | 9.50 - 10.99% |
| 13.01 - 17.0% | Very high, extremely poor diabetic control. | 11.88 - 14.91% | 11.00 - 13.99% |
| > 17.0% | Very high, uncontrolled diabetes. | > 14.91% | > 13.99% |

## 4.7 Column Life of the GHb-100 Analyser

Drew Scientific recommend that the column of the GHb-100 analyser should be replaced after analysing 500 samples. A study of extending the column life of the GHb-100 analyser was carried out, to determine whether more than 500 samples can be processed on one column. The column was not replaced after processing 500 samples during the evaluation and a maximum of 720 samples were analysed before the column had to be replaced due to ineffective separation of the glycohaemoglobin from the total haemoglobin.

## 4.8  Problems Encountered with the GHb-100 Analyser when in Routine Use

Once the GHb-100 analysers evaluation was successfully completed and good correlations were obtained with the Pierce method, the GHb-100 analyser was used to carry out GHb measurements routinely in our laboratory.

Before the GHb-100 analyser begins analysing samples, a background (deionised water) is run as the reagents themselves do cause a small signal in the detector during chromatography due to changes in refractive index. The GHb-100 analyser then subtracts this background from the actual sample traces, and more reproducible chromatograms are obtained. When the GHb-100 analyser was in routine use, it was noticed that occasionally, exaggerated peaks in the background chromatograms would cause a shift in the baseline. The shift in the baseline was a problem of concern as it would cause falsely elevated GHb results.

Drew Scientific were informed about the shifting baseline problem that we were encountering, and the chromatograms with shifting baselines were faxed to the GHb-100 analyser engineers as requested. The engineers solved our problem by altering the computer software to prevent the baseline shift, i.e. the baseline now stays at the base of the sample peaks. **Figure 12** shows a chromatogram of a background with no exaggerated peaks. This type of background did not cause a baseline shift. **Figure 13** shows a chromatogram of a background with slightly exaggerated peaks. This type of background did cause a baseline shift.

An experiment was carried out on the GHb-100 analyser to determine which peak of the slightly exaggerated background was causing the problem baseline shift. Deionised water was analysed as a sample and the slightly exaggerated background was subtracted automatically by the GHb-100 analyser. **Figure 14** shows a chromatogram of deionised water after the slightly exaggerated background was subtracted. It can be seen from Figure

86

14 that it was the first peak in the exaggerated background that was causing the baseline to shift.

Low and high carryovers were then analysed on the GHb-100 analyser to show the effect of the shifting baseline on GHb results. **Figure 15** shows a chromatogram of a low carryover with a baseline shift. The GHb result of the low carryover was 6.5%, the shifting baseline increased the GHb result to 7.1%. **Figure 16** shows a chromatogram of a high carryover with a baseline shift. The GHb result of the high carryover was 9.5%, the shifting baseline increased the GHb result to 10.3%.

**Figure 12** A chromatogram of a background with no exaggerated peaks.
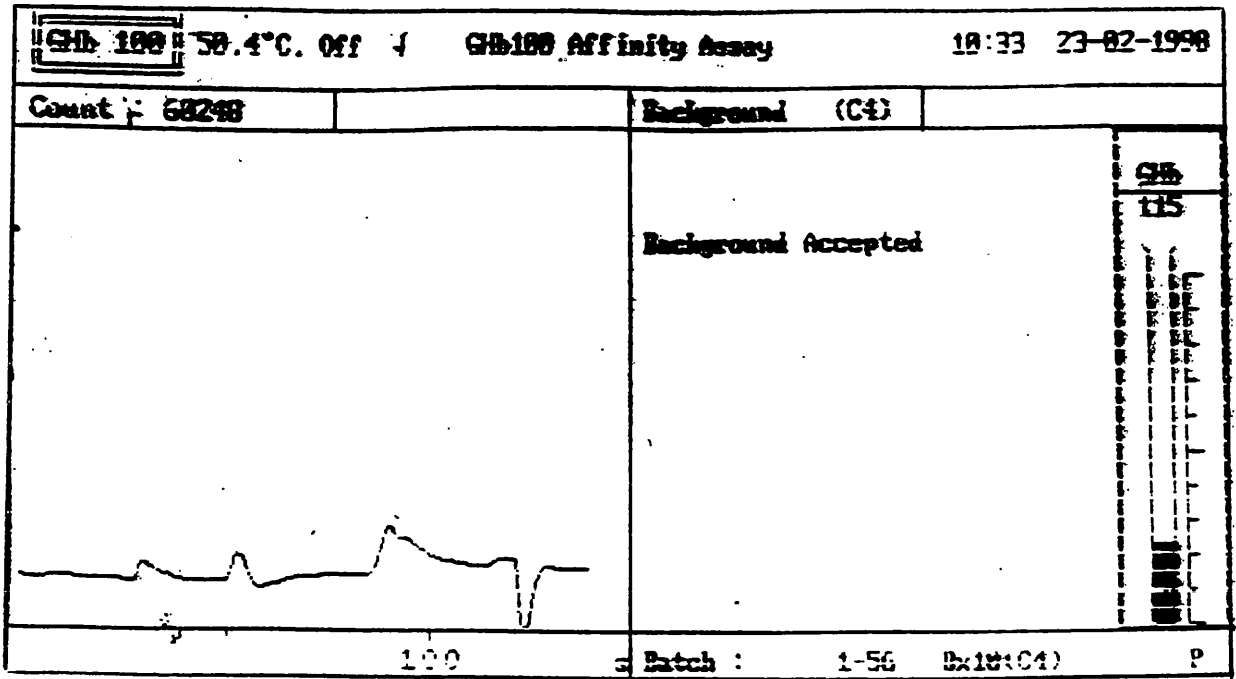


**Figure 13** A chromatogram of a background with slight exaggerated peaks.
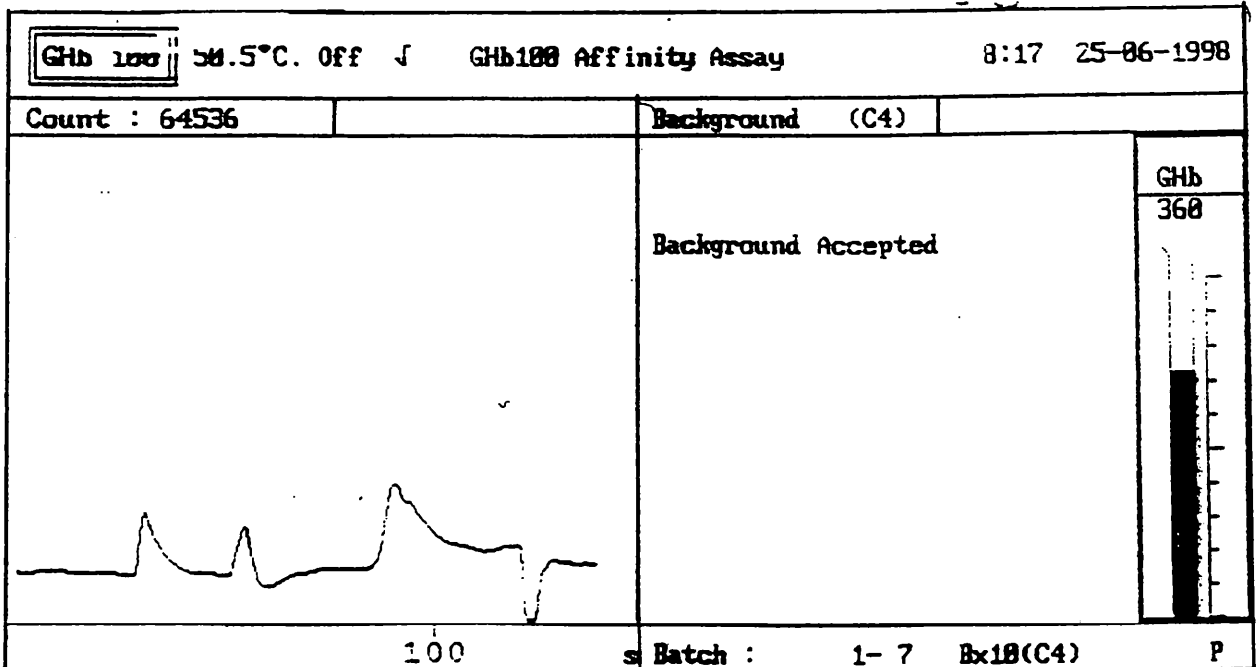
**Figure 14** A chromatogram of deionised water (analysed as a sample) after the slightly exaggerated background was subtracted.
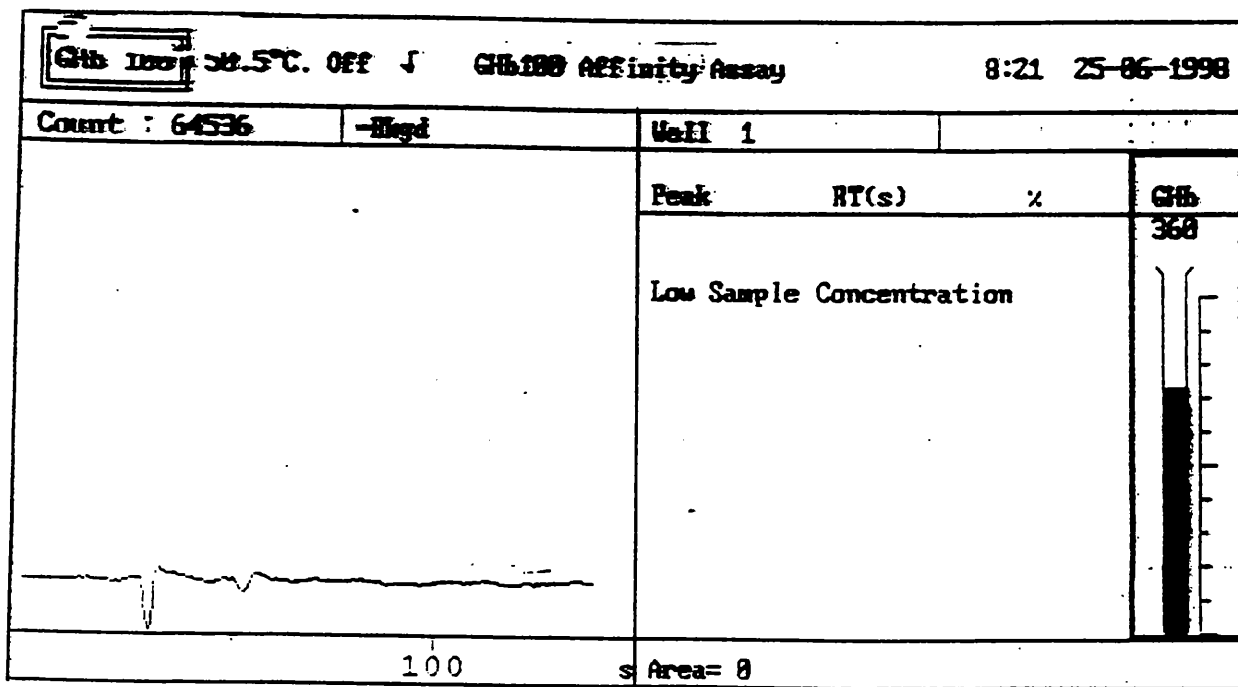
**Figure 15** A chromatogram of a low carryover with a baseline shift. The GHb result of the low carryover was 6.5%, the shifting baseline increased the GHb result to 7.1%.



| | | | |
|---|---|---|---|
| GHb 100 | 50.5°C. Heat √ | GHb100 Affinity Assay | 8:25  25-06-1998 |

| Count : 64537 | —Bkgd | Well 2 | |
|---|---|---|---|

| Peak | RT(s) | % | GHb |
|---|---|---|---|
| | | | 359 |
| 1  Hb | 55 | | |
| 2  GHb | 92 | 7.1 | |

1    2

100          s Area= 617
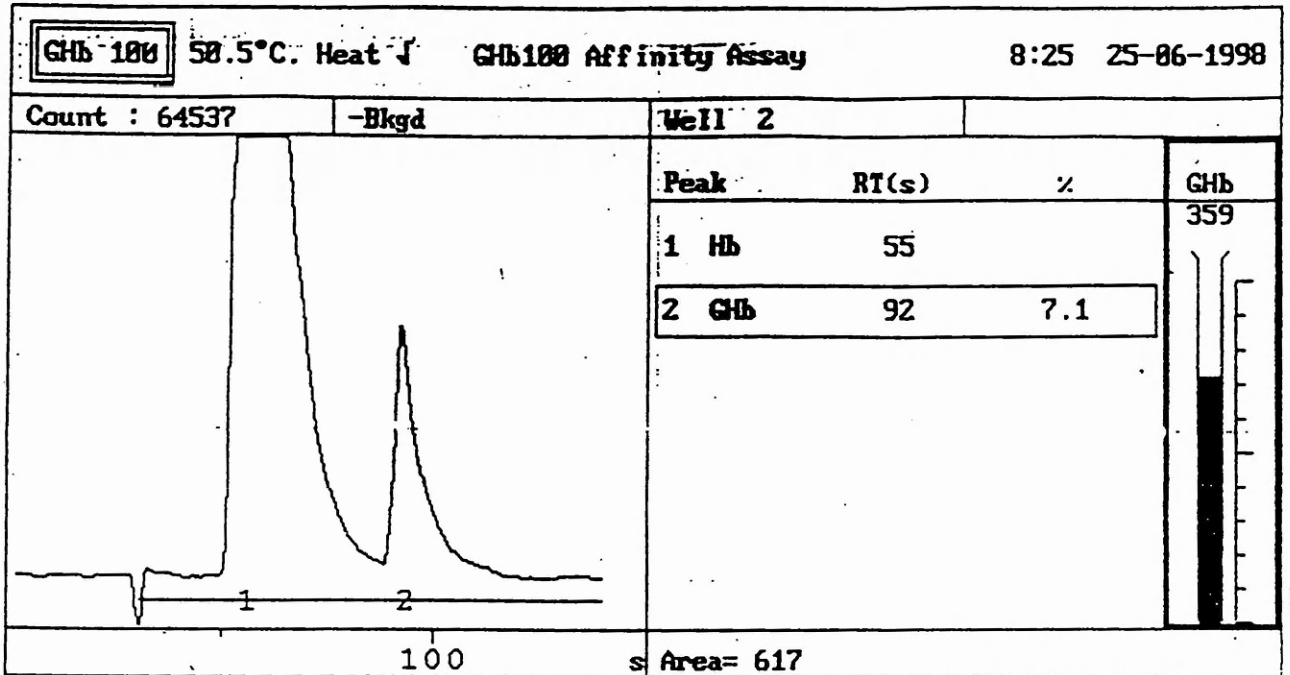
**Figure 16** A chromatogram of a high carryover with a baseline shift. The GHb result of the high carryover was 9.5%, the shifting baseline increased the GHb result to 10.3%.



| | | | |
|---|---|---|---|
| GHb 100 | 50.3°C. Heat √ | GHb100 Affinity Assay | 8:38  25-06-1998 |

| Count : 64540 | —Bkgd | Well 5 | |
|---|---|---|---|

| Peak | RT(s) | % | GHb |
|---|---|---|---|
| | | | 356 |
| 1  Hb | 55 | | |
| 2  GHb | 92 | 10.3 | |

1    2

100          s Area= 534
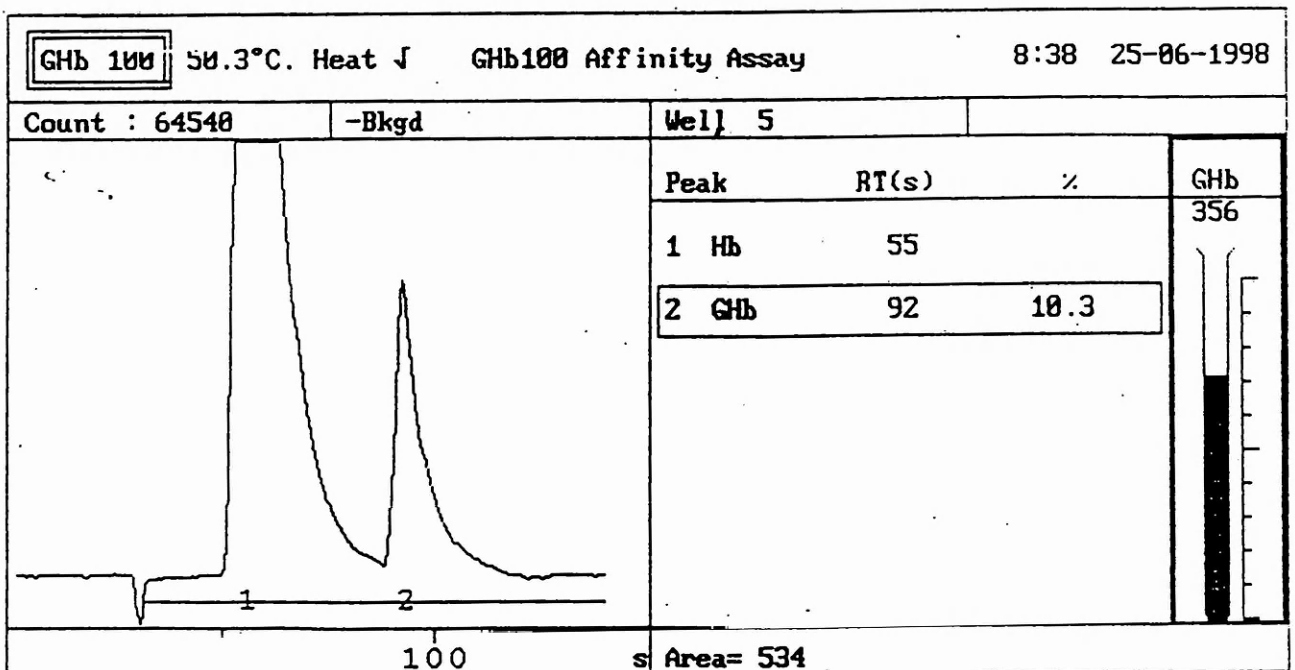
# 5.0 EVALUATION AND RESULTS OF THE RENAL PATIENTS ON CAPD TREATMENT

The continuous peritoneal absorption of glucose from glucose containing CAPD fluids in renal patients on CAPD treatment represents a unique situation that is not encountered in any other clinical situation. The reported average daily absorption of glucose in several studies varies between 100-200 g of glucose. This continuous supply of glucose may result in a variety of potentially harmful nutritional and metabolic alterations which have generated concern among nephrologists prescribing CAPD to their patients (8).

