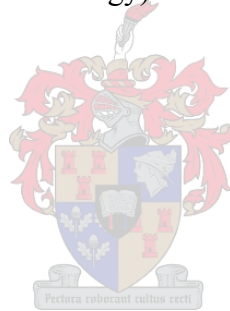# Lipoprotein X:

BIOCHEMICAL PREDICTORS AND DETECTION BY NON-DENATURING POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS

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Assignment presented in partial fulfilment of the requirements for the degree of Master of Medicine (Chemical Pathology) at Stellenbosch University.

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DECLARATION

I, the undersigned, hereby declare that the work contained in this assignment is my original work and that it has not previously in its entirety or in part been submitted at any university for a degree.

Signature:……………………….**Date**:………………………………..

SUMMARY

Lipoprotein X (LpX) is an abnormal cholesterol-containing particle that may be present in the serum of subjects with cholestasis, lecithin:cholesterol acyltransferase (LCAT) deficiency and parenteral nutrition. The biochemistry, metabolism, clinical significance and laboratory analysis of LpX is discussed in this study. This laboratory-based project investigated icteric samples received at the Chemical Pathology laboratory, Tygerberg Hospital, for serum predictors of LpX and the use of a modified non-denaturing polyacrylamide gradient gel electrophoresis system in the detection of LpX. The study showed that the non-denaturing polyacrylamide gradient gel electrophoresis system (2-8%) is a useful test in demonstrating LpX in icteric plasma and has potential for a screening test in LCAT deficiency. Serum concentration of conjugated bilirubin, alkaline phosphatase, gamma glutamyltransferase, free cholesterol, phospholipid, free cholesterol: total cholesterol ratio and conjugated bilirubin: total bilirubin ratio are all good predictors of LpX. The ratio of free cholesterol to total cholesterol (FC/TC > 0.6) was the best predictor of LpX. In the setting of obstructive liver disease LpX is seen in 66% of patients if total cholesterol is > 7.5 mmol/L.

OPSOMMING

Lipoproteien X (LpX) is 'n abnormale cholesterol-bevattende partikel wat teenwoordig mag wees in die serum van persone met cholestase, lesitien:cholesterol asieltransferase (LCAT) gebrek en parenterale voeding. Die biochemie, metabolisme, kliniese belang en laboratorium analise van LpX word bespreek in hierdie werkstuk. Hierdie laboratorium-gebaseerde projek het geelsugtige monsters ondersoek wat ontvang is by die Chemiese Patologie laboratorium, Tygerberg Hospitaal, vir serum voorspellers van LpX en die gebruik van 'n gemodifiseerde nie-denaturerende polie-akrielamied gradiënt gel elektroforese sisteem in die demonstrasie van LpX. Die bevindinge was dat die nie-denaturerende polie-akrielamied gradient gel elektroforese sisteem (2-8%) is 'n nuttige toets om LpX te demonstreer in geelsugtige plasma en het potensiaal as 'n siftingstoets in LCAT gebrek. Serum konsentrasie van gekonjugeerde bilirubien, alkaliese fosfatase, gamma glutamieltransferase, vry cholesterol, fosfolipied, vry cholesterol:totale cholesterol verhouding en gekonjugeerde bilirubien:totale bilirubien verhouding is alles goeie voorspellers van LpX. Die verhouding van vry cholesterol tot totale cholesterol (VC/TC > 0.6) was die beste voorspeller van LpX. In gevalle van obstruktiewe lewersiekte word LpX gesien in 66% van pasiente as die totale cholesterol meer as 7.5 mmol/l is.

