A NOVEL MEANS OF MONITORING HEPATIC DAMAGE

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By

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SUMMARY

Total serum ferritin levels are elevated in patients with varying types of liver disease. The elevation may be caused by leakage of the intracellular form of ferritin from damaged cells into the serum. Extracellular ferritin can be distinguished from intracellular ferritin by its greater carbohydrate content, as residues are probably added to ferritin during secretion from the cells. Normal serum ferritin is approximately 60-70% glycated, and this can be separated from non-glycated ferritin by its reaction with Concanavalin A, a plant lectin that specifically binds glycated residues. The resultant extract and Sepharose control are assayed by radioimmunoassay techniques to determine ferritin content. It is then possible to calculate the % glycation of the sample.

The aim of the study was to determine what changes in serum ferritin occured with different types of liver damage, and whether other physiological or pathological conditions resulted in an alteration in total or % glycated serum ferritin.

Total serum ferritin was demonstrated to be elevated, together with a reduction in % glycation, in cases of acute and chronic hepatocellular damage. There were no changes associated with cholestatic liver disease without hepatocellular involvment. Insufficient evidence was found to determine whether changes in serum ferritin were a more sensitive indicator of drug induced hepatotoxic reactions than standard liver function tests. There was a wide variation evident in total and % glycated serum ferritin within normal subjects. In addition, changes in ferritin were not specific for hepatocellular damage, and were also seen in cases of diabetes, myocardial infarction, and after alcohol consumption. In view of these findings it is unlikely that the test will have a wide diagnostic use.

Key words:

Concanavalin A

Ferritin

Hepatotoxicity.

To my parents

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Collaborative work in the studies

All studies were carried out by myself, with the exception of the routine investigations performed by the departments of Biochemistry and Haemotology, Selly Oak Hospital; the Department of Clinical Chemistry, Birmingham General Hospital; and the Department of Biochemistry, Dudley Road Hospital. The presentation and analysis of results set out in this thesis is entirely my own.

Ethical approval

All of the studies incorporated in this work were approved by the South Birmingham District Health Authority Ethical Comittee prior to commencement of the study.

Written informed consent was received from all volunteers who supplied blood samples other than for routine investigations.

List of abbreviations used

Alk.P.	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ASPT	Aspartate Transaminase
Ca	Calcium
Cl	Chlorine
ConA	Concanavalin A
CPK	Creatinine Phosphokinase
CV	Coefficient of Variation
DRH	Dudley Road Hospital
EDTA	Ethylenedinitrilotetraacetate
F	Female
$FADH_2$	Dihydroflavin Adenine Dinucleotide
Fe	Iron
$FMNH_2$	Dihydroflavin Mononucleotide
γ GT	Gamma Glutamyl Transferase
GHB	General Hospital Birmingham
GLDH	Glutamate Dehydrogenase
GOT	Glutamate Oxaloacetate Transaminase
HBD	Hydroxybutyrate Dehydrogenase
LAP	Leucine Aminopeptidase
LDH	Lactate Dehydrogenase
LFT	Liver Function Test
M	Male
MDH	Malate Dehydrogenase
MI	Myocardial Infarction
Mg	Magnesium
Mn	Manganese

mRNA	Messenger Ribonucleic Acid
Na	Sodium
5'NT	5' Nucleotidase
O	Oxygen
OCT	Ornithine Carbamoyl Transferase
PT	Prothrombin Ratio
PTTK	Partial Thromboplastin Time Kaolin-cephalin
RIA	Radioimmunoassay
SDH	Sorbitol Dehydrogenase
SOH	Selly Oak Hospital
t½	Elimination Half-life

INTRODUCTION

The liver is the largest single organ of the body and is the principal area of protein synthesis and drug metabolism. Minor hepatic injury can be absorbed by the large reserve functional capacity of the liver, but major hepatic injury is a life threatening condition. Injury may be chronic or acute, and caused by a pathological condition or toxic chemical. Methods currently available to monitor hepatic damage include the measurement of indirect physiological results of chemicals synthesised by the liver; such as prothrombin and blood clotting times, direct measurement of intracellular hepatic enzymes that are released into serum when cells are destroyed; such as aspartate transaminase, or by liver biopsy. These markers are broadly predictive of the extent of hepatic damage. However, serum liver enzyme changes are not specific, and changes in the synthetic capability of the liver is not sensitive to early hepatic damage. Therefore there is scope for the introduction of a more sensitive, specific marker of hepatic damage. The observation of early changes in total and % glycated serum ferritin levels in a patient suffering from a hepatotoxic reaction to sulphasalazine (1), suggested that this may be of use as a means of monitoring hepatic damage.

In order to examine the effects of potentially hepatotoxic drugs, it is first necessary to consider the structure and function of the liver.

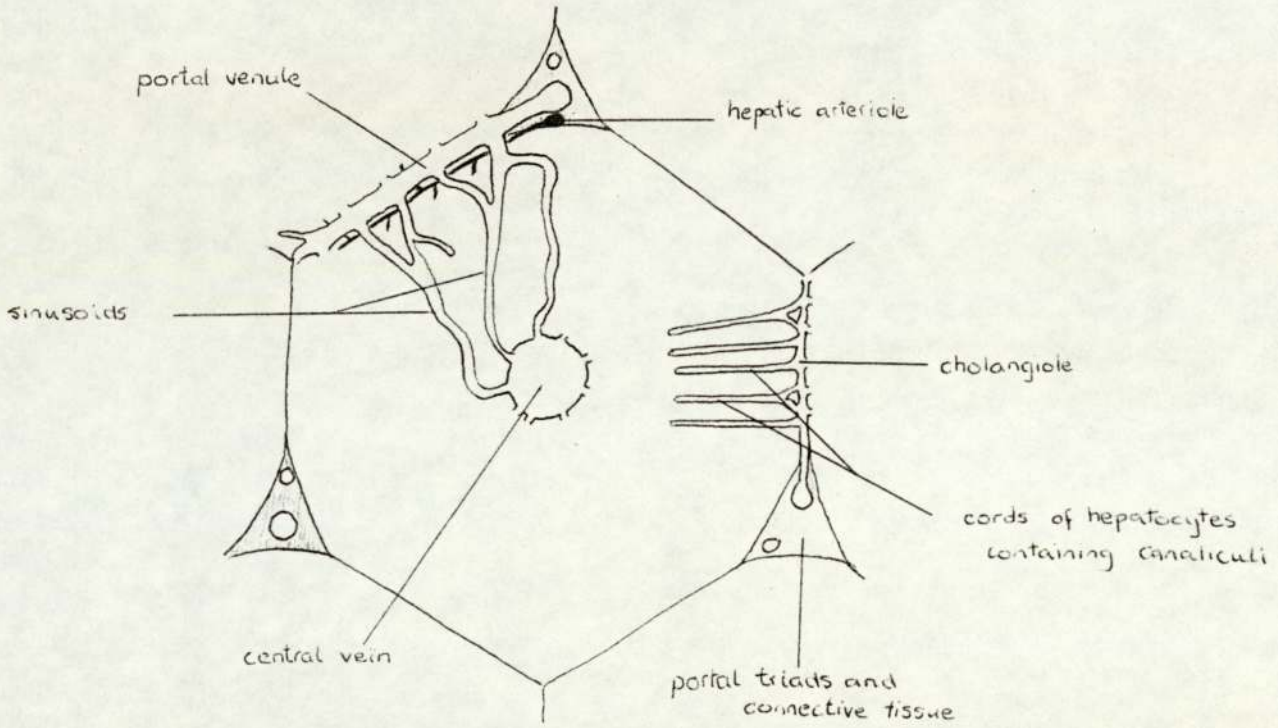
Structure of the liver

The liver is comprised of four main groups of cells : the parenchymal cells, or hepatocytes, that are the functional cells; reticuloendothelial cells (Kupfer cells), that are phagocytic; the cells lining the biliary tract, which delivers bile from the hepatocytes ultimately to the duodenum; and the cells of the blood vessels, namely the hepatic artery and the portal vein.

The functional unit of the liver is the lobule, of which there are between 50,000 and 100,000 in the human liver. The lobule is composed of a central vein running longitudinally, hepatocytes and sinusoids. Between lobules run branches of the portal vein, hepatic artery, and bile duct. Within

the lobule, hepatocytes radiate away from the central vein in planar sheets, between which run biliary canaliculi that remove bile secretions to the periphery. Between hepatocytes the hepatic sinusoids convey blood from branches of the portal vein and the hepatic artery through to the central vein. The sinusoids are coated with endothelial and reticuloendothelial cells (See Figure. 1).

Figure 1. The structural organisation of the hepatic lobes.



The biliary canaliculi drain into biliary ductules that run on the surface of the plates of hepatocytes, and these in turn drain into cholangioles running between adjacent lobules. The cholangioles drain into branches of the bile duct, and bile is stored in the gall bladder.

Within the hepatocyte, the mitochondria are involved with metabolism in the tri-carboxylic acid cycle, catabolism of fatty acids, oxidative phosphorylation, and the synthesis of urea and haem. Rough endoplasmic reticulum is concerned with the synthesis of enzymes, and also of proteins for extracellular utilisation, and the Golgi apparatus is concerned with their secretion. The purpose of smooth endoplasmic reticulum is the synthesis of cholesterol and its subsequent metabolism to bile salts, the conjugation of bilirubin, and the metabolism of steroids and other drugs.

Function of the liver

The functions of the liver are so numerous and complex that it is not possible or desirable to consider them in detail in this text. However, a brief overview would be an advantage for the understanding of later work. The functions mainly relate to metabolism and storage of substances in the body.

The liver is involved in the metabolism of carbohydrates, and is responsible for the conversion of glucose to glucuronic acid and for the synthesis of glycogen. It is also responsible for the conversion of fructose and galactose from the diet to glucose, and is the site of gluconeogenesis, occurring as a response to low serum glucose concentration.

The metabolism of fat may occur in most cells in the body, but will occur more rapidly in the liver. Specifically, fatty acids undergo ß -oxidation; lipoproteins, cholesterol and phospholipids are formed; and large quantities of carbohydrate and proteins are converted to fat, the majority of which is exported to adipose tissue for storage.

Amino-acids are transaminated and deaminated to ∝-keto acids and ammonia. Virtually all of the plasma proteins, including ferritin and the coagulation factors, are synthesised in the liver, with the exception of part of the ♂ globulins that are formed by cells in lymphoid tissue. In addition, urea is synthesised as a means of removing ammonia from the body.

Liver acts as a store for protein, fat, and carbohydrate, all of the fat soluble vitamins (A, D, E, and K), and the water soluble folic acid and cyanocobalamin. The liver is also the main storage area of iron in the body in the form of ferritin.

Cholesterol is converted to either cholic acid, or chenodeoxycholic acid, which are further converted into the bile salts. Bile salts are temporarily stored in the gall bladder, and delivered to the gastrointestinal tract to emulsify dietary fat and aid the absorbtion of fatty acids, glycerides and lipids through formation of soluble micelles.

Hepatic Damage

Damage to the liver can be caused either by disease or by drugs and toxins.

Jaundice is seen as a yellowing of the skin and tissues, usually caused by large quantities of bilirubin in the extracellular fluid. The bilirubin may be free or conjugated, depending upon its origin.

Haemolytic jaundice is associated with an increase in free, unconjugated bilirubin that is released from damaged red blood cells. There is no detrimental effect on the function of the liver except that it is unable to cope with the large influx of free bilirubin.

Obstructive jaundice can be caused by obstruction of the bile duct or by damage to liver cells. Bilirubin is produced at a normal rate, but cannot pass from the liver to the intestines. The obstruction may be caused extrahepatically by occlusion of the bile ducts by gall stones, or carcinoma of the head of the pancreas; or intrahepatically by lesions of the biliary canaliculi. In addition to an increase in serum conjugated bilirubin, there is an increase in serum alkaline phosphatase, cholesterol and bile salts. Accumulation of bile in the liver may lead to impairment of hepatocellular function, destruction of hepatocytes, and efflux of intracellular contents to the serum.

Viral hepatitis may occur in two forms : the A type, that occurs in unhygenic conditions and is spread by contaminated food and water; and the B type, that is transmitted by blood products and sexual contact. Both may be of variable severity and duration, although the B type carries a greater mortality. In the early phase, serum liver enzymes are raised, followed by cholestasis caused by a compression of the bile ducts. In the later phase, hepatocyte function is compromised leading to significantly raised serum liver enzyme and bilirubin levels. In the severe manifestations, there is hepatic necrosis and development of fibrosis, associated with the loss of intracellular contents to the serum.

Cirrhosis is characterised by the destruction of hepatocytes that are then replaced with fibrous connective tissue. It can be caused by viral hepatitis, biliary obstruction, hepatotoxic agents, malnutrition, congestive cardiac failure, and extensive long term alcohol abuse. There may be regeneration of parenchymal tissue, but the structure is disorganised and leads to functional insufficiency. Serum liver enzyme levels are raised, and in the advanced stages synthetic capabilities are impaired, including the production of blood clotting factors.

Hepatic failure may be acute, resulting from poisoning with a hepatotoxin, or viral hepatitis, or it may be a chronic condition. In the latter case it is often not noticed until the reserve capacity of one aspect of hepatic function has been exceeded. Serum liver function tests are elevated and synthetic capabilities impaired. In addition serum ammonia may increase to toxic levels that result in neurological complications.

Impairment of fat metabolism (e.g. in diabetes) will result in a 'fatty liver', where fat is deposited within hepatocytes. Hepatotoxins and viral hepatitis may increase the concentration of fat by interfering with the relevant metabolic pathways. Changes in serum liver enzyme levels are not observed until sufficiently advanced to cause hepatic necrosis.

Drug Induced Hepatotoxicity

Toxic reactions due to drugs or chemicals may be of two broad types: those that are predictable and arise from direct hepatocellular toxicity; and those that are idiosyncratic, and are thought to involve a hypersensitivity reaction.

Idiosyncratic hepatotoxic reactions only occur with a low incidence, are not dose dependent, and are often associated with a more generalised hypersensitivity reaction. The form that hepatic injury may take can be either cholestatic or hepatocellular, and in some cases is not confined to one or the other.

Intrahepatic cholestatic reactions may arise from simple plugging of biliary canaliculi and result in deposition of bile in the surrounding tissue. There is often no inflammatory damage, and no infiltration of the portal zones. If the drug is withdrawn the biliary system will return to normal. Serum liver enzyme levels remain normal, but serum bilirubin is often raised. The type of drugs implicated include methyltestosterone and the oral contraceptives.

Cholangiolitis involves portal cell infiltration of bile, cholestasis and scattered hepatocellular destruction. When drug induced, symptoms are seen between 10 and 21 days after starting the drug, and return to normal gradually after withdrawal. Serum liver enzyme levels may be slightly raised depending upon the extent of damage, and serum bilirubin levels are increased. The drugs often implicated are the phenothiazines, such as chlorpromazine, and some tri-cyclic antidepressants, such as amitriptyline.

Hepatocellular damage may vary in severity from minor impairment with fatty infiltration to necrosis and fibrosis. All serum liver enzyme levels, in addition to bilirubin, are raised to a varying degree. It may occur as part of a generalised hypersensitivity reaction as with gold salts or sulphasalazine, where withdrawal of the drug will lead to recovery of function. Alternatively the reaction may take the form of a hepatitis-like injury, characterised by jaundice evident between 1 and 3 weeks later, for example with halothane.

When taken in overdose, some drugs, such as paracetamol, are hepatic poisons that lead directly to cell death. Evidence of damage, by serum liver function tests, is often not seen until 3-5 days after ingestion. Outcome is dependent upon numerous factors including the time of medical intervention after ingestion, the quantity of paracetamol consumed, and whether the antidote has been administered.

The final category of hepatocellular damage can be seen as a result of hepatic venous obstruction, caused by endophlebitis in the hepatic vein.

Liver Function Tests

Circulating serum levels of certain chemicals and enzymes produced in the liver are often taken to be indicators of hepatic damage. However, other factors will often have a bearing on their concentration in the serum. The following are the most commonly reported liver function tests.

Aspartate transaminase (ASPT), or glutamate oxaloacetate transaminase (GOT), is an intracellular enzyme present in liver, heart and skeletal muscle. In the liver it exists in concentrations up to 9,000 times that of serum, and therefore if hepatic cells are damaged, the release of ASPT into the serum can be easily detected. It is also released, although in smaller quantities, after skeletal muscle exertion and cardiac cell damage.

Alkaline phosphatase (Alk.P.) is a membrane bound enzyme that is excreted in bile. It will appear in greater quantities in the serum in cases of hepatic obstruction, and also in liver cell disease. Alk.P. is present in bone, and elevated serum levels are seen in bone disease such as Paget's disease and osteomalacia. Alk.P. is also produced by the placenta and is consequently elevated in pregnancy.

Gamma glutamyl transferase (ɣ GT) is found in kidneys, pancreas, prostate and liver. It is a membrane bound enzyme that is elevated in chronic alcoholism, hepatitis, cholestasis and cirrhosis. Non-hepatic factors that increase ɣ GT serum levels include carcinoma of the pancreas, gastrointestinal disease, and lobar pneumonia.

Bilirubin is a breakdown product of haemoglobin that is taken up by the liver, conjugated, and excreted in the bile. Factors affecting serum bilirubin levels were discussed earlier. Serum bilirubin levels in haemolytic jaundice rarely reach higher than 85 μmol/L. In hepatocellular jaundice the usual range may be as high as 250-350 μmol/L, and in obstructive jaundice serum levels of 500 μmol/L are not uncommon. The extent of the rise in bilirubin levels is not necessarily proportional to the size of the obstruction.

Prothrombin ratio may be increased in liver cell disease where there is a deficiency in the synthesis of clotting factors. This does not usually occur until hepatic damage is well advanced.

The enzymes alanine aminotransferase (ALT), glutamate (GLDH), sorbitol (SDH), and malate (MDH) dehydrogenase, ornithine carbamoyltransferase (OCT), ß-glucuronidase, and arginase are all elevated in hepatocellular necrosis, but are rarely reported. The same is true of 5'-nucleotidase (5'-NT) and leucine aminopeptidase (LAP) that are elevated in obstructive jaundice.

ASPT, Alk.P, ɣ GT and bilirubin determinations are the most widely used indicators of hepatic damage. They are sensitive, and may be mildly abnormal in 5-10% of the population, most of whom do not have significant liver disease (2). The assay methods are simple, cheap and accurate, giving results that are useful as supportive evidence of the clinical findings of hepatic disease. Their main disadvantage is that changes are neither specific for general liver damage, nor for different types of liver damage, although the pattern of abnormalities may indicate the type of disorder.

Ferritin in Hepatic Damage

Elevation of total serum ferritin has been observed in varying types of liver disorder (3,4,5,6). Recent work has suggested that the increase is primarily due to leakage of intracellular ferritin from damaged cells into the serum. It may prove to be more specific for hepatic damage and provide a means to distinguish between hepatocellular and cholestatic damage. It has also been suggested that changes in the glycation of serum ferritin occur before conventional changes in liver function tests (1).

The human body contains between 45-55 mg/kg body weight of iron, representing approximately 3.15-3.85g in an average 70 kg male. 60-70 % of the total is associated with haemoglobin, 10% is associated with myoglobin, and the remaining 20-30 % is associated with the storage proteins; ferritin and haemosiderin. The amount associated with transferrin, the extracellular transport protein, is negligable at 0.1-0.2 % (7).

The stored iron acts as a reservoir for use in biochemical redox reactions and enzyme systems, where its conversion from Fe(II) to Fe(III) can be utilised.

The storage of iron within the cell is hampered by the toxic effects of free iron, which may lead to lipid peroxidation and ultimately cell destruction. The body has evolved a method of sequestering iron within the storage proteins, ferritin (which is soluble) and haemosiderin (which is insoluble). The exact relationship between the two is unclear, but it is thought that haemosiderin may be a chemically degraded form of ferritin (8,9).

Structure of ferritin

Ferritin consists of a protein shell made up of 24 subunits, surrounding a central core of hydrous ferric oxide phosphate. This is the form in which iron is stored, with a complement of approximately 4,500 atoms in each ferritin molecule. At any given time the system is rarely used to capacity, most molecules carrying some iron (1/3 - 1/2 of maximum) but very few being filled to capacity (10), allowing a functional reserve.

The protein shell, or apoferritin (molecule without iron), has a molecular weight of about 450,000g and is constructed from subunits that fall into two broad categories : L subunits, which are more basic; and H subunits, which are more acidic; having molecular weights of 19,000g and 21,000g respectively (11). The presence of a further subunit, G, apparent molecular weight of 23,000g was noted by Cragg et al (12), but it is thought to be a glycated derivative of the L subunit.

The relative proportion of these units in the complete shell governs the overall properties of the apoferritin or ferritin. Small variations in structure give rise to a heterogeneous population of isoferritins, characteristic of the organs in which they are found. The liver and spleen tend to produce ferritins that are predominantly of the basic L subunit, heart tissue ferritin contains predominantly the acidic H subunit, and the kidney contains a mixture of the two different types. Plasma isoferritins are rich in the L subunits and in the G subunit, and tend to be low in iron content (13).

The subunits fit tightly together to leave six channels running through the coat connecting the interior with its external environment. X-Ray diffraction techniques have estimated the channels to be between 0.8-1.3 nm wide in the crystalline state (10).

Opinion is divided on the abundance of intracellular glycated ferritin, ranging from 0% to 20% (14,15). In the normal population serum ferritin is approximately 60-70 % glycated. There is no evidence to suggest that ferritin is glycated extracellularly after secretion, which suggests that it is glycated during secretion into the serum (13).

Synthesis of ferritin

Ferritin molecules are mainly synthesised on free ribosomes in the cytosol, but it has been estimated that about 20 % of the subunits are made by ribosomes that are attached to the membrane of the endoplasmic reticulum (9,16).

In general, proteins that are synthesised for extra-cellular use are made exclusively on membrane bound ribosomes and secreted when manufactured.

Synthesis is initiated in response to increased levels of free iron, possibly by the following scheme. In normal circumstances, a subunit of ferritin is attached to the initiation site of the ferritin messenger-RNA (mRNA) in the cytosol, thus preventing its combination with ribosomes. The entry of iron into the cell alters an equilibrium between subunits and complete apoferritin molecules toward the completed molecule. This in turn causes a mobilisation of subunits, and the release of

mRNA for ribosome attachment and subsequent synthesis of more subunits. The completed molecules are then available for iron uptake (16).

The induction of ferritin synthesis by iron only applies to the free ribosome fraction, resulting in iron-poor apoferritin.

Incorporation of iron into the apoferritin molecule

Research work suggests that iron gains entry into ready formed apoferritin shells rather than have the protein form around the iron core (11).

Two possible methods for iron entry have been postulated, but neither has been conclusively proved.

The method formulated by Macara et al (17,18) is that iron, in the form of Fe(II) enters the channels in the protein structure with an oxidant species. Fe(II) is bound on the inner surface and is oxidised to Fe(III), with apoferritin acting as a catalyst. Fe(III) is then preferentially bound by carboxylate side chains on the inside of the apoferritin molecule. This acts as a basis for the formation of a microcrystalline iron core where subsequent iron additions can be made without the need for oxidation on the protein shell. In this scheme it is apparent that the surface area available for iron deposition would first increase to a maximum, and then decrease as some of the channels become blocked. Therefore it would be more efficient if ferritin molecules contained an optimum iron content, being neither full nor empty, which appears to be the case.

The second method, also proposed by Macara et al, and modified by Crichton (19), suggests that Fe(II) oxidation and crystallite nucleation occur on separate sites of the shell. A catalytic site binds two Fe(II) atoms with an oxygen molecule in the reaction scheme :

$$2Fe(II) + O_2 \text{ ----> } O_2^{2-} + 2Fe(III)$$

The site has a higher affinity for Fe(II), which displaces Fe(III) that then migrates to a heteronucleation site.

Whichever method is the closer representation, it is clear that iron enters the ferritin molecule in the reduced form, and is stored in the oxidised form.

The mechanism of release is not fully understood, but iron is likely to be removed from ferritin by a reductant; that is a species that is able to reduce stored Fe(III) to Fe(II). If this occurs within the protein shell, then the reducing species must be small enough to penetrate the channels. The principal reductants that have been implicated are $FMNH_2$, a dihydroflavin, and $FADH_2$, dihydroflavin adenine dinucleotide (16), however both are too large for the 0.8-1.3 nm gap. X-Ray diffraction data are collected in the crystalline state, where unusual rigid constraints may be imposed on the molecule. In vivo a more flexible structure can be adopted which would not exclude the entry of either species.

The alternative to penetration is that these species act as coenzymes, binding externally and altering the conformation so that prior oxidation sites may become reduction sites (16,19).

The removal of Fe(III) from the microcrystalline core must be by the 'first in, last out' principle, which has been confirmed by experiments involving radiolabelled iron atoms (20).

Clearance of ferritin from plasma is by uptake into the liver, and although the mechanism is unknown it may be related to a specific receptor site (21,22). The overall half-life for ferritin species in serum is in the region of 30 hours, which can be broken down into a half-life of 5 hours for non-glycated ferritin and 50 hours for glycated ferritin (23). Iron content has no effect on the rate of clearance, so that ferritin and apoferritin are the same in this respect (24). An explanation for the more rapid removal of non-glycated ferritin from serum is that the removal of carbohydrate may expose galactose residues, that are preferentially taken into the liver (25).