Glycation and subsequent advanced glycation end products (AGE) formation, arising from the non-enzymatic interaction of reducing sugars with amino groups of biomolecules, has been recognised as a major contributing factor to diabetic complications and the natural ageing process (74).

The use of icodextrin ( a glucose polymer) as the osmotic agent in peritoneal dialysis fluids has the potential to reduce glycation and hence AGE formation, due to the lower molar concentration of icodextrin required for successful ultrafiltration (34).

A total number of 67 renal patients on CAPD were studied in this project. The number of male CAPD patients = 42 ( 32 non-diabetic, 10 diabetic). The number of female CAPD patients = 25 (20 non-diabetic, 5 diabetic). Sixty samples from non-diabetic non-renal patients were used as controls. Number of male control patients = 40, number of female control patients = 20. Forty samples from diabetic, non-renal patients were also used as controls. Number of male control patients = 20, number of female control patients = 20.

Samples from renal patients on CAPD and controls were haemolysed; 10 microlitres of packed cells with 2 ml of deionised water, mixed thoroughly and the glycohaemoglobin concentration analysed on the Drew Scientific GHb-100 analyser using the assigned calibrant values of BR1 = 3.8% BR2 = 10.0%.

Three to five 2 - litre bags of commercially available dialysate solution were instilled

each day into the peritoneal cavity of the renal patients on CAPD by the method of Oreopolous et al. (174). The number of exchanges containing either (1.36%, 2.27%, 3.86%) glucose solutions and 7.5% w/v icodextrin was prescribed by the renal physician according to the need for fluid removal in each patient.

The results of the of the evaluation of GHb measurement on renal patients on CAPD are shown in the following tables:-

**Table 12** shows the clinical and laboratory data on the renal patients on CAPD treatment

**Table 13** shows the laboratory data on controls (non-diabetic, non-renal patients)

**Table 14** Laboratory data on controls (diabetic, non-renal patients)

**Table 15** Random plasma glucose concentrations (mmol/l) on renal patients on CAPD treatment

**Table 16** shows a summary of results (mean +/- 2SD) on renal patients on CAPD treatment and control patients

It can be seen from Table 16 that male controls (non-diabetic, non-renal patients) have a mean GHb result of 4.8% +/- 2.2 (+/- 2SD) when n = 40. Female controls have a mean GHb result of 4.5% +/- 1.6 when n = 20. As expected these results show that GHb results for non-diabetic, non-renal control patients are within the normal reference ranges given for GHb - see result and interpretation in **section 3.6.4.2**.

Male non-diabetic renal patients on glucose have a mean GHb result of 4.6% +/- 1.0 when n = 28, and a mean random plasma glucose concentration of 5.0 +/- 1.1. Female non-diabetic renal patients on glucose have a mean GHb of 4.9% +/- 1.1 when n = 15, and a mean random plasma glucose concentration of 5.8 +/- 2.1. The results of the non-diabetic renal patients on glucose are within the normal reference ranges given for the respective tests.

Male non-diabetic renal patients on icodextrin have a mean GHb result of 5.0% +/- 0.8 when n = 4, and a mean random plasma glucose concentration of 6.0 +/- 1.3. Female

92

non-diabetic renal patients on icodextrin have a mean GHb result of 5.0% +/- 0.9 when n = 5, and a mean random plasma glucose concentration of 6.0 +/- 2.8. The results of the non-diabetic renal patients on icodextrin are within the normal reference ranges given for the respective tests.

Male controls (diabetic, non-renal patients) have a mean GHb result of 8.6 +/- 4.6 when n = 20. Female controls have a mean GHb result of 8.8 +/- 4.7 when n = 20. As expected, these results show that GHb results for diabetic, non-renal control patients are above the normal reference ranges given for GHb.

Male diabetic renal patients on glucose have a mean GHb result of 8.9% +/- 7.0 when n = 8, and a mean random plasma glucose concentration of 13.2 +/- 11.1. Female diabetic renal patients on glucose have a mean GHb result of 8.7% +/- 6.7 when n = 5, and a mean random plasma glucose concentration of 11.4 +/- 4.0. As expected the results of the diabetic renal patients on glucose are above the normal reference ranges given for the respective tests. The mean GHb results for this group of patients was not 'clinically' different from results obtained from diabetic patients not being treated with CAPD.

Male diabetic renal patients on icodextrin have a mean GHb of 7.0% +/- 0 when n = 2, and a mean random plasma glucose concentration of 8.0 +/- 0.8. There were no female diabetic renal patients on icodextrin. The results show that GHb results of the two male diabetic renal patients on icodextrin are within the normal reference ranges given for GHb, and the mean random plasma glucose concentration was above the normal reference range for the test. However, the findings of this particular experiment was limited as only two male diabetic renal patients on icodextrin were studied. More male diabetic renal patients on icodextrin need to be studied in order to get a better average and female diabetic renal patients on icodextrin must also be studied.

# Table 12 Clinical and laboratory data on the renal patients on CAPD treatment.

| No. (Gp. No.) | Age (years) | Sex M/F | Diabetes type and duration (years) | CAPD type and duration (months) | Urea mmol/l (ref. range 3.3 - 10.2) | Creatinine µmol/l (ref. range 71 - 152) | GHb (%) |
|---|---|---|---|---|---|---|---|
| 1 (1) | 71 | M | ND | Glucose (26) | 18.7 | 1178 | 4.9 |
| 2 (5) | 56 | M | NIDDM (15) | Glucose (6) | 16.0 | 358 | 8.7 * |
| 3 (7) | 38 | M | IDDM (25) | Icodextrin (14) | 19.0 | 241 | 7.0 * |
| 4 (1) | 71 | M | ND | Glucose (15) | 17.4 | 652 | 4.3 |
| 5 (1) | 41 | M | ND | Glucose (19) | 23.0 | 982 | 4.4 |
| 6 (1) | 70 | M | ND | Glucose (17) | 16.3 | 1031 | 5.1 |
| 7 (2) | 66 | F | ND | Glucose (4) | 10.7 | 789 | 5.5 |
| 8 (2) | 74 | F | ND | Glucose (7) | 23.1 | 608 | 5.4 |
| 9 (2) | 49 | F | ND | Glucose (10) | 18.9 | 1012 | 4.5 |
| 10 (1) | 71 | M | ND | Glucose (4) | 12.3 | 729 | 4.4 |
| 11 (1) | 78 | M | ND | Glucose (20) | 18.0 | 809 | 5.4 |
| 12 (1) | 72 | M | ND | Glucose (17) | 22.2 | 1108 | 6.0 |
| 13 (4) | 68 | F | ND | Icodextrin (4) | 33.2 | 914 | 4.7 |
| 14 (1) | 70 | M | ND | Glucose (72) | 18.6 | 630 | 4.5 |
| 15 (1) | 56 | M | ND | Glucose (16) | 27.5 | 746 | 4.8 |
| 16 (1) | 61 | M | ND | Glucose (26) | 17.4 | 997 | 4.8 |
| 17 (2) | 76 | F | ND | Glucose (5) | 16.5 | 608 | 5.3 |
| 18 (2) | 74 | F | ND | Glucose (50) | 14.7 | 737 | 4.3 |
| 19 (5) | 37 | M | IDDM (19) | Glucose (8) | 23.1 | 622 | 16.6 * |
| 20 (5) | 71 | M | NIDDM (6) | Glucose (27) | 16.1 | 445 | 8.4 * |
| 21 (4) | 52 | F | ND | Icodextrin (103) | 9.5 | 855 | 5.4 |
| 22 (2) | 60 | F | ND | Glucose (11) | 14.7 | 981 | 4.4 |
| 23 (1) | 54 | M | ND | Glucose (11) | 19.2 | 901 | 4.1 |
| 24 (1) | 38 | M | ND | Glucose (17) | 22.9 | 1078 | 4.0 |
| 25 (2) | 79 | F | ND | Glucose (10) | 21.6 | 790 | 4.8 |
| 26 (1) | 74 | M | ND | Glucose (22) | 28.1 | 711 | 4.1 |
| 27 (2) | 57 | F | ND | Glucose (8) | 15.3 | 849 | 4.9 |
| 28 (1) | 69 | M | ND | Glucose (15) | 20.1 | 767 | 4.4 |
| 29 (2) | 65 | F | ND | Glucose (37) | 3.7 | 65 | 5.8 |
| 30 (3) | 71 | M | ND | Icodextrin (42) | 23.3 | 855 | 4.5 |
| 31 (2) | 74 | F | ND | Glucose (106) | 20.4 | 716 | 4.6 |
| 32 (2) | 59 | F | ND | Glucose (18) | 14.3 | 1039 | 5.0 |
| 33 (6) | 59 | F | IDDM (16) | Glucose (15) | 21.7 | 680 | 13.7 * |
| 34 (1) | 79 | M | ND | Glucose (11) | 23.3 | 957 | 5.0 |
| 35 (1) | 62 | M | ND | Glucose (13) | 23.0 | 868 | 4.4 |
| 36 (1) | 52 | M | ND | Glucose (39) | 19.1 | 1060 | 3.6 |
| 37 (1) | 45 | M | ND | Glucose (5) | 21.9 | 703 | 4.1 |
| 38 (2) | 66 | F | ND | Glucose (7) | 13.4 | 720 | 4.9 |
| 39 (2) | 67 | F | ND | Glucose (13) | 6.4 | 422 | 4.4 |
| 40 (4) | 72 | F | ND | Icodextrin (6) | 21.8 | 694 | 5.0 |
| 41 (1) | 55 | M | ND | Glucose (17) | 10.5 | 401 | 4.6 |
| 42 (5) | 42 | M | NIDDM (20) | Glucose (13) | 18.0 | 918 | 9.9 * |
| 43 (3) | 70 | M | ND | Icodextrin (21) | 22.6 | 1005 | 4.9 |
| 44 (1) | 80 | M | ND | Glucose (11) | 15.2 | 892 | 4.9 |
| 45 (7) | 72 | M | NIDDM (1) | Icodextrin (25) | 18.7 | 1131 | 7.0 * |
| 46 (1) | 73 | M | ND | Glucose (5) | 20.2 | 1054 | 4.5 |
| 47 (1) | 58 | M | ND | Glucose (10) | 21.9 | 823 | 4.2 |
| 48 (2) | 75 | F | ND | Glucose (15) | 11.0 | 804 | 5.5 |
| 49 (5) | 74 | M | NIDDM (1) | Glucose (35) | 15.5 | 901 | 8.0 * |
| 50 (6) | 71 | F | IDDM (15) | Glucose (4) | 8.1 | 374 | 8.1 * |
| 51 (1) | 63 | M | ND | Glucose (23) | 15.6 | 985 | 4.7 |

94

**Table 12 cont..d** Clinical and laboratory data on the renal patients on CAPD treatment.

| No. (Gp. No.) | Age (years) | Sex M/F | Diabetes type and duration (years) | CAPD type and duration (months) | Urea mmol/l (ref. range 3.3 - 10.2) | Creatinine μmol/l (ref. range 71 - 152) | GHb (%) |
|---|---|---|---|---|---|---|---|
| 52 (1) | 51 | M | ND | Glucose (23) | 22.0 | 614 | 5.2 |
| 53 (6) | 56 | F | IDDM (29) | Glucose (17) | 11.0 | 480 | 4.4 * |
| 54 (2) | 67 | F | ND | Glucose (35) | 18.3 | 603 | 4.0 |
| 55 (1) | 57 | M | ND | Glucose (16) | 11.5 | 401 | 4.4 |
| 56 (4) | 78 | F | ND | Icodextrin (9) | 12.8 | 680 | 5.4 |
| 57 (4) | 78 | F | ND | Icodextrin (31) | 19.6 | 1119 | 4.3 |
| 58 (1) | 77 | M | ND | Glucose (19) | 22.6 | 941 | 4.7 |
| 59 (5) | 49 | M | IDDM (39) | Glucose (6) | 21.6 | 788 | 8.7 * |
| 60 (6) | 46 | F | NIDDM (11) | Glucose (37) | 20.0 | 655 | 7.8 * |
| 61 (6) | 54 | F | IDDM (12) | Glucose (5) | 17.3 | 588 | 9.3 * |
| 62 (5) | 60 | M | IDDM (10) | Glucose (4) | 24.9 | 954 | 5.8 * |
| 63 (1) | 77 | M | ND | Glucose (27) | 17.9 | 982 | 3.8 |
| 64 (1) | 84 | M | ND | Glucose (16) | 11.9 | 709 | 4.9 |
| 65 (5) | 77 | M | NIDDM (1) | Glucose (47) | 15.3 | 489 | 5.2 * |
| 66 (3) | 55 | M | ND | Icodextrin (10) | 30.1 | 869 | 5.4 |
| 67 (3) | 81 | M | ND | Icodextrin (23) | 20.6 | 601 | 5.2 |

| | |
|---|---|
| ND = | non-diabetic |
| IDDM = | insulin-dependent diabetes mellitus |
| NIDDM = | non-insulin-dependent diabetes mellitus |
| * | denotes patients with diabetes mellitus |

Gp. No = Group number
Patients were put into 8 groups for statistical analysis (see Table 16):-

| | |
|---|---|
| 1 = | male non-diabetic on glucose |
| 2 = | female non-diabetic on glucose |
| 3 = | male non-diabetic on icodextrin |
| 4 = | female non-diabetic on icodextrin |
| 5 = | male diabetic on glucose |
| 6 = | female diabetic on glucose |
| 7 = | male diabetic on icodextrin |
| 8 = | female diabetic on icodextrin |

**Table 13** Laboratory data on controls (non-diabetic, non-renal patients).

| No. | Age (years) | Sex M/F | Urea mmol/l (ref. range 3.3 - 10.2) | Creatinine μmol/l (ref. range 71 -152) | GHb (%) |
|---|---|---|---|---|---|
| 1 | 63 | M | 4.2 | 103 | 5.1 |
| 2 | 69 | M | 6.0 | 108 | 5.1 |
| 3 | 80 | M | 4.5 | 63 | 4.7 |
| 4 | 59 | M | 5.7 | 85 | 3.7 |
| 5 | 80 | M | 8.9 | 88 | 4.4 |
| 6 | 89 | M | 9.5 | 124 | 6.8 |
| 7 | 82 | M | 4.6 | 70 | 5.8 |
| 8 | 81 | M | 5.4 | 79 | 4.6 |
| 9 | 66 | M | 3.7 | 84 | 6.8 |
| 10 | 83 | M | 5.9 | 83 | 4.6 |
| 11 | 86 | M | 4.8 | 79 | 4.2 |
| 12 | 64 | M | 6.4 | 95 | 5.0 |
| 13 | 66 | M | 5.1 | 102 | 4.2 |
| 14 | 84 | M | 7.9 | 78 | 4.7 |
| 15 | 67 | M | 9.4 | 89 | 4.7 |
| 16 | 78 | M | 8.2 | 116 | 6.7 |
| 17 | 84 | M | 6.1 | 113 | 4.1 |
| 18 | 63 | M | 3.7 | 122 | 4.5 |
| 19 | 81 | M | 6.3 | 70 | 7.5 |
| 20 | 41 | M | 4.0 | 71 | 3.2 |
| 21 | 25 | M | 3.0 | 89 | 3.5 |
| 22 | 62 | M | 7.1 | 83 | 3.7 |
| 23 | 73 | M | 5.3 | 99 | 5.5 |
| 24 | 70 | M | 3.7 | 74 | 5.1 |
| 25 | 36 | M | 3.7 | 86 | 3.5 |
| 26 | 51 | M | 5.0 | 96 | 3.7 |
| 27 | 66 | M | 3.8 | 77 | 6.2 |
| 28 | 81 | M | 7.0 | 120 | 4.6 |
| 29 | 59 | M | 3.9 | 80 | 6.9 |
| 30 | 94 | M | 3.5 | 71 | 4.8 |
| 31 | 81 | M | 4.8 | 105 | 6.6 |
| 32 | 65 | M | 5.0 | 100 | 4.3 |
| 33 | 79 | M | 4.7 | 93 | 4.3 |
| 34 | 65 | M | 4.0 | 89 | 4.0 |
| 35 | 67 | M | 7.3 | 108 | 4.1 |
| 36 | 56 | M | 6.2 | 117 | 3.8 |
| 37 | 84 | M | 6.1 | 97 | 3.8 |
| 38 | 41 | M | 4.8 | 108 | 4.1 |
| 39 | 83 | M | 5.1 | 86 | 4.4 |
| 40 | 84 | M | 3.4 | 86 | 4.6 |
| 41 | 93 | F | 9.7 | 115 | 6.9 |
| 42 | 88 | F | 6.0 | 99 | 4.5 |
| 43 | 85 | F | 5.5 | 73 | 5.1 |
| 44 | 88 | F | 3.7 | 57 | 3.8 |
| 45 | 95 | F | 6.1 | 94 | 5.0 |
| 46 | 73 | F | 3.0 | 70 | 4.8 |
| 47 | 32 | F | 2.5 | 70 | 4.0 |
| 48 | 88 | F | 5.8 | 80 | 5.4 |
| 49 | 43 | F | 4.0 | 74 | 3.7 |
| 50 | 73 | F | 5.5 | 76 | 4.8 |
| 51 | 90 | F | 5.3 | 90 | 3.8 |