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LIST OF ABBREVIATIONS

ACAT	acylCoA:cholesterol acyltransferase
AGE	agarose gel electrophoresis
AGS	Alagille syndrome
ALP	alkaline phosphatase
ALT	alanine transaminase
ApoB	apolipoprotein B
ApoE	apolipoprotein E
AST	aspatate transaminase
ATP	adenosine triphosphate
BA	bile acid
CABG	coronary artery bypass graft
CB	conjugated bilirubin
CETP	cholesteryl ester transfer protein
c-IMT	carotid intimal-medial thickness
CM	chylomicrons
CV	coefficient of variation
CVD	cardiovascular disease
c-WS	carotid wall stiffness
DAOS	N-ethyl-N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline sodium
DMSO	dimethylsulphoxide
FC	free cholesterol
FH	familial hypercholesterolaemia
GGE	gradient gel electrophoresis
GGT	gamma glutamyltransferase
HDL	high-density lipoproteins
HDL-C	HDL-cholesterol
HL	hepatic lipase
HMG	hydroxymethylglutaryl
Hb	haemoglobin
Hp	haptoglobin
IDL	intermediate-density lipoproteins
LCAT	lecithin:cholesterol acyltransferase
LDH	lactate dehydrogenase
LDL	low-density lipoproteins
LDL-C	LDL-cholesterol
LP	lipoproteins
LpA	lipoprotein A
LpB	lipoprotein B
LPDS	lipoprotein deficient serum
LpX	lipoprotein X
MF	mid-focussed band on GGE
NAD	nicotinamide adenine dinucleotide

NADP	nicotinamide adenosine dinucleotide phosphate
NBT	nitroblue tetrazolium
OD	optical density
PEG	polyethylene glycol
PBC	primary biliary cirrhosis
PFIC	progressive familial intrahepatic cholestasis
PL	phospholipid
PLTP	phospholipid transfer protein
PTB	pulmonary tuberculosis
Rf	retardation factor
SDS	sodium dodecyl sulphate
TB	total bilirubin
TC	total cholesterol
TG	triglyceride
VLDL	very-low-density lipoproteins

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ACKNOWLEDGEMENTS

1. INTRODUCTION

As a result of the high prevalence of atherosclerosis in the developed and developing world, and the clear link of hypercholesterolaemia with the atherosclerosis, blood is frequently analysed for hyperlipidaemia. It is also recognized that (cholestatic) liver disease is a cause of secondary dyslipidaemia. During investigation of liver disease it may be necessary to discriminate between primary and secondary dyslipidaemia. A more detailed analysis of lipoprotein fractions can aid in the aetiological diagnosis of dyslipidaemia.

Lipoprotein X (LpX) is an abnormal cholesterol-containing particle that was first described in the 1950s. It may be present in the serum of subjects with obstructive jaundice, lecithin:cholesterol acyltransferase (LCAT) deficiency and parenteral nutrition.[1] The biochemistry, metabolism, clinical significance and laboratory analysis of LpX is reviewed in this study. This laboratory-based project investigated icteric samples received at the Chemical Pathology laboratory, Tygerberg Hospital, for serum predictors of LpX and the use of a non-denaturing polyacrylamide gradient gel electrophoresis system in the detection of LpX.

2. LITERATURE REVIEW

In 1952, the occurrence of abnormal "lipoproteins" in the serum of patients with liver disease was detected on electrophoresis by Kunkel and Slater.[1] In 1956, Russ et al identified this abnormal lipoprotein as low-density lipoprotein (LDL). It was lacking in cholesterol ester, and was called the "obstructive lipoprotein". It has subsequently been named lipoprotein X (LpX). LpX was first believed to be specific for cholestasis, but later it was also identified in the serum of patients with familial LCAT deficiency, a disorder in which cholestasis is not a feature.

2.1 Biochemistry and metabolism of LpX

LpX is in fact not a true lipoprotein. The name *lipovesicle X* would be more appropriate. It consists of a lipid bilayer, enclosing an aqueous compartment, in a structure that measures 40 to 100 nm.[2] Unlike lipoproteins it does not have a neutral lipid core. It appears as separate entities or rouleaux aggregates under electron microscopy. Although the density of LpX is in the LDL range, the physical size is in the range of very-low-density lipoproteins (VLDL) or larger. LpX has a high content of phospholipid ($\pm$ 65%) and unesterified (free) cholesterol ($\pm$ 25%), as well as some apolipoproteinE (apoE), apoC and albumin.[3] Albumin, other than trace quantities, is not present in any other known lipoprotein. LpX is devoid of apoB. It does not interact with the LDL receptor and cannot deliver cholesterol to the liver or down-regulate cholesterol synthesis.[4]