Factors affecting serum ferritin levels

Serum ferritin is a reliable indicator of body iron stores, showing a good correlation between log serum level and storage iron per kg body weight assessed by other methods (26). Normal

ranges have been documented between 15-300 μg/l, usually higher for men than for women up to the age of 65 (13,27,28).

Absolute ferritin levels can be altered by pathological changes in the body. Inflammation and infection lead to increased retention of iron in the reticuloendothelial system. It is probable that under these circumstances ferritin will act as an acute phase protein, and there will be a direct stimulus to increase its rate of synthesis (15,29).

Iron deficiency anaemia will be accompanied by a low serum ferritin level, that is indicative of reduced serum iron stores. Conversely, iron overload will be accompanied by an increase in serum ferritin (30,31).

Malignancy can also result in an increase in serum ferritin levels, either because of an anaemia with iron accumulation in reticuloendothelial cells, because of tissue necrosis and release of ferritin from damaged cells, or because of a compromised mechanism in the hepatic clearance of serum ferritin (13).

The factors affecting total serum ferritin levels may have no effect on the % glycation, however it would be expected that situations that result in hepatocellular damage would elicit a low % glycated serum ferritin, whereas cases of increased synthesis would result in a normal % glycation.

It is possible to distinguish between intracellular, non-glycated ferritin, and the extracellular glycated species by incubation with Concanavalin A (ConA), a plant lectin that specifically binds to glycated residues (14,32). The resultant extract can be assayed for ferritin content using radioimmunoassay techniques, and the relative proportions of the two species calculated.

The normal range for % glycated ferritin in serum has been documented between 60-70 %, and significantly lower with hepatocellular damage (14,33). It is important to assess the value of serum ferritin as an early marker for hepatic damage with consideration of how ferritin reacts in different disease states, and with potentially hepatotoxic drugs.

AIMS AND OBJECTIVES

The concept of this work originated from an observation made while investigating serum and synovial fluid ferritin changes in Rheumatoid Arthritis. One patient, treated with the disease modifying agent sulphasalazine, developed an idiosyncratic hepatotoxic reaction. On review of biochemical measurements, an increase in total serum ferritin together with a reduction in % glycation was evident chronologically before clinical signs of hepatotoxicity, or changes in conventional liver function tests (1). These observations led to the main aim of this work, namely to determine whether changes in serum ferritin parameters could be a novel means of monitoring hepatic damage.

The initial observation was recorded in only one subject, and although there are reports of similar changes associated with both chronic (3) and acute (6) hepatocellular damage, there has been no study of possible applications as an indicator of hepatic damage.

The measurement of total serum ferritin by radioimmunoassay is well established, but the separation of the glycated from the non-glycated form of ferritin is not widely employed. Therefore it was necessary to investigate the reliability and reproducibility of the assay in order to validate the methods used.

It was important to eliminate any artefacts that could have been introduced as a result of either collection or storage of subject material, so a part of this work was devoted to investigation of these conditions.

There are reports in the literature regarding normal physiological variation in total serum ferritin (34,35), but no reports on variation in % glycation. This would be of relevance in the use of serum ferritin parameters as an indicator of hepatic damage if significant variation existed.

It was necessary to investigate the specificity of the test by consideration of other pathological conditions that had no hepatic involvement. It was conceivable that there would be abnormal serum glycated ferritin levels in sufferers of diabetes mellitus, a disease associated with abnormal

glycation of serum proteins. Similarly, ferritin is also present in cardiac tissue, which may then be released from damaged cells following a myocardial infarction. Changes in total and % glycated serum ferritin have previously been reported in cases of systemic infection (15,36), which may prove relevant in assessing the specificity of the test.

If the test is specific, reliable and reproducible, it would have a useful application in the monitoring of potentially hepatotoxic drugs. Current monitoring methods include changes in serum liver enzyme levels and liver biopsy (2). The former is non-specific, and the latter carries a definite morbidity. There is a suggestion that ferritin changes are more sensitive and it is against the measurement of serum liver enzyme levels that the usefulness of changes in ferritin must be evaluated.

MATERIALS

Chemicals And Reagents

The following were required for the glycated protein extraction process:

a) Concanavalin-A (ConA) Sepharose 4B gel, No. C-9017, containing approximately 14mg ConA /ml packed gel suspended in 0.1M acetate buffer, pH 6.0; 1M NaCl, 1mM of each of $MnCl_2$, $CaCl_2$, $MgCl_2$, with 0.01% thiomersal as a preservative.

b) Sepharose 4B-200 gel. Bead size in swollen state : 60-140 μM. Aqueous suspension with 0.01% thiomersal and 0.1% dimethoxane as preservatives. Agarose concentration : 4%. Exclusion limit of an average molecular weight of 20×10^6 g.

Both were supplied by Sigma Chemical Company, Poole, Dorset, England.

c) Barbitone buffer:

Sodium barbitone: 50 mmol/l

Sodium chloride: 500 mmol/l

Sodium azide: 3.1 mmol/l

adjusted to pH 8.0 with concentrated hydrochloric acid.

The solution was used within two weeks of preparation. All chemicals were obtained from BDH Chemicals Ltd, Poole, Dorset, England, and were of analytical grade.

In addition, sterile sodium chloride 0.9% w/v (Normasol) was used, supplied by Travenol, Thetford, Norfolk, England.

Ferritin ^{125}I Radioimmunoassay.

This assay was performed using Ferritin ^{125}I Radioimmunoassay kits, supplied by Becton Dickinson & Co., Cowley, Oxford, and an LKB 1280 Ultrogamma gamma counter.

The kit consisted of:

Seven standard solutions containing ferritin in the concentrations of 0, 5, 10, 25, 75, 200, and 500 μg/l.

^{125}I-Ferritin

Ferritin antiserum (rabbit) I -for high end sensitivity

Ferritin antiserum (rabbit) II -for low end sensitivity

Precipitating antiserum solution

The ferritin standards were supplied in 0.1M phosphate buffer containing human serum albumin with 0.02% sodium azide and other preservatives.

The ferritin tracer was supplied in 0.1M phosphate buffer containing 370 kBq ferritin ^{125}I per 75 ml and bovine albumin, normal rabbit serum, 0.02% sodium azide, other preservatives and blue dye.

The ferritin antisera (rabbit) were supplied in 0.1M phosphate buffer containing human serum albumin and 0.02% sodium azide, other preservatives and dye, yellow for high end sensitivity, and red for low end sensitivity.

The precipitating antiserum was supplied in 0.1M phosphate buffer containing goat anti- rabbit antiserum, precipitating aids, human serum albumin and sodium chloride with 0.02% sodium azide and other preservatives.

Glucose determinations were conducted using a Glucose Auto and Stat GA1120 machine marketed by Kyoto of Japan. Anhydrous dextrose was supplied by Evans Medical Ltd, Horsham, England.

Alcohol determinations were conducted using a Perkin Elmer 8310 Gas Chromatograph. The reagents, absolute alcohol and propan-2-ol were obtained from BDH Chemicals Ltd.

Alkaline phosphatase (Alk.P.) and aspartate transaminase (ASPT) concentrations were determined using a Vickers SP120 Continuous Flow Multichannel Autoanalyser. Gamma-glutamyl transferase (γ-GT) concentrations were assayed using a Technicon RA-1000. These determinations were conducted as part of the routine clinical chemistry in the Biochemistry Department of Selly Oak Hospital, Birmingham.

The pH meter used was an Orion Research 811 microprocessor pH/millivolt meter, calibrated before use with colour key buffer solutions of pH 7 +/- 0.02, and pH 10 +/- 0.02 at 20° C, supplied by BDH Chemicals Ltd, Poole Dorset, England.

Pipettes used were of four different types :

glass Pasteur pipettes with rubber bulbs

glass 10ml pipettes, class B.

positive pressure displacement pipettes calibrated to 500 μl and 200 μl supplied by Gilson, France.

autopipette supplied by Eppendorf, Hamburg, W.Germany.

Tubes used were:

Sarstedt monovette tubes and needles, Numbrecht, W.Germany.

64 x 11mm round bottom tubes, supplied by Biomedical Laboratory Supplies, Birmingham, England.

30ml universal tubes, supplied by Sterilin, England.

Other items of equipment used were :

A 30/160g 0.01/0.1mg electronic balance, supplied by Sartorius, W.Germany.

A magnetic stirrer and thermostat hotplate, supplied by Gallenkamp, England.

A heated water bath, supplied by Grant Instruments, Cambridge, England.

A rotamixer, supplied by Hook and Tucker Ltd, England.

An Indesit freezer deluxe deep freeze.

A Lec super deluxe refrigerator.

A Heraeus Christ centrifuge, supplied by VA Howe, London, England.

Two blood tube rotators, supplied by Eschmann, Shoreham, Sussex, England, and Jencons (Scientific) Ltd, Leighton Buzzard, Bedfordshire, England.

Micro-touch rubber gloves, supplied by Surgicos Ltd, England.

Volumetric flasks.

METHODS

Specimen Collection

Venous blood was collected from patients and volunteers in a plain tube, containing neither heparin nor EDTA.

All samples from patients were collected at a similar time of day.

The blood was allowed to clot, then serum separated from packed cells by centrifugation at >3,000 rpm for 5 minutes, and stored at -20° C.

Serum samples taken for routine clinical investigation by medical and biochemistry staff were stored at 4° C after separation for up to one week, and then frozen.

Samples obtained from the Regional Poisons Unit at Dudley Road Hospital from cases of paracetamol overdose were stored at 4° C as whole blood for up to two weeks before separation and storage.

Prior to assay or extraction samples were allowed to thaw at room temperature and gently mixed by hand.

Procedure for extraction of glycated proteins

Based on the method of Worwood et al (14).

Concanavalin A (ConA) is a lectin isolated from the jack bean, that specifically binds to α-D-glucopyranose, α-D-mannopyranose, α- and ß-D-fructofuranose and α- and ß-D-arabofuranose or to polysaccharides that have these residues in a terminal position (32,37).

Many serum proteins contain mannose, and hence bind to ConA. If the ConA is in turn bound to an inert gel such as sepharose, then the glycated proteins are retained in the gel matrix and the non-glycated proteins remain in the liquid phase after a suitable period of incubation. The binding

proceeds rapidly during the first 30 minutes, is complete by about 90 minutes and stable for at least 3 hours.

The liquid phase can be separated from the gel by centrifugation and assayed for non-glycated ferritin. Concurrently, an identical sample is incubated with plain sepharose gel as a control. The supernatant from this extraction will give an estimate of total ferritin in the sample. It will also allow for any non-specific binding of ferritin to the gel matrix.

The percentage of ferritin that is glycated can be calculated from the quantities of non-glycated and total ferritin using the equation :

$$\%\text{glycated ferritin} = 1 - \frac{\text{non glycated ferritin}}{\text{total ferritin}} \times 100$$

Preliminary washing of gel

The procedure applies to both Concanavalin-A and the Sepharose 4B control.

Barbitone buffer was added to packed gel in the proportion of 3 volumes of buffer to 1 volume of gel.

Gel and buffer were mixed well, and then separated by centrifugation at 3,000 rpm for 5 minutes.

The supernatant was decanted from the packed gel and discarded.

The process was repeated a further four times.

The gel was finally resuspended to twice the volume of packed gel with barbitone buffer, which was accurately measured using a graduated pipette.

Extraction process

Sample tubes were numbered consecutively, the active gel (ConA) in odd numbers, with control sepharose in even numbers.

Two 0.5ml serum samples were placed in separate paired tubes. 1ml of ConA gel suspension was then added to one of the pair of samples, and 1ml of Sepharose suspension was added to the other sample. Both suspensions consisted of 0.5ml of packed gel with 0.5ml of barbitone buffer. In order to prevent saturation of the binding sites of the gel, samples were previously assayed for ferritin content, and then diluted if necessary with 0.9% w/v sodium chloride solution to produce a ferritin content in the range of 50-200 μg/l.

Volumetric measurements were made with a standard Gilson displacement pipette.

The samples were then incubated on a roller mixer for 2 hours at room temperature to allow thorough mixing and binding to take place between glycated residues and ConA.

After incubation samples were centrifuged for 15 minutes at 3,000 rpm to separate the gel from the supernatant.

Using a pasteur pipette,the supernatant was carefully removed without disturbing the sediment, and then stored at -20° C until assayed.

Samples were allowed to thaw at room temperature before assay. They were mixed gently, then their ferritin content determined by radioimmunoassay.

Procedure for radioimmunoassay

A standard curve was prepared by addition of 200 μl of different concentrations of ferritin, between 1-200 μg/l, to duplicate tubes. Samples were placed in further tubes to be assayed in duplicate.

700 μl aliquots of ferritin ^{125}I tracer were added to all sample tubes, and a pair of empty tubes for determination of background radioactivity. In addition the same volume of tracer was added to a further pair of empty tubes to be used for estimation of total radioactivity count.

100 μl of ferritin antiserum (rabbit) was then added to all tubes except those set aside for total count and the pair for background radiation count.

All tubes (except the pair for total count) were mixed well and incubated at 37° C +/- 1° C for 90 minutes.

Following incubation, the precipitating antiserum (goat anti-rabbit) was thoroughly mixed and 500μl was added to each of standard solutions and samples, which were then vortexed.

The resultant solutions were centrifuged at > 1,000g for 15 minutes either at room temperature or in the cold.

They were then immediately decanted and supernatants discarded from all tubes. Care was taken to wipe away any excess liquid with absorbent paper.

Tubes were counted in sequence for 60 seconds on a gamma counter. Total count was ideally between 25,000-40,000 counts per minute.

Table 1 : Preparation of standard curve for ferritin radioimmunoassay

Ferritin μg/l	Ferritin ^{125}I tracer μl	Antiserum μl	ppt.antiserum μl
Total count	700	nil	nil
Background	700	nil	500
0	700	100	500
1	700	100	500
5	700	100	500
10	700	100	500
25	700	100	500
75	700	100	500
200	700	100	500

There is no commercially available standard solution containing 1μg/l of ferritin. This strength was achieved by a 1 in 5 dilution of 5μg/l with ferritin 0 μg/l standard solution.

All radioisotope work complied with the South Birmingham Health District guide-lines.

Calculation of results

Logit-log standard curve

Tube 1 duplicates : Total count

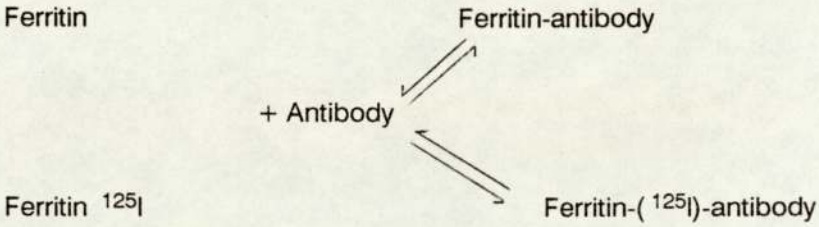
Tube 2 duplicates : Background count (contains no antiserum)

Tube 3 duplicates : Blank, containing no ferritin and is a measure of the maximum quantity of ferritin, in the form of ^{125}I tracer, able to be bound by the antibody.

Tubes 4-9 in duplicate : Standard human liver ferritin concentrations from 1-200 μg/l as detailed.

Subtract background count from all tubes.

Principle of radioimmunoassay



From the scheme above (28), in the pair of blank tubes, all ferritin-antibody complexes will be in the form of ferritin-(^{125}I)-antibody as no exogenous ferritin has been added. The radioactivity in the complex is therefore the maximum radioactivity that can be bound to the antibody, and hence represents the maximum binding capacity of the antibody. This is expressed as a percentage of the total count and called Bo, the trace binding, calculated from the equation (1) :

$$Bo = \frac{\text{average corrected count for blank samples}}{\text{average corrected total count}} \times 100$$

Addition of exogenous ferritin in the form of either standards or sample will compete with ferritin ^{125}I for the available binding sites on the antibody. The corresponding reduction in radioactivity will be proportional to the amount of free ferritin added.

This can be quantified by expressing B, the binding of free ferritin, as a percentage of the total count, demonstrated in the equation (2) :

$$B = \frac{\text{average corrected count for sample tubes}}{\text{average corrected total count}} \times 100$$

The ratio B/Bo represents the percentage of total available binding sites occupied by free ferritin. Division of equation 2 by equation 1 yields :

$$\frac{B}{Bo} \times 100 = \frac{\text{average corrected count for sample tubes}}{\text{average corrected count for blank samples}} \times 100$$

$$= \% \text{ trace binding}$$

If % trace binding is calculated for each standard and plotted along the logit axis against concentration on the log axis, the result should be a straight line calibration curve (Appendix 1).

The process was repeated for each sample and ferritin concentrations interpolated from the graph.

Alternative method

Plot uncorrected counts per minute of the standard samples on a linear axis against ferritin concentration on semi-log paper. This should produce a sigmoid curve. Interpolate sample results directly from the curve (Appendix 1).

RESULTS

Laboratory results

Quality Control Data

Precision: Coefficient of variation (CV)

$$CV = \frac{\text{standard deviation (sd)}}{\text{mean}} \times 100$$

Intra-assay variation

Ten 0.5ml aliquots of pooled serum were incubated with ConA and ten with Sepharose according to the methods documented.

Ferritin content of the twenty supernatants was assayed in duplicate. The duplicates were averaged and used to calculate separate coefficients of variation for both ConA and Sepharose. The percentage glycation was also calculated for each of the ten aliquots and these results were used to calculate a further coefficient of variation representative of percentage glycation.

In addition, ten separate serum samples (five pairs) were assayed for total ferritin content, and cv calculated (Table 2).

Table 2 : Intra-assay variation of gel extraction and ferritin determination.

	mean ferritin content (μg/l) +/-sd	number of samples	CV (%)
Serum	57.8 +/- 2.8	5 pairs	4.8
ConA	22.2 +/- 1.8	10 pairs	8.1
Sepharose	48.5 +/- 3.8	10 pairs	7.8
% glycated	54.0 +/- 4.3	10 pairs	8.0

Inter-assay variation

Two 0.5ml samples of pooled serum were incubated with ConA and two with Sepharose according to the documented methods. The supernatants were assayed in duplicate for ferritin content and mean values calculated. This process was repeated on five separate occasions, and the results used to calculate inter-assay coefficients of variation for ConA and Sepharose samples, and a further coefficient of variation for % glycated ferritin (Table 3).

Table 3 : Inter-assay variation of gel extraction and ferritin determination.

	mean ferritin content (μg/l) +/- sd	number of samples	CV (%)
ConA	24.9 +/- 3.1	6 x 2 pairs	12.4
Sepharose	41.3 +/- 6.2	6 x 2 pairs	15.0
% glycated	39.1 +/- 6.4	6 x 2 pairs	16.3

The serum pools used for inter- and intra- assay CV were of different composition.

Discussion

The coefficients of variation calculated represent the variability of the whole process from extraction of glycated proteins to assay of ferritin content of those extracted portions.

The manufacturers of the Ferritin ^{125}I Radioimmunoassay Kit, Becton Dickinson, quote an inter-assay CV of between 5.7-11.4 % (mean = 8.0, sd = 2.2) obtained from six different serum pools assayed between 8-20 times, each time with a different operator and different batch of reagent.

The published figures for intra-assay CV are between 3.5-8.5 % (mean = 5.6, sd = 1.9) obtained from five different serum pools,each assayed 15 times (28).

The precision data quoted by the manufacturers of the radioimmunoassay kit was calculated from the assay of serum ferritin samples. The precision data calculated for this experiment included an extraction stage, so that the coefficient of variation represents the dual process of extraction and assay, compared with the assay alone. An intra-assay variation of 4.8% was calculated under the same laboratory conditions and compared favourably with the data published by Becton-Dickinson. It was to be expected that introduction of a further stage in the assay would result in a greater variability which was understandably more pronounced for the inter-assay figure.

In most circumstances, healthy and pathological samples that were related in a series were assayed as a single batch to reduce experimental variation. On the rare occasions that single members of a series had to be re-extracted and re-assayed the results were annotated as such.

Sample Storage

A sample of pooled serum from subjects undergoing routine biochemical investigations was divided into twenty-four aliquots. Eight were set aside for assay of total serum ferritin, eight were incubated with ConA, and eight with sepharose. The supernatants from the ConA and Sepharose gel extractions were separately re-pooled to ensure that all were identical, then divided into volumes of approximately 1ml.

One sample of each of serum, ConA, and Sepharose extracts were assayed immediately for ferritin content and % glycation calculated. The remaining samples were frozen and stored at -20° C. Two samples of each were allowed to thaw at room temperature, gently mixed, then assayed in duplicate after 2, 5, 8, 12, and 24 weeks. The results are expressed in table 4.

Table 4 : To show the effect on total and % glycated ferritin of storage of samples at -20° C over a six month period.

Time stored (weeks)	Ferritin (μg/l)			%glycation
	Serum	ConA fraction	Sepharose fraction	
0	98	30.0	43.5	31.0
2	90	24.0	39.5	39.2
5	90	25.8	41.0	37.1
8	86	21.8	39.0	44.2
12	87	30.5	51.0	40.2
24	98	17.5	30.2	42.1

Serum ferritin: mean = 91.5, standard deviation = 5.28, cv = 5.77 %

% glycated ferritin: mean = 39.0, standard deviation = 4.60, cv = 11.8 %

Stability of sample

A sample of each of serum, ConA, and Sepharose extracts was allowed to thaw at room temperature, then immediately re-frozen. This cycle was repeated eight times in less than eight hours. Ferritin content was then determined, % glycation calculated, and compared with baseline levels. The results are presented in table 5.

Table 5 : To show the effect of a vigorous freeze/thaw cycle on total and % glycated ferritin of a pooled sample.

	Ferritin Concentration μg/l			% glycation
	serum	ConA fraction	Sepharose fraction	
control	98	30.0	43.5	31.0
freeze/thaw	96	26.0	37.0	29.7

There does not appear to be any difference between both the total serum ferritin and % glycated ferritin of the samples that have undergone a freeze/thaw cycle and the control. Therefore one can conclude, in agreement with published work (38), that ferritin is a robust protein, and unlikely to denature following a single freeze and thaw.

The effect of sample haemolysis on % glycated ferritin

A 30ml sample of blood was taken and divided into three 10ml portions. One of these portions was allowed to clot, then centrifuged to separate cells from serum which was stored at -20° C (Sample A, Table 6).

The second sample was allowed to stand for twenty-four hours at room temperature before centrifugation, and serum stored at -20° C (Sample B, Table 6).

The third sample was allowed to clot, then frozen immediately, to be stored as whole blood (Sample C, Table 6).

All samples were retrieved from storage, allowed to thaw, and gently mixed. The sample stored as whole blood was centrifuged to separate cells from serum. 0.5ml of each sample was extracted with ConA and Sepharose gels by the prescribed method. The resultant extracts were assayed for ferritin content, and % glycation calculated. The results are presented in Table 6.

Table 6 : The effect of sample haemolysis on % glycated ferritin

	ferritin concentration (μg/l)			% glycated
	Serum	ConA fraction	Sepharose fraction	
Sample A	87	11.2	25.2	55.6
Sample B	83	9.8	26.6	63.2
Sample C	-	15.9	40.0	60.3

To investigate the effects of allowing the samples to haemolyse more completely, further blood samples of 20ml were taken on two separate occasions. In each case one 10ml sample was allowed to clot, then centrifuged to separate cells from serum, which was then stored at -20° C for three weeks.The other sample was stored as whole blood at 4° C for three weeks.

After the time period had elapsed the whole blood was centrifuged, and serum thawed. 0.5ml of each was extracted in duplicate with ConA and Sepharose gels. The extracts were assayed for ferritin content, and % glycation calculated. The results are presented in Table 7.

Table 7 : The effect of haemolysis on total and % glycated ferritin

	ferritin concentration (μg/l)			% glycated
	Serum	ConA fraction	Sepharose fraction	
Sample 1				
Control	71	12.3	29.2	57.9
Haemolysed	105	20.8	34.5	39.7
Sample 2				
Control	84	10.9	32.0	65.9
Haemolysed	96	17.0	36.5	53.4

Dilution Experiments

The purpose of the dilution experiments was to investigate whether changes in sample volume added to the gels for extraction resulted in any variation in apparent % glycation. A standard volume of 0.5ml was used for all samples, but it may have been necessary to use less if only small samples were available, or more if the sample contained a low endogenous ferritin level.

Different volumes of a pooled serum sample were extracted with a standard volume of ConA and Sepharose gels. The volumes used were 0.1-1ml in 0.1ml increments. All samples were made up to a constant volume of 2ml with barbitone buffer. The resultant extracts were assayed for ferritin and % glycation calculated. The results are displayed in Table 8.