**Table 13 cont..d** Laboratory data on controls ( non-diabetic, non-renal patients).

| No. | Age (years) | Sex M/F | Urea mmol/l (ref. range 3.3 - 10.2) | Creatinine μmol/l (ref. range 71 -152) | GHb (%) |
|-----|-------------|---------|-------------------------------------|----------------------------------------|---------|
| 52  | 83          | F       | 3.7                                 | 55                                     | 4.1     |
| 53  | 87          | F       | 8.5                                 | 57                                     | 4.2     |
| 54  | 81          | F       | 6.2                                 | 77                                     | 4.6     |
| 55  | 78          | F       | 3.8                                 | 58                                     | 4.1     |
| 56  | 80          | F       | 6.5                                 | 56                                     | 3.7     |
| 57  | 56          | F       | 4.3                                 | 51                                     | 3.8     |
| 58  | 78          | F       | 3.3                                 | 86                                     | 4.5     |
| 59  | 87          | F       | 9.1                                 | 81                                     | 5.4     |
| 60  | 97          | F       | 4.6                                 | 69                                     | 4.0     |

**Table 14** Laboratory data on controls (diabetic, non-renal patients).

| No. | Age (years) | Sex M/F | Diabetes IDDM/ NIDDM | Urea mmol/l (ref. range 3.3 - 10.2) | Creatinine µmol/l (ref. range 71 -152) | GHb (%) |
|---|---|---|---|---|---|---|
| 1 | 76 | M | NIDDM | 3.7 | 76 | 6.2 |
| 2 | 46 | M | IDDM | 4.5 | 88 | 10.8 |
| 3 | 25 | M | IDDM | 3.9 | 76 | 5.9 |
| 4 | 35 | M | NIDDM | 5.1 | 91 | 6.3 |
| 5 | 71 | M | IDDM | 8.6 | 133 | 10.6 |
| 6 | 69 | M | NIDDM | 6.2 | 114 | 10.2 |
| 7 | 63 | M | NIDDM | 6.1 | 78 | 7.9 |
| 8 | 73 | M | NIDDM | 5.6 | 94 | 7.3 |
| 9 | 61 | M | NIDDM | 5.5 | 76 | 9.2 |
| 10 | 62 | M | NIDDM | 9.9 | 117 | 8.7 |
| 11 | 71 | M | NIDDM | 4.8 | 77 | 8.2 |
| 12 | 64 | M | NIDDM | 4.7 | 116 | 6.2 |
| 13 | 46 | M | NIDDM | 5.4 | 85 | 10.9 |
| 14 | 70 | M | NIDDM | 5.3 | 117 | 8.3 |
| 15 | 59 | M | NIDDM | 5.3 | 95 | 8.5 |
| 16 | 61 | M | NIDDM | 8.9 | 134 | 5.6 |
| 17 | 68 | M | NIDDM | 4.3 | 87 | 8.5 |
| 18 | 64 | M | NIDDM | 4.0 | 93 | 7.3 |
| 19 | 66 | M | IDDM | 4.6 | 98 | 15.3 |
| 20 | 60 | M | NIDDM | 4.5 | 81 | 10.0 |
| 21 | 73 | F | NIDDM | 5.0 | 72 | 8.8 |
| 22 | 34 | F | IDDM | 4.1 | 80 | 12.1 |
| 23 | 68 | F | IDDM | 4.3 | 77 | 10.1 |
| 24 | 71 | F | NIDDM | 8.1 | 83 | 6.8 |
| 25 | 69 | F | IDDM | 4.8 | 66 | 13.9 |
| 26 | 78 | F | NIDDM | 7.6 | 67 | 11.6 |
| 27 | 86 | F | NIDDM | 5.6 | 92 | 5.3 |
| 28 | 88 | F | NIDDM | 2.8 | 65 | 9.5 |
| 29 | 53 | F | IDDM | 3.6 | 70 | 10.7 |
| 30 | 55 | F | NIDDM | 3.8 | 65 | 5.1 |
| 31 | 76 | F | IDDM | 5.8 | 58 | 7.5 |
| 32 | 37 | F | IDDM | 3.8 | 71 | 9.4 |
| 33 | 67 | F | IDDM | 6.6 | 79 | 9.8 |
| 34 | 71 | F | NIDDM | 6.3 | 76 | 10.0 |
| 35 | 52 | F | NIDDM | 6.4 | 67 | 7.9 |
| 36 | 41 | F | IDDM | 4.8 | 98 | 5.4 |
| 37 | 60 | F | NIDDM | 5.3 | 60 | 6.8 |
| 38 | 62 | F | IDDM | 3.5 | 73 | 9.0 |
| 39 | 71 | F | NIDDM | 3.3 | 68 | 9.0 |
| 40 | 88 | F | NIDDM | 5.1 | 64 | 7.2 |

IDDM =        insulin-dependent diabetes mellitus
NIDDM =       non-insulin-dependent diabetes mellitus

**Table 15** Random plasma glucose concentrations (mmol/l) on renal patients on CAPD treatment.

| No. (Gp. No.) | 1 (Most recent) | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 (Least recent) | Mean +/-2SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Monthly plasma glucose concentrations (mmol/l) ref.range 3.6 - 6.1 mmol/l | | | | | | | | |
| 1 (1) | 5.6 | 4.9 | 5.0 | 3.9 | 5.6 | 4.6 | 5.7 | 5.0 | 3.9 | 5.6 | 5.0 +/-1.36 |
| 2 (5) | 6.1 | 20.4 | 22.8 | 12.6 | 13.9 | 13.0 | 7.2 | 19.4 | 10.8 | 8.5 | 13.5 * +/-11.5 |
| 3 (7) | 4.5 | 5.4 | 11.0 | 6.6 | 6.5 | 4.0 | 7.3 | 8.7 | 11.2 | 11.9 | 7.7 * +/- 5.7 |
| 4 (1) | 5.4 | 5.7 | 6.3 | 3.3 | 4.2 | 3.9 | 4.0 | 3.6 | 4.2 | 5.1 | 4.6 +/- 2.0 |
| 5 (1) | 5.6 | 6.2 | 4.9 | 4.6 | 5.2 | 5.6 | 3.9 | 4.7 | 4.6 | 3.8 | 4.9 +/- 1.5 |
| 6 (1) | 6.1 | 4.3 | 7.4 | 4.4 | 6.2 | 3.8 | | | | | 5.4 +/- 2.8 |
| 7 (2) | 6.4 | 6.5 | 5.5 | 5.0 | 4.4 | 5.8 | 6.0 | 5.3 | 5.3 | 7.1 | 5.7 +/- 1.6 |
| 8 (2) | 5.8 | 5.4 | 5.0 | 4.8 | 3.9 | | | | | | 5.0 +/- 1.4 |
| 9 (2) | 4.6 | 5.2 | 6.7 | 4.9 | 10.4 | 4.7 | 4.4 | 4.4 | 4.6 | | 5.5 +/- 3.9 |
| 10 (1) | 5.6 | 7.3 | 6.0 | 5.4 | 6.2 | 8.5 | 4.8 | 7.6 | 5.0 | 7.1 | 6.4 +/- 2.4 |
| 11 (1) | 5.0 | 4.4 | 5.2 | 4.5 | 3.7 | 3.9 | 3.9 | | | | 4.4 +/- 1.2 |
| 12 (1) | 5.4 | 4.1 | 9.5 | 4.1 | | | | | | | 5.8 +/- 5.1 |
| 13 (4) | 4.9 | 6.2 | 6.3 | 5.7 | 5.1 | 5.4 | 5.8 | | | | 5.6 +/- 1.1 |
| 14 (1) | 5.5 | 4.5 | 5.7 | 4.9 | 4.5 | 4.8 | 6.7 | 5.0 | 4.8 | | 5.2 +/- 1.4 |
| 15 (1) | 7.3 | 4.7 | 5.9 | 4.8 | 3.7 | 5.0 | 5.0 | 4.7 | | | 5.1 +/- 2.1 |
| 16 (1) | 5.1 | 4.5 | 5.1 | 5.3 | 6.4 | 4.2 | 5.2 | 3.6 | 4.7 | 3.3 | 4.7 +/- 1.8 |
| 17 (2) | 8.0 | 8.6 | 8.8 | 9.0 | 7.3 | | | | | | 8.3 +/- 1.4 |
| 18 (2) | 4.4 | 5.5 | 6.6 | | | | | | | | 5.5 +/- 2.2 |
| 19 (5) | 28.4 | 20.2 | 39.8 | 22.1 | 17.0 | | | | | | 25.5 * +/- 18.0 |
| 20 (5) | 12.6 | 14.1 | 24.4 | 10.9 | 8.8 | 10.5 | 6.8 | 19.0 | 14.7 | 8.5 | 13.0 * +/- 10.7 |
| 21 (4) | 5.7 | 4.2 | 4.4 | | | | | | | | 4.8 +/- 1.6 |
| 22 (2) | 6.7 | 5.4 | 4.9 | 7.8 | 7.9 | | | | | | 6.5 +/- 2.7 |
| 23 (1) | 3.3 | 3.6 | 3.5 | 4.2 | 6.2 | | | | | | 4.2 +/- 2.4 |
| 24 (1) | 5.4 | 5.4 | 7.1 | 6.5 | 5.9 | 5.2 | 7.2 | 5.9 | 5.2 | 5.3 | 5.9 +/- 1.5 |
| 25 (2) | 4.9 | 6.2 | 5.3 | 4.8 | 6.2 | 5.3 | 4.1 | 5.4 | 5.2 | 4.6 | 5.2 +/- 1.3 |

**Table 15 cont..d** Random plasma glucose concentrations (mmol/l) on renal patients on CAPD treatment.

| No. (Gp. No.) | 1 (Most recent) | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 (Least recent) | 10 | Mean +/-2SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Monthly plasma glucose concentrations (mmol/l) ref.range 3.6 - 6.1 mmol/l | | | | | | | | |
| 26 (1) | 6.1 | 6.4 | 4.5 | 4.7 | 5.2 | 5.3 | 3.9 | 5.2 | 4.4 | 4.8 | 5.1 +/- 1.5 |
| 27 (2) | 5.3 | 5.9 | 4.7 | | | | | | | | 5.3 +/- 1.2 |
| 28 (1) | 6.3 | 4.8 | 4.3 | 7.0 | 5.9 | 4.9 | 4.9 | 5.1 | 4.9 | | 5.3 +/- 1.7 |
| 29 (2) | 10.0 | 5.9 | 5.9 | 5.8 | 5.7 | 6.3 | 6.2 | 6.1 | 5.4 | 6.4 | 6.4 +/- 2.6 |
| 30 (3) | 5.2 | 4.2 | 6.0 | 4.9 | 4.1 | 5.0 | 7.2 | 4.9 | 5.1 | 8.6 | 5.5 +/- 2.8 |
| 31 (2) | 5.3 | 5.8 | 5.0 | 6.2 | 5.0 | 6.4 | 6.5 | | | | 5.7 +/- 1.3 |
| 32 (2) | 4.3 | 4.6 | 4.0 | 4.3 | 5.8 | 4.4 | 3.7 | 5.1 | 5.0 | 4.7 | 4.6 +/- 1.2 |
| 33 (6) | 6.1 | 17.9 | 19.3 | 13.0 | 13.5 | 14.0 | | | | | 14.0 * +/- 9.3 |
| 34 (1) | 5.1 | 5.5 | 4.0 | 4.0 | 4.5 | 3.7 | 3.7 | 3.7 | 4.0 | 4.6 | 4.3 +/- 1.3 |
| 35 (1) | 5.4 | 8.8 | 4.1 | 5.7 | 5.1 | 6.2 | 4.3 | 6.5 | | | 5.8 +/- 3.0 |
| 36 (1) | 5.0 | 3.6 | 4.5 | 4.4 | 5.4 | 2.7 | 3.7 | 3.1 | 4.1 | 4.7 | 4.1 +/- 1.7 |
| 37 (1) | 6.3 | 7.0 | 4.3 | 4.3 | 4.1 | 4.6 | 4.8 | 6.1 | | | 5.2 +/- 2.2 |
| 38 (2) | 6.2 | 5.2 | 5.5 | 4.8 | 6.0 | 4.2 | 3.6 | 4.0 | 5.2 | 3.1 | 4.8 +/- 2.1 |
| 39 (2) | 11.0 | 7.3 | 7.6 | 9.8 | 13.1 | 5.9 | 5.7 | 7.3 | 6.0 | 5.7 | 7.9 +/- 5.1 |
| 40 (4) | 6.4 | 6.3 | 8.0 | 10.5 | 8.8 | | | | | | 8.0 +/- 3.5 |
| 41 (1) | 6.9 | 4.9 | 5.3 | 4.3 | 5.1 | 5.6 | 5.5 | 5.9 | | | 5.4 +/- 1.5 |
| 42 (5) | 9.0 | 3.6 | 9.9 | 5.5 | | | | | | | 7.0 * +/- 5.9 |
| 43 (3) | 4.6 | 5.8 | 6.3 | 6.7 | 6.3 | 5.1 | 4.7 | 4.7 | 4.5 | 4.6 | 5.3 +/- 1.7 |
| 44 (1) | 4.7 | 4.5 | 4.7 | 4.1 | 4.5 | 6.2 | 5.1 | | | | 4.8 +/- 1.4 |
| 45 (7) | 7.6 | 6.9 | 12.3 | 8.1 | 9.8 | 7.5 | 7.8 | 7.9 | 8.2 | 6.5 | 8.3 * +/- 3.3 |
| 46 (1) | 3.8 | 5.1 | 5.1 | 5.1 | 4.2 | 4.9 | | | | | 4.7 +/- 1.1 |
| 47 (1) | 3.6 | 4.5 | 4.0 | 4.3 | 4.8 | | | | | | 4.2 +/- 0.9 |
| 48 (2) | 5.1 | 4.9 | 4.5 | 7.5 | 4.9 | 6.5 | 4.9 | 4.4 | 5.1 | 5.4 | 5.3 +/- 1.9 |
| 49 (5) | 16.0 | 15.8 | 23.7 | 28.4 | 10.2 | 5.6 | 6.9 | 4.8 | 6.2 | 4.0 | 12.2 * +/- 17.1 |
| 50 (6) | 9.5 | 6.8 | 5.4 | 7.7 | 11.3 | 10.7 | 10.8 | 24.9 | 5.1 | 6.4 | 9.9 * +/- 11.5 |