Patsch et al described the subtypes LpX_1, LpX_2, and LpX_3, with densities of 1.038, 1.049 and 1.058, and Stokes radii of 339, 343, and 294 Å, respectively.[5] These investigators studied five patients with documented extrahepatic biliary disease and in each patient all three species of LpX was present. Bile acid content in LpX_1, LpX_2 and LpX_3 was low, at 0.04%, 0.04%, and 0.06% (w/w), respectively. The hepatotoxic lithocholic acid is the major bile acid in LpX.[6] High-density lipoprotein (HDL) carries about 20% of total serum bile salts, and albumin carries the rest.[7] In cholestasis there is a shift of bile salts from albumin to HDL. Studies with labelled taurocholate have demonstrated that LpX does not play a significant role in bile salt transport in cholestasis.

The subtypes of LpX differ significantly in their content of apoAI and apoE. Each of the three subtypes contains albumin, but the antigenic determinant(s) of albumin are not accessible to the exterior of the particle. Paramagnetic resonance studies indicate that all three LpX fractions are extremely rigid.[5] This rigidity is attributable to the high unesterified cholesterol and saturated fatty acid (myristic acid) content. The phospholipid fraction of LpX is predominantly lecithin.[6]

The mechanism of formation of LpX is still poorly understood. It was initially believed to be an intestinal lipoprotein, normally catabolised by the liver, and accumulating as a

result of liver dysfunction.[8] The possibility of bile salt inhibition of catabolic reactions has also been suggested, but the finding of similar bile salt concentrations in large duct obstruction, whether or not LpX was present, does not support this theory.[8] Precursor lipoprotein complexes (containing phospholipids, unesterified cholesterol and albumin) are found in bile. In cholestasis, it is thought that these complexes are regurgitated into plasma.[9] Experimentally, the addition of albumin or serum to bile results in the formation of particles indistinguishable from LpX. Class III p-glycoprotein (the canalicular translocator for phosphatidylcholine) on the hepatocyte canaliculi mediates the generation of LpX in biliary obstruction.[2] Biliary vesicles are "re-transcytosed" to the sinusoidal surface to enter the circulation. Lipoprotein production rate is a modulator of the biogenesis of LpX. In LCAT deficiency, LpX probably forms secondary to the accumulation of phospholipids and unesterified cholesterol in serum.

LpX is taken up and catabolised by the reticuloendothelial system, primarily by the spleen, unlike most other lipoproteins that are taken up by the liver.[10] Receptor-binding studies indicate that isolated hepatocytes or fibroblasts exhibit a low degree of binding and uptake of LpX, whereas in lymphocytes high rates are observed. The exact mode of uptake is unknown. These cells show a marked reduction in the activity of the hydroxymethylglutaryl (HMG)-CoA-reductase when in contact with LpX. The reductase activity is greatly depressed in lymphocytes from cholestatic patients.

2.2 Clinical Settings where LpX may be present in serum

2.2.1 Cholestasis

The liver plays a central role in lipoprotein synthesis and clearance (Table 1).[11] Hepatic disease is accompanied by major qualitative and quantitative derangements of lipoprotein metabolism. The basic defects seen in liver disease are disturbed biosynthesis of lipoproteins, disturbed hepatic removal of lipoproteins from the circulation and disturbed plasma lipolytic activity.

Cholestasis may be defined as a functional disorder of bile flow in which the characteristic chemicals (bile salts and bilirubin) spill over into the systemic circulation and induction of enzymes in the biliary canalicular lining may occur. Clinical and laboratory findings in patients with cholestasis include jaundice, dark urine, pale stools, pruritus, elevation of biliary tract enzymes in the plasma and conjugated hyperbilirubinaemia. Dyslipidaemic features include hypertriglyceridaemia with persisting $apoB_{48}$, increased plasma phospholipids, decreased LCAT activity, and hypercholesterolaemia. The hypercholesterolaemia is mainly due to unesterified cholesterol, which is principally transported in the form of LpX. Hypercholanaemia is not regularly documented but is typical of cholestasis.