Table 8 : To show the effects of different sample volume on total and % glycated serum ferritin

sample volume	buffer volume	ferritin (μg/l)				% glycation
(ml)	(ml)	ConA	Average	Sepharose	Average	
1.0	0	38.0 36.5	37.25	49.0 45.0	47.0	20.7
0.9	0.1	30.0 25.0	27.50	42.0 45.0	43.5	36.8
0.8	0.2	26.0 24.2	25.10	42.0 40.0	41.0	38.8
0.7	0.3	25.0 23.7	24.35	42.0 43.0	42.5	42.7
0.6	0.4	(13.0 28.5)	(20.75)	(11.5 32.5)	(22.0)	(5.7)
0.5	0.5	14.0 11.2	12.60	31.0 29.0	30.0	58.0
0.4	0.6	11.7 11.7	11.70	22.0 23.0	22.5	48.0
0.3	0.7	(7.0 3.8)	(5.40)	19.5 17.5	(18.5)	(70.8)
0.2	0.8	5.8 5.3	5.55	10.5 13.5	12.0	53.8
0.1	0.9	1.9 1.8	1.85	(5.5 10.5)	(8.0)	(76.9)

( ) -denotes figures eliminated because of poor reproducibility within a pair of duplicates.

The experiment was repeated with a different serum pool, and the results are shown below in Table 9.

Table 9 : To show the effects of different sample volume on total and % glycated serum ferritin

sample volume	buffer volume	ferritin (μg/l)				% glycation
(ml)	(ml)	ConA	Average	Sepharose	Average	
1.0	0	74.0 75.0	74.5	80.0 85.0	82.5	9.7
0.9	0.1	52.0 52.0	52.0	(63.0 53.0)	(58.0)	(10.3)
0.8	0.2	51.0 50.0	50.5	74.0 68.0	71.0	28.9
0.7	0.3	52.0 53.0	52.5	68.0 61.0	64.5	18.6
0.6	0.4	44.0 44.O	44.0	65.0 62.0	63.5	30.7
0.5	0.5	41.0 40.0	40.5	54.0 60.0	57.0	28.9
0.4	0.6	35.0 33.0	34.0	46.0	46.0	26.1
0.3	0.7	22.5 25.5	24.0	27.0 30.0	28.5	15.8
0.2	0.8	25.5 19.5	22.5	(23.5 37.5)	(30.5)	(26.2)
0.1	0.9	5.0 5.0	5.0	(13.4 20.0)	(16.7)	(70.0)

( ) -denotes figures eliminated due to poor reproducibility within a pair of duplicates.

Discussion

The overall view of the storage data would suggest that there was no tendency for the measured values of either total serum ferritin or % glycated ferritin to change as storage time progressed.

Calculation of coefficients of variation of the data resulted in figures of 5.8% and 11.8% for total serum ferritin and % glycated ferritin respectively. Neither figure exceeded the cv of the assay.

Statistical analysis using the Pearson correlation coefficient yielded figures of $r = 0.21$ and $r = 0.61$, with 4 degrees of freedom (df.4), for total serum ferritin and % glycated ferritin respectively.

At a level of $p = 0.05$, $r = 0.811$ (df.4), which in both cases exceeds the correlation coefficient obtained, and suggests that neither are significantly different from the situation where there is no correlation between storage time and total serum ferritin, or % glycated ferritin.

However, the square of the correlation coefficient can be regarded as the percentage of variation that can be related to a different length of time in storage. $r^2 = 0.04$ (4%) for total serum ferritin, and 0.37 (37%) for % glycated ferritin. The relatively large degree of variation related to time of storage for figures of % glycated ferritin would be relevant in cases where an extract had been assayed, then repeated after a period of storage of up to six months. This did not occur during the course of practical work.

The vigorous freeze/thaw cycle to which samples of serum ferritin and ferritin extracted with ConA and Sepharose gels were exposed, was designed to test the stability of the protein. If a marked decrease in levels were observed when compared with initial measurements, this would suggest that the protein had denatured during the experiment. It was not possible to obtain meaningful results from statistical analysis because of the small sample size, but there did not appear to be any noticeable difference, that could not be attributed to the assay method, between the values obtained after the freeze/thaw cycle and the initial control values. The results do not contradict the work of Smith-Johannsen and Drysdale, who suggested that ferritin was extremely resistant to denaturation by heat and chemical attack (39).

The results of the experiment to determine the influence of haemolysis showed an average increase of 32.5% in total serum ferritin measurements and with an average decrease of 25% in % glycated ferritin in samples from haemolysed blood compared with control values.

The reduction in % glycated ferritin could be due to either a genuine reduction in the quantity of glycated ferritin, caused by enzymic degradation or oxidation reactions, or an increase in the amount of non-glycated ferritin relative to glycated ferritin. The latter explanation is supported by the increase in total serum ferritin, suggesting that intracellular non-glycated ferritin could be released into serum from haemolysed, disrupted blood cells. It has been previously demonstrated

that erythrocytes and leucocytes contain acidic and basic ferritins, although their glycation status has not been clarified (40,41).

The practical application for these observations occurred with some haemolysed blood samples from paracetamol overdoses received from the Regional Poisons Centre at Dudley Road Hospital. It was important when analysing the overdose data to bear in mind that some ferritin changes may have been due to sample haemolysis. All samples assayed that were haemolysed were annotated as such.

In the experiment carried out to determine any influence of sample volume on the result of % glycated ferritin estimation there appeared to be a trend towards higher glycated ferritin values from smaller volumes of sample. This was not confirmed statistically, resulting in a Pearson correlation coefficient of $r = -0.28$ (df5), $p = 0.754$, and therefore not significant. r^2 , the percentage of variability caused by variation in sample volume = 0.078 (7.8%).

Repetition of the experiment yielded a straight line on graphical evidence, with a correlation coefficient of $r = 0.31$ (df5), $p = 0.754$, and therefore not significant. $r^2 = 0.096$ (9.6%).

The results confirm that different volumes of sample may be used with no adverse effect on apparent % glycated ferritin. However, in most cases the standard volume of 0.5 ml was used.

The Effect Of Glucose On The Binding Capacity Of ConA Gel

Glucose extraction

The purpose of this work was to investigate the specific and non-specific binding of glucose to the ConA and Sepharose gel matrices. Specific binding of glucose occurs when the molecules bind to a specific receptor for glucose or glycated residues. Non-specific binding occurs when the molecules bind to, or become trapped within, the gel matrix.

Standard solutions of glucose in barbitone buffer were prepared as detailed below.

1.8g of anhydrous dextrose (Evans Medical) was dissolved in 20ml of freshly prepared barbitone buffer to give a stock solution of 500mmol/l. This solution was used to produce further dilutions of 100, 50, 25, 10, 5, 1, 0.5, and 0.1 mmol/l of glucose in barbitone buffer.

In addition two standard solutions of 20mmol/l of glucose in barbitone buffer and distilled water were prepared by adding 20ml of buffer or water respectively to 144mg of anhydrous dextrose.

Two aliquots of 0.5ml of each solution were added to 1ml of ConA and Sepharose gel suspensions, together with 0.5ml of barbitone buffer to yield a total volume of 2ml. (0.5ml sample, 0.5ml buffer, 0.5ml packed gel, and a further 0.5ml buffer). The amount of glucose (mg) added to each gel suspension was calculated. The supernatants were assayed for glucose concentration using the Glucose Auto and Stat GA 1120 apparatus. From this the quantity of glucose present in the 1.5ml aqueous phase and hence the quantity of glucose remaining in the gelatinous phase was calculated. The results are detailed in Tables 10, 11, 12 and 13.

Table 10 : To show the different concentrations of glucose after incubation with ConA gel

initial glucose conc. (mmol/l)	amount of glucose added to gel (mg)	measured glucose conc. in extract (mmol/l)	amount of glucose in aqueous phase (mg/1.5ml)	amount of glucose in gel phase (mg/0.5ml)
0	0	0.3 0.2	0	-
0.1	0.009	0.2 0.2	0.05	-
0.5	0.018	0.3 0.3	0.08	-
1	0.09	0.4 0.4	0.11	-
5	0.45	1.3 1.2	0.34	0.11
10	0.90	2.1 2.4	0.61	0.29
25	2.25	5.7 5.7	1.54	0.71
50	4.50	11.7 11.1	3.08	1.42
100	9.00	23.1 23.6	6.30	2.70
500	45.00	119.5 115.0	31.66	13.34

Table 11: To show the different concentrations of glucose after incubation with Sepharose gel

initial glucose conc. (mmol/l)	amount of glucose added to gel (mg)	measured glucose conc. in extract (mmol/l)	amount of glucose in aqueous phase (mg/1.5ml)	amount of glucose in gel phase (mg/0.5ml)
0	0	0.2 0.2	0	-
0.1	0.009	0.2 0.2	-	-
0.5	0.018	0.3 0.3	-	-
1	0.09	0.4 0.4	-	-
5	0.45	1.3 1.2	0.34	0.11
10	0.90	2.4 2.5	0.66	0.24
25	2.25	6.0 5.9	1.61	0.64
50	4.50	11.9 11.7	3.19	1.31
100	9.00	22.8 23.7	6.28	2.72
500	45.00	124.0 121.0	33.08	11.92

Table 12 : To show the variation in ratio of ConA:Sepharose with different glucose concentrations.

Conc. glucose (mmol/l)	0	0.1	0.5	1	5	10	25	50	100	500
Ratio Sepharose: ConA	-	-	-	-	1	0.83	0.90	0.92	1.01	0.89

The two control solutions were not extracted, but assayed for glucose content. Both had an approximate glucose concentration of 20 mmol/l, and yielded results of 18.6 and 18.6 mmol/l for distilled water, and 19.8 and 19.8 mmol for barbitone buffer.

The effect on % glycated serum ferritin of added glucose

A stock solution of 300 mmol/l glucose in 0.9% sodium chloride was prepared by dissolving 2.7g of anhydrous dextrose (Evans Medical) in 50ml of diluent. This solution was further diluted as detailed in table 13.

Table 13 : To show the final concentration of glucose added to each sample.

Volume of stock solution of glucose 300 mmol/l	Volume of sodium chloride 0.9%	final glucose concentration (mmol/l)	
		solution	sample
undiluted	-	300	25
9	1	270	22.5
8	2	240	20
7	3	210	17.5
6	4	180	15
5	5	150	12.5
4	6	120	10
3	7	90	7.5
2	8	60	5
1	9	30	2.5

0.1ml of each concentration of glucose solution was added to 1.1 ml aliquots of serum. The serum was a single subject pooled sample with a measurable ferritin content and low endogenous glucose concentration.

Each sample was assayed for accurate glucose concentration and then extracted as paired samples with ConA and Sepharose gels. Ferritin concentrations were determined for the extracts, and % glycation calculated. The results are expressed in table 14.

Table 14 : The effect of exogenous glucose on % glycated serum ferritin.

glucose concentration (mmol/l)	ferritin concentration (μg/l)		% glycated
	ConA fraction	Sepharose fraction	
4.1	13.0	34.9	62.8
6.3	14.7	38.5	61.8
8.6	13.7	37.7	63.6
10.8	14.9	34.5	56.8
11.8	13.5	37.0	63.5
15.1	17.0	37.0	54.1
17.1	13.6	34.8	60.9
19.8	11.1	33.0	66.4
21.9	13.9	37.0	62.4
24.1	14.5	36.9	60.7
26.7	14.7	37.7	61.0

Discussion

The presence of barbitone buffer in the assay of glucose concentration did not have any noticeable effect on the determination of glucose level, as demonstrated by using distilled water as a control.

The range of glucose concentrations used in this experiment were 0.1-500 mmol/l, which far exceeded the concentrations of ferritin expected in samples of healthy sera for extraction (0.32-6.24 x 10^{-7} mmol/l). The minimum glucose concentration used was restricted by the sensitivity of the detection equipment (minimum value of 0.5 mmol/l), however it has served as a model for investigation of the binding characteristics of the respective ConA and Sepharose gels.

In the case of glucose, 25-30% of the total sample remained in the gel matrix, and of this a minimum of 80% was bound non-specifically. Therefore a maximum of 20% is bound specifically to receptors for glycated residues. This represents approximately 6% of the total sample.

As the amount of glucose was increased up to 0.25 mmol there was no tendency to reach a maximum concentration bound within the gel and therefore no apparent saturation of binding sites was reached.

Similarities between the relatively large percentage of non-specific binding of glucose (molecular weight 180g) cannot be directly made to ferritin, which is a much larger molecule (molecular weight 480,000g), with a completely different structure. However, in order to take account of non-specific binding, all calculations of % glycated ferritin are to be made with reference to Sepharose gel as a control for ConA-Sepharose.

The addition of glucose in varying concentrations that may be expected in pathological sera to a sample of healthy serum resulted in no significant difference between % glycated serum ferritin levels, yielding a Pearson correlation co-efficient of $r = -0.008$, $p << 0.05$. r^2 , the percentage of observations that may be due to variation of glucose concentration = 0.000064 (or 0.0064%).

In summary, the binding sites for glucose and glycated residues are not saturable within the likely ranges to be found in healthy or pathological sera.

For glucose, there is a relatively large amount of non-specific binding to the gel matrix. If this also applies to glycated ferritin then it is imperative to use inert sepharose gel as a control when calculating % glycated ferritin.

The results of Warren et al (42) that there is no apparent change in % glycated ferritin as measured by laser nephelometry with addition of exogenous glucose up to a concentration of 50 mmol/l are confirmed for this assay method. However it should be stressed that the same may not hold true for cases with a prolonged raised serum glucose concentration.

Clinical results

Normal Population Data

Samples for estimation of normal population data for total and % glycated serum ferritin were obtained from healthy volunteers amongst colleagues and staff at Selly Oak Hospital. In addition some samples were obtained pre-operatively from otherwise healthy day case patients presenting for minor surgery. Informed written consent was received from all subjects, together with a history of medication and alcohol use.

For the purpose of calculations, all figures recorded as <1 μg/l were considered to equal 1 μg/l. Results are displayed in Tables 15, 16, 17, 18 and Figures 2, 3 and 4.

Table 15 : To show total and % glycated serum ferritin in healthy male subjects.

Number	Age(yr)	Total serum ferritin (μg/l)	% glycated serum ferritin
1	74	21	-
2	64	130	47.2
3	40	64	75.9
4	38	18	-
5	59	<1	-
6	43	66	46.0
7	31	28	83.7
8	49	35	63.4
9	23	64	62.2
10	30	17	-
11	25	90	66.7

Table 16 : Statistical analysis of serum ferritin parameters in healthy male subjects.

	samples (n)	mean ($\bar{x}$)	standard deviation (sd)	standard error of the mean (sem)
Age (yr)	11	43.3	16.6	-
Total serum ferritin (μg/l)	11	48.6	38.3	11.5
% glycated ferritin	7	63.6	13.8	5.2

Figure 2. Normal population distribution of total serum ferritin for healthy male subjects

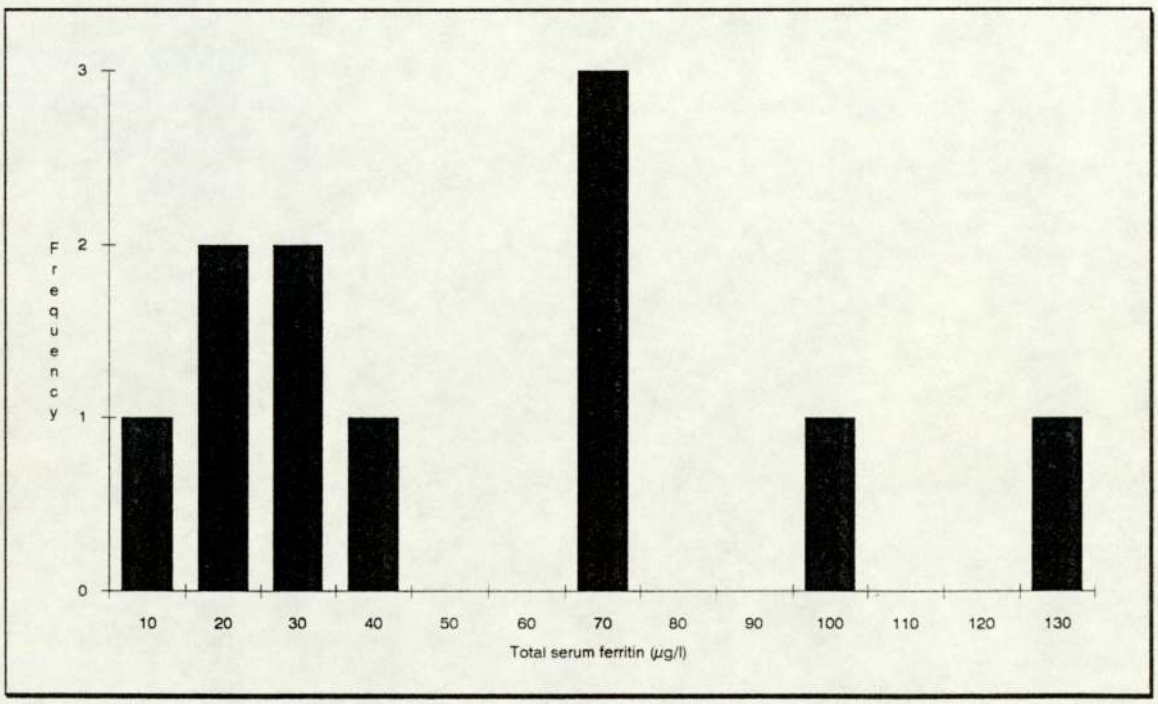


Table 17 : To show total and % glycated serum ferritin in healthy female subjects.

Number	Age(yr)	Total serum ferritin (μg/l)	% glycated serum ferritin
1	24	40	70.7
2	26	6.1	-
3	27	20.5	-
4	41	<1	-
5	34	<1	-
6	58	56	66.3
7	51	9.2	-
8	20	10.5	-
9	54	34	71.1
10	50	59	68.9
11	37	14.7	-
12	38	12.3	-
13	55	<1	-
14	64	33.5	76.1
15	23	19.5	47.8
16	22	28.5	71.5
17	26	35	61.2

Table 18 : Statistical analysis of serum ferritin parameters in healthy female subjects.

	samples (n)	mean ($\bar{x}$)	standard deviation (sd)	standard error of the mean (sem)
Age (yr)	17	38.2	14.5	-
Total serum ferritin (μg/l)	17	22.5	18.3	4.4
% glycated ferritin	8	66.7	8.8	3.1

Figure 3. Normal population distribution of total serum ferritin for healthy female subjects

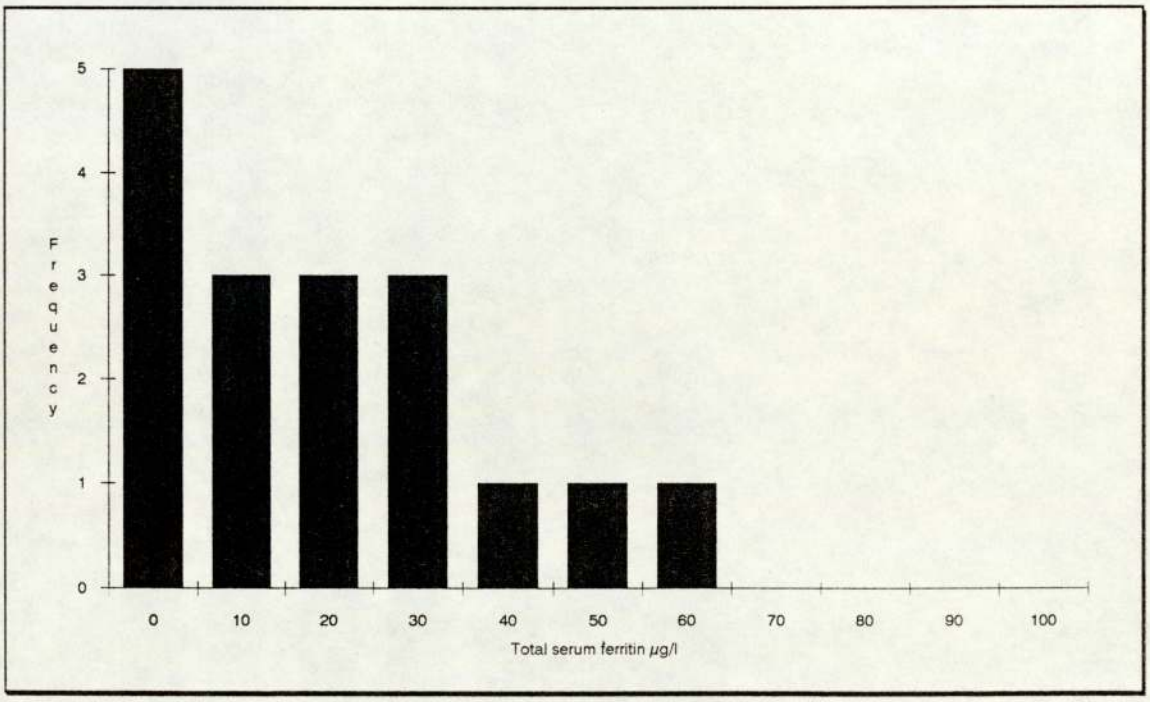
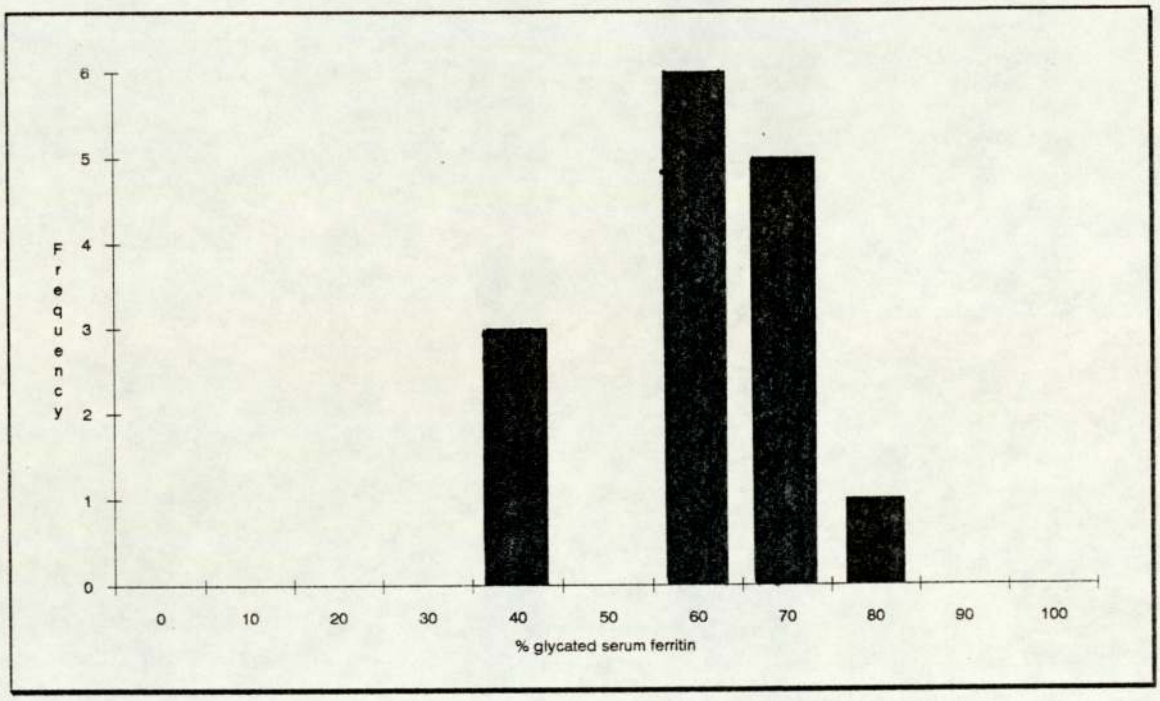


Figure 4. Population distribution of the % glycated serum ferritin for healthy male and female subjects



Discussion

The two populations chosen were statistically similar with regard to age distribution. The male population had a range of 23-74 years (mean = 43.3 yrs) and the female population had a range of 20-64 years (mean = 38.2 yrs). Calculation of standard error of difference (se diff.) resulted in a figure of 6.1, which when divided into the difference between the means = 0.8 standard deviations. At a level of $p = 0.05$, $0.8 < 1.96$, therefore there was no significant difference between the two samples, and both were likely to have come from the same population.

From graphical evidence, the total serum ferritin observed followed a skew distribution for female subjects,with the tail to the right. This was not so apparent for male subjects (Figure 2). Jacobs et al (30) have previously documented a skew distribution for both male and female subjects.

Most samples appear towards the lower end of the accepted range of serum ferritin values for healthy subjects, (15-300 μg/l) (13). There was nothing obviously unusual about the samples

selected, so it may be possible that the radioimmunoassay used has a tendency to lower values than other commercial kits available.

Statistical comparison of the two samples by calculation of standard error of difference resulted in a significant difference at the 0.05 level, se diff. = 12.4, difference between means = 26.1. difference between means / se diff. = 2.1 standard deviations 2.1 > 1.96, therefore total serum ferritin values in male subjects are significantly higher than those found in female subjects at the 0.05 level.

Present data cannot be compared with that of Walters et al (27) for which standard deviations were unpublished, and sample sizes relatively small. However, it is possible to make comparisons with the work of Jacobs et al (30).

For male samples : difference between means / se diff. = 1.78

For female samples : difference between means / se diff. = 2.73

$p = 0.05$; sd = 1.96, $p = 0.01$; sd = 2.58.

Statistically there is no significant difference between observed data and published data for male samples, but there is a significant difference, $p = 0.01$, between observed and published data for female samples.