**Table 15 cont..d** Random plasma glucose concentrations (mmol/l) on renal patients on CAPD treatment.

| No. (Gp. No.) | 1 (Most recent) | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 (Least recent) | 10 | Mean +/-2SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Monthly plasma glucose concentrations (mmol/l) ref.range 3.6 - 6.1 mmol/l | | | | | | | | |
| 51 (1) | 4.8 | 4.7 | 4.8 | 4.2 | 4.4 | 4.0 | 4.3 | 5.4 | 7.5 | 3.8 | 4.7 +/- 2.1 |
| 52 (1) | 4.0 | 5.4 | 6.2 | 4.5 | 3.6 | 5.5 | 4.5 | 4.4 | 4.2 | 5.1 | 4.7 +/- 1.6 |
| 53 (6) | 4.2 | 4.5 | 18.3 | 4.1 | 7.6 | 24.3 | 5.8 | 5.2 | 9.8 | 7.3 | 9.1 * +/- 13.6 |
| 54 (2) | 5.9 | 5.6 | 4.6 | 5.1 | 5.9 | 5.2 | 4.8 | 5.0 | 5.0 | 4.6 | 5.2 +/- 1.0 |
| 55 (1) | 5.7 | 5.6 | 6.2 | 6.5 | 5.1 | 4.3 | 6.1 | 4.8 | | | 5.5 +/- 1.5 |
| 56 (4) | 6.0 | 5.3 | 6.4 | 5.7 | 5.1 | 7.7 | 11.1 | | | | 6.8 +/- 4.2 |
| 57 (4) | 5.0 | 5.5 | 4.8 | 5.1 | 6.3 | 4.3 | 4.7 | 4.1 | 3.6 | 4.4 | 4.8 +/- 1.5 |
| 58 (1) | 4.9 | 4.9 | 4.0 | 5.4 | 4.2 | 5.5 | | | | | 4.8 +/- 1.2 |
| 59 (5) | 5.9 | 11.3 | 5.1 | 3.6 | 23.5 | 15.3 | 34.0 | 14.7 | 6.3 | 18.3 | 13.8 * +/- 19.2 |
| 60 (6) | 12.4 | 13.3 | 7.8 | 15.3 | 9.2 | 8.3 | 11.3 | 14.3 | 15.1 | 6.3 | 11.3 * +/- 6.5 |
| 61 (6) | 8.4 | 15.7 | 14.0 | | | | | | | | 12.7 * +/- 7.6 |
| 62 (5) | 8.4 | 8.0 | 10.9 | 10.4 | 5.0 | 6.0 | | | | | 8.1 * +/- 4.7 |
| 63 (1) | 5.9 | 4.7 | 4.7 | 5.0 | 6.0 | 5.3 | 5.6 | 7.0 | 6.4 | 4.5 | 5.5 +/- 1.6 |
| 64 (1) | 8.6 | 6.8 | 5.1 | 4.5 | 4.8 | 5.0 | 4.6 | 5.3 | 4.6 | 5.3 | 5.5 +/- 2.6 |
| 65 (5) | 11.1 | 14.3 | 14.0 | 12.0 | 14.8 | 17.5 | 9.4 | 15.2 | 9.8 | 9.6 | 12.8 * +/- 5.6 |
| 66 (3) | 7.7 | 5.3 | 6.9 | 10.7 | 6.0 | 6.9 | 5.6 | 6.1 | 4.4 | 5.9 | 6.6 +/- 3.5 |
| 67 (3) | 8.1 | 5.8 | 6.1 | 8.9 | 6.6 | 5.5 | 4.3 | 5.4 | 6.5 | 6.5 | 6.4 +/- 2.7 |

*       denotes patients with diabetes mellitus

**Gp. No =**       Group number
Patients were put into 8 groups for statistical analysis (see **Table 16**):-

| | |
|---|---|
| 1 = | male non-diabetic on glucose |
| 2 = | female non-diabetic on glucose |
| 3 = | male non-diabetic on icodextrin |
| 4 = | female non-diabetic on icodextrin |
| 5 = | male diabetic on glucose |
| 6 = | female diabetic on glucose |
| 7 = | male diabetic on icodextrin |
| 8 = | female diabetic on icodextrin |

**Table 16** Summary of results (mean +/- 2SD) on renal patients on CAPD treatment and control patients.

| (Gp. No.) Sex (M/F) (D/ND) Osmotic agent | No. | Age (years) | CAPD duration (months) | Urea mmol/l (ref range 3.3 - 10.2) | Creatinine mmol/l (ref range 71 - 152) | GHb (%) | Random monthly plasma glucose concentration (ref range 3.6 - 6.1 mmol/l) |
|---|---|---|---|---|---|---|---|
| (1) M/ND glucose | 28 | 65 +/- 26 | 20 +/- 26 | 19.1 +/- 8.9 | 852 +/- 397 | 4.6 +/- 1.0 | 5.0 +/- 1.1 |
| (2) F/ND glucose | 15 | 66 +/- 19 | 23 +/- 52 | 15.2 +/- 10.7 | 712 +/- 476 | 4.9 +/- 1.1 | 5.8 +/- 2.1 |
| (3) M/ND icodextrin | 4 | 69 +/- 21 | 24 +/- 27 | 24.2 +/- 8.3 | 833 +/- 337 | 5.0 +/- 0.8 | 6.0 +/- 1.3 |
| (4) F/ND icodextrin | 5 | 70 +/- 21 | 31 +/- 84 | 19.4 +/- 18.4 | 852 +/- 360 | 5.0 +/- 0.9 | 6.0 +/- 2.8 |
| (5) M/D glucose | 8 | 56 +/- 29 | 16 +/- 32 | 19.3 +/- 7.7 | 653 +/- 474 | 8.9 +/- 7.0 | 13.2 +/- 11.1 |
| (6) F/D glucose | 5 | 60 +/- 15 | 10 +/- 13 | 14.5 +/- 12.3 | 531 +/- 265 | 8.7 +/- 6.7 | 11.4 +/- 4.0 |
| (7) M/D icodextrin | 2 | 55 +/- 48 | 20 +/- 16 | 18.9 +/- 0.4 | 686 +/- 1258 | 7.0 +/- 0 | 8.0 +/- 0.8 |
| (8) F/D icodextrin | 0 | ---- | ---- | ---- | ---- | ---- | ---- |
| M/ND control N/A | 40 | 70 +/- 31 | N/A | 5.4 +/- 3.4 | 92 +/- 32 | 4.8 +/- 2.2 | |
| F/ND control N/A | 20 | 79 +/- 34 | N/A | 5.4 +/- 4.0 | 74 +/- 33 | 4.5 +/- 1.6 | |
| M/D control N/A | 20 | 61 +/- 26 | N/A | 5.5 +/- 3.4 | 96 +/- 40 | 8.6 +/- 4.6 | |
| F/D control N/A | 20 | 65 +/- 32 | N/A | 5.0 +/- 2.9 | 73 +/- 20 | 8.8 +/- 4.7 | |

M = male    ND = non-diabetic    N/A = not applicable
F = female    D = diabetic

# 6.0 DISCUSSION

## 6.1 Effect of Haemoglobin Concentration on GHb Results

The measurement of GHb by the GHb-100 analyser from Drew Scientific appears to be relatively independent of the haemoglobin concentration. The GHb-100 analyser measures total glycohaemoglobin by boronate affinity chromatography and the results are expressed as a ratio and not in absolute terms. It can be seen from **Table 7** that the PCV of 30% to 70% shows a very small increasing trend in GHb results (0.3% GHb). A PCV of 30% gives a GHb of 8.7%, while a PCV of 70% gives a GHb of 9.0%.

## 6.2 Effect of Using 1% Triton X-100 as a Haemolysing Reagent

The results obtained for the study of the effect of using 1% Triton X-100 as a haemolysing reagent is shown in **Figure 4**. It can be seen from Figure 4 that Triton X-100 should not be used as a haemolysing reagent for the GHb-100 analyser as it causes irreversible damage to the column, thus preventing the effective separation and measurement of GHb. The use of 1% Triton as a haemolysing reagent was discontinued after the results of its affect were known. Drew Scientific were informed of our results of the effects of using Triton X-100 as a haemolysing reagent. They repeated our experiment and confirmed our results.

Deionised water (**Figure 3**) does not interfere with or adversely affect the GHb separation on the GHb-100 analyser. We agree with Drew Scientifics recommendation of using deionised water as the haemolysing reagent for the GHb-100 analyser.

## 6.3 Packed Cells versus Whole Blood

The results of the study of packed cells versus whole blood is shown in **Figure 5**. It can be seen from Figure 5 that there is a very good correlation between the GHb results from packed cells and those from whole blood. n = 40, y = 0.9572x - 0.0987 where y = whole blood GHb (%) x = packed cells GHb (%), r = 0.9904.

103

Drew Scientific recommend 10 microlitres of whole blood to be haemolysed with 1 ml of deionised water. This study showed that 10 microlitres of packed cells can be haemolysed with 2 ml of deionised water to obtain similar readings. Our laboratory now uses packed cells for the preparation of haemolysates for the measurement of GHb on the GHb-100 analyser, as we find the use of packed cells easier and more convenient than whole blood.

## 6.4   Precision-run Study

The results of the precision-run are shown in **Tables 8** and **9.** It can be seen from Tables 8 and 9 that the GHb-100 analyser is a very precise instrument for the measurement of GHb. The calculated % CV for the low GHb precision-run (mean = 5.7125) was 1.74% when n = 40. The calculated % CV for the high GHb precision-run (mean = 10.885) was 1.76% when n = 53.

The Pierce method of measuring GHb is reported to have a coefficient of variation of about 2% for replicate estimations done on normal and diabetic patients (166). Our results show that the % CV obtained for the precision-run study on the GHb-100 analyser are within the recommendations of the National Institute of Health Diabetes Group Expert Committee on Glycosylated Haemoglobin which suggest an intra assay CV of 5% (171), and are an improvement to the Pierce method.

## 6.5   Between-batch Precision Study

The results obtained for the between-batch precision study is shown in **Table 10**. It can be seen from Table 10 that the between-batch precision for the GHb-100 analyser is very good.

The results of the in-house low and high controls were as follows:-

In-house low control  n = 89  mean = 6.3697  % CV = 4.69

In-house high control  n = 89  mean = 9.6831  % CV = 3.39

The Pierce method of measuring GHb is reported to have a between-batch coefficient of variation of about 5-6% (167). Our results show that the % CV obtained for the between-batch precision study are within the recommendations of The National Institute of Health Diabetes Group Expert Committee on Glycosylated Haemoglobin which suggest an inter assay CV of 5% (171) and are an improvement to the Pierce method.

By comparison with published precision studies on other methods for determining GHb (**Table 6**) the Drew GHb-100 method is in the most precise group, comparing favourably with other fully automated chromatography systems.

## 6.6    Correlation Study

The best correlation of GHb results between the Pierce and Drew method was obtained for the correlation study of assigned calibrant values of BR1 = 3.8% BR2 = 10.0%. The results are shown in **Figures 8** and **9**. It can be seen from these figures that the correlation of Pierce and Drew GHb results was very good. $n = 50$, $y = 1.018x - 0.1034$ where $y$ = Drew GHb (%)  $x$ = Pierce GHb (%), $r = 0.9806$, $p = <0.0001$. The percentage difference of GHb results between the Pierce and Drew methods showed no overall positive or negative bias.

The mean difference of GHb results (%) between the methods (Drew-Pierce) = 0.0440.

The mean percentage difference of GHb results (%) between the methods (Drew-Pierce/ Pierce x 100) = 0.432.

For a brief period our laboratory calibrated the GHb-100 analyser using the assigned calibrant values of BR1 = 3.8%   BR2 = 10.0%. The advantage of using this calibrant value was that it allowed us to use the reference range given for the Pierce method, therefore causing no disruption to our existing system of reporting GHb results - hence making the change in analytical method invisible to the requesting clinician.

The GHb-100 analyser was calibrated using the assigned calibration value BR1 = 3.8%, BR2 = 10.0%. Primus 1 and 2 were then analysed as samples four times each. The mean GHb result for Primus 1 = 5.2%, the mean GHb result for Primus 2 = 14.8%.

DCCT values for glycohaemoglobin are derived from assay by ion-exchange chromatography and consequently give different results from affinity methods. **Figures 10** and **11** show the difference. The correlation of GHb results obtained for the calibrant values BR1 = 3.8%, BR2 = 10.0% versus Primus 1 = 5.8%, Primus 2 = 13.3% (DCCT values) was n = 139, y = 0.7585x + 2.0154 where y = Primus GHb (%)  x = Biorad GHb (%), r = 0.9981, p = <0.0001. An intercept and slope change caused the percentage difference of GHb results between Biorad and Primus calibrants to show a positive bias for low GHb values and a negative bias for high GHb values relative to the affinity method. The DCCT $HbA_{1c}$ values were established on a first generation ion-exchange analyser (Biorad, Diamat), and it is now known that carbamylated and acetylated Hb co-eluted with the $HbA_{1c}$ peak. This probably accounts for the intercept shown on **Figure 10**.

The diabetes control and complications trial (DCCT) (173), demonstrated that development and progression of the chronic complications of diabetes are related to the degree of altered glycaemia as determined by determinations of glycohaemoglobin. Thus, the use of glycohaemoglobin testing for routine diabetes care provides an objective measure of a patients risk for developing diabetic complications. Results of this test alert patients and health providers to the need for change in the treatment plan. Optimal use of glycohaemoglobin testing for diabetes care would benefit from universal standardisation of the test results.

Standardisation of test results to DCCT numbers would be particularly attractive given that the DCCT GHb values predict the risk of diabetic complications. In addition, data from the DCCT were used to establish a relationship between GHb value and the mean

blood glucose concentration, a task that would be extremely difficult for most laboratories to do independently for each method.

There is now a greatly increased need for accurate and precise assays that reflect blood glucose concentrations integrated over time. Although glycohaemoglobin is an excellent measure of average glycaemia, as already mentioned, clinical laboratories employ many diverse methods to quantify it. Some methods are labour-intensive, slow, relatively expensive, and imprecise. Furthermore, most assays are not well standardised (177-179), such that results from one laboratory are not easily interchangeable with those from another. Therapeutic alterations based on such technical discrepancies may place patients in intensive treatment regimes at significant risk of life-threatening hypoglycaemia.

Recent research clearly shows that calibration and standardisation results in better long-term precision, decreases lot-to-lot variability, and improves interassay precision - as well as allowing direct comparison of data produced by the various methods (177-179)

All the major manufacturers of GHb and $HbA_{1c}$ analysers have recently recommended calibrating their instruments using DCCT referenced standards. Many U.K. hospital laboratories have now adopted this approach.

Our method of calibration, referenced to the Pierce method, gives us intra-laboratory consistency, but the trend is continuing towards DCCT calibrants. We have now also decided to change in this direction. This has necessitated changes to our existing reference ranges - see **Table 11**, but in the long-run patient management will benefit from better inter-laboratory agreement.

## 6.7    Column Life of the GHb-100 Analyser

The results of the study of extending the column life of the GHb-100 analyser showed that a maximum of 720 samples can be processed with one column. However, we found that the practice of exceeding Drew Scientifics recommendation of 500 samples

should not be encouraged as the precision and separation of GHb deteriorates with the over use of the column.

Some competing ion-exchange chromatography systems have a maximum column life of 150 tests. The 500 tests per column offered by the GHb-100 analyser is an important advantage and should result in lower running costs.

## 6.8 Problems Encountered with the GHb-100 Analyser when in Routine Use

When the GHb-100 analyser was used routinely in our laboratory, after several months use, a shifting baseline was found to give falsely elevated GHb results. **Figures 15** and **16** show the shifting baseline of the low and high carryover samples respectively. A low carryover of 6.5% was found to give a GHb result of 7.1%, and a high GHb carryover of 9.5% was found to give a GHb result of 10.3%.

Drew Scientific were informed about our findings and they have now changed their software program to prevent the baseline from shifting.

## 6.9 Improvements to the GHb-100 Analyser - Barcode Reading and Download Facilities

The GHb-100 analyser has a facility for reading bar coded patient samples using a light pen attached to a special keyboard. The data is read directly into the sample identification field and printed on the results sheet and summary sheets. The GHb-100 analyser also has a facility to send a summary of the results to another computer, for example, a central laboratory computer.

The GHb-100 analyser accumulates a summary of the results for each sample in a batch. It can output this summary list via the RS232 serial port on the rear of the instrument. Checksum characters are added to the results so that the laboratory can check there have been no errors in transmission. This facility is included as standard, though it may

have to be tailored to suit the requirements of our laboratory. The system hardware is fitted as standard (169).

The use of the barcode reading and downloading facilities will ease and quicken reporting of our GHb results. In addition it would reduce sample identification and transcription errors, therefore greatly improving the standard and service of our GHb measurements.

## 6.10  Renal Patients on CAPD Treatment

Glucose intolerance is common in uraemic patients and is mainly due to an impaired peripheral sensitivity to the action of insulin. One potential hazard with CAPD is that the peritoneal glucose load could lead to a depletion of the pancreatic beta-cells. This could create a diabetic state, which would add to the already impaired glucose tolerance in uraemia (180).

Lameire et al. (1) studied the long-term effects of CAPD treatment on carbohydrate and lipid metabolism. They concluded that the long-term follow-up of CAPD patients indicated that these patients were at relatively low risk of developing *de novo* diabetes mellitus. Transperitoneal glucose absorption and the blood glucose and serum insulin response to the glucose load remained relatively unchanged with time.