Table 1: Role of the liver in lipid metabolism (from ref 11)

Lipoproteins	Apolipoproteins	Enzymes and receptors
VLDL secretion Discoid HDL secretion IDL, LDL removal Chylomicron remnant removal IDL-LDL interconversion HDL_2-HDL_3 interconversion Excretion of cholesterol returned to the liver by reverse cholesterol transport	Synthesis of apolipoproteins AI, AII, B_{100}, C, E	LCAT Hepatic lipase Cholesterol ester transfer protein LDL receptor Chylomicron remnant receptor

The precise cause of the hypercholesterolaemia of cholestasis is unknown, but several factors may contribute. Deficiencies of hepatic lipase, lipoprotein lipase and LCAT may occur in cholestasis.[12] One study showed that LpX fails to inhibit HMG CoA reductase in cultured human hepatoma cells, and therefore may play a role in the pathogenesis of hypercholesterolaemia in the cholestatic patient by on-going biosynthesis of cholesterol even in the state where cholesterol may not be re-absorbed.[4] However, LpX does not interfere with the ability of LDL to decrease reductase activity. In another study, LpX infused into rats did not reduce hepatic cholesterol synthesis.[13] In fact, enhanced HMG CoA reductase activity (suggesting stimulation of cholesterol synthesis) and depressed cyp7 activity (suggesting less efficient conversion of cholesterol into bile acids) occurs in cholestatic liver disease.[14] As LpX carries apoE, it may competitively inhibit chylomicron remnant uptake in hepatocytes, contributing to the dyslipidaemia of cholestasis.

In α-naphthylisothiocyanate-treated mice, where cholestasis is induced by this hepatotoxin, phospholipids and free cholesterol (corresponding to the appearance of LpX) increase promptly, peaking at 48 hours.[14] In experimental cholestasis, LpX may be detected and quantified as early as 24 hours after ligation of the common bile duct. In cases of proven large duct extrahepatic obstruction, LpX had disappeared within two weeks of surgery.[8]

LpX is regarded as the most sensitive and specific biochemical parameter for the diagnosis of intra- and extrahepatic cholestasis.[15] Detection of LpX is of high specificity (1) and predictive value (1), but lacks sensitivity (0,57) in intrahepatic cholestasis.[15] In extrahepatic cholestasis however, the diagnostic sensitivity is 100%. Causes of *intrahepatic cholestasis* include primary biliary cirrhosis, alcoholic and viral hepatitis, and drug-induced cholestasis. *Extrahepatic cholestasis* results from obstruction of major bile ducts commonly due to gallstones and carcinoma of the pancreas or bile ducts. The

distinction between intrahepatic and extrahepatic cholestasis is of critical clinical importance, as it influences management. This distinction is best done by ultrasound imaging. Investigations into the utility of LpX have not been encouraging for discrimination of intrahepatic versus extrahepatic cholestasis.

Magnani and Alaupovic utilised quantitative assays of LpX in the differentiation of extrahepatic and intrahepatic cholestasis.[16] They claimed that LpX measurements may be more reliable than standard liver function tests in separating these disorders and stated that LpX-cholesterol values over 300 mg/dL (7.8mmol/L) are highly suggestive of extrahepatic obstruction. Ritland concluded that values over 400 mg/dL (10.4mmol/L) are highly indicative of extrahepatic biliary obstruction.[17] Other investigators reported that the quantitative assay of LpX levels has little or no clinical value in differentiating extrahepatic from intrahepatic cholestasis.[18] Wengeler et al claimed that the combined detection of LpX and determination of LCAT activity enables differentiation of extrahepatic from intrahepatic cholestasis.[19] Degenaar et al suggested that the combination of LpX and the $\alpha_2\beta$ fraction of gamma glutamyltransferase (GGT) permits this differentiation.[20] Schut and Diaz concluded that the combined determination of high molecular mass alkaline phosphatase (ALP) and LpX offered the best results.[21] Seidel et al analysed LpX in 1481 subjects, concluding that LpX has a specificity of 0.98 to demonstrate or exclude cholestasis.[22] LpX was positive in all cases of extrahepatic biliary obstruction, even though some cases had normal ALP activities. Tazawa et al found that the combination of LpX and GGT level differentiated biliary atresia from intrahepatic cholestasis in infants.[23]