The accepted reference range for total serum ferritin in healthy subjects lies between 15-300 μg/l, lower for females than males. The figures recorded under these conditions did not approach this magnitude, so a more realistic reference range would be from 1-60 μg/l for females and 1-125 μg/l for males. These figures are based on the respective means +/- 2 standard deviations, which should account for 95% of the population. Klockars et al reported a reference range of 20-120 μg/l for women, and 40-240 μg/L for men (43).

Data recorded for both male and female normal population samples for % glycated ferritin appeared exclusively between 46-83.7%, with the majority in the region of 60-80%. There was no significant difference between male and female samples.

Difference between means / se diff. = 0.5 standard deviations 0.5 < 1.96, therefore there is no significant difference between % glycated ferritin for male and female subjects.

It should be noted that % glycated ferritin could only be calculated for samples of > 25 μg/l under these circumstances, as this represented the lower limit of the assay after dilution factors and non-specific gel binding had been considered.

The mean value of the combined male and female samples of x = 65.2% (sd = 11.1) correlated with observations of approximately 60% made by Worwood et al (14).

In summary, the current data suggests a skew distribution for female total serum ferritin, the mean of which is significantly lower than that of the male total serum ferritin value. There is no significant difference between the means of samples of % glycated ferritin for male and female subjects. For the purpose of this work, the reference range of 1-60 μg/l for females and 1-125 μg/l for males has been adopted.

Physiological Variation In Serum Ferritin Measurements

Informed written consent was received from all healthy volunteers, together with a history of medication and alcohol use.

Diurnal variation

The diurnal variation experiment involved one male subject from whom blood was collected at four hourly intervals throughout a 24 hour period. There were no restrictions with regard to food, but consumption of alcohol was prohibited. As normal a lifestyle as possible continued.

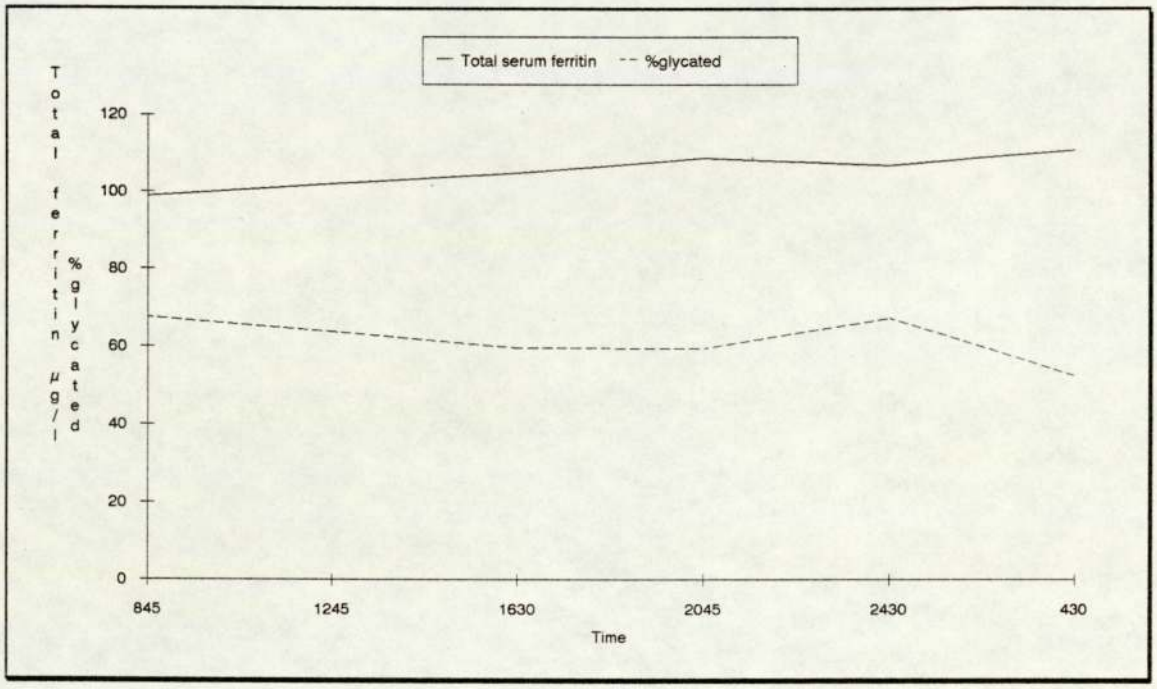
The blood samples were centrifuged and resultant serum stored at -20° C, until required for gel extractions to be made, ferritin content to be estimated, and % glycated ferritin to be calculated.

The results are displayed graphically in Figure 5, which demonstrates no apparent rhythmic variation.

Total serum ferritin : mean = 105.5 μg/l, sd = 4.5 μg/l, CV = 4.3%

% glycated serum ferritin : mean = 61.8 %, sd = 5.7 %, CV = 9.2%

Figure 5. Diurnal variation of total and % glycated serum ferritin in a healthy subject



Weekly variation

Four female and two male volunteers each had a single blood sample taken on five occasions, at approximately weekly intervals. In all cases, serum was separated from packed cells immediately by centrifugation, then stored at -20° C until required. All five samples from each individual subject were assayed together to eliminate inter-assay variation.

All individual samples were taken at a similar range of times, and no subject complained of any illness or minor ailment at the time of sample collection.

Female subject 2 regularly took the combined oral contraceptive pill (Trinordiol). None of the others were taking any medication.

Two subjects, female subject 4 and male subject 6, were regular cigarette smokers.

All admitted to social alcohol consumption (4-10 units/week), although female subject 4 admitted to up to 20 units/week.

One sample, from female subject 3, proved to have too low a total serum ferritin level for detection after extraction, and therefore only appeared in the total serum ferritin results.

One sample, from female subject 2, was lacking one total serum ferritin measurement and one % glycated serum ferritin measurement from the five week period.

Female subject 1 agreed to repeat the experiment, having a single blood sample taken approximately once a week over an extended period of eight weeks. The samples were taken as close to weekly intervals as possible allowing for holidays. The samples were allowed to clot, then serum separated from packed cells. The serum was stored at -20° C until required for extraction and assay. To eliminate inter-assay variation, all total serum ferritin estimations were assayed together in the same batch, similarly all estimations of ferritin for calculation of % glycation were also assayed together in the same batch.

All results are displayed graphically in Figures 6, 7 and 8.

Figure 6. To show variation in total serum ferritin in 6 healthy subjects over a 4 week period

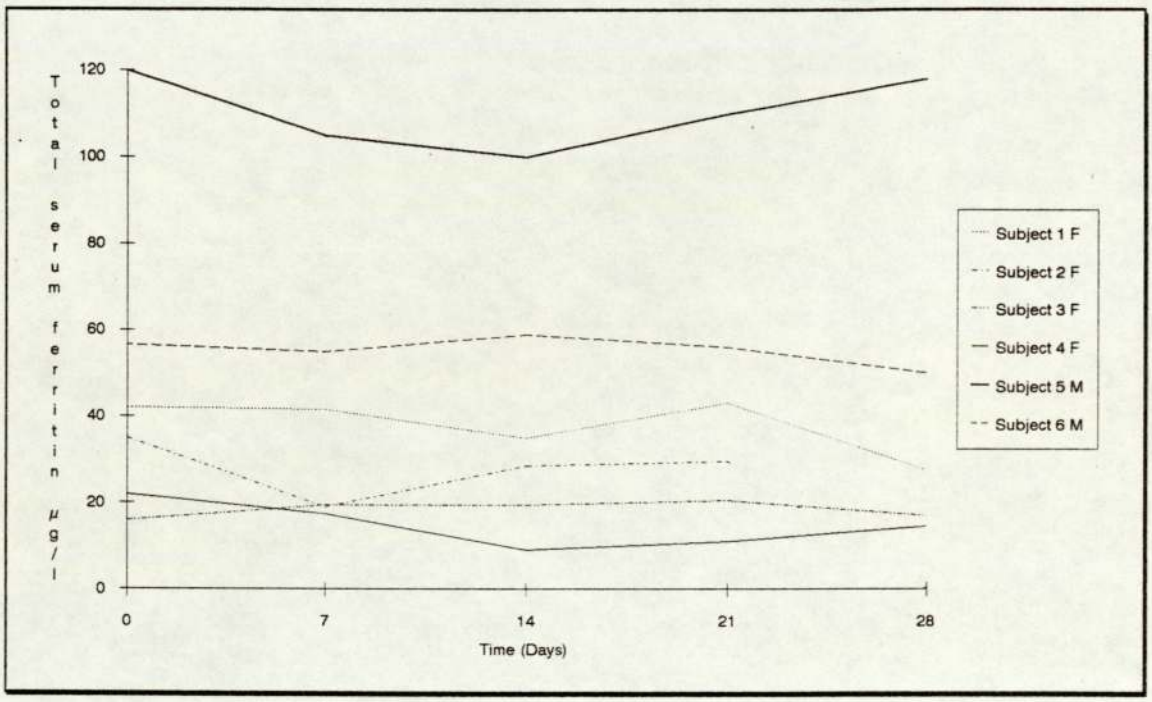


Figure 7. To show variation in % glycated serum ferritin in 6 healthy subjects over a 4 week period

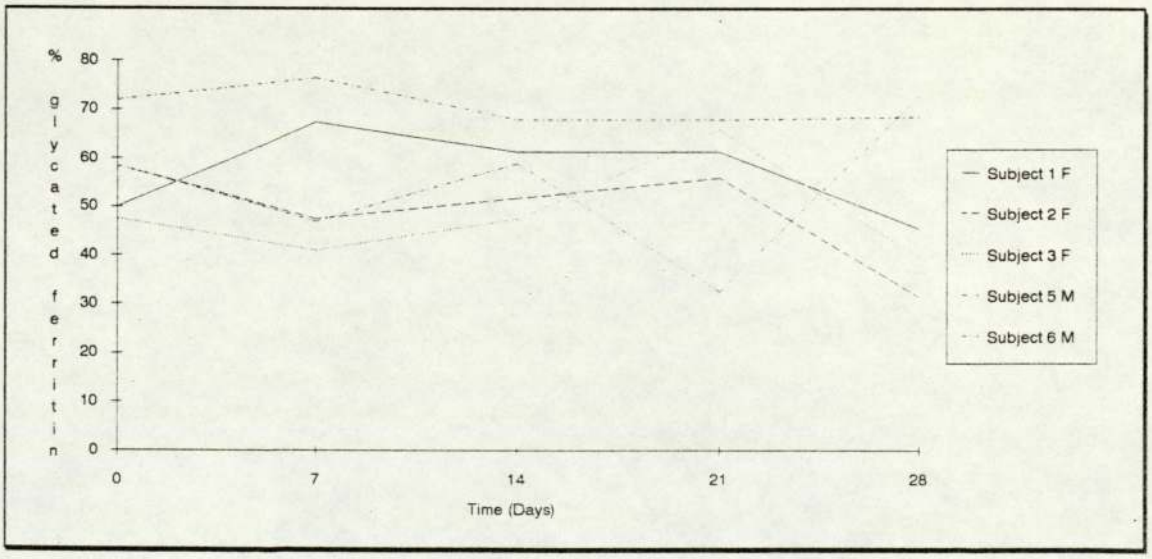
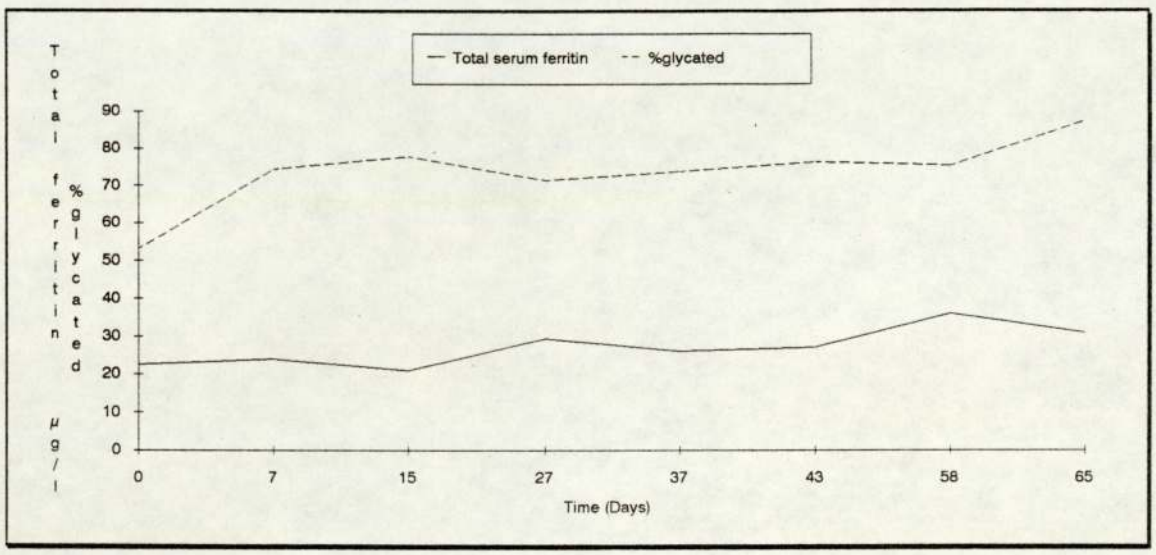


Figure 8. To show variation in total and % glycated serum ferritin in a healthy subject over an 8 week period.



Discussion

There were no apparent trends in either total serum ferritin or % glycated serum ferritin for one subject throughout a 24 hour period. The coefficients of variation calculated were below the cv of the assay for total serum ferritin, and slightly above for % glycated serum ferritin.The data does not appear to show any diurnal variation for either total serum ferritin or % glycated serum ferritin. No definitive conclusion can be drawn concerning diurnal variation on the basis of a study involving only one subject over a single 24 hour period, however it is in agreement with results published by Siimes et al (35), and Casale et al (44) for total serum ferritin. % glycated serum ferritin has not previously been investigated for diurnal variation.

Total serum ferritin levels for individual subjects demonstrated no apparent trends or rhythms over the period of time investigated. All remained individually within the same order of magnitude, but this varied between subjects. The observations noted in the normal population data were supported, higher values were recorded for male subjects than for female subjects. The total serum ferritin levels of the volunteer who participated in the extended 8 week investigation compared with her 5 week variation were within similar ranges (27-48 μg/l for the 5 week study and 21-36 μg/l for the eight week study).

The results recorded for % glycation demonstrated no apparent rhythm and a far greater variability than for total serum ferritin over the same period. There were no sex related trends. The maximum range recorded for one subject was from 26-73% (male subject 6) and the minimum range was 68-76% (male subject 5).

The observed variability for both total serum ferritin and % glycated ferritin in most cases appeared to exceed the inherent variability of the assay technique. There is no obvious explanation for the weekly variation in total serum ferritin and % glycated ferritin amongst healthy individuals, so it may be assigned to normal physiological phenomena. This has not been investigated before, and may need further research.

In summary, the diurnal variation experiment showed a minimum variation throughout a 24 hour period, inferring that the time a sample is taken during the day would have no effect on the results obtained. Within healthy individual subjects there exists marked variation in % glycated and total serum ferritin levels, with no apparent explanation.

To determine the effect of pathological hepatic damage on serum liver enzyme levels and ferritin parameters

Introduction

Total serum ferritin has been shown to be elevated in cases of hepatocellular damage due to a variety of causes (13). It is often accompanied by a reduction in % glycation, implying a release of intra-cellular ferritin into the serum from damaged cells (14).

The aim of this part of the study was twofold. Firstly, to confirm changes in serum ferritin parameters concurrent with liver damage, and secondly, to investigate the value of serum ferritin as an indicator in the diagnosis of jaundice of an obstructive or hepatocellular cause.

Methods

All serum liver enzyme and bilirubin estimations were carried out by the Department of Biochemistry, Selly Oak Hospital as a part of routine investigations. Prothrombin ratios were determined by the Department of Haematology, Selly Oak Hospital, as a part of routine investigations.

All patients were admitted to Selly Oak Hospital between March 1987 and February 1988. Serum samples were obtained by the Biochemistry Department for routine investigations, then retrieved from storage at 4° C to be stored at -20° C until required for ferritin determinations.

Results

All patients were admitted to Selly Oak Hospital between May 1987 and February 1988, and all results are displayed in Table 19.

Subject number 1 was admitted to the Rheumatology unit with an exacerbation of rheumatoid arthritis in August 1987. She was commenced on sulphasalazine, initially at a dose of 500mg daily,

increasing by 500mg each week up to a maintenance dose of 2g daily. She was discharged after 14 days while still on an increasing dose of sulphasalazine. She was re-admitted on 16.9.87 complaining of bruising on the upper thighs and buttocks. Drug therapy at that time was sulphasalazine 1g twice a day, indomethacin 75mg twice a day, and fybogel, all of which were discontinued. Serum liver enzyme levels were all raised on admission, decreasing throughout the stay in hospital. ASPT reached a maximum of 3 times normal values, Alk.P. reached 5 times normal and bilirubin was slightly raised. Total serum ferritin concentration was also raised to 6 times normal, with a corresponding reduction in % glycation, to a third of normal. She was diagnosed as suffering from a hepatotoxic reaction to sulphasalazine, and made a complete recovery after withdrawal of the drug.

Subject number 2 was a known epileptic controlled by phenytoin 100mg three times a day and carbamazepine 200mg five times a day, who presented to the casualty department on 19.2.88 with signs of phenytoin intoxication. He went on to develop renal failure and some degree of hepatic impairment. Renal failure was attributed to an acute interstitial nephritis and although there was no apparent pathological cause of the hepatic impairment, phenytoin could not be discounted. The patient made a full recovery and was followed up through an outpatient appointment six weeks later. On admission ASPT was raised to 400 times normal levels, decreasing rapidly over the next 3 days. Alk.P. and bilirubin levels were also raised, to 2 and 1½ times normal respectively. Serum bilirubin levels continued to rise to 4 times normal over the short period that they were monitored. Total serum ferritin was greatly raised, decreasing as time progressed, and % glycated serum ferritin levels were reduced to one sixth of normal values.

Subject number 3 was admitted with jaundiced alcoholic hepatitis bordering on hepatic encephalopathy on 2.7.87. Drug therapy on admission was prochlorperazine 5mg three times a day. While in hospital he was started on vitamin K, and multivitamins to correct deficiencies, lactulose and neomycin to reduce the risk of hepatic encephalopathy, and frusemide, spironolactone and amiloride to treat accumulation of ascitic fluid. All serum liver enzyme levels were raised, ASPT reaching 7 times normal levels, Alk.P. reaching 1½ times normal and bilirubin

eventually reaching 30 times normal. Prothrombin ratio was raised. In addition, a high total serum ferritin level corresponded to a low % glycation of just under one half of normal.

Subject number 4 had a four year history of primary biliary cirrhosis and had been admitted on several occasions to treat complications such as bleeding oesophageal varices and advancing hepatic encephalopathy. She was admitted on 2.3.88 and at the time of the ferritin determination she was hospitalised because of end stage hepatic failure. Drug therapy was lactulose, spironolactone, neomycin and ranitidine. All serum liver enzyme levels were raised, ASPT to 7 times normal levels, Alk.P. to 5 times normal, and bilirubin to 4 times normal. Total serum ferritin was only slightly elevated, and % glycated within normal limits. The patient died two weeks later from hepatic failure.

Subject number 5 had a history of alcoholic liver disease and was admitted on 6.5.87 for routine drainage of gross ascites. While in hospital drug therapy consisted of parentrovite, spironolactone, vitamin K and chlormethiazole. All liver enzyme levels were elevated, ASPT and bilirubin to 5 times normal levels, and Alk.P. to 4 times normal. Prothrombin ratio was also raised. Total serum ferritin levels were raised to 9 times normal, and % glycated reduced to one third of normal values. He was discharged on 29.5.87.

Subject number 6 was admitted on 11.2.88 for investigation of jaundice and abdominal swelling. The diagnosis of active alcoholic cirrhosis and cholestasis was made after liver biopsy. Drug treatment on admission was spironolactone and frusemide. All serum liver enzyme levels were elevated, ASPT to 8 times normal levels, Alk.P. to 4 times normal, and bilirubin to 13 times normal. Prothrombin ratio was raised, and total serum ferritin was raised to 5-6 times normal values, but % glycation was only slightly low. The patient was discharged on 3.3.88.

Subject number 7 had a one year history of Parkinson's disease and was admitted with jaundice. Drug therapy on admission was Sinemet Plus (levodopa & carbidopa), diazepam, and mianserin. The diagnosis was of a carcinoma of the pancreas causing an obstruction of the pancreatic and common bile ducts. All serum liver enzyme levels were raised, ASPT to 13 times

normal levels, and Alk.P. and bilirubin to 14 times normal. Prothrombin ratio was normal, total serum ferritin was markedly raised and % glycated ferritin reduced to one half of normal.

Subject number 8 had a history of ulcerative collitis, and seropositive rheumatoid arthritis and was admitted for investigation of jaundice. Drug therapy on admission was Frumil (frusemide & amiloride), and sulphasalazine. All serum liver enzyme levels were raised, ASPT to 8 times normal levels, Alk.P. to 6 times normal and bilirubin to 7 times normal. Prothrombin ratio and partial thromboplastin time were increased. Both total and % glycated serum ferritin were within normal limits. He was diagnosed as suffering from sclerosing cholangitis and discharged approximately one month after admission.

Subject number 9 was admitted on 15.6.87 for investigation of an obstructive jaundice, which was diagnosed as a carcinoma of the common bile duct or gall bladder. Drug therapy on admission was methyldopa for hypertension. ASPT and Alk.P. were slightly elevated, to 2 and 3 times normal levels respectively, and bilirubin elevated to 16 times normal. Prothrombin ratio was normal, total serum ferritin slightly raised, with a % glycation of two thirds of normal values.

Subject number 10 was admitted on 19.4.87 for investigation of jaundice. There was neither an obstructive or infective cause, and no evidence of alcohol abuse. He took no medication. The diagnosis was of a chronic liver disease of auto-immune origin. All serum liver enzyme levels were elevated, ASPT to 20 times normal levels, Alk.P. to 4 times normal, and bilirubin to 8 times normal. Total serum ferritin was raised to 14 times normal values, but % glycation was only slightly reduced. The patient died three months later.

Subject number 11 was admitted on 30.3.87 with an acute jaundice of obstructive origin. At the time of admission she was not taking any medication. Investigation revealed the cause to be carcinoma of the pancreas. All serum liver enzyme levels were elevated, ASPT and Alk.P. to 3 times normal values, and bilirubin to 18 times normal. Total serum ferritin was raised to 3 times normal levels, however the % glycated was only slightly low.

Table 19 : To show the effect of hepatic disorder on serum liver enzyme and ferritin values

Subject	Age	Sex	Date	ASPT	Alk.P.	Bilirub	ɣGT	PT	Ferritin	% glyc
Units	yrs		dd.mm	IU/l	KA unit	µmol/l	IU/l		µg/l	
Normal range				5-35	3-12	<22	5-55	0.8-1.2	1-125	60-80
1. BC	59	F	17.9	127	61	23	-	-	150	-
			21.9	48	53	10	328	-	900	17.6
			25.9	23	29	7	-	-	520	24.8
			29.9	14	27	8	180	-	315	17.0
			29.10	-	-	7	-	-	190	-
2. JP	50	M	19.2	13750	22	36	-	1.2	2000+	19.1
			20.2	4466	-	-	-	-	2000+	9.8
			21.2	780	18	67	-	-	1430	17.9
			22.2	321	18	90	-	-	1050	22.4
			8.4	23	11	11	-	-	-	-
3. TH	51	M	2.7	237	17	306	-	-	-	-
			8.7	129	15	346	280	2.2	2000+	-
			10.7	129	14	756	210	2.2	2000+	27.3
4. MB	79	F	7.3	252	61	94	-	-	230	59.0
5. MK	55	M	12.5	154	54	98	-	1.3	1160	18.8
6. NP	75	F	21.2	289	50	286	-	1.6	710	44.4
7. MA	74	F	29.4	450	174	300	-	1.1	2000+	32.7
8. EM	79	M	16.7	277	72	155	-	2.1	29	65.3
9. ES	66	F	16.6	60	38	378	-	1.1	160	36.8
10. FW	86	M	20.4	732	45	164	-	-	1820	44.4
11. KT	71	F	13.4	120	33	396	-	-	400	48.2

Discussion

It was only possible to serially follow patient numbers 1 and 2 throughout the course of their stay in hospital. It was unfortunate that no information was gathered from the period immediately prior to the hepatotoxic reaction. Both appear to support the theory that raised total serum ferritin allied to a reduction in % glycation is an indication of hepatocellular damage (5,14). There is no evidence available to suggest that there were any prior changes in serum ferritin parameters before disruption of serum liver enzyme levels.

Total and % glycated serum ferritin was measured on only one sample from each of the remaining subjects, and was considered representative of their condition.

All three subjects with cases of alcoholic hepatitis (numbers 3,5, and 6) demonstrated elevated total serum ferritin levels, which in subject numbers 3 and 5 was accompanied by a low % glycation. Subject number 3 demonstrated the highest total serum ferritin and was also the subject with the most serious clinical manifestations of hepatitis. The % glycated serum ferritin of subject number 6 was higher than expected, but remained lower than that found in the normal population. The evidence from these subjects further supports other recorded observations of ferritin species in hepatocellular damage (5,14).