Smith et al. (181) determined glycohaemoglobin and carbamylated haemoglobin in patients with uraemia and/or diabetes mellitus. Glycohaemoglobin measured by ion-exchange chromatography ($HbA_1$, $HbA_{1c}$, $HbA_{1\ a+b}$) was elevated in non-diabetic patients, while colorimetrically determined glycohaemoglobin was similar to controls. Patients with diabetes mellitus and normal renal function had similar glycohaemoglobin concentrations to those with renal failure, in patients with diabetes mellitus. Both methods showed an excellent correlation, independent of renal function, in patients with diabetes mellitus. The $HbA_{1c}$ component was more influenced by diabetes and the $HbA_{1\ a+b}$

component was relatively more dependent on renal function. Carbamylated haemoglobin was detected in all subjects, but was grossly elevated in uraemia. Carbamylated haemoglobin significantly correlated with renal function and chromatographically determined glycohaemoglobin. Data from this study strongly suggested that the apparent elevation of chromatographically determined glycohaemoglobin in uraemia was due to the increased formation of carbamylated haemoglobin. However, in patients with diabetes mellitus, independent of renal function, both the chromatographic and colorimetric methods of determining glycohaemoglobin were equally valuable and reliable.

Tzamaloukas et al. (182) measured glycohaemoglobin concentrations by affinity chromatography in the blood of 43 diabetic and non-diabetic patients on long-term dialysis therapy (CAPD and haemodialysis) to determine the usefulness of this method of estimating glycaemic control in diabetic persons on dialysis therapy. In non-diabetic patients, glycohaemoglobin concentrations were within their normal reference range (4.0%-6.8%) for both continuous ambulatory peritoneal dialysis and haemodialysis.

Morgan et al. (183) measured glycohaemoglobin by affinity chromatography in 91 non-diabetic subjects with chronic renal failure (25 on CAPD, 22 on haemodialysis, and 22 on renal transplantation). Results were compared with those in a control group of 43 non-diabetic subjects with normal renal function. Mean glycohaemoglobin measured by affinity chromatography was not significantly different from controls in any of the groups with chronic renal failure. They concluded that glycohaemoglobin measured by affinity chromatography appears to be unaffected by chronic renal failure.

The summary of my results of CAPD treatment on GHb concentration are shown in **Table 16**. Male non-diabetic, non-renal control patients had a mean GHb result of 4.8% +/- 2.2 (+/- 2SD) when n = 40. Female non-diabetic, non-renal control patients had a mean GHb result of 4.5% +/- 1.6 when n = 20. As expected the mean GHb results of non-diabetic non-renal control patients were within the normal reference.

110

Male non-diabetic renal patients on glucose containing CAPD fluid had a mean GHb result of 4.6% +/- 1.0 (n = 28). Female non-diabetic renal patients on glucose containing CAPD fluid had a mean GHb result of 4.9% +/- 1.1 (n = 15). These results were within the normal reference range.

Lindholm et al. (12) did not indicate any change in the beta-cell response after 4 months on CAPD. Their results were in accordance with those of Baeyer et al. (14), but in another study by Armstrong et al. (184) a further deterioration in glucose tolerance as well as a decreased rate of insulin secretion was observed.

There are few reports concerning the effects of dialysate glucose load on plasma glucose and insulin levels in CAPD patients. Armstrong et al. (185) have studied the effects of an exchange with 1.5% and 4.25% dialysate, respectively, and they found that plasma glucose and insulin levels increased above basal values only in the case of the 4.25% solutions. The authors concluded that if the use of hypertonic solutions is restricted there will be no continual stimulations of the pancreatic beta-cells due to the dialysate glucose alone during CAPD. De Santo et al. (17) and Splendiani et al. (16) found similar results, and also that glucagon levels decrease during a dwell with the 4.25% solution.

Short-term treatment with CAPD does not seem to lead to any further disturbances of the already abnormal glucose metabolism, but the long-term effects are yet unknown. The absorption of large quantities of glucose from glucose containing peritoneal fluids may contribute to hyperlipidaemia and obesity. Hypercholesterolaemia is a risk factor that may lead to the development of atherosclerosis.

Male non-diabetic renal patients on icodextrin had a mean GHb result of 5.0% +/- 0.8 (n = 4). Female non-diabetic renal patients on icodextrin had a mean GHb result of 5.0% +/- 0.9 (n = 5). These results were within the normal reference range.

Male diabetic non-renal control patients had a mean GHb result of 8.6% +/- 4.6 (n = 20). Female diabetic non-renal control patients had a mean GHb result of 8.8% +/- 4.7. As

expected these results were above the normal reference range.

Male diabetic renal patients on glucose containing CAPD fluid had a mean GHb result of 8.9% +/- 7.0 (n = 8). Female diabetic renal patients on glucose containing CAPD fluid had a mean GHb result of 8.7% +/- 6.7 (n = 5). As expected these results were above the normal reference range. It is not surprising that the diabetic renal patients with hyperglycaemia and poor glycaemic control have a higher than normal GHb result. The authors of the MIDAS study (34) suggested the use of icodextrin for CAPD treatment on diabetic renal patients as it leads to less carbohydrate absorption, and the small group of diabetic renal patients in the MIDAS trial did perform well.

Table 12 shows the clinical and laboratory data on the renal patients on CAPD treatment. Patient no. 19, a male diabetic renal patient on glucose containing CAPD fluid, had a GHb result of 16.6%, and patient no. 33, a female diabetic renal patient on glucose containing CAPD fluid, had a GHb result of 13.7%. Table 15 shows the individual and mean (+/- 2SD) results of the random plasma glucose concentrations. These two patients who have very high GHb concentrations and extremely poor diabetic control have now been put on icodextrin by the physician of the renal ward to help improve their glycaemic control and normalise their GHb result.

Male diabetic renal patients on icodextrin had a mean GHb result of 7.0% +/- 0 (n = 2). Unfortunately there were no female diabetic renal patients on icodextrin in the renal ward at the time of the study to evaluate for this project. The results of the male diabetic renal patients on icodextrin were within the normal reference range. The results of this particular experimentation were therefore limited, as only two male diabetic renal patients on icodextrin were studied. More diabetic renal patients on icodextrin must be studied and female diabetic renal patients on icodextrin must be studied in order to get a better average GHb result to determine the effect of icodextrin in these patients.

# 7.0 CONCLUSION

The new Drew Scientific GHb-100 analyser has proved to be a rapid and precise instrument with a within-batch and between-batch precision of less than 5% CV. Whole blood or packed cells may be analysed, and small sample volumes are required (10 microlitres). Each sample takes approximately 4 minutes to complete with a maximum of 99 samples per batch and 1 space for an urgent sample. The GHb-100 analyser uses the boronate affinity low pressure liquid chromatography technique for the measurement of glycohaemoglobin. The boronate affinity method for measuring total glycohaemoglobin is not affected by the labile Schiff base, or abnormal haemoglobins. The methodology is not significantly affected by the haemoglobin concentration, and the temperature is controlled by the GHb-100 analysers internal computer at $50^{o}C$, so rigorous external temperature control by the laboratory worker is not required. During the trial period the use of the GHb-100 analyser has provided Brighton Health Care NHS Trust with a good quality and timely service for the measurement of GHb.

GHb is now a well recognised tool in helping to monitor the long-term treatment of diabetic patients. Consistently raised concentrations of GHb are associated with vascular damage attributed to formation of advanced glycation end products. Because hyperglycaemia has been reported in CAPD patients, I thought it would be interesting to study GHb in this group of patients.

The 67 patients studied were not selected in any conscious way and represented a typical cross-section of CAPD patients. There was no significant 'clinical' difference between the non-diabetic CAPD group and the control group. This could mean that the non diabetic patients on CAPD are not at risk from repeated exposure to high peritoneal cavity glucose concentrations or that GHb is not, in this case, an appropriate indicator of risk.

# 8.0 REFERENCES

1. Lameire N, Matthys D, Matthys E, Beheydt R. Effects of long-term CAPD on carbohydrate and lipid metabolism. *Clin. Nephrol.* 1988: **30**: 53-58

2. Kumar P, Clarke M. Clinical Medicine. 3rd rev. ed. London Bailliere Tindall, 1994

3. Bloom A, Ireland JA. A Colour Atlas of Diabetes. 2nd ed. London Wolfe publishing LTD, 1992

4. De Fronzo RA, Andres R, Edgar P, Walter WG. Carbohydrate metabolism in uraemia; a review. *Medicine* 1973; **52**: 469-81

5. Emmanuel DS, Lindheimer MD, Katz AI. Endocrine abnormalities in chronic renal failure; pathogenic principles and clinical implications. *Seminars in Nephrology* 1981; **1**: 151-75

6. De Fronzo RA, Alvestrand A, Smith D, Hendler R, Hendler E, Wahren J. Insulin resistance in uraemia. *J Clin Invest* 1981; **67**: 563-8

7. Schmitz O. Glucose metabolism in non diabetic and insulin dependent diabetic subjects with end stage renal failure. *Danish Medical Bulletin.* 1991; **38:1** 36-52

8. Grodsteine GP, Blumenkrantz MJ, Kopple JD, Moran JK, Coburn JW. Glucose absorption during continuous ambulatory peritoneal dialysis. *Kidney Int.* 1981; **19**: 567-7

9. Spitz I, Rubensteine AH, Bersohn I, Lawrence AM, Kirsteins L. The effect of dialysis on the carbohydrate intolerance of chronic renal failure. *Horm Metab. Res.* 1970; **2**: 86-93

10. Brighton MW, Furth AJ. Protein glycation after return to normoglycaemia; implications for mild, repetitive hyperglycaemia. *Diabetologia* 1989; **32**: 700-1

11. Pyle WK, Popovich RP, Moncrief JW. Mass transfer evaluation in peritoneal dialysis. In Moncrief JW, Popovich RP eds. CAPD update. Masson NY 1981 p.35

12. Lindholm B, Karlander SG, Norbeck HE, Bergstrom J. Hormonal and metabolic adaption to the glucose load of CAPD in non diabetic patients. In Keen H, Legrain M (eds) Prevention and treatment of diabetic nephropathy. MPT Press, Boston, p 353 1983

13. Grodstein GP, Blumenkrantz MJ, Kopple JD, Morran JK, Coburn JW. Glucose absorption during continuous ambulatory peritoneal dialysis. *Kidney Int.* 1981; **19**: 564

14. Baeyer V. Gahl H, Reidinger H, Borowzak R, Rudiger A, Schurig R, Kessel M. adaption of CAPD patients to the continuous peritoneal uptake. *Kidney Int.* 1983; **23**: 29

15. Keusch G, Bammatter F, Mordasini R, Bingswanger U. Serum lipoprotein concentrations during continuous ambulatory peritoneal dialysis (CAPD). In Gahl GM, Kessel M, Nolph KD (eds) Advances in Peritoneal Dialysis. Amsterdam, Oxford and Princeton. Excerpta Medica 1981; 421-423

16. Splendiani G, Acitelli S, Albano V, Gianforte A, Tancredi M. Metabolic aspects of CAPD. In Gahl GM, Kessel M, Nolph KD (eds) Advances in Peritoneal Dialysis. Amsterdam, Oxford and Princeton. Excerpta Medica 1981; 449-51

17. De Santo NG, Capodicasa G, Senatore R, Cicchetti T, Cirillo D, Damiano M, Torella R, Gingliano D, Importa L, Giordano C. Glucose utilization from dialysate in patients on continuous ambulatory peritoneal dialysis (CAPD). *Int. J. Artif. Organs* 1979; **2**: 119

18. Hain H, Kessel M. Aspect of new solutions for peritoneal dialysis. *Nephrol. Dial. Transplant* 1987; **2**: 67

19. Breckenridge WC, Roncari DAK, Khanna R, Oreopolous PG. The influence of continuous ambulatory peritoneal dialysis on plasma lipoproteins. *Atherosclerosis* 1982; **45**: 249

20. Chan MK, Raftery MJ, Baillod RA, Sweny P, Varghese Z, Moorehead JF. Three years experience of continuous ambulatory peritoneal dialysis. *Lancet* 1981; **ii**; 1409

21. Khanna R, Breckenridge C, Roncari D, Digenis G, Oreopolous PG. Lipid abnormalities in patients undergoing continuous ambulatory peritoneal dialysis. *Perit. Dial Bull.* 1983 **3**: 13

22. Turgan C, Feehally J, Bennet S, Davies TJ, Walls J. Accelerated hypertriglyceridaemia in patients on continuous ambulatory peritoneal dialysis - a preventable abnormality. *Int. J. Artif. Organs* 1981; **4**: 158

23. Ota K, Mineshima M, Watanabe N, Naganuma S. Functional deterioration of the peritoneum; does it occur in the absence of peritonitis? *Nephrol. Dial. Transplant* 1987; **2**: 30

24. Raja RM, Kramer MS, Manchandra R, Lazaro N, Rosenbaum JL. Peritoneal dialysis with fructose dialysate - prevention of hyperglycaemia and hyperosmolality. *Ann. Intern. Med.* 1973; **79**: 511

25. Bazzato G, Coli U, Landinis S, Fracasso A, Morachiello P, Righetto F, Scanferla F. Xylitol and low dosage of insulin; new perspectives for diabetic uraemic patients on CAPD. *Perit. Dial. Bull.* 1982; **2**: 161

26. Raja RM, Moros JG, Kramer MS, Rosenbaum JL. Hyperosmotic coma complicating peritoneal dialysis with sorbitol dialysate. *Ann. Intern. Med.* 1970; **73**: 993

27. Heaton A, Ward KD, Johnston DG, Alberti KGM, Kerr DNS. Evaluation of glycerol as an osmotic agent for continuous ambulatory peritoneal dialysis in end stage renal failure. *Clin. Science* 1986; **70**: 23

28. Matthys E, Dolkart R, Lameire N. Potential hazards with the use of glycerol dialysate in diabetic CAPD patients. *Perit. Dial. Bull.* 1987; **7**: 16

29. Matthys E, Dolkart R, Lameire N. Extended use of a glycerol containing dialysate in the treatment of diabetic CAPD patients. *Perit. Dial. Bull* 1987; **7**: 10

30. Oreopolous DG, Crassweller P, Kartirtzoglou A, Orgilvie R, Zellerman G, Rodella H, Vas ST. Amino acids as an osmotic agent in continuous peritoneal dialysis. In Legrain M. Eds. Continuous ambulatory peritoneal dialysis. Excerpta Medica Amsterdam 1979; p 335

31. William PF, Marliss EB, Anderson GH, Oren A, Stein AN, Khanna R, Pettit J, Brandes L, Rodella H, Mupas L, Dombros N, Oreopolous DG. Amino acid absorption following intraperitoneal administration in CAPD patients. *Perit. Dial. Bull.* 1982; **2**: 124

32. Gjessing J. Use of dextran as a dialysing fluid in peritoneal dialysis. *Acta. Med. Scand.* 1969; **185**: 237

33. Twardoswski ZJ, Moore H, McGary T, Poskuta M, Hirszel P, Stathakis C. Polymer as osmotic agents in peritoneal dialysis. *Perit. Dial. Bull* 1984; **4**: 125

34. Mistry CD, Gokal R, Peers E, and the MIDAS Study Group. A randomised multicentre clinical trial comparing isosmolar icodextrin with hyperosmolar glucose solutions in CAPD. *Kidney International* 1994; **46**: 496-503

35. Cameron S, Davidson MA, Grunfeld JP, Kerr D, Ritz E. Oxford textbook of clinical nephrology. Oxford medical publications 1992; **2**: 1481

36. Baxter Health Care LTD. Patient leaflet on Dianeal PD4 (glucose 1.36, 2.27, 3.86% p/v. 13.6, 22.7, 38.6 mg/ml.). Caxton Way, Thetford, Norfolk, UK. IP24 3SE. 1997

37. Baxter Health Care LTD. Patient leaflet on Extraneal (icodextrin 7.5%). Caxton Way, Thetford, Norfolk, UK. IP24 3SE. 1997

38. ADA Position Statement. Urine glucose and ketone determinations. *Diabetes Care* 1996; **19 (suppl 1.)**: 35

39. Ohlsen P, Danowski TS, Rosenblum DH, Mrieden T, Fisher ER, Surier JH. Discrepancies between glycosuria and home estimates of blood glucose in insulin-treated diabetes mellitus. *Diabetes Care* 1980; **3**: 178-83

40. Hayford JT, Weydert DA, Thompson RG. Validity of urine glucose measurements for estimating plasma glucose concentration. *Diabetes Care* 1983; **6**: 40-4

41. Csako G. Causes, consequences and recognition of false-positive reactions for ketones. *Clin Chem* 1980; **36**: 1388-9

42. Gonen B, Rochman H, Rubenstein AH. Metabolic control in diabetic patients; assessment by haemoglobin $A_1$ values. *Metabolism* 1979; **28**: 448-52

43. McCrance DR, Richie CM, Kennedy L. Is $HbA_1$ measurement superfluous in NIDDM? *Diabetes Care* 1988; **11**: 512-14

44. Holman RR, Turner RC. Maintenance of basal plasma glucose and insulin concentrations in maturity onset diabetes. *Diabetes* 1979; **28**: 227-30