The administration of cholestyramine helps in differentiating intrahepatic and extrahepatic cholestasis. Disappearance of LpX after a 2-week course of cholestyramine is consistent with intrahepatic cholestasis.[24] However, cholestyramine is contraindicated in complete biliary obstruction and compliance is usually poor owing to the coarse texture of this powder. Witt and Ober found that LpX was present in the serum of about 50% of infants during the first weeks of life and persisted for several months.[25] In neonates, LpX levels do not correlate with bilirubin, cholesterol or GGT levels. LpX is more prevalent in premature neonates. The presence of LpX in newborns is thought to be due to immature liver function. Thus LpX can only be used as a marker of cholestasis after the first year of life.

2.2.2 LCAT Deficiency

The plasma enzyme lecithin: cholesterol acyl transferase (LCAT) uses lecithin and free cholesterol as substrates to produce lysolecithin and cholesterol esters. LCAT deficiency may be primary, due to a rare inborn error of metabolism or secondary to hepatocellular necrosis and cholestasis. Familial LCAT deficiency is a rare autosomal recessive genetic disorder which may be complete or partial (Fish Eye Disease).[26] The lack of LCAT activity leads to an increase in the plasma concentrations of unesterified cholesterol and phosphatidylcholine, resulting in hypoalphalipoproteinemia and other lipoprotein

abnormalities. Clinical manifestations of complete LCAT deficiency include corneal opacities, corneal arcus, xanthomata, haemolytic anaemia and proteinuria. The majority of patients develop progressive glomerulosclerosis, which may lead to chronic renal failure. Heterozygotes display approximately 50% of normal plasma LCAT activity and none of the biochemical or clinical manifestations of the disorder.[27]

Low HDL-cholesterol (HDL-C) concentration is an independent cardiovascular risk factor. The protective effect of HDL is thought to be mainly through reverse cholesterol transport. Despite reduced HDL, most patients with Familial LCAT deficiency or Fish Eye Disease do not appear to have increased risk for developing cardiovascular disease (CVD). However, in a subset of these patients premature CVD has been described.

In a study of LCAT knockout mice, LpX was only present in a subset of mice, after being on a high fat, high cholesterol diet.[28] LCAT deficiency reduces plasma levels of the atherogenic apoB containing lipoproteins by modulating the hepatic expression of the LDL receptor and apoE, which leads to reduced atherogenic risk. The study also showed that LCAT knockout mice had a dramatic increase in their plasma cholesterol ester levels on the atherogenic diet, suggesting enhanced contribution by the enzyme acylCoA:cholesterol acyltransferase (ACAT) responsible for the intracellular esterification of cholesterol.

Paradoxically, a study of a Canadian family with LCAT deficiency showed that increased HDL-C in heterozygote LCAT deficient individuals may be associated with enhanced progression of atherosclerosis.[26] This may be related to "dysfunctional" HDL. Heterozygotes for LCAT deficiency have a highly atherogenic lipoprotein profile, and it is possible that this defect may be underdiagnosed in the general population.

2.2.3 Parenteral Nutrition

During intravenous lipid emulsion infusions, liposome-rich emulsions, such as 10% or 20% Intralipid® or Lipovenous®, induce plasma lipid accumulation, and the appearance of LpX-like particles.[29] The ratio of medium-chain triglycerides to long-chain triglycerides in fat emulsions used in total parenteral nutrition influence the appearance of LpX.[30] Cholestasis is also a possible complication of parenteral nutrition.