The four cases of obstructive jaundice consisted of one sclerosing cholangitis, two carcinomas of the pancreas, and one of the common bile duct. A carcinoma of hepatocellular origin is accompanied by raised total serum ferritin, often with a reduction in % glycation. Suggested causes include the increased production of the acidic isoferritin, leakage of ferritin from damaged cells, and a reduction in the uptake of ferritin by the damaged liver (4,5). Normal serum contains the basic isoferritin, but, as there is no information on the specificity of the radioimmunoassay for the individual isoferritin species the observed ferritin increase cannot be characterised without electrophoretic separation. Leakage of serum ferritin from damaged cells would result in an increase in total serum ferritin with a relative decrease in % glycation. Reduced uptake of ferritin by the liver will result in an increase in total serum ferritin with no change in the % glycated. Despite an apparent impairment of synthetic liver function, evident by an increase in prothrombin ratio,

and evidence of cellular damage shown by raised ASPT and Alk.P levels in the serum, the ferritin parameters in the case of sclerosing cholangitis remained within normal limits. The total serum ferritin levels seen in all cases of carcinoma ranged from slightly elevated in subject number 9, to significantly elevated in subject number 7, all accompanied by reduced % glycation, although to a lesser degree in subject number 11. Despite the small numbers of subjects studied, it would suggest that in cases of obstructive jaundice with no malignant cause, serum ferritin parameters remain within normal limits. Additional support is found from subject number 4, who suffered from primary biliary cirrhosis, an obstructive cause, in whom serum ferritin parameters could be considered to be within normal limits.

In summary, the results obtained suggest that abnormal ferritin parameters arise from cases of hepatic damage of cellular origin, as previously demonstrated (5,14), but this is not mirrored in hepatic obstruction. Therefore, determination of serum ferritin may be of some benefit to differentiate between jaundice of an obstructive or cellular origin.

To determine the effect of moderate alcohol ingestion on total serum ferritin, % glycated ferritin, and standard liver function tests

Introduction

Chronic alcohol intake is known to induce hepatic micro-enzyme systems, however, acute alcohol intake has been shown to inhibit hepatic enzymes (45).

The extent of damage caused to the liver by chronic alcohol intake is governed by the dosage of alcohol taken and the period of time for which it has been abused. In chronic, well nourished alcoholics, hepatotoxicity is manifest as a fatty degeneration of the liver that is associated with hyperlipidaemia. Cirrhosis is more common in poorly nourished alcoholics, and is related to dietary deficiencies (46).

The purpose of this experiment was to determine whether a moderate single oral dose of ethanol, in the form of gin, would have any effect on the established indicators of liver function, compared with total and % glycated serum ferritin levels over a period of 108 hours.

Methods

Two healthy volunteers, one male (26 yrs), and one female (25 yrs), who were both social drinkers, agreed to consume 0.8g/kg of ethanol, as gin made up to 200ml with tonic water, over a period of one hour. Single 10ml samples of venous blood were taken prior to ingestion, and then at approximately 0.5, 2, 12, 18, 24, 36, 60, 108 hours post ingestion.

The blood samples were allowed to clot, and the serum separated by centrifugation immediately where possible. However, overnight samples (ie those taken at times 0, 0.5, 2, and 24 hours) were stored at 4° C in a refrigerator, and serum separated the next morning. Those concerned were the initial level, 0.5, 2, and 24 hours. Serum samples were frozen and stored at -20° C until required for assay.

A third volunteer, a 26 year old female social drinker, agreed to a similar experiment involving three doses of 0.8g/kg of ethanol taken as gin and tonic on three consecutive nights. Baseline measurements were taken before ingestion of alcohol on the first night, and the collection of blood samples proceeded after alcohol ingestion on the third night at the same intervals as for the other volunteers.

Informed written consent was received from all volunteers, and the study received the approval of the local hospital ethical committee. None complained of any minor ailment, or admitted to taking any medication.

There were no dietary restrictions, although all abstained from alcohol for four days prior to the experiment and during the follow-up period.

Individual samples were batched for extraction and assay to eliminate inter-assay variation. The supernatants from the gel extraction process were frozen and stored at -20° C. Serum was assayed for total ferritin content, then passed to the Biochemistry department at Selly Oak Hospital for routine determination of ɣGT, ASPT and Alk.P. Where possible, alcohol estimations were made with the residual serum by gas chromatographic techniques.

Serum alcohol determination

Stock solutions of absolute ethanol and propan-2-ol were prepared by accurately weighing 10g of the respective alcohol and diluting to 100ml with distilled water using volumetric flasks. 10ml of each of these solutions were pipetted into volumetric flasks and made up to 1 litre with distilled water. The resultant stock solutions were both 0.1% w/v (100mg / 100ml).

A reference sample was created by taking 50 μl of each solution, measured with a Gilson displacement pipette, and mixing well. The reference sample contained 50 μl of a 0.1 % solution of ethanol as a standard, and 50 μl of a 0.1 % solution of propan-2-ol as an internal standard.

50 μl of each sample was then diluted with 50 μl of 0.1% propan-2-ol and assayed for ethanol content using the Perkin Elmer 8310 Gas Chromatograph.

The injection volume was 5 μl, the oven temperature was 120° C, the injector temperature was 230° C and the detector temperature was 230° C. The carrier gas was oxygen-free nitrogen, passing at a rate of 20ml/minute, and the stationary phase was Poropak Q, 80 mesh, packed in a 2 metre column. The detector was of the flame ionisation type.

The Perkin Elmer 8310 Gas Chromatograph has an integral computer that is capable of calculating concentrations of sample materials from 'area under the curve' data that is compared to the known concentration of the ethanol standard.

Results

The average retention time for ethanol was 7.18 minutes, and for propan-2-ol was 14.62 minutes. One other peak, not present in the initial reference sample, but which appeared in each sample had an average retention time of 3.82 minutes.

Sample runs using methanol and acetaldehyde resulted in retention times of 2.85 and 3.85 minutes respectively. The unknown peak was therefore acetaldehyde, a breakdown product of ethanol in the body.

Serum ethanol levels are displayed in table 20

Table 20 : To show serum ethanol levels (mg/100ml) up to 18 hours after ingestion

Time (h)	0	0.5	2	12	18
Subject 1	-	64.1	8.7	-	-
Subject 2	-	-	26.7	-	-
Subject 3	-	24.0	30.7	-	-

- denotes level undetectable

The results of ɣ GT, ASPT, and Alk.P. levels obtained from the Biochemistry Department, together with the total and % glycated serum ferritin results are displayed graphically in figures 9, 10 and 11.

Figure 9. The effect of alcohol ingestion on liver function tests and total and % glycated serum ferritin: Subject 1.

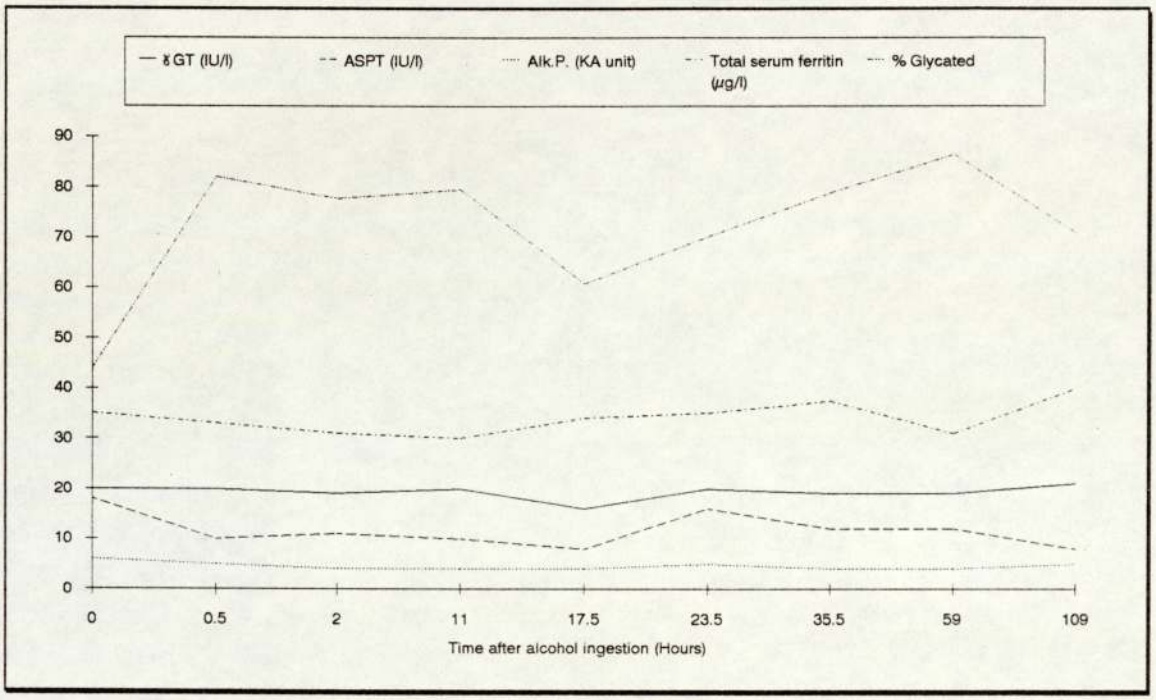


Figure 10. The effect of alcohol ingestion on liver function tests and total and % glycated serum ferritin: Subject 2.

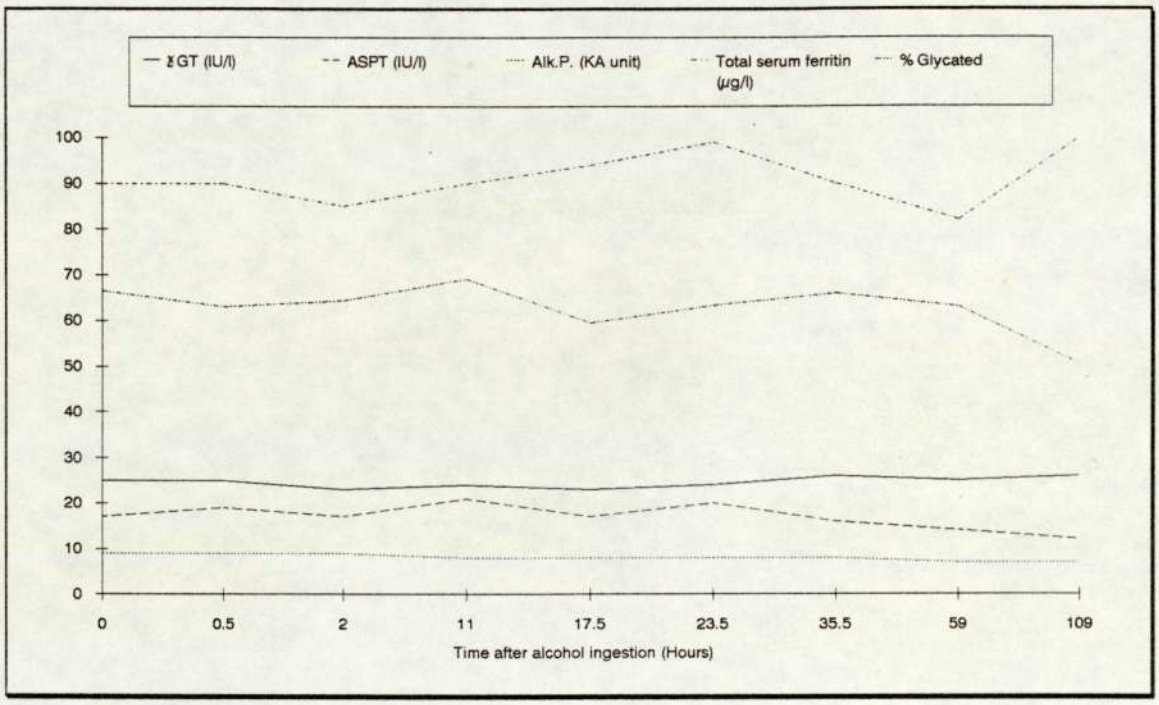
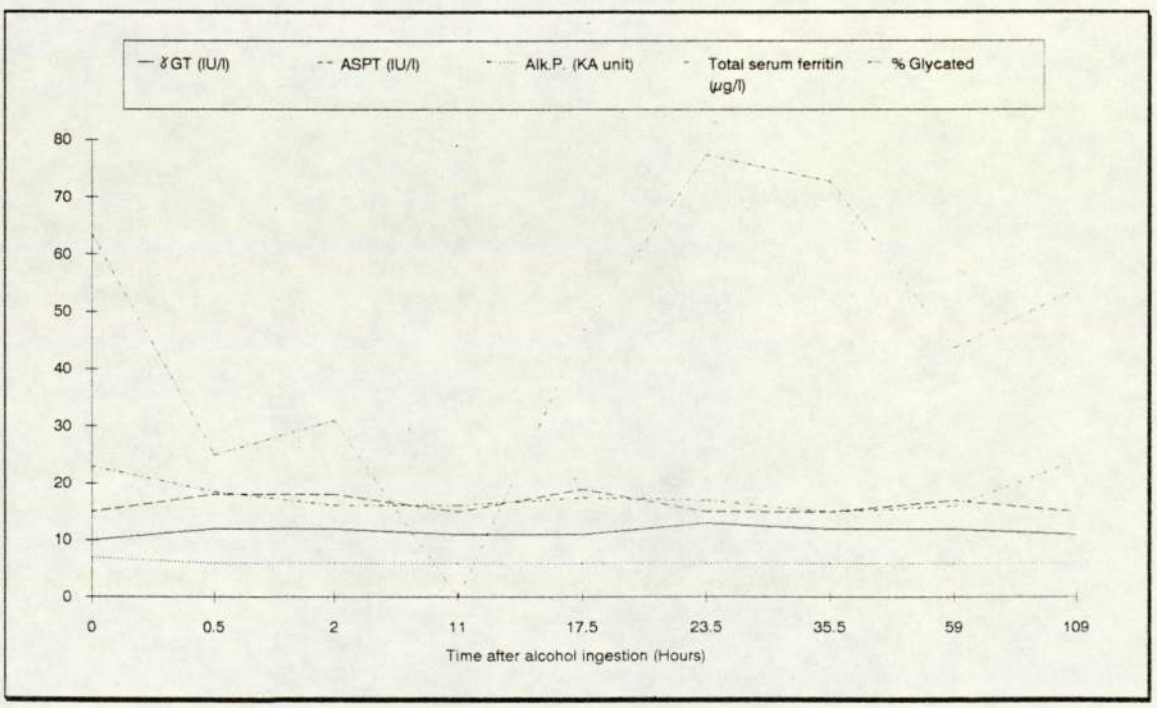


Figure 11. The effect of alcohol ingestion on liver function tests and total and % glycated serum ferritin: Subject 3.



All serum liver enzyme and total and % glycated ferritin levels remained within the normal range throughout the course of the experiment for subject number 1. However, within the normal range, ASPT reached a maximum after 24 hours, and % glycated ferritin reached a minimum after 18 hours. All other levels fluctuated with no significant maxima or minima.

All serum ferritin and enzyme levels remained within the normal ranges for subject number 2. ASPT reached a maximum after approximately 12 hours, and % glycated ferritin reached a minimum 18 hours after ingestion. None of the other parameters measured demonstrated any apparent maxima or minima.

Subject number 3 demonstrated a significant reduction in % glycated serum ferritin approximately 12 hours post ingestion. All other levels remained within the normal range, and ASPT reached a maximum 18 hours after ingestion of alcohol.

Discussion

The liver enzyme results for subjects 1 and 2 were known before subject 3 undertook the experiment. As there was no apparent change in levels for 1 and 2 it was decided to alter the conditions of the test to repeat the ethanol dose on three consecutive nights for subject 3.

The ingestion of a standardised quantity of alcohol of 0.8g/kg body weight by three different subjects produced different ethanol serum levels. There may be a number of factors which affect alcohol absorbtion, including ingestion of food, difference between the sexes, and individual variation.

Alcohol may separate from serum on repeated freeze/thaw storage, but containers were tightly stoppered and contents mixed thoroughly to reduce any losses.

Examination of the graphical data does not support the observations of Freer and Statland (47) who dosed volunteers with 0.75g/kg body weight of alcohol on three consecutive nights. They reported changes in γGT and ASPT after alcohol consumption by healthy volunteers. This was not even observed in subject number 3 who mirrored their experiment. There is some suggestion of a peak in ASPT concentration at approximately 18-24 hours, more pronounced in subject 1, but also present in subjects 2 and 3.

All subjects showed minimal variation in total serum ferritin throughout the experiment, but all exhibited a drop in % glycated ferritin after 12-18 hours, especially noticeable in subject 3, who had a three day alcohol intake. Previous work has not detected any diurnal variation in % glycated serum ferritin (See physiological variation in serum ferritin measurements).

The fact that none of these changes is accompanied by any alteration in total serum ferritin warrants an explanation.

Glycated and non-glycated serum ferritin are both cleared by uptake into the liver, however, non-glycated ferritin is cleared more quickly ($t_{1/2}$ = 5hrs) compared with glycated ferritin ($t_{1/2}$ = 50 hrs) (23).

A reduction in % glycated ferritin could arise from either an increase in the non-glycated form relative to glycated in the serum, or from a reduction in the glycated form. The former would result in an increase in total serum ferritin, and the latter in a reduction in total serum ferritin. As there is no apparent change in the levels of total serum ferritin,the change must be from either a combination of both possibilities, or an alteration in ferritin clearance.

The results had returned to normal within a further 12-18 hours.

The presence of acetaldehyde was to be expected as a principal breakdown product of ethanol metabolism. Its continued presence over 18 hours could be an artifact, retained by the column, or a tendency to persist in the body (48).

To determine the effect of an acute infection on total and % glycated serum ferritin

Introduction

Acute infection in man causes a rise in serum ferritin levels that starts within 24-30 hours and may persist for several weeks (15). The rise in serum ferritin could be a consequence either of leakage from damaged cells or augmented ferritin synthesis. The leakage of ferritin from damaged cells would result in an increase in the proportion of the non-glycated ferritin detectable in the serum, together with an increase in total serum ferritin. Augmented ferritin synthesis, however, would result in increased total serum ferritin, while maintaining the same percentage glycation (approximately 60-80 %). Work done by Birgegård (36) suggests that augmented ferritin synthesis is the cause of the increase. The purpose of this study was to further investigate the effect of pathological conditions on serum ferritin parameters, and if possible, add to the findings of Birgegård.

Methods

All serum enzyme level determinations were carried out as a part of routine investigations requested from the Department of Biochemistry, Selly Oak Hospital.

Results

All patients were admitted to Selly Oak Hospital between March and May 1987.

Subject number 1 was admitted through the Casualty Department on 8.5.87 with an unspecified infection. Further investigation suggested a tubercular meningitis, and therapy was started on 12.5.87 with Rimactazid 300, (rifampicin 300mg & isoniazid 150mg) two daily, ethambutol 800mg daily, pyrazinamide 1500mg daily and pyridoxine 10mg daily. Diagnosis was confirmed by liver biopsy on 13.5.87, although it was not possible to isolate acid-fast bacilli

characteristic of tuberculosis. Despite active treatment, the patient died almost one month after admission. There were no changes in serum Alk.P. or bilirubin levels throughout the course of the illness, but ASPT levels reached a maximum of 8 times normal levels nine days after admission. Total serum ferritin levels increased gradually, while % glycation dropped suddenly between days 2 and 9 to stabilise at a low level.

Subject number 2 was admitted on the request of his General Practitioner on 28.3.87 with a history of chronic obstructive airways disease and a current chest infection. Drug therapy on admission was theophylline 800mg at night, frumil (frusemide 40mg & amiloride 5mg), two daily, and salbutamol and ipratropium bromide inhalers. While in hospital, both gram +ve and gram -ve cocci were isolated from samples of sputum and the infection was treated with erythromycin 500mg four times a day and trimethoprim 200mg twice a day. The patient was discharged approximately one month after admission. On admission, serum ASPT levels were slightly elevated, returning to within the normal range over the following 7-10 days. Alk.P. and bilirubin levels remained within normal limits. The only ferritin measurement taken was elevated to 3 to 4 times normal, with a normal % glycation.

Subject number 3 was also admitted at the request of his General Practitioner with a history of chronic obstructive airways disease and current chest infection. Drug therapy on admission was prednisolone 20mg daily, frusemide 40mg daily, ranitidine 150mg twice a day and amoxycillin 500mg three times a day. While in hospital he was started on nebulised salbutamol, erythromycin 500mg four times a day, and frusemide was increased to 80mg twice a day. Sputum cultures were negative and the patient was discharged two weeks after admission. Throughout the time spent in hospital, ASPT, Alk.P. and bilirubin levels remained within normal limits. The only ferritin measurement taken was slightly elevated, accompanied by a reduced % glycation, to approximately one third of normal.

Subject number 4 was admitted for a minor surgical procedure (hernia repair), with one blood sample taken pre-operatively, one post -operatively, and one follow-up ten days later. At the time of the planned follow-up sample the patient had contracted gastroenteritis of unknown origin. No

serum enzyme estimations were made, but total serum ferritin levels taken during the infection were 4 times the value taken pre-operatively, and % glycation had fallen markedly.

All results are displayed in Table 21.

Table 21 : To show the effect of systemic infection on serum enzyme and ferritin values

Subject	Age	Sex	Date dd.mm	ASPT IU/l	Alk.P. KA unit	Bilirubin µmol/l	Ferritin µg/l	% glycated
Normal range				5-35	3-12	<22	1-125	60-80
1. DM	42	M	9.5	78	9	11	-	-
			11.5	76	11	9	140	> 98
			18.5	265	10	13	185	20.8
			20.5	125	10	15	-	-
			22.5	56	10	11	-	-
			25.5	157	13	10	-	-
			26.5	69	11	13	-	-
			27.5	53	11	13	-	-
			28.5	38	10	10	195	22.5
			29.5	37	9	10	-	-
			1.6	79	10	9	190	27.5
			2.6	30	11	8	-	-
			3.6	32	11	11	220	17.2
			4.6	26	10	15	-	-
2. WT	71	M	28.3	42	11	6	-	-
			2.4	40	11	9	-	-
			6.4	31	9	8	-	-
			7.4	35	9	8	-	-
			9.4	19	10	6	-	-
			12.4	19	12	13	425	64.3
			13.4	21	11	10	-	-
			14.4	22	10	10	-	-
3. WM	70	M	19.4	31	11	17	-	-
			29.4	29	9	7	175	22.4
4. WK	29	M	9.4	-	-	-	28	83.7
			9.4	-	-	-	-	86.7
			18.4	-	-	-	130	35.4

Discussion

The attempt to determine the effect of systemic infection on the ferritin parameters of subject number 1 was frustrated by the administration of isoniazid and rifampicin, both of which are drugs with a proven ability to damage hepatic cells (48), and the invasive nature of the liver biopsy. Within 5-6 days of the administration of both drugs and the liver biopsy, there was a transient increase in ASPT levels; lasting for approximately 7 days. Alk.P. and bilirubin levels were unaffected, but the increase in ASPT was accompanied by a slight rise in the total serum ferritin level together with a profound fall in % glycated serum ferritin, indicative of hepatocellular damage, that persisted until the demise of the patient. The initial, very high % glycated serum ferritin result taken on 11.5.87 was repeated and found to be reproducible. It is unclear in this subject whether the net effect of administration of hepatotoxic drugs, or liver biopsy, together with systemic infection resulted in the characteristic change in serum ferritin parameters suggestive of hepatocellular damage. It may be argued, with reference to the initial ferritin values, that the majority of the effect was unlikely to have been caused by the infection, and that the persistence of the results implicated the medication. It would have been interesting to be able to further chart the change in serum ferritin if the patient had made a recovery.

Subject numbers 2 and 3 presented conflicting evidence. Neither demonstrated any change in serum Alk.P. or bilirubin levels, although patient number 2 did initially show slightly raised ASPT levels. Total serum ferritin was increased to 3-4 times normal levels for subject number 2 but remained at the upper limits of the normal range for subject number 3. However, % glycated serum ferritin was within the normal range for patient number 2, indicative of augmented synthesis, but was reduced for patient number 3, indicative of cellular damage. The difficulty in isolating a causative organism for patient number 3 did not negate the diagnosis of a bacterial infection.

The increase in total serum ferritin coupled to a reduction in % glycation observed in subject number 4, a case of gastroenteritis, supports the theory of non-specific cellular damage, caused

by an infective agent rather than augmented ferritin synthesis. These changes were not observed in other healthy subjects admitted for minor surgical procedures.

The interpretation of results is difficult in this study because of the very small sample size, however the limited evidence available is more supportive of the theory of leakage of ferritin into the serum from damaged cells, rather than augmented ferritin synthesis.

To determine the effect of an acute myocardial infarction on total and % glycated serum ferritin

Introduction

A myocardial infarction occurs when blood flow ceases in a part of the coronary vessels beyond an occlusion. The area of muscle receives either no blood supply, or too little blood to sustain cardiac function, and the area becomes infarcted.