45. Soksen PH, Judd SL, Lowry C. Home monitoring of blood glucose. *Lancet* 1978; **i**: 729-32

46. ADA Consensus Statement. Self-monitoring of blood glucose. *Diabetes Care* 1996; **19** (suppl 1): 62-6

47. Harris MI, Cowie CC, Howie LJ. Self-monitoring of blood glucose by adults with diabetes in the United States population. *Diabetes Care* 1993; **16**: 1116-23

48. Bunn HF, Gabbay KH, Gallop MP. The glycosylation of haemoglobin; relevance to diabetes mellitus. *Science* 1978; **200**: 21-27

49. Roth M. 'glycated haemoglobin', not 'glycosylated' or 'glucosylated' [lett]. *Clin Chem* 1983; **29**: 1991

50. IUPAC - IUB Joint Commission on Biochemical Nomenclature. Nomenclature of glycoproteins, glycopeptides and peptidoglycans. (recommendations 1985). *Eur J. Biochem*. 1986; **159**: 1-6

51. Goldsteine DE, Little RR, Lorenz RA. Tests of glycaemia in diabetes. *Diabetes Care* 1995: **18**: 896-909

52. Schliecher E, Wieland OH. Protein glycation; measurement and clinical relevance. *Clin Chem* 1989; **27**: 577-587

53. Whitby LG, Smith AF, Beckett GJ, Waller SW. Lecture notes on clinical biochemistry. 5th rev. ed. London, Blackwell Scientific, 1994

54. Miedema K, Casparie T. Glycosylated haemoglobins; biochemical evaluation and clinical utility. *Ann. Clin. Biochem*. 1984; **21**: 2-15

55. Lester E. The clinical value of glycated haemoglobin and glycated plasma proteins. *Ann. Clin. Biochem*. 1989; **26**: 213-219

56. Leslie RDG, John PN, Pyke DA. Haemoglobin $A_1$ in diabetic pregnancy. *Lancet* 1978: ii: 958-9

57. Miller E, Hare JH, Cloherty JP. Elevated maternal haemoglobin $A_{1c}$ in early pregnancy and major congenital anomalies in infants of diabetic mothers. *New Eng. J. Med*. 1981; **304**: 1331-4

58. Hinen K, Raivio K, Teramo K, Haemoglobin $A_{1c}$ predicts the perinatal outcome in insulin dependent pregnancies. *Br. J. Obstret Gynaecol* 1981; **88**: 961-7

59. Jovanovic L, Drozin M, Peterson M. Effect of euglycaemia on the outcome of pregnancy in insulin dependent diabetic women as compared with normal control subjects. *Amer. J. Med*. 1981; **71**: 921-7

60. Madsen H, Kjaergaard JJ, Ditzel J. Relationship between glycosylation of haemoglobin and the duration of diabetes; a study during the third trimester of pregnancy. *Diabetologia* 1982: **22**: 37-40

61. Armbruster DA. Fructosamine; structure, analysis and clinical usefulness. *Clin Chem* 1987; **33**: 2153-63

62. Ashby JP, Frier BM. Is serum fructosamine a clinically useful test ? *Diab. Med.* 1988; **5**: 118-21

63. Johnson RN, Metcalfe PA, Baker JR. Fructosamine; a new approach to the estimation of serum glycosylprotein. An index of diabetic control. *Clin. Chem. Acta.* 1982; **127**: 87-95

64. Johnson RN, Metcalfe PA. Relationship between albumin and fructosamine concentration in diabetic and non diabetic sera. *Clin. Chem. Acta.* 1987; **164**: 151-162

65. Howey JEA, Browning MCK, Fraser GC. Assay of serum fructosamine that minimises standardisation and matrix problems; use to assess components of biological variation. *Clin. Chem.* 1987; **33**: 269-272

66. Van Dreijen-Visser MP, Seynaeve C, Brombacher PJ. Influence of variations in albumin or total protein concentration on serum fructosamine concentration. *Clin. Chem.* 1986; **32**: 1610

67. Constanti C, Simo JM, Joven J, Camps J. Serum fructosamine concentrations in patients with nephrotic syndrome and with cirrhosis of the liver; influence of hypoalbuminaemia and hypergammaglobulinaemia 1992; **29**: 437-42

68. Henrichs HR, Lehmann P, Vorberg E. An improved fructosamine assay for monitoring blood glucose control. *Diabet. Med.* 1991; **8**: 580-4

69. Lloyd DR, Nott M, Marples J. Comparison of serum fructosamine with glycosylated serum protein (determined by affinity chromatography) for the assessment of diabetic control. *Diabet. Med.* 1985; **2**: 474-8

70. Gordon A, Glasser B, Wald M. Glycosylated serum protein levels assayed with highly sensitive immunoradiometric assay accurately reflects glycaemic control of diabetic patients. *Diabetes Care* 1992; **15**: 645-50

71. Roberts AB, Court DJ, Henley P, Baker JR, James AG, Ronayne ID. Fructosamine in diabetic pregnancy. *Lancet* 1993; **ii**: 998-100

72. Ardawi MSM, Nasrat HN, Mira SA, Fatini HH. Comparison of glycosylated fibrinogen, albumin and haemoglobin as indices of blood glucose control in diabetic patients. *Diabet. Med.* 1990; **7**: 819-24

73. Furth AJ. Glycated proteins in diabetes. *British Journal of Biomedical Science.* 1997; **54**: 192-200

74. Furth AJ, Harding J. Why sugar is bad for you. *New scientist* 1989; **23rd Sept.** : 44-47

75. Ahmed N, Furth AJ. Failure of common glycation assays to detect glycation by fructose. *Clin. Chem.* 1992; **38**: 1301-3

76. Miller JA, Gravallese E, Bunn HF. Nonenzymatic glycosylation of erythrocyte membrane proteins. Relevance to diabetes. *J. Clin. Invest.* 1980; **65**: 896-901

77. Bunn HF. Evaluation of glycosylated haemoglobin in diabetic patients. *Diabetes* 1981; **30**: 613-617

78. Dolhofer R, Wieland OH. Glycosylation of serum albumin; elevated glycosyl albumin in diabetic patients. *FEBS lett* 1979; **103**: 282-286

79. Witzum JL, Mahoney EM, Branks MJ. Nonenzymatic glycosylation of low-density lipoprotein alters its biological activity. *Diabetes* 1982; **31**: 283-291

80. Witzum JL, Fisher M, Pietro T. Nonenzymatic glycosylation of high-density lipoprotein accelerates its catabolism in guinea pigs. *Diabetes* 1982; **31**: 1029-1032

81. Kaneshiga H. Nonenzymatic glycosylation of serum IgG and its effect on antibody activity in patients with diabetes mellitus. *Diabetes* 1987; **36**: 822-828

82. Cerami A, Stevens VJ, Monnier VM. Role of nonenzymatic glycosylation in the development of the sequelae of diabetes mellitus. *Metabolism* 1979; **28 (suppl 1)**: 431-437

83. Cohen MP, Urdanivia E, Surma M. Increased glycosylation of glomerular basement membrane collagen in diabetes. *Biochem. Biophys. Res.* 1980; **95**: 765-769

84. Garlick RL, Bunn HF, Spiro RG. Nonenzymatic glycation of basement membranes from human glomeruli and bovine sources. *Diabetes* 1988; **37**: 1144-1150

85. Summerfield JA, Vergall J, Jones EA. Modulation of glycoprotein recognition system on rat hepatic endothelial cells by glucose and diabetes mellitus. *J. Clin. Invest* 1982; **69**: 1337-1347

86. Villanueva GB, Allen N. Demonstration of altered antithrombin III activity due to nonenzymatic glycosylation at glucose concentration expected to be encountered in severely diabetic patients. *Diabetes* 1988; **37**: 1103-1107

87. McVerry BA, Fisher C, Hopp A. Production of pseudodiabetic renal glomerular changes in mice after repeated injections of glycosylated proteins. *Lancet* 1980; **i**: 738-740

88. Jeraj KP, Michael AF, Mauer SM. Glycosylated and normal human and rat albumin do not bind to renal basement membranes of diabetic and control rats. *Diabetes* 1983; **32**: 380-382

89. Brownlee M, Vlassara M, Cerami A. Nonenzymatic glycosylation reduces the susceptibility of fibrin to degradation by plasmin. *Diabetes* 1983; **32**: 680-684

90. Davies MJ, Woolf N, Carstairs KC. Immunohistochemical studies in diabetic glomerulosclerosis. *J. Pathol. Bacteriol.* 1966; **92**: 441-445

91. Beisswenger PJ, Spiro RG. Human glomerular basement membrane; chemical alteration in diabetes mellitus. *Science* 1970; **168**: 596-598

92. Schnider SL, Glucosylation of human collagen. *Diabetes* 1982: **31: (suppl 3)**: 47-51

93. Rohrbach DH, Hassell JR, Kleinman HK. Alterations in the basement membrane (heparan sulphate) proteoglycan in diabetic mice. *Diabetes* 1982: **31**: 185-188

94. Bassiouny AR, Rosenberg H, McDonald TL. Glucosylated collagen is antigenic. *Diabetes* 1983; **32**: 1182-1184

95. Schnider SL, Kohn RR. Effects of age and diabetes mellitus on the solubility and nonenzymatic glycosylation of human skin collagen. *J. Clin. Invest.* 1981; **67**: 1630-1635

96. Rosenblum Al, Silverstein JH, Riley WJ. Limited joint mobility in childhood diabetes; family studies. *Diabetes Care* 1983; **6**: 370-373

97. Peterson CM, Jones RL, Koeing RJ. Reversible haematological sequelae of diabetes mellitus. *Ann. Intern. Med* 1977; **86**: 425-429

98. McMillan DE, Utterback NG, La Puma J. Reduced erythrocyte deformability in diabetes. *Diabetes* 1978; **27**: 895-901

99. Vlassara H, Brownlee M, Cerami A. Excessive nonenzymatic glycosylation of peripheral and central nervous system myelin components in diabetic rats. *Diabetes* 1983; **32**: 670-674

100. De Fronzo RA, Hendler R, Simpsonson D. Insulin resistance is a prominent feature of insulin dependent diabetes. *Diabetes* 1982; **31**: 795-801

101. Scarlett JA, Gray RS, Griffin J. Insulin treatment reverses the insulin resistance of type II diabetes mellitus. *Diabetes Care* 1982; **5**: 353-363

102. Muwat AG, Baum J. Chemotaxis of polymorphonuclear leukocytes from patients with diabetes mellitus. *New Eng. J. Med* 1971; **284**: 621-627

103. Bagdade JD. Phagocytic and microbicidal function in diabetes mellitus. *Acta. Endocrinol.* 1976; **83 (suppl 201)**: 27-34

104. Casey JI, Heeter BJ, Klyshevich KA. Impaired response of lymphocytes of diabetic subjects to antigen of staphylococcus aureus. *J. Infect. Dis.* 1977; **136**: 495-501

105. Selam JL, Clot J, Andary M. Circulating lymphocyte subpopulations in juvenile insulin dependent diabetes. Correction of abnormalities by adequate blood glucose control. *Diabetologia* 1979; **16**: 35-40

106. Jones RL, Peterson CM. Haematological alterations in diabetes mellitus. *Am. J. Med.* 1981; **70**: 399-352

107. Brownlee M, Cerami A, Vlassara H. Advanced products of nonenzymatic glycation and the pathogenesis of diabetic vascular disease. *Diabetes Metab. Rev.* 1988; **5**: 437-451

108. Winegrad AI. Banting lecture 1986. Does a common mechanism induce the diverse complications of diabetes? *Diabetes* 1986; **36**: 396-406

109. Kinoshita JH. Mechanisms initiating cataract formation. *Invest. opthalmol* 1974; **13**: 713-724

110. Robinson WG Jr., Kador PF, Kinoshita JH. Retinal capillaries; basement membrane thickening by galactosaemia prevented with aldose reductase inhibition. *Science* 1983; **221**: 1177-1179

111. Greene DA, Lattimer SA, Sima AAF. Are disturbances of sorbitol, phosphoinositide, and Na+,- K+ - ATpase regulation involved in pathogenesis of diabetic neuropathy? *Diabetes* 1988; **37**: 688-693

112. Morrison AD, Clements RS Jr., Winegrad AI. Effects of elevated glucose concentrations on the metabolism of the aortic wall. *J. Clin. Invest.* 1972; **51**: 3114-3123

113. Greene DA, Lattimer SA. Impaired rat sciatic nerve sodium-potassium adenosine triphosphatase in acute streptozotocin diabetes and its correction by dietary myo-inisitol supplementation. *J. Clin. Invest.* 1983; **72**: 1058-1063

114. Greene DA, Lattimer SA. Action of sorbinil in diabetic peripheral nerve; relationship of polyol (sorbitol) pathway inhibition to a myo-inisitol mediated defect in sodium-potassium ATpase activity. *Diabetes* 1984; **33**: 712-716

115. Dyck PJ, Zimmerman BR, Viles TH. Nerve glucose, fructose, sorbitol, myo-inisitol and fibre degeneration and regeneration in diabetic neuropathy. *New Eng. J. Med.* 1988; **319**; 542-548

116. Young RJ, Ewing DJ, Clarke BF. A controlled trial of sorbinil, an aldose reductase inhibitor, in chronic painful diabetic neuropathy. *Diabetes* 1983; **32**: 938-942

117. Sima AAF, Bril V, Nathaniel V. Regeneration and repair of myelinated fibres in sural nerve biopsy specimens from patients with diabetic neuropathy treated with sorbinol. *New. Eng. J. Med* 1988; **319**: 548-555

118. Suarez G, Rajaram R. Oronsky AL. Nonenzymatic glycosylation of bovine serum albumin by fructose (fructation). Comparison with the Malliard reaction initiated by glucose. *J. Biol. Chem.* 1989; **264**: 3674-3679

119. Parving H-H, Viberti GC, Keen H. Haemodynamic factors in the genesis of diabetic microangiopathy. *Metabolism* 1983; **32**: 943-949

120. Anderson S, Brenner B. Pathogenesis of diabetic glomerulopathy; haemodynamic considerations. *Diabetes Metab Rev.* 1988; **4**: 163-177

121. Eckerbom S, Bergquist Y, Jeppsson. Improved method for analysis of glycated haemoglobin by ion-exchange chromatography. *Ann. Clin. Biochem.* 1994; **31**: 355-360

122. Dix D, Cohen P, Kingsby S, Senkbeil J, Sexton K. Evaluation of a glyco-haemoglobin kit [letter]. *Clin. Chem. Invest* 1983; **71**: 1062-72

123. Arnqvist H, Hermansson G, Ludvigsson J, Wettre S. A chromatographic method for measuring haemoglobin $A_1$; comparison with two commercial kits. *Ann. Clin. Biochem* 1981; **18**: 240-2

124. Rosenthal PK, Vazquez DA, Seckinger DL. Determination of glycohaemoglobin by rapid batch separation. *Am. J. Clin. Pathol.* 1981; **75**: 45-9

125. Davis RE, Nicol DJ. A rapid method for routine measurement of glycosylated haemoglobin. *Lancet* 1978; ii: 350-1

126. Maquart FX, Gillery P, Bernard JF, Borel JP. A method for specifically measuring haemoglobin $A_{1c}$ with a disposable commercial ion-exchange column. *Clin. Chem. Acta.* 1980; **108**: 329-32

127. Jones MD, Koler RD, Jones RT. Microcolumn method for the determination of haemoglobin minor fractions $A_{1a}+_b$ and $A_{1c}$. *Haemoglobin* 1978; **2**: 53-8

128. Clark JT, Canivet J. Haemoglobin $A_{1c}$ separation by microcolumn chromatography; a new rapid method of assay. *Diabetes Metab.* 1979; **5**: 293-6

129. Fielden P, Frost PG. An assessment of Drew Glycomat haemoglobin $A_1$ analyser! Proceedings of the ACB National Meeting Focus 1990. 1990; **38**: 43

130. Standing SJ, Taylor RP. Glycated haemoglobin; an assessment of high capacity liquid chromatographic and immunoassay methods. *Ann. Clin. Biochem.* 1992; **29**: 494-505

131. Menard L, Dempsey ME, Blankstein LA, Aleyassine H, Wacks M, Soeldness JS. Quantitative determination of glycosylated haemoglobin $A_1$ by agar gel electrophoresis. *Clin. Chem.* 1980; **26**: 1598-1602

132. Thornton WE, Schellekans AMP, Sanders GTB. Assay of glycosylated haemoglobin using agar electrophoresis. *Ann. Clin. Biochem.* 1981; **18**: 182-184

133. Puukka R, Leppilampi M. Electrophoretically determined haemoglobin $A_1$ concentrations during short-term changes in glucose concentration. *Ann. Clin. Biochem.* 1982; **19**: 350-353

134. Keating J, Pattison A, Sherwood RA. Evaluation of the Helena REP automated electrophoresis instrument for the measurement of glycated haemoglobin. *Ann. Clin. Biochem.* 1991; **28**: 185-186

135. Saibene B, Brembilla A. Chromatographic and colorimetric determination of glycosylated haemoglobin; a comparative analysis of two different methods. *Clin. Chem. Acta* 1979; **93**: 199-205

136. Simon M, Cuan J. Haemoglobin $A_{1c}$ by iso-electric focusing. *Clin. Chem.* 1982; **28**: 9-12

137. Simon M, Eissler J. Critical factors in the chromatographic measurement of glycohaemoglobin ($HbA_1$). *Diabetes* 1980; **29**: 467-74

138. Schellekens APM, Sanders GTB, Thornton W, Von Groenestein T. Sources of variation in the column-chromatographic determination of glycohaemoglobin ($HbA_1$). *Clin. Chem.* 1981; **27**: 94-99

139. Svenden PA, Christiansen JS, Welinder B, Nerup J. Fast glycosylation of haemoglobin. *Lancet* 1979; i: 603

140. Karamanos B, Christacopoulos P, Zacharious N, Korkoliss S. Rapid changes of haemoglobin A$_{1c}$ fraction following alteration of diabetic control. *Diabetologia* 1977; **13**: 406

141. Svenden PA, Christiansen JS, Soegaard U, welinder BS, Nerup J. Rapid changes in chromatographically determined haemoglobin A$_{1c}$ induced by short-term changes in glucose. *Diabetologia* 1980; **19**: 130-6

142. Bisse E, Berger W, Fluckiger R. Quantitation of glycosylated haemoglobin. Elimination of labile glycohaemoglobin during sample haemolysis at pH 5. *Diabetes* 1982; **31**: 630-3

143. Nathan DM, Avezzano ES, Palmer JL. A rapid chemical means for removing labile glycohaemoglobin. *Diabetes* 1981; **30**: 700-1

144. Panzer S, Kronik G, Lechner K, Neumman E, Dudczak R. Glycosylated haemoglobin (GHb); an index of red cell survival. *Blood* 1982; **59**: 1348-50

145. Lind T, Cheyne GA. Effect of normal pregnancy upon the glycosylated haemoglobins. *Br. J. Obstret. Gynaecol.* 1979; **86**: 210-3

146. Sosenko JM, Fluckiger R, Platt OS, Gabbay KH. Glycosylation of varient heamoglobins in normal and diabetic subjects. *Diabetes Care* 1980; **3**: 590-3

147. Vesley DL, Sanders LL. False elevation of haemoglobin A$_1$ by hereditary persistence of foetal haemoglobin. *J. Med.* 1987; **18**: 81-92

148. Mullis P, Schuler J, Zuppinger K. Increased prevalance of foetal haemoglobin in type I (insulin-dependent) diabetes mellitus. *Diabetologica* 1989; **32**: 227-30

149. Klenk DC, Hermanson GT, Randall KI, Edward K. Determination of glycosylated haemoglobin by affinity chromatography; comparison of with colorimetric and ion-exchange methods, and effects of common interferences. *Clin. Chem.* 1982; **28(10)**: 2088-2094

150. Hughes-Jones NC, Wickramasinghe SN. Lecture notes on haemotology. 5th ed. London, Blackwell Scientific 1991

151. Dix D, Cohen D, Kingsley S, Lea MJ, Senkbeil J, Sexton K. Influence of lactescence in glycohaemoglobin analysis. *Clin. Chem.* 1979; **25**: 494-5

152. Ceriello A, Giugliano D, Dello Russo P, Sgambato S, D' Onofrio F. Increased glycosylated haemoglobin A$_1$ in opiate addicts. Evidence for hyperglycaemic effects of morphine. *Diabetologia* 1982; **22**: 379

153. Gabbay KH, Sosenko JM, Banuchi GA, Mininshan MJ, Fluckiger R. Glycosylated haemoglobins; increased glycosylation of haemoglobin A$_1$ in diabetic patients. *Diabetes* 1979; **28**: 337-40

154. Fluckiger R, Harmon W, Meier W, Loo S, Gabbay K. Haemoglobin carbamylation in uraemia. *New Eng. J. Med.* 1981; **304**: 823-7

155. Simon M. Hoover JD. Effect of sample instability of glycohaemoglobin (HbA$_1$) measured by cation exchange chromatography. *Clin. Chem.* 1982; **28**: 195-8

156. Little RR, England JD, Wiedmeyer H, Goldstein DE. Effects of whole blood storage on results for glycosylated haemoglobins as measured by ion-exchange chromatography, affinity chromatography and colorimetry. *Clin. Chem.* 1983; **29**: 1113-5

157. Fluckiger R, Winterhalter KH. In vitro synthesis of haemoglobin A$_{1c}$. *FEBS Letters* 1976; **71**: 356-60

158. Fischer RW, De Jong C, Voigt E. The colorimetric determination of HbA$_{1c}$ in normal and diabetic subjects. *Clin. Lab. Haemat.* 1980; **2**: 129-38

159. Pecoraro RE, Graf RF, Halter JB. Comparison of a colorimetric assay for glycohaemoglobin with ion-exchange chromatography. *Diabetes* 1979; **28**: 1220-5

160. Menez JF, Mestar A, Lucas D. Glycosylated haemoglobin and serum proteins; a semi-automated estimation. *Clin. Chem* 1981; **27**: 1947-8

161. Postmes TJ, Halders S, Herpers H. Colorimetry vs column chromatography in HbA$_1$ assay. *Clin. Chem* 1981; **27**: 535-6

162. Parker KM, England JD, Dacosta J. Improved colorimetric assay for glycosylated haemoglobin. *Clin. Chem.* 1981; **27**: 669-72

163. Dolholfer R, Wieland OH. Improvement of the thiobarbituric assay for serum glycosylprotein determination. *Clin. Chem. Acta.* 1981; **112**: 197-204

164. Sudhaker S, Pattabiranam TN. A new colorimetric method for the estimation of glycosylated haemoglobin. *Clin. Chem. Acta.* 1981; **109**: 267-74

165. Mallia AK, Hermanson GT, Krohn RI, Fujimoto EK, Smith PK. Preparation and use of a boronic acid affinity support for separation and quantitation of glycosylated haemoglobins. *Ann. Lett.* 1981; **14**: 649-61

166. Hall PM, Cook JGH, Gould BJ. An inexpensive, rapid and precise affinity chromatography method for the measurement of glycosylated haemoglobins. *Ann. Clin. Biochem.* 1983; **20**: 129-135

167. Pierce and Warriner. Glycotest II affinity chromatography kit insert. Cambridge, UK. 1997

168. Holt J. New approaches to the affinity separation of glycohaemoglobin. MSc thesis, University of Surrey. 1993

169. Drew Scientific LTD. GHb-100 analyser operator manual. Cumbria, UK. 1997

170. Gould BJ, Hall PM, Cook JGH. Measurement of glycosylated haemoglobins using an affinity chromatography method. *Clin Chem Acta.* 1982; **125**: 41-48

171. Baynes JW, Bunn HF, Goldstein DE. National Diabetes Data Group; report of the expert committee on glycosylated haemoglobin. *Diabetes Care* 1984: **7**: 602-6

172. United Kingdom National External Quality Assurance Scheme (NEQAS) for glycohaemoglobin. Birmingham UK 1997

173. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin dependent diabetes mellitus. *New Eng. J. Med.* 1993; **329**: 977-86

174. Oreopoulas DG, Robson M, Clayton S, Verber GA. A simple and safe technique for CAPD. *Transactions of the American Society for Artificial Internal Organs* 1978; **24**: 484-9

175. Dix D, Cohen P, Kingsley S. Glycohaemoglobin and OGTT compared as indicators of borderline diabetes mellitus. *Clin. Chem.* 1979; **25**: 877-9

176. Primus CLC330. Primus Corporation, 7325 Central Avenue, Kansas City, USA.

177. Little RR, Wiedmeyer HM, England JD, Wilke AL, Rohlfing CL, Wians FH. Interlaboratory standardisation of measurements of glycohaemoglobin. *Clin. Chem.* 1992; **38**: 2472-8

178. Bodor GS, Little RR, Garett N, Brown W, Goldsteine DE. Standardisation of glycohaemoglobin determinations in the clinical laboratory; three years of experience. *Clin. Chem.* 1992; **38**: 2414-8

179. Bruns DE. Standardisation, calibration, and the care of diabetic patients [Editorial]. *Clin. Chem.* 1992; **38**: 2363-4

180. Lindholm B, Karlander SG. Glucose tolerance in patients undergoing continuous ambulatory peritoneal dialysis. *Acta. Med. Scand.* 1986; **220**: 477-83

181. Smith WG, Holden M, Benton M, Brown CB. Glycosylated and carbamylated haemoglobin in uraemia. *Nephrol. Dial. Transplant.* 1989; **4(2)**: 96-100

182. Tzamaloukas AH, Hsi KC, Quintana BJ, Merlin TL, Avasthi PS. Glycosylated haemoglobin measured by affinity chromatography in diabetic and nondiabetic patients on long-term dialysis therapy. *West J. Med.* 1989 Apr; **150(4)**: 415-419

183. Morgan LJ, Marenah CB, Morgan AG, Burden RP, John WG. Glycated haemoglobin and fructosamine in non-diabetic subjects with chronic renal failure. *Nephrol. Dial. Transplant.* 1990; **5(10)**: 868-873

184. Armstrong VW, Buschmann ER, Fuchs C, Reiger J, Scheler F. Biochemical investigations of CAPD; plasma leves of trace elements and amino acids and impaired glucose tolerance during the course of treatment. *Int. J. Artif. Organs* 1980; **3**: 237

185. Armstrong VW, Fuchs C, Scheler F. Effect of dialysate glucose load on plasma glucose and insulin levels in CAPD patients. In Gahl GM, Kessel M, Nolph KD (eds) Advances in Peritoneal Dialysis. Amsterdam, Oxford and Princeton. Excerpta Medica 1981; 421-423

# APPENDIX

**Table 1** Correlation between GHb results determined from packed cells and from whole blood

**Table 2** Correlation study of assigned calibrant values of BR1 = 4.3%  BR2 = 11.3%

**Table 3** Correlation study of assigned calibrant values of BR1 = 3.8%  BR2 = 10.0%

**Table 4**  Correlation study of Biorad versus Primus Calibrants

Table 1 Correlation between GHb results determined from packed cells and from whole blood

| n | Packed Cells (x) | Whole Blood (y) | y-x |
|---|---|---|---|
| 1 | 9.8 | 10 | 0.2 |
| 2 | 5 | 5 | 0 |
| 3 | 6.4 | 5.9 | -0.5 |
| 4 | 8.1 | 7.2 | -0.9 |
| 5 | 8.6 | 8.5 | -0.1 |
| 6 | 9.8 | 9.9 | 0.1 |
| 7 | 10.8 | 9.7 | -1.1 |
| 8 | 11.2 | 10.8 | -0.4 |
| 9 | 12 | 11.7 | -0.3 |
| 10 | 13.5 | 13.2 | -0.3 |
| 11 | 14.7 | 13.6 | -1.1 |
| 12 | 5.1 | 4.6 | -0.5 |
| 13 | 7.1 | 6.5 | -0.6 |
| 14 | 8.9 | 8.6 | -0.3 |
| 15 | 10.5 | 10.3 | -0.2 |
| 16 | 9.3 | 8.8 | -0.5 |
| 17 | 4.7 | 3.8 | -0.9 |
| 18 | 8.8 | 7.7 | -1.1 |
| 19 | 5.8 | 5.3 | -0.5 |
| 20 | 11.3 | 10.4 | -0.9 |
| 21 | 14.9 | 14 | -0.9 |
| 22 | 8.7 | 8.1 | -0.6 |
| 23 | 5.2 | 4.9 | -0.3 |
| 24 | 7.9 | 7.7 | -0.2 |
| 25 | 10.3 | 10.1 | -0.2 |
| 26 | 7.2 | 6.8 | -0.4 |
| 27 | 8.9 | 8.2 | -0.7 |
| 28 | 11.4 | 10.9 | -0.5 |
| 29 | 11 | 10 | -1 |
| 30 | 8.6 | 7.9 | -0.7 |
| 31 | 9.6 | 9 | -0.6 |
| 32 | 8.3 | 7.7 | -0.6 |
| 33 | 10.5 | 10.1 | -0.4 |
| 34 | 6.3 | 6 | -0.3 |
| 35 | 9.3 | 9.5 | 0.2 |
| 36 | 8.7 | 8.7 | 0 |
| 37 | 6.2 | 6 | -0.2 |
| 38 | 13 | 12 | -1 |
| 39 | 7.9 | 7.5 | -0.4 |
| 40 | 12.6 | 11.6 | -1 |

Table 2  Correlation Study of assigned calibrant values of BR1=4.3% BR2=11.3%

| n | Pierce (x) | Drew (y) | y-x | (y-x/x) X100 |
|---|---|---|---|---|
| 1 | 17.4 | 18.2 | 0.8 | 4.6 |
| 2 | 11 | 12.2 | 1.2 | 10.9 |
| 3 | 8.5 | 9.4 | 0.9 | 10.6 |
| 4 | 7.4 | 8.1 | 0.7 | 9.5 |
| 5 | 9.5 | 10.5 | 1 | 10.5 |
| 6 | 8.1 | 8.8 | 0.7 | 8.6 |
| 7 | 5.3 | 5.7 | 0.4 | 7.5 |
| 8 | 7.8 | 9 | 1.2 | 15.4 |
| 9 | 7 | 7.7 | 0.7 | 10.0 |
| 10 | 9.3 | 10.6 | 1.3 | 14.0 |
| 11 | 5.8 | 6.6 | 0.8 | 13.8 |
| 12 | 7.6 | 8.7 | 1.1 | 14.5 |
| 13 | 9.1 | 10.8 | 1.7 | 18.7 |
| 14 | 7.3 | 8.3 | 1 | 13.7 |
| 15 | 8.5 | 9.5 | 1 | 11.8 |
| 16 | 11.8 | 12.6 | 0.8 | 6.8 |
| 17 | 4.7 | 5.4 | 0.7 | 14.9 |
| 18 | 8.4 | 9.1 | 0.7 | 8.3 |
| 19 | 7.5 | 8.3 | 0.8 | 10.7 |
| 20 | 9.5 | 10.9 | 1.4 | 14.7 |
| 21 | 4.6 | 5.2 | 0.6 | 13.0 |
| 22 | 8.2 | 8.5 | 0.3 | 3.7 |
| 23 | 10 | 10.9 | 0.9 | 9.0 |
| 24 | 4.2 | 4.7 | 0.5 | 11.9 |
| 25 | 7.6 | 8.4 | 0.8 | 10.5 |
| 26 | 10.2 | 10.8 | 0.6 | 5.9 |
| 27 | 10 | 11.1 | 1.1 | 11.0 |
| 28 | 7.1 | 7.4 | 0.3 | 4.2 |
| 29 | 5.6 | 6.5 | 0.9 | 16.1 |
| 30 | 9.1 | 10.1 | 1 | 11.0 |
| 31 | 7 | 7.9 | 0.9 | 12.9 |
| 32 | 9.4 | 11.7 | 2.3 | 24.5 |
| 33 | 7.7 | 8.1 | 0.4 | 5.2 |
| 34 | 11 | 11.9 | 0.9 | 8.2 |
| 35 | 11.2 | 10.8 | -0.4 | -3.6 |
| 36 | 9.4 | 10.2 | 0.8 | 8.5 |
| 37 | 8.7 | 10.4 | 1.7 | 19.5 |
| 38 | 9 | 10.3 | 1.3 | 14.4 |
| 39 | 7.7 | 8.9 | 1.2 | 15.6 |
| 40 | 6.9 | 7.6 | 0.7 | 10.1 |
| 41 | 5.5 | 6.3 | 0.8 | 14.5 |
| 42 | 7.3 | 8 | 0.7 | 9.6 |
| 43 | 8.5 | 9.7 | 1.2 | 14.1 |
| 44 | 8.1 | 8.6 | 0.5 | 6.2 |
| 45 | 6.3 | 7.2 | 0.9 | 14.3 |
| 46 | 6.2 | 6.3 | 0.1 | 1.6 |
| 47 | 9.4 | 10.9 | 1.5 | 16.0 |
| 48 | 10.1 | 10.9 | 0.8 | 7.9 |
| 49 | 11.2 | 12.6 | 1.4 | 12.5 |
| 50 | 5.5 | 6.3 | 0.8 | 14.5 |
| 51 | 8.4 | 9 | 0.6 | 7.1 |
| 52 | 10.8 | 11.3 | 0.5 | 4.6 |