Serum levels of three enzymes: creatinine phosphokinase (CPK), aspartate transaminase (ASPT), and lactate dehydrogenase (LDH, HBD) follow a characteristic and distinctive pattern after a myocardial infarction.

An increase in serum CPK levels is detectable within 4-8 hours, reaching a maximum between 24-48 hours and remaining raised for 3-5 days after infarct. Increases in serum ASPT are detectable within 6-8 hours, reaching a maximum in 24-48 hours, and remaining raised for 4-6 days. Increases in serum HBD levels are detectable within 12-24 hours, reaching a maximum after 48-72 hours, and having a duration of 7-12 days. The extent of the increase in serum enzyme levels is proportional to the size of the infarction. If the infarction becomes extended, or congestive cardiac failure develops there may be a second, subsequent increase in ASPT levels.

Ferritin in human tissue consists of two subunit types, H and L. The H subunits are predominant in the acidic isoferritins isolated from heart tissue, while the L subunits predominate in the more basic isoferritins of liver and spleen. The subgroups are distinguishable immunologically. (13,49)

In theory, an acute myocardial infarction should result in the release of non-glycated intracellular ferritin of the H subunit type into the serum. This may be detected depending on the specificity of the radioimmunoassay method.

The purpose of this study is to investigate the effects of a myocardial infarction on serum ferritin parameters.

Methods

All determinations of enzyme levels were carried out by the Department of Biochemistry, Selly Oak Hospital, and were part of routine investigations. Serum creatinine phosphokinase levels fall outside routine work, and were not assayed.

Estimations of prothrombin ratio and partial thromboplastin time (PTTK) were carried out as part of routine investigations by the Department of Haematology, Selly Oak Hospital.

Results

All patients were admitted to Selly Oak Hospital between March and May 1987.

Patient number 1 was admitted through the Casualty Department with heart failure, and suffered a myocardial infarction while in hospital. His drug therapy on admission consisted of Frumil (frusemide + amiloride), digoxin, nebulised salbutamol, aminophylline and amoxycillin. Salbutamol, aminophylline and amoxycillin were discontinued, and the dose of diuretic increased. The patient was anti-coagulated with heparin, and given pain relief in the form of intramuscular diamorphine. One week after admission Haemophilus influenzae was isolated from samples of sputum. The patient died 11 days after admission from intractable heart failure. Total serum ferritin was elevated and % glycated serum ferritin reached a minimum 4 days post infarction. The changes in serum ASPT and HBD levels were consistent with those observed in a myocardial infarction. Elevated bilirubin levels together with raised ASPT levels could be suggestive of hepatic congestion secondary to heart failure. Prothrombin ratio and partial thromboplastin times were elevated beyond normal levels by heparin anticoagulation therapy.

Patient number 2 was admitted from Casualty with a myocardial infarction and sinus bradycardia. His drug therapy consisted of isosorbide dinitrate when required for angina. While in

hospital he was treated with Frumil, and diamorphine for pain relief. He entered into complete heart block that necessitated electrical pacing and was discharged 9 days after admission. Serum enzyme levels were consistent with a myocardial infarction. Alk.P. and bilirubin remained within normal limits, and total serum ferritin levels increased twofold over a three day period, but remained within normal limits. The maximum total serum ferritin level corresponded with the minimum % glycated serum ferritin level.

Patient number 3 was admitted from Casualty with a myocardial infarction. There was no drug therapy on admission. While in hospital he was treated with heparin and diamorphine. Serum enzyme levels were consistent with a myocardial infarction and he was discharged 8 days after admission. Total serum ferritin levels increased threefold, but remained within normal limits. Changes in % glycated serum ferritin were inconsistent.

Patient number 4 was admitted from Casualty with a myocardial infarction. Drug therapy was Tenoretic (atenolol + chlorthalidone) daily. Treatment with Frumil was initiated, and in addition the patient received heparin and diamorphine. The serum enzyme changes were consistent with a myocardial infarction and the patient was discharged 7 days after admission. Alk.P. levels remained within the normal range, but total serum ferritin levels were markedly elevated, and % glycated levels were slightly reduced.

Patient number 5 was admitted from Casualty with a myocardial infarction. The only drug therapy was indomethacin for osteoarthritis. While in hospital she was anti-coagulated with heparin, and pain relief was supplied by diamorphine, then later co-dydramol. Serum enzyme levels were consistent with a myocardial infarction; Alk.P. was elevated, and bilirubin was within normal limits, but insufficient ferritin measurements were obtained to allow interpretation.

All results are displayed in Table 22.

Table 22 : To show the effect of a myocardial infarction on serum liver enzyme and ferritin parameters

Subject	Age yr	Sex	No days post MI	ASPT IU/l	Alk.P. KA unit	Bili μmol /l	HBD IU/l	PT	PTTK secs	Ferr μg/l	%glyc
Normal range				5-35	3-12	<22	70-170	0.8-1.2		1-125	60-80
1. KM	60	M	0	111	10	20	160	2.2	50.4 (45.4)	>200	30.5
			4	1092	12	28	460	1.7	52.3 (40.8)	>500	8.6
			5	528	12	38	300	2.1	175.1 (40.6)	-	-
			9	86	12	56	-	2.0	135.9 (45.0)	130	85.4
2. VH	68	M	0	65	13	6	140	-	-	58	-
			1	340	14	10	536	-	-	62	49.8
			2	320	18	16	686	-	-	109	29.3
3. EC	59	M	0	396	8	9	760	-	-	50	24.0
			1	371	8	8	1320	-	-	78	46.3
			2	165	10	9	855	-	-	160	29.3
4. MS	63	M	1	292	5	-	940	-	-	-	-
			2	143	5	-	800	-	-	900	37.6
			3	74	6	-	680	-	-	-	-
			4	46	8	-	-	-	-	-	-
			8	36	8	-	-	-	-	1880	28.4
5. ES	82	F	3	114	15	10	-	-	-	50	52.5
			4	56	-	-	720	-	-	-	-
			5	38	25	12	563	-	-	-	-
			13	5	26	4	173	-	-	-	-

Discussion

Total serum ferritin levels have been demonstrated to increase as a result of an acute systemic inflammatory reaction, caused either by myocardial infarction or as a response to bacterial endotoxins.(50,51)

There is no evidence on the effect of a myocardial infarction on % glycated ferritin. In addition there is no reason to suspect liver involvement with myocardial infarction, unless if it is accompanied by congestive cardiac failure that may lead to hepatic congestion. Therefore if the radioimmunoassay kit is specific for the L subunit type of ferritin commonly found in the liver and spleen, then there should not be any large changes in serum ferritin levels. Unfortunately there is no information available on the specificity of the Becton Dickinson Radioimmunoassay kit for the different isoferritins.

All patients exhibited a clinically and biochemically proven myocardial infarction, but patient number 5 had to be excluded from the analysis of results because of an insufficient number of ferritin measurements.

Patient numbers 1 and 4 demonstrated large increases in total serum ferritin levels, accompanied by reductions in % glycated serum ferritin. It is possible that the ferritin changes seen in patient number 1 were associated with liver involvement from congestive cardiac failure. There are no apparent explanations for patient number 4. The elevated PT ratio and PTTK time evident in patient number 1 can be attributed to anti-coagulation therapy.

Patient numbers 2 and 3 demonstrated progressively increasing total serum ferritin levels, while % glycated levels were slightly reduced, but with no discernible pattern.

The results for total serum ferritin levels correlate with those of Birgegård et al, (51) showing an increase in levels, although not always above the normal range, coincident with myocardial infarction. There does not appear to be any pattern to changes in % glycated serum ferritin, although most were slightly below normal values.

To determine the effect of diabetes mellitus on total and % glycated serum ferritin

Introduction

Diabetes mellitus is a disease characterised by hyperglycaemia that is a result of a partial or complete deficiency of insulin activity. One of the consequences of an elevated serum glucose level is that plasma proteins, notably haemoglobin, may become glycated. Over a period of time, the poorer the control of diabetes, the greater the carbohydrate content of plasma proteins (52,53,54).

The purpose of this work was to determine whether the % glycated serum ferritin in the diabetic population differed significantly from that seen in the population as a whole.

Methods

All glucose determinations were carried out by the Department of Biochemistry, Selly Oak Hospital, and were part of routine investigations.

Results

Patient sample numbers 1-5 inclusive were obtained from Selly Oak Hospital Biochemistry Department and chosen for their elevated glucose levels. All samples were from diabetic patients; some were from diabetic out-patient clinics, and some had been submitted by General Practitioners in the South Birmingham Health District for analysis. It was only possible to obtain further medical information from the group of patients seen at the hospital.

Sample number 6 was from a pool of samples chosen on the criteria of elevated serum fructosamine levels, and therefore indicative of long term poor diabetic control.

Sample numbers 7 and 9 were both taken from otherwise healthy Type II (maturity onset) diabetics well controlled by oral medication, admitted to hospital for unrelated surgical procedures.

Sample number 8 was taken from a well controlled Type II diabetic who was admitted to a surgical ward with a gangrenous foot.

Sample number 10 was taken from a Type II diabetic who was well controlled by insulin while in hospital following a surgical procedure.

All results are displayed in Table 23.

Table 23 : To show the effect of serum glucose concentration in diabetics on serum ferritin parameters

Subject	Sex	Glucose mmol/l	Total serum ferritin. μg/l	% glycated
Normal range		3.9-5.6	1-125	60-80
1. LR	M	26.9	200	34.1
2. GM	F	27.4	45	53.8
3. EM	M	>20.0	90	13.2
4. WH	M	35.5	201	21.7
		20.0	160	20.3
5. MD	M	>20.0	140	50.0
6. Pooled sample	-	-	88	46.9
7. AH	M	<10.0	74	12.8
8. BP	M	<10.0	500	37.6
9. CG	M	<10.0	163	24.2
10. HS	M	10.0	234	48.3

The type of diabetes exhibited by patient numbers 1-5 was unknown, but patient numbers 7-10 were all type II diabetics. Sample number 6 was a pooled sample taken from patients with a high serum fructosamine concentration, indicative of poor long term diabetic control.

Statistical Analysis

Table 24 : Statistical parameters for total serum ferritin in diabetic subjects.

	Well controlled	Poorly controlled
Total serum ferritin	n = 4	n = 6
	$\bar{x}$ = 242.8	$\bar{x}$ = 124.0 μg/l
	sd = 183.6	sd = 60.0

Student t test : t = 1.51, df = 8 p(0.05), df = 8, t = 2.306 therefore not significant

Table 25 : Statistical parameters for % glycated serum ferritin in diabetic subjects.

	Well controlled	Poorly controlled
% glycated serum ferritin	n = 4	n = 6
	$\bar{x}$ = 30.4	$\bar{x}$ = 36.5
	sd = 15.6	sd = 16.6

Student t test : t = 0.55, df = 8 p(0.05), df = 8, t = 2.306 therefore not significant

The sample taken from the population of poorly controlled diabetics included the pooled sample and a mean of the values recorded for subject number 4.

As there did not appear to be any significant difference between the samples taken from both well controlled and poorly controlled diabetics, it was decided to combine these two groups as

being representative of the diabetic population and compare them with a representative sample of the normal population taken from healthy male colleagues.

Table 26 : Statistical comparison of total serum ferritin in diabetic and healthy subjects.

	Diabetic population	Normal population
Total serum ferritin	n = 10	n = 11
	x = 171.5	x = 48.6 μg/l
	sd = 130.3	sd = 38.3

Student t test : t = 2.99, df = 19 p(0.01), df = 19,

t = 2.86 therefore significant

Table 27 : Statistical comparison of % glycated serum ferritin in diabetic and healthy subjects.

	Diabetic population	Normal population
% glycated serum	n = 10	n = 7
	x = 34.2	x = 63.6 %
	sd = 15.6	sd = 13.8

Student t test : t = 4.00, df = 15 p(0.01), df = 15,

t = 2.95 therefore significant

Discussion

Eight of the nine diabetic subjects considered were male, one was female and one was a mixed sex pooled sample. The preponderance of males was by chance alone, so it was decided to compare results with the normal values for male subjects.

All but one of the total serum ferritin levels fell within normal limits, although most were at the upper end of the range. The only exception was for subject number 8, which was 4 times the

normal figure. One possible explanation for this is that the subject was suffering from a gangrene infection, which may have resulted in an increase in total serum ferritin levels. This phenomenon has been demonstrated to occur with infection both in this work and work by Birgegård et al (15).

Statistical analysis suggested that there was no difference between poorly controlled and well controlled diabetics for either total serum ferritin, $p > 0.05$, or % glycated serum ferritin, $p > 0.05$. It was considered that elevated blood glucose levels might result in a greater percentage of glycated serum ferritin. This was not observed, and is probably a reflection of the time taken to effect a glycation reaction under physiological conditions compared to the relatively short plasma elimination half-life (5 hours) of non-glycated serum ferritin. Non-enzymic glycation of haemoglobin occurs slowly by a chemical reaction that takes place between glucose and the amine groups of the amino acids valine and lysine to form a ketoamine linkage. If the same reaction scheme is involved in the glycation of ferritin, then the same time factors will apply.

Comparison of ferritin parameters in samples of diabetic and the normal population revealed total serum ferritin levels to be significantly higher in diabetics ($p < 0.01$) and % glycated serum ferritin to be significantly lower ($p < 0.01$) than in the normal population.

Previous work presented in the quality control section has confirmed that this is not related to the effects of glucose on the assay method, and must therefore be attributable to a physiological difference. A raised total serum ferritin together with a reduced % glycated serum ferritin is usually indicative of cellular damage.

Hyperglycaemia characteristic of diabetes results from hepatic overproduction of glucose by glycogenolysis and gluconeogenesis together with a decreased ability to remove glucose from the serum into cells. These biochemical pathways are unlikely to have a direct effect on the integrity of cells, however in extreme cases there is a deficiency in the production of pyruvate and consequent reduction in the energy made available by the tricarboxylic acid cycle. The result is an increased amount of protein degradation that leads to cell destruction of adipose and skeletal muscular tissues. This would perhaps be of greater significance in the poorly controlled diabetic, although no statistical difference was observed. An avenue of further investigation would be to

determine the effect of muscular injury on serum ferritin parameters, and discover whether it mimics the effect seen in diabetes.

In summary, it is apparent that there is no statistical difference between the serum ferritin parameters of poorly controlled and well controlled diabetics, but that there is a difference between diabetics and healthy subjects, with no conclusive explanation.

To determine the effect of acute hepatocellular damage caused by a self-administered overdose of paracetamol on serum liver enzyme, and total and % glycated ferritin levels

Introduction

Paracetamol is a commonly used analgesic and anti-pyretic agent that was introduced in the late nineteenth century. It is administered as tablets, elixir or suppositories at a suggested adult dose of 0.5-1 gram every 4 hours up to a recommended maximum of 4 g in 24 hours.

It is rapidly absorbed from the gastrointestinal tract, resulting in peak plasma concentrations approximately 30 minutes - 1 hour after ingestion. It is metabolised in the liver and excreted mainly in the urine as the glucuronide and sulphate conjugates. The half-life is in the region of 1-4 hours (55).

When taken in therapeutic doses, paracetamol does not have any appreciable side effects, however detectable hepatocellular damage may occur from as little as 10g ingested as a single dose. This is manifest biochemically by an increase in circulating liver enzyme levels (γ-glutamyl transferase, aspartate transaminase, and alkaline phosphatase) within 24-48 hours of the event and an increase in the elimination half-life of paracetamol.

The purpose of this study was to determine whether the acute liver damage also caused changes in both total serum ferritin as reported by Eastham (6) and % glycated serum ferritin levels, and in addition whether ferritin estimates hold any predictive advantages over the conventional estimates of liver damage.

The nature of paracetamol induced hepatocellular damage

Paracetamol is conjugated to the glucuronide and sulphate forms in the liver but it is also converted by the cytochrome P450 dependant pathway to a number of reactive intermediates,

including electrophiles and free radicals. Under normal circumstances, these will react with glutathione, an intracellular free radical scavenger, to form further inactive metabolites.

After a large dose of paracetamol the glutathione system becomes saturated, allowing the concentration of toxic metabolites to build up (56).

The method by which cells are disrupted is unclear, but it is thought that electrophiles can react with nucleophilic cellular macromolecules present in proteins, lipids, and nucleic acids. Free radical metabolites can give rise to the superoxide anion $O^{\cdot}_2$ that can produce hydrogen peroxide and ultimately the peroxide radical, $OH^{\cdot}$, that can oxidise membrane lipids (57).

Disruption of the cell wall will result in influx of Ca^{2+} ions and lead ultimately to cell death and efflux of intracellular contents (58).

The aim of acute treatment with sulphydryl compounds (methionine and N-acetylcysteine) is to replenish stocks of glutathione and provide alternative substrates for S-conjugation, or act as a source of inorganic sulphate (59).

The clinical decision on how to treat a paracetamol overdose will depend on a number of factors including time of presentation, serum paracetamol level, and half-life of paracetamol. Guide-lines are available for South Birmingham Health District, and are presented with permission in appendix 3.

Methods

Patient numbers 1, 2, and 3 were admitted to Selly Oak Hospital, and the remaining patients were admitted to Dudley Road Hospital.

The method of quantitative paracetamol determination varied between the two hospitals. At Selly Oak the Glynn-Kendal method was used which is based on the reaction of paracetamol with nitrous acid, which is then ionised to the phenoxide ion that may be detected spectroscopically

with an absorbtion maximum at 430nm (60). Dudley Road Hospital employ either a polarised fluorescence immunoassay or an enzyme colourimetric assay.

Liver enzyme levels and prothrombin times were presented as a service by each laboratory. The units and reference ranges vary between the two laboratories, so that abnormal results could only be compared as multiples of normal results, not as absolute figures.

All of the samples from Selly Oak Hospital were centrifuged to separate serum from packed cells within hours of collection. This also applied to samples from Dudley Road Hospital for serum liver enzyme determination, but not for all ferritin and % glycated ferritin determinations. Some of these samples were stored at 4° C for a period of up to 2-3 weeks before centrifugation. This resulted in haemolysis, and where this occurred, samples are annotated with a * and consideration given in the analysis of results.

Results

All of the overdose cases were considered sufficiently severe to warrant administration of the appropriate antidote provided that the patient presented at a time when treatment could prove beneficial (appendix 3). Those for whom treatment would not have been beneficial were patient numbers 5, 7, 8, and 9, and consequently no antidote was administered.

The quantities of paracetamol ingested varied from 15-20 to in excess of 100 tablets.

None of the patients took any preparations containing barbiturates concurrent with their overdose, but patient number 5 did consume approximately 200ml of whisky.

Patient numbers 1 and 2 were early presentations, treated promptly with the antidote, who did not progress to any abnormalities in liver function tests or serum ferritin parameters. The only indication of any damage was a raised prothrombin ratio, representing impaired synthetic capabilities of the liver.

Patient number 3 was also treated promptly but showed a peak increase of 40 times normal values of ASPT two days post ingestion. This was mirrored by a 15 times increase in total serum ferritin on previous levels and a reduction in the % glycated serum ferritin level compared with later values. All parameters started to return towards normal ranges over the following days.

Patient number 4 was treated with the antidote 12 hours post ingestion of an unknown quantity of paracetamol tablets. ASPT levels reached a maximum of 100 times normal two days later. The level of bilirubin was slightly raised, but Alk.P. remained within normal limits. It was possible to obtain only two results for serum ferritin parameters. Total serum ferritin appeared slightly elevated approximately 36 hours post ingestion, rising to a maximum two days after admission. These results were reflected by a slightly low % glycated serum ferritin after admission, reaching a minimum approximately one day later. Both samples for ferritin estimation were haemolysed on arrival.

Patient number 5 presented to the Casualty Department at Dudley Road hospital approximately 48 hours after self-administration of 100 paracetamol 500mg tablets together with 200ml of whisky. No antidote was administered. A peak ASPT level of 140 times normal was recorded between 60-72 hours post ingestion. Serum bilirubin reached a peak of 2-3 times normal 84-96 hours post ingestion. Alk.P. was slightly raised on admission, decreasing to within normal limits whilst in hospital. Three ferritin levels were obtained, at approximately 72, 96 and 120 hours post ingestion. All samples were haemolysed. Total serum ferritin levels decreased over the three day period, but % glycated serum ferritin which was initially normal, reached a minimum 24 hours later.

Patient number 6 presented 4½ hours after consuming an unknown quantity of paracetamol tablets, and was treated with the antidote. ASPT reached a maximum of 90 times normal levels 2 days after the overdose. The serum level of bilirubin was raised to 10 times normal, and Alk.P. to 3 times normal three days post ingestion. All results returned to nearly normal within three weeks. All three samples for ferritin determination were haemolysed.The first, taken within 24 hours of

ingestion showed a vast increase in total serum ferritin but no change in % glycated serum ferritin. This followed, reaching a minimum 24 hours later.

Patient number 7 presented 12 hours after ingestion of an unknown quantity of paracetamol tablets. No treatment was administered. This subject demonstrated a mild increase in ASPT, reaching a maximum of 20 times normal within 2-3 days of ingestion. All other results remained within normal limits. One ferritin sample was haemolysed.

Patient number 8 presented 12 hours post ingestion of an unknown quantity of paracetamol tablets. ASPT was at a peak of 170 times normal on admission. Similarly bilirubin was at a maximum of 3-4 times normal and prothrombin time was at a maximum on admission. Alk.P. reached a maximum of 1½ times normal 3-4 days post ingestion. Ferritin samples were not obtained until 5 days post ingestion. Total serum ferritin appeared marginally raised and % glycated serum ferritin marginally low. Both remained so while the patient was followed over a period of 15 days.

Patient number 9 presented 18 hours post ingestion of 100 Co-proxamol tablets (containing 325mg paracetamol and 32.5mg dextropropoxyphene in each tablet). No treatment was administered. ASPT was at a maximum of 170 times normal, and prothrombin time was at a maximum on admission. Serum bilirubin and Alk.P. were slightly raised. One sample for ferritin determination was haemolysed. Total serum ferritin reached a peak approximately 48 hours after ingestion, which corresponded with a minimum value for % glycated ferritin.

Patient number 10 presented 12 hours post ingestion of an unknown quantity of paracetamol tablets and was treated with the antidote. On admission all estimates of liver damage were at a maximum, ASPT reached 100 times normal, bilirubin 3 times normal, and Alk.P. twice normal. All samples for ferritin determination were haemolysed. All total serum ferritin levels were twice the normal reference range, and all % glycated serum ferritin levels were within normal limits.

All results are displayed in Table 28.

Table 28 : To show the affect of acute hepatocellular injury in the form of a paracetamol overdose, on serum liver enzyme and ferritin parameters

Subject	Age yrs	Sex M/F	Treat	Date	Parac mg/l	ASPT IU/l	Bili µmol/l	Alk.P. IU/l	PT	Ferr µg/l	%glyc
Normal Range SOH						5-35	<22	3-12	0.8-1.2	1-125	60-80
1. LD	16	F	YES	14.3	200	14	9	9	1.5	<10	>80
SOH			M/A	15.3	(6.5 HRS)	18	6	13	1.3	37	>80
				16.3		10	6	8	1.1	-	-
2. DW	22	M	YES	24.2	344	17	24	8	1.4	84	66.0
SOH			A	25.2	(4 HR)	16	12	7	1.4	125	69.0
				26.2		15	10	8	1.2	-	-
3. LS	19	F	YES	16.6	376	14	10	6	-	10	-
SOH			M/A	17.6	(4 HR)	24	26	7	-	10	-
				18.6		1448	23	7	-	138	43.4
				20.6		404	24	7	-	66	82.0
				21.6		156	24	7	-	54	58.2

Table 28 (Continued)

Subject	Age yrs	Sex M/F	Treat	Date	Parac mg/l	ASPT IU/l	Bili µmol/l	Alk.P. IU/l	PT	Ferr µg/l	%glyc
Normal Range DRH						<45	5-17	20-130	15/15	1-125	60-80
4. EE	24	F	YES	19.12	162	77	11	101	-	-	-
DRH				19.12	(12 HRS)	160	20	86	-	-	-
				20.12		1534	26	94	-	214	42.0 *
				20.12		4834	19	88	-	-	-
				21.12		4172	17	86	-	>2000	16.0 *
				21.12		2358	18	86	-	-	-
				22.12		1015	25	78	-	-	-
				23.12		370	14	74	-	-	-
5. WG	36	M	NO	29.2	-	4140	35	150	-	-	-
DRH				1.3		6140	31	141	-	>2000	76.0 *
				2.3		1150	58	118	-	>2000	27.0 *
				3.3		340	32	112	-	1500	34.0 *
				4.3		180	22	115	-	-	-
				5.3		96	19	110	-	-	-
				6.3		65	14	108	-	-	-
				7.3		54	13	117	-	-	-
6. PS	20	M	YES	20.2	176	894	87	172	-	-	-
DRH			A	20.2	(4.5 HR)	2937	106	187	-	>2000	68.1 *
				22.2		4015	156	296	-	>2000	19.1 *
				23.2		-	210	344	-	570	25.5 *
				24.2		714	154	301	-	-	-
				25.2		220	145	267	-	-	-
				26.2		130	158	232	-	-	-
				27.2		116	200	281	-	-	-
				1.3		103	120	315	-	-	-
				2.3		94	91	256	-	-	-
				11.3		45	30	132	-	-	-

Table 28 (Continued)

Subject	Age yrs	Sex M/F	Treat	Date	Parac mg/l	ASPT IU/l	Bili µmol/l	Alk.P. IU/l	PT	Ferr µg/l	%glyc
Normal Range DRH						<45	5-17	20-130	15/15	1-125	60-80
7. JH	22	F	NO	14.2		169	14	71	20/15	-	-
DRH				15.2		570	12	72	19/15	167	56.0
				16.2		830	13	75	16/15	200	63.9
				17.2		235	16	68	17/15	180	77.3
				18.2		152	8	62	-	220	43.4 *
8. HM	54	F	NO	3.2		7620	75	-	23/15	-	-
DRH				5.2		1275	68	205		-	
				6.2		455	72	208	17/15	-	-
				7.2		112	47	177	N	-	-
				8.2		56	35	161	-	480	59.4+
				9.2		27	31	167	-	-	-
				10.2		26	29	159	-	-	-
				11.2		19	17	152	-	-	-
				12.2		14	12	153	-	426	42.7
				16.2		14	10	161	-	410	52.9
				18.2		-	-	-	-	400	43.0
9. NS	58	F	NO	19.1		7770	29	115	24/15	-	-
DRH				20.1		3450	23	116	27/15	>2000	5.2 *
				21.1		893	34	145	19/15	-	-
				22.1		323	20	151	16/15	380	52.2+
				23.1		167	16	132	-	345	62.8
				24.1		99	11	125	-	318	45.1
10.GM	20	M	YES	14.12		4800	60	235	32/15	-	-
DRH				15.12		1600	53	194	22/15	215	68.2 *
				16.12		-	39	152	-	190	62.8 *
				17.12		260	34	191		232	63.3 *
				18.12		160	33	189	-	-	-

Key

* = haemolysed sample

+ = sample assayed separately.