Table 2 cont..d Correlation Study of assigned calibrant values of BR1=4.3% BR2=11.3%

| n | Pierce (x) | Drew (y) | y-x | (y-x/x) X100 |
|---|---|---|---|---|
| 53 | 7.6 | 8.5 | 0.9 | 11.8 |
| 54 | 8.2 | 8.8 | 0.6 | 7.3 |
| 55 | 6.5 | 7.6 | 1.1 | 16.9 |
| 56 | 9.6 | 10.3 | 0.7 | 7.3 |
| 57 | 11.6 | 12 | 0.4 | 3.4 |
| 58 | 4.7 | 4.8 | 0.1 | 2.1 |
| 59 | 7.2 | 8 | 0.8 | 11.1 |
| 60 | 7.5 | 8.7 | 1.2 | 16.0 |
| 61 | 12 | 13.1 | 1.1 | 9.2 |
| 62 | 8.3 | 9.4 | 1.1 | 13.3 |
| 63 | 9.2 | 9.8 | 0.6 | 6.5 |
| 64 | 9.8 | 10.7 | 0.9 | 9.2 |
| 65 | 14.8 | 15.2 | 0.4 | 2.7 |
| 66 | 9.6 | 10.2 | 0.6 | 6.3 |
| 67 | 7.5 | 7.5 | 0 | 0.0 |
| 68 | 10 | 10.6 | 0.6 | 6.0 |
| 69 | 7.4 | 7.2 | -0.2 | -2.7 |
| 70 | 7.6 | 8.2 | 0.6 | 7.9 |
| 71 | 8.7 | 9.1 | 0.4 | 4.6 |
| 72 | 9.1 | 9 | -0.1 | -1.1 |
| 73 | 7 | 7 | 0 | 0.0 |
| 74 | 5.2 | 5.4 | 0.2 | 3.8 |
| 75 | 8.2 | 9.3 | 1.1 | 13.4 |
| 76 | 6.5 | 7.1 | 0.6 | 9.2 |
| 77 | 7.2 | 7 | -0.2 | -2.8 |
| 78 | 8 | 8.2 | 0.2 | 2.5.. |
| 79 | 13.2 | 13.6 | 0.4 | 3.0 |
| 80 | 6.5 | 7.5 | 1 | 15.4 |
| 81 | 8.7 | 10.3 | 1.6 | 18.4 |
| 82 | 4.5 | 4.7 | 0.2 | 4.4 |
| 83 | 9.5 | 10.7 | 1.2 | 12.6 |
| 84 | 8.3 | 8.9 | 0.6 | 7.2 |
| 85 | 5 | 5.5 | 0.5 | 10.0 |
| 86 | 9.4 | 10.3 | 0.9 | 9.6 |
| 87 | 8.8 | 9.9 | 1.1 | 12.5 |
| 88 | 14.9 | 16.3 | 1.4 | 9.4 |
| 89 | 6.1 | 6.7 | 0.6 | 9.8 |

Table 3 Correlation Study of assigned calibrant values of BR1=3.8% BR2=10.0%

| n | Pierce (x) | Drew (y) | y-x | (y-x/x) X100 |
|---|---|---|---|---|
| 1 | 7.6 | 7.9 | 0.3 | 3.9 |
| 2 | 8.4 | 8.3 | -0.1 | -1.2 |
| 3 | 8.5 | 8.2 | -0.3 | -3.5 |
| 4 | 8 | 7.6 | -0.4 | -5.0 |
| 5 | 9.5 | 9.2 | -0.3 | -3.2 |
| 6 | 8.9 | 8.6 | -0.3 | -3.4 |
| 7 | 6.1 | 7 | 0.9 | 14.8 |
| 8 | 5.5 | 4.8 | -0.7 | -12.7 |
| 9 | 10 | 10.1 | 0.1 | 1.0 |
| 10 | 6.3 | 6.9 | 0.6 | 9.5 |
| 11 | 7.3 | 7.7 | 0.4 | 5.5 |
| 12 | 8.6 | 8.9 | 0.3 | 3.5 |
| 13 | 6.7 | 7.4 | 0.7 | 10.4 |
| 14 | 10.8 | 10.8 | 0 | 0.0 |
| 15 | 4.9 | 5.3 | 0.4 | 8.2 |
| 16 | 4.5 | 4.4 | -0.1 | -2.2 |
| 17 | 6.6 | 6.7 | 0.1 | 1.5 |
| 18 | 11.5 | 12.4 | 0.9 | 7.8 |
| 19 | 9.8 | 9.5 | -0.3 | -3.1 |
| 20 | 6.4 | 6.8 | 0.4 | 6.2 |
| 21 | 9.7 | 9.7 | 0 | 0.0 |
| 22 | 7.9 | 8.5 | 0.6 | 7.6 |
| 23 | 7.5 | 7.3 | -0.2 | -2.7 |
| 24 | 7 | 7 | 0 | 0.0 |
| 25 | 10.4 | 10.4 | 0 | 0.0 |
| 26 | 6.9 | 7.1 | 0.2 | 2.9 |
| 27 | 5.3 | 5.1 | -0.2 | -3.8 |
| 28 | 6.1 | 6.4 | 0.3 | 4.9 |
| 29 | 12.2 | 11.4 | -0.8 | -6.6 |
| 30 | 13.1 | 13.7 | 0.6 | 4.6 |
| 31 | 8.2 | 8.6 | 0.4 | 4.9 |
| 32 | 10.1 | 11.5 | 1.4 | 13.9 |
| 33 | 11 | 11.5 | 0.5 | 4.5 |
| 34 | 11.1 | 10.9 | -0.2 | -1.8 |
| 35 | 7.1 | 6.9 | -0.2 | -2.8 |
| 36 | 10.2 | 10.5 | 0.3 | 2.9 |
| 37 | 4.9 | 4.5 | -0.4 | -8.2 |
| 38 | 9 | 8.6 | -0.4 | -4.4 |
| 39 | 7.1 | 6.7 | -0.4 | -5.6 |
| 40 | 6.1 | 5.9 | -0.2 | -3.3 |
| 41 | 11.1 | 11 | -0.1 | -0.9 |
| 42 | 9.6 | 9.1 | -0.5 | -5.2 |
| 43 | 7.2 | 6.9 | -0.3 | -4.2 |
| 44 | 6 | 5.7 | -0.3 | -5.0 |
| 45 | 9.2 | 9.2 | 0 | 0.0 |
| 46 | 8 | 7.8 | -0.2 | -2.5 |
| 47 | 5.4 | 5.3 | -0.1 | -1.9 |
| 48 | 10.4 | 10.2 | -0.2 | -1.9 |
| 49 | 5.4 | 5.2 | -0.2 | -3.7 |
| 50 | 10.4 | 10.6 | 0.2 | 1.9 |

Table 4 Correlation study of Biorad versus Primus Calibrants

| n | BIORAD (X) | PRIMUS (Y) | Y - X | (Y-X/X) X 100 |
|---|---|---|---|---|
| 1 | 5.2 | 6.1 | 0.9 | 17.3 |
| 2 | 9.6 | 9.5 | -0.1 | -1.0 |
| 3 | 8.5 | 8.5 | 0 | 0.0 |
| 4 | 8.1 | 8 | -0.1 | -1.2 |
| 5 | 12.5 | 11.4 | -1.1 | -8.8 |
| 6 | 6.7 | 7.1 | 0.4 | 6.0 |
| 7 | 13.7 | 12.3 | -1.4 | -10.2 |
| 8 | 5.7 | 6.2 | 0.5 | 8.8 |
| 9 | 8 | 7.9 | -0.1 | -1.3 |
| 10 | 6.2 | 6.6 | 0.4 | 6.5 |
| 11 | 9.4 | 9.1 | -0.3 | -3.2 |
| 12 | 13.9 | 12.5 | -1.4 | -10.1 |
| 13 | 10.7 | 10.1 | -0.6 | -5.6 |
| 14 | 6.7 | 7.1 | 0.4 | 6.0 |
| 15 | 6.5 | 6.9 | 0.4 | 6.2 |
| 16 | 8.1 | 8.1 | 0 | 0.0 |
| 17 | 5 | 5.7 | 0.7 | 14.0 |
| 18 | 8.8 | 8.7 | -0.1 | -1.1 |
| 19 | 13.4 | 12.1 | -1.3 | -9.7 |
| 20 | 11.5 | 10.6 | -0.9 | -7.8 |
| 21 | 10.1 | 9.7 | -0.4 | -4.0 |
| 22 | 8.5 | 8.4 | -0.1 | -1.2 |
| 23 | 5.5 | 6.2 | 0.7 | 12.7 |
| 24 | 7.3 | 7.4 | 0.1 | 1.4 |
| 25 | 6.8 | 7.1 | 0.3 | 4.4 |
| 26 | 7.4 | 7.5 | 0.1 | 1.4 |
| 27 | 7.6 | 7.7 | 0.1 | 1.3 |
| 28 | 7.1 | 7.4 | 0.3 | 4.2 |
| 29 | 8.6 | 8.5 | -0.1 | -1.2 |
| 30 | 16.6 | 14.3 | -2.3 | -13.9 |
| 31 | 10.1 | 9.4 | -0.7 | -6.9 |
| 32 | 9.1 | 8.8 | -0.3 | -3.3 |
| 33 | 7.4 | 7.5 | 0.1 | 1.4 |
| 34 | 6.5 | 6.6 | 0.1 | 1.5 |
| 35 | 5.8 | 6.2 | 0.4 | 6.9 |
| 36 | 13.1 | 11.9 | -1.2 | -9.2 |
| 37 | 7.8 | 7.8 | 0 | 0.0 |
| 38 | 12.8 | 11.6 | -1.2 | -9.4 |
| 39 | 5.9 | 6.3 | 0.4 | 6.8 |
| 40 | 6.6 | 6.9 | 0.3 | 4.5 |
| 41 | 5.1 | 5.8 | 0.7 | 13.7 |
| 42 | 7.8 | 7.8 | 0 | 0.0 |
| 43 | 8.7 | 8.5 | -0.2 | -2.3 |
| 44 | 11.3 | 10.5 | -0.8 | -7.1 |
| 45 | 6.8 | 7.2 | 0.4 | 5.9 |
| 46 | 9.1 | 8.7 | -0.4 | -4.4 |
| 47 | 7.9 | 7.9 | 0 | 0.0 |
| 48 | 12.9 | 11.7 | -1.2 | -9.3 |
| 49 | 9.3 | 8.9 | -0.4 | -4.3 |
| 50 | 11.8 | 10.9 | -0.9 | -7.6 |
| 51 | 9.1 | 8.8 | -0.3 | -3.3 |
| 52 | 8.3 | 8.2 | -0.1 | -1.2 |
| 53 | 6.7 | 6.9 | 0.2 | 3.0 |
| 54 | 5.5 | 6 | 0.5 | 9.1 |
| 55 | 16.9 | 14.7 | -2.2 | -13.0 |

Table 4 cont..d Correlation study of Biorad versus Primus Calibrants

| n | BIORAD (X) | PRIMUS (Y) | Y - X | (Y-X/X) X 100 |
|---|---|---|---|---|
| 56 | 14.3 | 12.6 | -1.7 | -11.9 |
| 57 | 8.4 | 8.2 | -0.2 | -2.4 |
| 58 | 9.9 | 9.4 | -0.5 | -5.1 |
| 59 | 7.3 | 7.5 | 0.2 | 2.7 |
| 60 | 9.6 | 9.2 | -0.4 | -4.2 |
| 61 | 6 | 6.6 | 0.6 | 10.0 |
| 62 | 10 | 9.5 | -0.5 | -5.0 |
| 63 | 6.9 | 7.2 | 0.3 | 4.3 |
| 64 | 8.8 | 8.5 | -0.3 | -3.4 |
| 65 | 6.8 | 7.2 | 0.4 | 5.9 |
| 66 | 5.5 | 6.1 | 0.6 | 10.9 |
| 67 | 9.7 | 9.5 | -0.2 | -2.1 |
| 68 | 10.9 | 10.4 | -0.5 | -4.6 |
| 69 | 9.6 | 9.3 | -0.3 | -3.1 |
| 70 | 6.9 | 7.5 | 0.6 | 8.7 |
| 71 | .7 | 7.5 | 0.5 | 7.1 |
| 72 | 10.7 | 10.5 | -0.2 | -1.9 |
| 73 | 6.6 | 7 | 0.4 | 6.1 |
| 74 | 9.3 | 9.4 | 0.1 | 1.1 |
| 75 | 11.7 | 10.9 | -0.8 | -6.8 |
| 76 | 9.6 | 9.3 | -0.3 | -3.1 |
| 77 | 14.1 | 12.7 | -1.4 | -9.9 |
| 78 | 8.2 | 8.3 | 0.1 | 1.2 |
| 79 | 16.1 | 14.4 | -1.7 | -10.6 |
| 80 | 6.5 | 7.1 | 0.6 | 9.2 |
| 81 | 4.8 | 6 | 1.2 | 25.0 |
| 82 | 16.8 | 15.1 | -1.7 | -10.1 |
| 83 | 13.2 | 12.2 | -1 | -7.6 |
| 84 | 6.6 | 7.2 | 0.6 | 9.1 |
| 85 | 5.2 | 6.2 | 1 | 19.2 |
| 86 | 12.9 | 11.8 | -1.1 | -8.5 |
| 87 | 5.9 | 6.6 | 0.7 | 11.9 |
| 88 | 6.8 | 7.2 | 0.4 | 5.9 |
| 89 | 6.6 | 7.2 | 0.6 | 9.1 |
| 90 | 7.3 | 7.7 | 0.4 | 5.5 |
| 91 | 6.9 | 7.2 | 0.3 | 4.3 |
| 92 | 6.4 | 6.9 | 0.5 | 7.8 |
| 93 | 14.5 | 13 | -1.5 | -10.3 |
| 94 | 15.9 | 14.2 | -1.7 | -10.7 |
| 95 | 9.6 | 9.3 | -0.3 | -3.1 |
| 96 | 3.9 | 5.1 | 1.2 | 30.8 |
| 97 | 10.2 | 9.7 | -0.5 | -4.9 |
| 98 | 7.5 | 7.8 | 0.3 | 4.0 |
| 99 | 7.2 | 7.5 | 0.3 | 4.2 |
| 100 | 8 | 8.2 | 0.2 | 2.5 |
| 101 | 4.7 | 5.5 | 0.8 | 17.0 |
| 102 | 16.2 | 14.4 | -1.8 | -11.1 |
| 103 | 9.6 | 9.4 | -0.2 | -2.1 |
| 104 | 11.8 | 11.1 | -0.7 | -5.9 |
| 105 | 11.4 | 10.9 | -0.5 | -4.4 |
| 106 | 5.5 | 6.2 | 0.7 | 12.7 |
| 107 | 13.4 | 12.1 | -1.3 | -9.7 |
| 108 | 8.1 | 8.4 | 0.3 | 3.7 |
| 109 | 6.8 | 7.4 | 0.6 | 8.8 |

Table 4 cont..d Correlation study of Biorad versus Primus Calibrants

| n | BIORAD (X) | PRIMUS (Y) | Y - X | (Y-X/X) X 100 |
|---|---|---|---|---|
| 110 | 4.4 | 5.5 | 1.1 | 25.0 |
| 111 | 8.3 | 8.5 | 0.2 | 2.4 |
| 112 | 7.4 | 7.7 | 0.3 | 4.1 |
| 113 | 7.6 | 7.9 | 0.3 | 3.9 |
| 114 | 8.2 | 8.4 | 0.2 | 2.4 |
| 115 | 9.7 | 9.5 | -0.2 | -2.1 |
| 116 | 8.5 | 8.5 | 0 | 0.0 |
| 117 | 10.7 | 10.3 | -0.4 | -3.7 |
| 118 | 8.8 | 8.7 | -0.1 | -1.1 |
| 119 | 9.3 | 9.1 | -0.2 | -2.2 |
| 120 | 4.9 | 5.8 | 0.9 | 18.4 |
| 121 | 5.1 | 5.8 | 0.7 | 13.7 |
| 122 | 10.2 | 9.8 | -0.4 | -3.9 |
| 123 | 9.9 | 9.5 | -0.4 | -4.0 |
| 124 | 7.7 | 7.9 | 0.2 | 2.6 |
| 125 | 5.1 | 5.9 | 0.8 | 15.7 |
| 126 | 8.3 | 8.4 | 0.1 | 1.2 |
| 127 | 8.4 | 8.5 | 0.1 | 1.2 |
| 128 | 10.8 | 10.4 | -0.4 | -3.7 |
| 129 | 4.9 | 5.7 | 0.8 | 16.3 |
| 130 | 4.1 | 5 | 0.9 | 22.0 |
| 131 | 7.4 | 7.7 | 0.3 | 4.1 |
| 132 | 10.1 | 9.7 | -0.4 | -4.0 |
| 133 | 7.8 | 8 | 0.2 | 2.6 |
| 134 | 9.2 | 9 | -0.2 | -2.2 |
| 135 | 7.3 | 7.6 | 0.3 | 4.1 |
| 136 | 7.2 | 7.5 | 0.3 | 4.2 |
| 137 | 7.2 | 7.5 | 0.3 | 4.2 |
| 138 | 5.5 | 6 | 0.5 | 9.1 |
| 139 | 9.6 | 9.4 | -0.2 | -2.1 |