M= methionine

A= N-acetylcysteine

Discussion

Previous work presented in the quality control section has shown that the haemolysis of samples has been associated with a marginally raised serum ferritin level together with a slightly reduced % glycated serum ferritin level. In the case of patient numbers 4, 5, 6, and 10 all samples were haemolysed, presumably to the same extent. This was not an impediment to comparison of successive samples within individual patients, although absolute values could not be relied upon, and it remained possible to examine trends throughout the period of investigation.

The situation became more complex for patient numbers 7 and 9 because, in each case, only one sample was haemolysed. The single haemolysed sample from patient 7 was not markedly different from the other samples, particularly when account is taken of haemolysis. However, the haemolysed sample from patient number 9 was significantly different from the remaining results and could not be explained by haemolysis alone.

It has been previously demonstrated that an increase in total serum ferritin together with a reduction in the % glycated serum ferritin may be indicative of hepatic damage (14). Subject numbers 1 and 2 did not show either any apparent variation in serum liver enzyme levels, or any changes in the nature of serum ferritin. The only indication of any hepatic injury was the decrease in the synthesis of prothrombin, manifest by an increased prothrombin ratio. Both subjects presented for treatment shortly after ingestion of the overdose, so it was probable that this averted serious hepatic damage. The only exception was patient number 6, who continued to develop hepatic injury that consisted of elevated ASPT, bilirubin and total serum ferritin levels despite treatment 4½ hours post ingestion with N-acetyl cysteine. One possible explanation is that the estimated time of the overdose in his case was incorrect.

In most of the patients who demonstrated liver damage by elevated serum liver enzyme levels, there was a corresponding increase in total serum ferritin and a reduction in % glycated serum ferritin levels from normal values. The only exceptions were patient numbers 8 and 10. Both exhibited large changes in serum liver enzyme levels, but only small changes in serum ferritin

parameters. Both presented 12 hours after ingestion, patient number 10 was treated and patient number 8 was not. The earliest available serum ferritin estimates were at approximately 120 and 36 hours post ingestion respectively. It is possible that ferritin levels were abnormal during the period for which there was no data available, and had since returned towards normal.

In the cases where changes were observed in serum ferritin levels, the maximum recorded total serum ferritin corresponded to the minimum % glycated serum ferritin levels, indicating that the total ferritin increase is made up of a vast increase in intracellular, non-glycated ferritin released into the circulation from damaged cells.

Patient numbers 5 and 6 demonstrated an initial increase in total serum ferritin with the % glycated level remaining in the normal range before reaching a minimum. This could be explained as an acute phase response characterised by increased ferritin synthesis, and hence production of the glycated form, as a response to injury, followed by the release of non-glycated ferritin from damaged cells.

In the situations where concurrent results of liver function tests and serum ferritin levels were available, the maximum levels of ASPT corresponded in time to maximum total serum ferritin levels. In addition, the magnitude of the serum levels of ASPT corresponded to those of total serum ferritin. There is insufficient data available to ascertain whether both sets of parameters returned to normal values over the same time course. However, the results from patient number 8 would suggest that total serum ferritin levels remain elevated after liver function tests have returned to normal.

In summary, the data presented provides further evidence on the efficacy of early treatment of paracetamol overdose with sulphydryl compounds. It has been demonstrated that acute hepatocellular injury results in an increase in the level of circulating serum ferritin, and that the majority of the ferritin is of the intra-cellular form. One interpretation of the evidence presented by patient numbers 5 and 6 is that changes in serum ferritin follow a pattern of initial increased synthesis followed by cell destruction and leakage of intra-cellular contents into the serum. There is no evidence to suggest that serum ferritin changes are either a more sensitive indicator, or offer

any predictive advantages over the conventional use of serum liver enzyme levels in cases of acute hepatocellular damage.

To determine the effect of methotrexate therapy on serum liver enzyme and ferritin parameters

Introduction

Methotrexate is a compound that competitively inhibits the enzyme dihydrofolate reductase, preventing the formation of tetrahydrofolate from dihydrofolic acid. Tetrahydrofolate is required for the synthesis of purines and pyrimidines and consequently the formation of DNA and RNA. The primary use of methotrexate is as an antineoplastic, cytotoxic agent, but it has also been used in lower doses to treat psoriasis and rheumatoid arthritis (61). The mode of action in these conditions remains unknown, but is probably related to the anti-proliferative effects of the drug on cells in the dermis (in psoriasis) and cells in the synovium (in rheumatoid arthritis). Other suggestions favour the immunosuppressive actions of methotrexate (62,63).

Psoriasis is a disease characterised by an excessively rapid turnover of cells in the epidermis, particularly in the psoriatic lesions. It is genetically determined but often triggered by exogenous factors, such as stress. There is evidence to suggest that it is not exclusively a disease of the skin, but that there are systemic manifestations. Mild cases can be effectively treated topically, however patients with very severe disease may require systemic treatment. Methotrexate has proved to be effective, often administered as a single dose of between 5mg and 25mg weekly (63). The most troublesome side effect is the tendency to cause hepatic fibrosis and cirrhosis in 5-10% of patients treated (64).

Rheumatoid arthritis is a disease characterised by local joint inflammation and erosion, and systemic auto-immune complications. Methotrexate is used in patients with severe disease who have proved unresponsive to the standard second line agents; gold salts, penicillamine, sulphasalazine or the chloroquines. The dosage regime is the same as for psoriasis, as is the spectrum of adverse reactions (63).

The incidence of hepatic fibrosis may be related to the cumulative dose of methotrexate consumed, therefore it is desirable to be able to monitor the patient for any signs of hepatic disorder. The damage seen is of a non-aggressive type, and in mild cases is not an indication for withdrawal of the drug (65). The current methods available for monitoring hepatic damage are inadequate. Serum liver enzyme levels alone are not a reliable indicator of alteration of hepatic structure, ultra-sound scans do not provide a detailed picture of hepatic damage, and liver biopsies carry up to a 10% morbidity (66). Current recommendations are to regularly monitor serum liver enzyme levels with each visit to clinic and to take a routine biopsy every 2 years, when a cumulative dose of 1.5g is reached or at any unexplained increase in liver function tests (63,65). Alternative monitoring methods that have been tried and found to be ineffective include radioactive technetium scans and galactose tolerance tests (66,67).

The aim of this work was to investigate the value of serum ferritin parameters as indicators of hepatic damage, achieved by a longitudinal study of patients treated with low dose methotrexate.

Methods

All serum enzyme determinations were carried out as part of routine investigation by the Departments of Clinical Chemistry and Biochemistry at Birmingham General and Selly Oak Hospitals respectively . The patients involved attended either the Rheumatology out-patient clinic at Selly Oak Hospital, or the Dermatology out-patient clinic at Birmingham General Hospital.

Samples collected at Selly Oak Hospital were either taken in clinic or on the ward, and retrieved from the Biochemistry Department following routine investigations. The samples collected from Birmingham General Hospital were all taken in clinic, centrifuged to separate serum from packed cells, and stored at -20° C until required for assay.

The units used when reporting Alk.P. levels differed between the two hospitals. Selly Oak use the King-Armstrong units, with a normal range of 3-12 KA units/l, and Birmingham General use International units, with a normal range of 70-350 IU/l.

Results

Patient number 1 had a twenty year history of psoriasis and a 5 year history of seropositive rheumatoid arthritis. He was admitted to Selly Oak Hospital as a rheumatoid patient with a view to initiating methotrexate therapy. Drug treatment on admission was fenbufen 300mg three times a day, and etretinate 50mg daily. He had a previous record of mild alcohol abuse. Prior to initiating methotrexate therapy on 17.8.87, the patient submitted to a liver biopsy, revealing no significant abnormalities. The starting dose of methotrexate was 7.5mg weekly, taken by mouth, and his progress was recorded over a ten month period by regular monthly attendance at the rheumatology out-patient clinic. Serum ASPT levels were initially within the normal range, increasing to a maximum of 2½ times normal in 3 months, then returning to the normal range in the following 2 months. All bilirubin levels were within the normal range. Alk.P. levels were marginally elevated throughout the course of treatment. ɣGT levels remained elevated throughout the period of study. The level of total serum ferritin changed very little, although there was greater fluctuation in % glycated ferritin.

Patient number 2 had a seven year history of seropositive, erosive rheumatoid arthritis and presented to Selly Oak Hospital with an exacerbation of her condition. Drug therapy on admission was ranitidine 300mg at night, fenbufen 300mg three times a day, prednisolone 7.5mg and 5mg on alternate days, and salbutamol and beclomethasone inhalers. Methotrexate therapy was started on 20.7.87 at a dose of 7.5mg per week, administered orally. This continued throughout the period of study, but was discontinued nine months after the study had been completed due to the development of a cellulitis. Methotrexate therapy was discontinued for a period of three weeks from 2.2.88 to 22.2.88 when the patient acquired a chest infection. Serum bilirubin, Alk.P. and ɣGT all remained within the normal range throughout the course of the study. However, serum ASPT gradually increased, reaching a peak of twice the normal maximum before returning to within normal limits. Total and % glycated serum ferritin levels were unremarkable.

Patient numbers 3-7 inclusive were all under the care of the department of Dermatology, General Hospital, Birmingham.

Patient number 3 had a history of psoriasis, treated with methotrexate 10mg orally each week for the preceding three years. She did not take any other medication. The dose of methotrexate was reduced to 7.5mg weekly on 9.6.87, and therapy was subsequently discontinued on 1.12.87 because of persistent nausea. At this time an ultra-sound scan of the liver revealed no abnormalities. Serum bilirubin levels remained within normal limits, serum ASPT levels were at the upper end of the normal range, and Alk.P. levels were all slightly higher than normal. Total serum ferritin remained constant, while there was a reduction in % glycated serum ferritin on 20.10.87.

Patient number 4 had a history of psoriasis treated with oral methotrexate at a dose of 20mg for the previous seven years. The only other drug therapy was folic acid, started on 14.8.87. Before the start of the study the subject had one abnormal ultra-sound scan of the liver in February 1986, which had resolved by March 1988 when there was record of a normal ultra-sound scan. Serum bilirubin and Alk.P. remained within the normal ranges, but the ASPT levels were slightly elevated. Of the available serum ferritin results, total serum ferritin increased, accompanied by a reduction in the % glycated serum ferritin.

Patient number 5 was started on oral methotrexate therapy five years prior to the study at a dose of 5mg weekly. There was evidence of a normal ultra-sound scan of the liver one year before the study. The subject was not treated with any other medication, and all serum liver enzyme levels and ferritin parameters were within normal limits.

Patient number 6 had a 45 year history of psoriasis and was started on oral methotrexate 10mg weekly on 21.1.87. All serum liver enzyme levels remained within normal limits, and the single % glycated and total serum ferritin levels measured were normal, although % glycated could be considered slightly low.

Patient number 7 had a history of psoriasis and had been treated with oral methotrexate for the previous eight years. There was evidence of a normal liver biopsy two years prior to the study, and the patient did not take any other medication. All serum liver enzyme levels and total serum ferritin levels were within normal limits, but % glycated levels appeared slightly low.

All results are displayed in Table 29.

Table 29 : To show the effect of methotrexate administration on serum liver enzyme and ferritin values

Subject	Age	Sex	Date	ASPT	Alk.P.	Bili	ɣGT	Ferritin	%gly
Units	yrs		d.m.y.	IU/l	KA unit,	µmol/l	IU/l	µg/l	
Normal Range				5-35	3-12	<22	5-55	1-125	60-80
1. EU. SOH	37	M	24.8.87	21	13	3	90	-	-
			23.9.87	77	13	7	70	53.5	20.6
			12.10.87	51	13	5	84	53.0	30.9
			10.11.87	89	13	5	100	50.0	56.8
			30.11.87	96	11	4	79	-	-
			11.1.88	38	12	3	83	-	-
			8.2.88	30	15	5	-	49.0	39.5
			7.3.88	-	-	-	-	55.0	33.8
			25.4.88	41	13	8	-	-	-
			20.6.88	15	14	6	-	-	-
2. OB. SOH	61	F	22.6.87	22	11	2	-	-	-
			24.7.87	25	11	4	-	38.0	37.0
			31.7.87	-	-	-	-	27.5	23.7
			10.8.87	17	11	3	-	14.0	33.0
			7.9.87	30	11	4	25	-	-
			28.9.87	28	10	4	22	18.0	-
			4.1.88	57	10	5	33	6.5	-
			25.1.88	71	11	4	37	-	-
			22.2.88	15	10	5	27	-	-

Table 29 (Continued)

Subject	Age	Sex	Date	ASPT	Alk.P.	Bili	ɣGT	Ferritin	%gly
Units	yrs		d.m.y.	IU/l	IU/l,	μmol/ll	IU/l	μg/l	
Normal Range				5-35	70-350	<22	5-55	1-125	60-80
3. DC	60	F	9.6.87	27	410	4	-	90.0	67.1
GHB			20.10.87	36	415	8	-	87.0	51.3
			1.12.87	29	355	7	-	87.0	60.2
4. KJ	62	M	27.3.87	62	280	8	-	-	-
GHB			10.4.87	67	270	13	-	-	-
			19.6.87	21	295	7	-	24.0	52.9
			4.12.87	36	255	9	-	54.0	37.0
5. KR	36	F	1.4.87	19	230	6	-	27.0	70.6
GHB			30.9.87	17	198	5	-	45.0	65.3
6. MS	74	F	3.3.87	28	305	6	35	-	-
GHB			7.4.87	23	310	7	-	85.0	44.7
			8.9.87	21	280	7	-	-	-
7. LJ	43	M	11.3.87	36	250	13	-	-	-
GHB			7.10.87	20	240	10	-	60.0	30.0
			13.1.87	21	225	10	-	49.0	31.2

Discussion

Hepatic injury caused by methotrexate has been well documented during the treatment of psoriasis and rheumatoid arthritis (62,68). Although well recognised, it remains a rare occurrence among the total number of patients treated with the drug. Estimates of the incidence vary from 3-5% in psoriatics, to 10-30% in rheumatoid patients who take the drug for 2-4 years (69). The likelihood of finding an advancing case of methotrexate induced hepatic damage from a small sample was low, especially among the rheumatoid patients who had only recently started the drug. However, there may have been evidence of sub-clinical hepatic impairment characterised by changes in serum ferritin parameters.

The two patients suffering from rheumatoid arthritis had a relatively low % glycated serum ferritin level compared with both that of healthy subjects previously documented, and the samples

of the psoriatic patients. This is in agreement with the work of Brailsford et al (unpublished), who demonstrated a low % glycated serum ferritin with an increased total serum ferritin in rheumatoid patients compared with the normal population (70).

Patient numbers 1 and 2 both exhibited significantly raised serum ASPT levels, accompanied in the case of patient number 1 by raised serum γ GT levels. All other serum liver enzyme levels remained within normal limits. There were no apparent changes in serum ferritin parameters for patient number 1. The enzyme changes for patient number 1 may be accounted for by the suggestion of continued over use of alcohol. There was no direct proof for this, but it was suspected by the clinician concerned with the patient's therapy. The total serum ferritin levels that corresponded to raised ASPT for patient number 2 were too low to allow determination of % glycation. This is not indicative of hepatic damage, where there would be an increase in total serum ferritin concurrent with a reduction in % glycation.

There remains insufficient evidence to confirm or deny the value of serum ferritin parameters as a means of monitoring for hepatic damage in patients maintained on methotrexate.

DISCUSSION

The methods employed in separation of glycated and non-glycated ferritin and subsequent assay were based on the method of Worwood et al (14), adapted by Brailsford at Birmingham University. These methods were transferred and adapted to suit the available facilities in the Biochemistry Department at Selly Oak Hospital.

There were a number of disadvantages related to the complexity of the process. It is an assay that contains several separate stages, at each of which errors may be introduced. However, it was considered outside the scope of this work to significantly modify the assay, and the methods were adopted virtually unchanged. Satisfactory results were obtained as judged from coefficient of variation data that testify to the reproducibility of the method, but give no information on the possibility of a single mistake that would invalidate a particular assay.

Another disadvantage stems from the time taken to perform the assay. The whole process from the washing of the gel in buffer until the final print-out of radioactivity counts from sample tubes may take up to eight hours, exclusive of the calculation of results.

From the moment of manufacture, the Ferritin ^{125}I-Tracer has a finite shelf life, as radioactive decay takes place. The kit is probably viable for up to 4-6 weeks, but as time progresses it becomes less sensitive. The differences in the age of the kits used was a contributory factor to the higher inter-assay coefficient of variation.

A further failing of the radioimmunoassay kit is the lack of information on its specificity for different human ferritin species. It is possible to detect changes in total and % glycated serum ferritin, but not to categorise the isoferritins involved.

Samples were stored and assayed in batches, partly to eliminate inter-assay variability in results from related samples, but also to ensure economic usage of the radioimmunoassay kit. The most economic usage of a Ferritin RIA kit involves just one calibration curve and approximately 42 duplicates, resulting in a cost of obtaining one total and % glycated ferritin result

of approximately £8.50 in materials alone. If a single determination was to be carried out the cost would be considerably higher. This compares with a total cost of £3.84 for estimation of serum liver enzyme levels performed using an Auto-Analyser machine.

The coefficients of variation reflected the complexity of the assay procedure. Within-assay variation was acceptable, but between-assay variation was significantly higher, reflecting factors such as batch variation of the RIA kits, concanavalin A and sepharose gels. Consequently, in most cases related samples were determined within the same assay. The inter-assay results compared favourably with the data published by Becton Dickinson for the RIA kit alone (28).

There has been no previously documented work on the effects of prolonged storage on sample stability. This was approached as a matter of urgency, as the results were likely to have a bearing on any further investigations.

There was no trend observed in total or % glycated serum ferritin in samples that had been stored for up to 24 weeks at -20° C. However, there was a greater variability in the % glycated serum ferritin. This was unlikely to have had any practical relevance, as most samples were collected over a short period of time, stored, and then assayed. The only exceptions were some samples taken for the methotrexate study, and for the prolonged variation over 8 weeks. These cases have been considered separately.

The investigation into the effect of sample haemolysis on ferritin parameters yielded an interesting result. The apparent increase in total serum ferritin with a reduction in % glycation was caused by a release of predominantly non-glycated ferritin from haemolysed red blood cells. Although previous work has stated that erythrocytes and leucocytes contain both acidic and basic ferritin species (40,41), there is no previous reference to whether the isoferritin species are glycated. Despite the small sample size, these results suggest that intracellular ferritin in blood cells is predominantly non-glycated.

The results of the study to determine whether dilution of sample prior to assay influenced the determination of % glycated ferritin allowed for the possibility of using a non-standard sample

volume for separation of glycated and non-glycated ferritin. This would have been advantageous in cases where there was an insufficient quantity of the original serum sample. In practice the situation rarely arose when other than 0.5ml of serum was taken for gel extraction.

The study to investigate the binding capacity of Concanavalin A and Sepharose gels was carried out using glucose, which is a significantly different molecule from ferritin. However, the results obtained suggested that there was a large degree of non-specific binding evident in the gel matrix. This emphasised the importance of using Sepharose gel as a control for samples extracted with ConA-sepharose gel. In addition, as the concentration of glucose increased there was no apparent saturation of available specific binding sites.

Normal population data was accumulated under the conditions of the assay, which prompted the adoption of a reference range slightly lower than that usually quoted of 15-300 μg/l (13). The overall range of 1-125 μg/l is closer to that quoted by Klockers et al (43), and may reflect the type of radioimmunoassay kit.

It was important to discover whether the time of day that a sample was collected had any bearing on the total and % glycated serum ferritin results. Siimes et al (35) and Casale et al (44) both reported an absence of any diurnal variation in total serum ferritin, confirmed in this study, which also revealed an absence of diurnal variation in % glycation that was previously unreported. Sequential variation in total and % glycated serum ferritin within individual subjects has not previously been investigated. The results of repeated measurements at weekly intervals revealed some variation in total and considerable variation in % glycated serum ferritin within individual subjects over the period of study. The fluctuations did not reflect any trend, or correlate with any other physiological cycles. Careful documentation was made of any concurrent illness or situation that could have affected ferritin parameters, and none was found.

One of the aims of this study was to determine whether changes in serum ferritin would be a useful indicator of hepatic damage. In order to evaluate this it was necessary to look at the specificity of the ferritin response.

In cases of hepatocellular damage due to drugs or disease, the total serum ferritin concentration was elevated, concomitant with a reduction in % glycation, implying release of non-glycated ferritin from damaged cells. This is in agreement with published work (5,14). However, alterations in total and % glycated serum ferritin levels were not confined to hepatic damage, but were also manifest in cases of infection, myocardial infarction, diabetes mellitus, and prior alcohol consumption.

It is conceivable that the evidence discovered in sufferers of diabetes could be related to fatty infiltration of hepatic tissue, and represent a mild cellular disruption. This appears to be a genuine physiological effect rather than an artifact, as any result of exogenous glucose on the assay was eliminated by a series of control experiments.

The source of the ferritin changes in myocardial infarction is difficult to characterise with the current methodology. The most likely explanation is the release of non-glycated, heart specific isoferritin from damaged cardiac cells, that are detected by the non-specific RIA kit. The only available manufacturer's data deals with specificity to human ferritin as opposed to equine ferritin, and not individual isoferritins. The alternative explanation, outlined by Birgegård et al, suggests a generalised increase in the synthesis of serum ferritin (51). Unfortunately it was not possible to investigate these possibilities further without an isoferritin specific assay.

The prevalence of alcohol use in society makes the effect of alcohol ingestion on serum ferritin levels an important factor when considering the usefulness of the test. There was little variation observed in total serum ferritin in the three volunteers, but all demonstrated a reduction in % glycation between 12 and 18 hours after alcohol consumption. The reason for this was not clear, but it was possibly related to changes in the clearance of serum ferritin.

It is difficult to prove that serum ferritin changes are more sensitive indicators of hepatocellular damage due to drugs than changes in serum liver enzyme levels. The incidence of these type of reactions is relatively low (48). If it were higher the drug in question would not have been granted a product licence for use in this country. In addition there are ethical implications involved. If the study had been ongoing rather than retrospective, any suspicion of a reaction demonstrated by

an unproven monitoring procedure would raise the question of whether treatment should be continued. The situation did not arise in this work because the study was retrospective, and no serious toxic reactions to methotrexate were observed in the subjects recruited into the study.

The only suggestion that measurement of serum ferritin offered greater sensitivity compared with conventional liver function tests in the detection of hepatic damage came from the study of diabetes, as previously discussed, where changes in ferritin were observed, possibly due to fatty infiltration of the liver, without any alteration in LFT's.

The results of the survey of pathological jaundice revealed a possible use for the monitoring of serum ferritin parameters in preference to serum liver enzyme levels. It is apparent that changes in serum ferritin are not observed in cases of cholestatic jaundice, but are observed in hepatocellular disease. The test may be of use to differentiate between the two, as liver function tests of hepatocellular and cholestatic damage are similar in the early stages.

CONCLUSIONS

The initial work suggesting that hepatocellular damage resulted in an increase in total serum ferritin concentration with a concurrent reduction in % of ferritin glycated was confirmed. The most likely explanation is that the increase in total serum ferritin is caused by the release of non-glycated intracellular ferritin from damaged cells. This occurred irrespective of the cause of the hepatocellular damage, and was manifest in cases of drug toxicity and pathological conditions.

The method of gel extraction and radioimmunoassay to detect total and % glycated serum ferritin proved to be both reliable and reproducible under laboratory conditions.

The test is not widely available and has the disadvantages of a high cost and a long and complicated method, particularly when compared with conventional liver function tests. It is not specific for hepatic damage and ferritin levels are affected by both physiological and pathological conditions.

During the course of this work it was not possible to determine whether the test is more sensitive than changes in serum liver enzyme levels as an indicator of hepatic damage because of an insufficient number of the necessary type of samples.

A possible application that did emerge for the use of serum ferritin parameters is to distinguish between the early stages of cholestasis, where there are no ferritin changes, and hepatocellular damage, that is accompanied by an increase in total serum ferritin with a reduction in % glycation.

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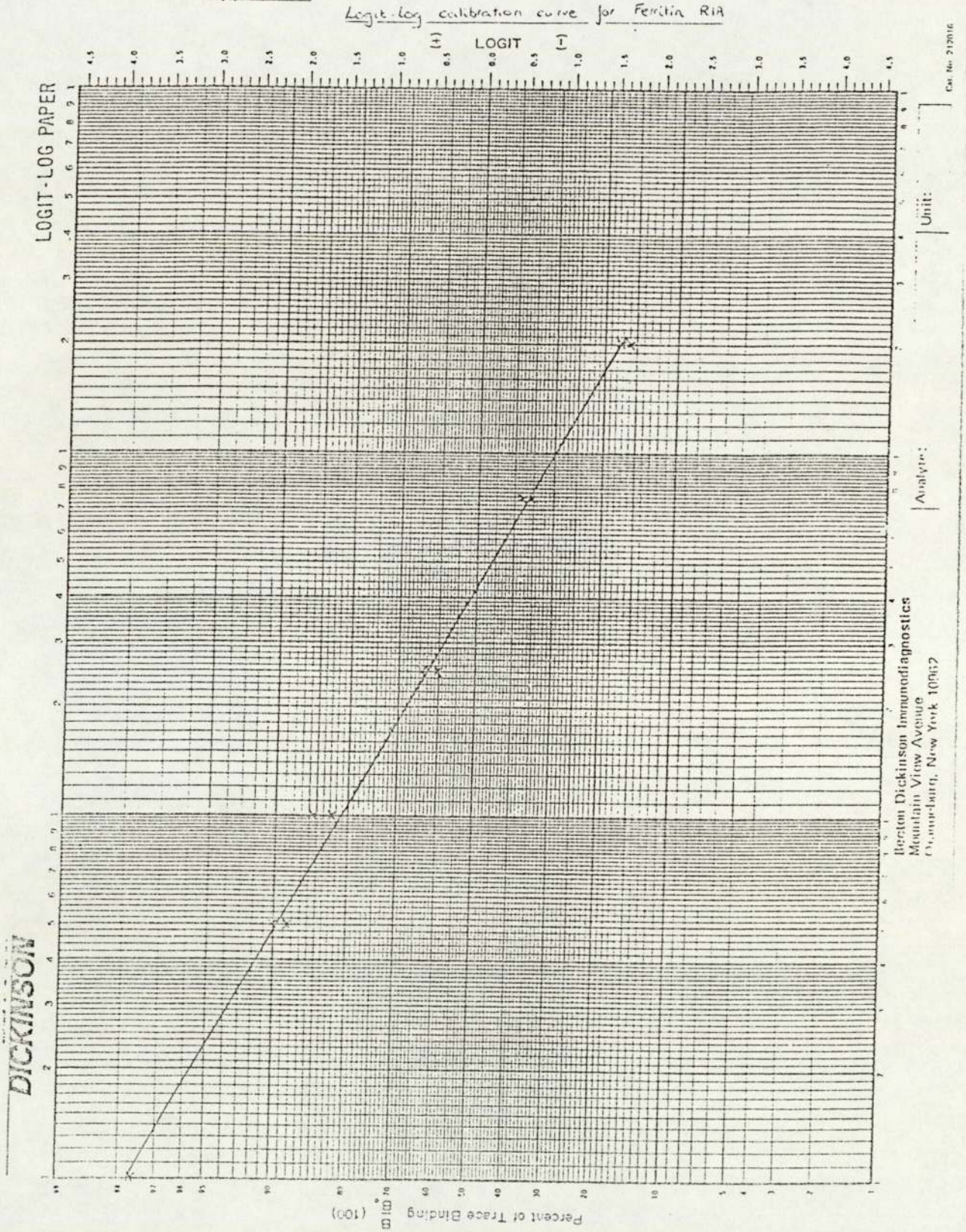
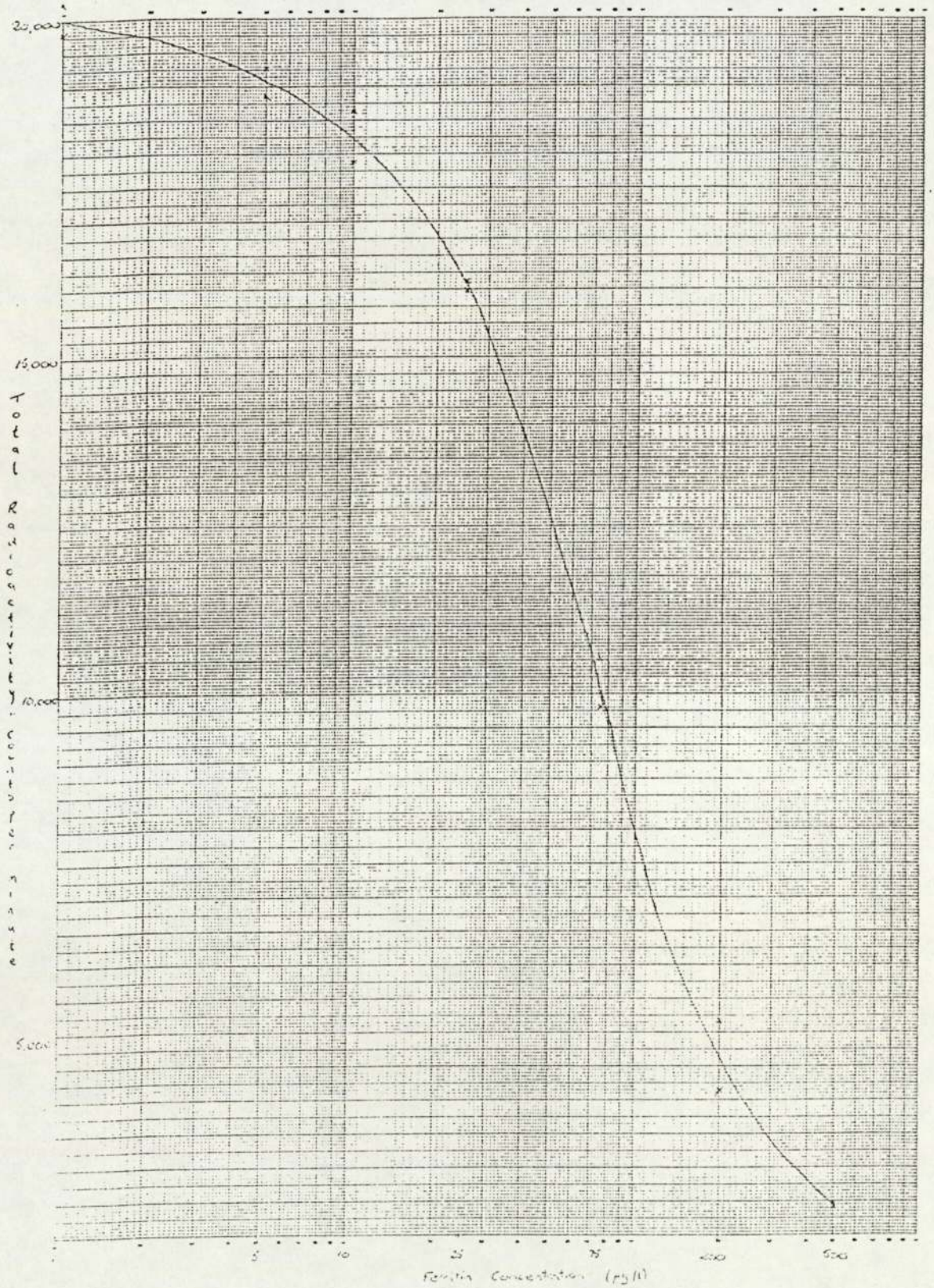
Appendix 1

Logit-log calibration curve for Ferritin RIA



Log Calibration Curve for Ferritin RIA



Appendix 2

A novel means of monitoring drug induced liver damage - variation study

A small number of drugs are known to cause a form of reversible drug damage. Although this is very rare, patients taking these drugs have regular blood tests that can warn the physician of the occurrence of any liver problems, with the consequence that the medication is stopped.

The aim of the study is to develop a more sensitive test that may alert the physician to any liver changes at an earlier stage than conventional methods. The test involves taking a 10ml sample of blood to measure one of the constituents, the iron storage protein ferritin.

To confirm that the new test is valid, it is necessary to collect blood samples from a control group of individuals who are not treated with any drugs that can cause liver damage. For it to be any practical use, the values for healthy individuals should remain within a narrow range over both a 24 hour period and on a longer week to week basis.

The investigation into diurnal variation will involve taking a single 10ml sample of venous blood at each of the following times:

08.00, 12.00, 16.00, 20.00, 24.00, and 04.00

in a given 24 hour period.

To assess variation over a longer period it is intended to take a single 10ml sample of venous blood at the same time each week for 5 consecutive weeks.

Thank you for your help with this study.

A novel means of monitoring drug induced liver damage - alcohol study

A small number of drugs are known to cause a form of reversible drug damage. Although this is very rare, patients taking these drugs have regular blood tests that can warn the physician of the occurrence of any liver problems, with the consequence that the medication is stopped.

The aim of the study is to develop a more sensitive test that may alert the physician to any liver changes at an earlier stage than conventional methods. The test involves taking a 10ml sample of blood to measure one of the constituents, the iron storage protein ferritin.

Ingestion of alcohol has been known over the short term to induce the activity of certain liver enzymes, and habitual abuse in the long term to cause hepatocellular damage.

The purpose of this study is to determine whether there is an element of hepatocellular damage, measured by changes in the nature of serum ferritin, from a single ingestion of a moderate amount of alcohol (equivalent to approximately 5 'shorts').

It will be necessary for each volunteer to abstain from alcohol for three days prior to the experiment, and during the follow up period. 10ml of venous blood will be taken before alcohol ingestion, and a further 10ml sample at the following times post-ingestion:

30mins, 2, 12, 18, 24, 36, 60, and 108 hours.

For example, if alcohol consumption is completed by 9pm Sunday, then blood samples will be taken at the following times:

Sunday at 9.30 pm

Sunday at 11.30 pm

Monday at 9.00 am

Monday at 3.00 pm

Monday at 9.00 pm

Tuesday at 9.00 am

Wednesday at 9.00 am

Friday at 9.00 am

Each sample will be assayed for biochemical profile, GT, alcohol level, and % glycation serum ferritin.

Thank you for your help with this study.

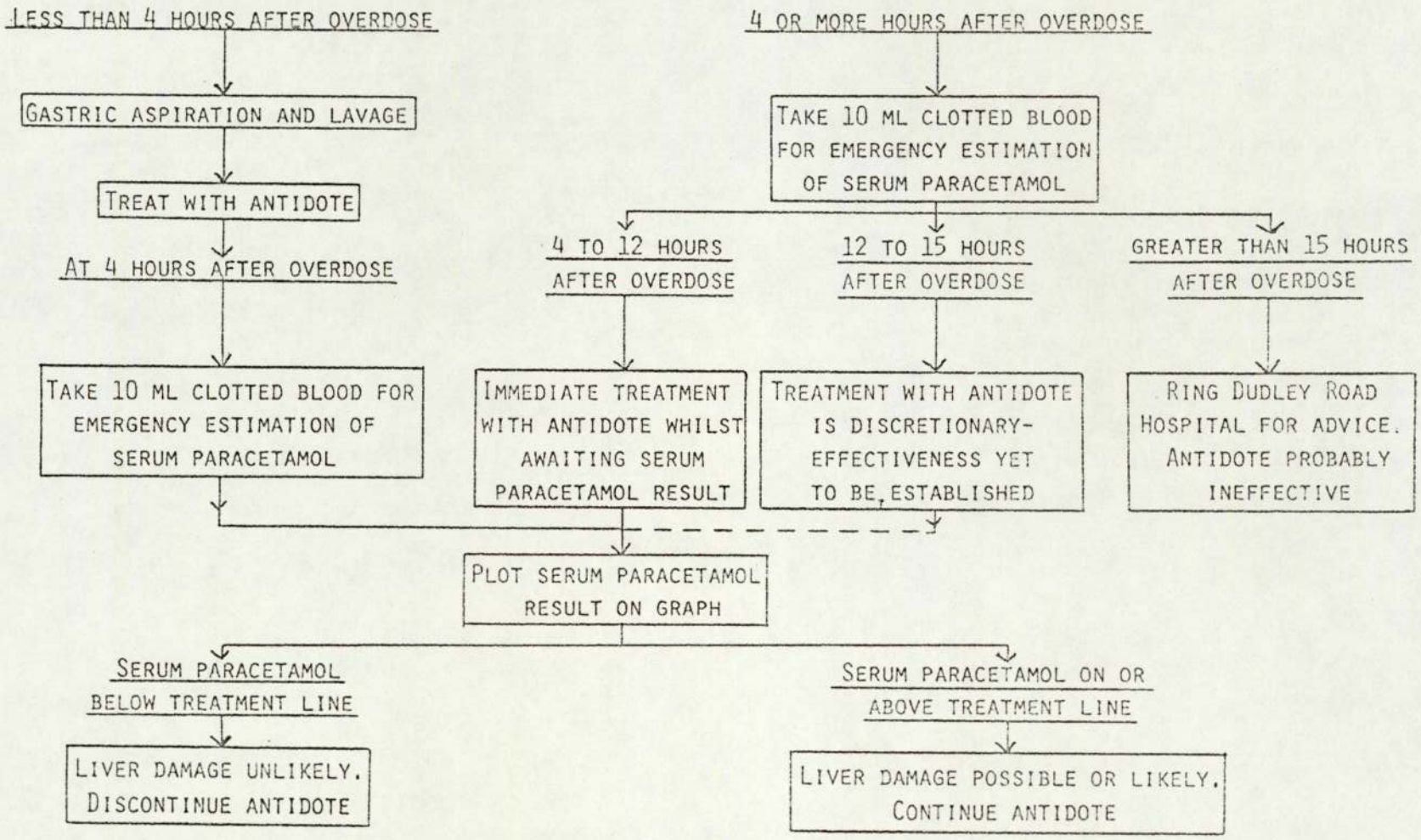
Appendix 3

TREATMENT OF PARACETAMOL OVERDOSE IN ADULTS USING METHIONINE OR N-ACETYLCYSTEINE (PARVOLEX)

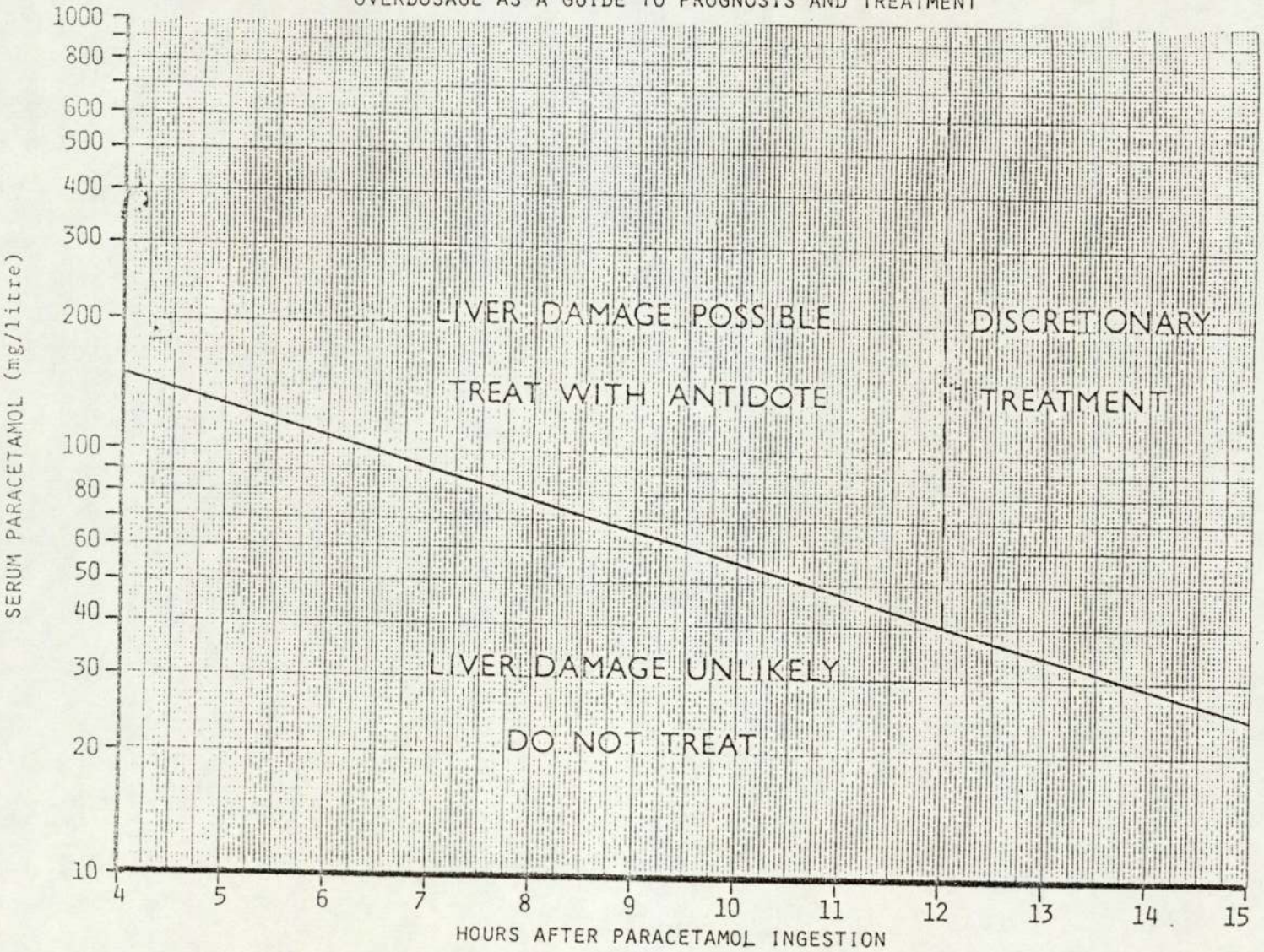
April, 1983

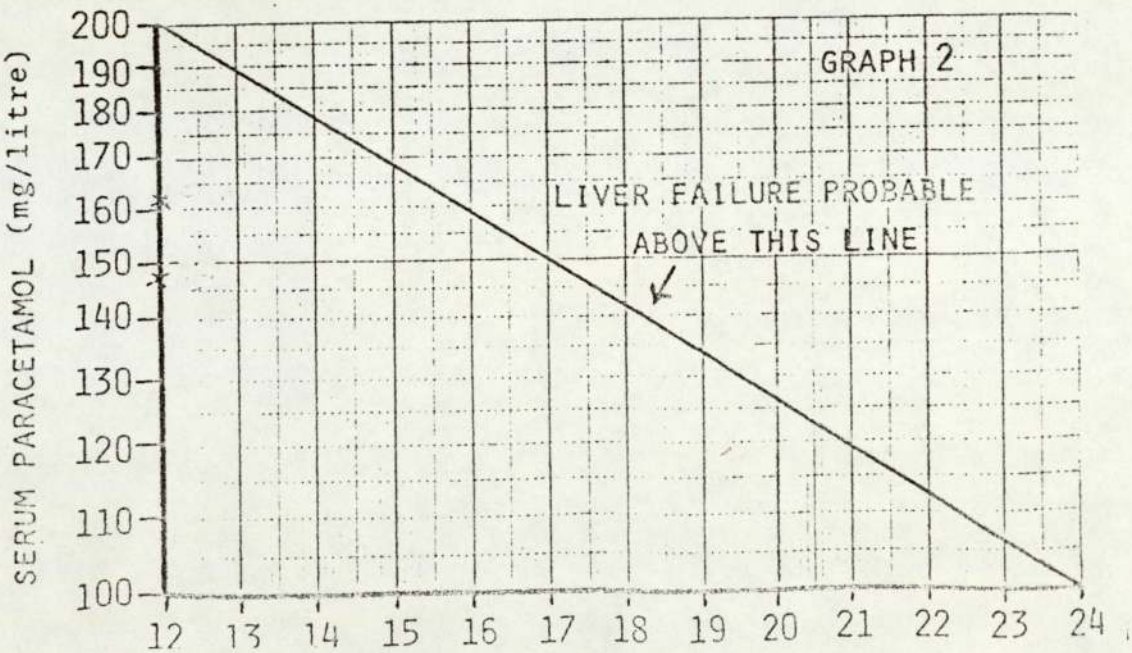
MANAGEMENT OF PARACETAMOL OVERDOSE DEPENDING ON TIME SINCE INGESTION

ORAL METHIONINE IS THE ANTIDOTE OF CHOICE UNLESS THE PATIENT IS VOMITING. WHEN USED, DO NOT GIVE CHARCOAL AFTER GASTRIC LAVAGE. INTRAVENOUS PARVOLEX IS INDICATED FOR PATIENTS WHO ARE VOMITING ON ARRIVAL OR DURING METHIONINE TREATMENT.



SERUM PARACETAMOL CONCENTRATION IN RELATION TO TIME AFTER PARACETAMOL OVERDOSAGE AS A GUIDE TO PROGNOSIS AND TREATMENT




GRAPH 2
LIVER FAILURE PROBABLE
ABOVE THIS LINE
SERUM PARACETAMOL (mg/litre)
200
190
180
170
160
150
140
130
120
110
100
12
13
14
15
16
17
18
19
20
21
22
23
24

I METHIONINE

Oral methionine is the treatment of choice for patients who are not vomiting since it is cheaper than Parvolex.

Dose

TEN 250mg tablets to be taken every FOUR hours for FOUR doses. Total dose = 10g.

Side Effects

Nausea and vomiting.

II PARVOLEX (N-Acetyl Cysteine)

Dose

Initial dose: 150mg per kg bodyweight in 150ml 5% Dextrose Injection intravenously over 15 minutes,

Followed IMMEDIATELY by:-

Second dose: 50mg per kg bodyweight in 500ml 5% Dextrose Injection over 4 hours,

then

Third dose: 100mg per kg bodyweight in 1 litre 5% Dextrose Injection over the following 16 hours.

Body Weight kg	Initial Dose* (ml)	Second Dose* (ml)	Third Dose* (ml)
50	38	13	25
55	41	14	28
60	45	15	30
65	49	16	32
70	53	18	35
75	56	19	38
x	0.75x	0.25x	0.5x

* dose calculated to nearest ml

Side Effects

Nausea and vomiting, minor transient rises in blood pressure. An anaphylactoid reaction has been reported but responded to conventional treatment with steroids and antihistamines.

Notes

1. Serum paracetamol levels estimated within 4 hours of ingestion cannot be interpreted.

2. If the serum paracetamol level is "on the line" a repeat level 2 to 4 hours later may be helpful in determining the outcome.

3. If the patient is unconscious, or the time of overdose unknown, treatment is discretionary. If the serum paracetamol level is less than 40 mg per litre, treatment is probably unnecessary or will be ineffective. Neither methionine nor Parvolex should be given if gross disturbance of hepatic function is already present since there is a theoretical possibility of precipitating hepatic encephalopathy.

4. If the patient has also taken dextropropoxyphene (as in Distalgesic) and has not been given naloxone, paracetamol absorption may be delayed and interpretation of serum results confounded. A repeat blood sample taken 4 hours later may be helpful.

5. Indications of likely liver damage are:-

   (a) a paracetamol level exceeding the line on Graph 2 below

   (b) a serum paracetamol half life ($T\frac{1}{2}$) exceeding 4 hours. $T\frac{1}{2}$ determination requires two serum paracetamol estimations, taken 3 hours apart. It is usually only necessary when:-

      (i) the initial paracetamol level is high (twice that given by the treatment line on Graph 1)

   or (ii) when the time of ingestion is unknown
   If $T\frac{1}{2}$ exceeds 10 hours, death is probable.

   (c) the most sensitive early indicator of hepatic dysfunction is a prothrombin time greater than 20 secs. in the first 24 hours post ingestion.

(Graph 2 to be inserted after Note 5)

6. Dr. J. Vale, Consultant Physician and Toxicologist, Dudley Road Hospital, is interested in accepting any poisoned patient and may be contacted directly (Ext. 4123) or via a member of his firm.

7. The accuracy and precision of the assay for the estimation of serum paracetamol levels is such that 95% of the results should be within 10 mg per litre of the true paracetamol concentration.

EFFICACY

The time interval between ingestion of overdose and the initiation of therapy is critical.

Methionine and Parvolex are effective up to 8 hours after overdose
have some effect between 8 and 12 hours
are of unknown value between 12 and 15 hours
and are completely ineffective after 15 hours, and possibly even harmful.