2.3 Clinical consequences of LpX

2.3.1 Renal Disease

Renal failure represents the major cause of morbidity and mortality in subjects with LCAT deficiency. The presence of LpX has been noted to be closely associated with the

occurrence of renal failure in LCAT deficiency, and the level of LpX has been suggested as a biological marker of the evolution of renal disease in this condition.[27]

The histological findings of the glomerulopathy seen in familial LCAT deficiency include glomerular lipid deposition. The direct deposition of LpX is the main cause for the accumulation of lipids in perfused rat kidney.[31,32] Studies suggest that the accumulation of LpX may be responsible for progressive glomerulosclerosis by inducing a pro-inflammatory response and causing glomerular capillary endothelial damage.[28,33,34] Glomerular lipid deposition is also seen in patients with chronic cholestatic liver disease, for instance in Alagille syndrome.[35]

2.3.2 Atherosclerosis

LpX is thought not to be atherogenic, but cases of severe cardiovascular disease associated with LpX have been described. In contrast, certain findings suggest that LpX may protect against atherosclerosis. Patients with primary biliary cirrhosis (PBC) have a low incidence of atherosclerosis, in spite of significant hypercholesterolaemia.[36] PBC is a chronic, progressive disease characterized by immune-mediated destruction of intrahepatic bile ducts, eventually terminating in cirrhosis and liver failure. High HDL-C and low lipoprotein(a) seen in PBC protect against atherosclerosis, but other factor(s) may also prevent atherosclerosis in these patients.[36] In a recent study, LpX isolated from patients with PBC prevented LDL from becoming oxidised by $CuSO_4$ and also prevented oxidised LDL from disrupting the fibroblast growth factor 2-dependent survival mechanism in vascular endothelial cells.[37] It is thought that LpX is resistant to copper oxidation due to the high rigidity of the particle, thereby preventing penetration of copper ions into the hydrophobic region of the bilayer, where oxidizeable unsaturated fatty acids are localized. LpX also has a high affinity for copper ions. Although bilirubin is a potent antioxidant, there was a lack of detectable bilirubin in the LpX fractions isolated. It may also be that what is regarded as atherogenic hyperlipidaemia due to LDL-C actually represents LpX. One study in particular, which is often quoted to show that high LDL-C in PBC does not cause an elevated atherosclerosis risk used LDL-C calculated by the Friedewald equation, which does not apply because LpX is thereby incorrectly included in the LDL-C concentration.[38]

Alagille syndrome (AGS) is an autosomal-dominant disorder characterised by paucity of interlobular bile ducts with cholestasis, and abnormalities of the heart, eye and vertebrae, as well as a characteristic facial appearance. Patients usually present with cholestasis within the first year of life, with episodes of jaundice interspersed by periods of remission. High cholesterol levels with hypoalphalipoproteinemia and LpX are detected during episodes of severe jaundice, while hyperalphalipoproteinaemia occurs during mild jaundice. A study of 22 children with AGS revealed that the distribution of excess cholesterol, and presence of LpX, depends on LCAT activity, and not on bilirubin level.[39] LCAT activity may be decreased due to decreased hepatic synthesis or the presence of an inhibitor during cholestasis. Decreased LCAT activity leads to an accumulation of

unesterified cholesterol, which may associate with phospholipids to form LpX. In contrast, another study of 5 children with AGS demonstrated LpX in 2 patients with normal LCAT activities.[40]

HDL enrichment with apoE is seen in AGS. It has been hypothesised that apoE-enriched HDL facilitates reverse cholesterol transport by promoting cellular efflux and influx of sterols.[40] HDL enriched with apoE may also help reverse cholesterol transport because of their recognition of the hepatic apoE receptor. The HDLs are also enriched with phospholipids, and the amount and composition of phospholipids present on HDL may determine their ability to remove cellular free cholesterol. ApoAI is important in reverse cholesterol transport. Since apoAI levels are sufficient in AGS and familial LCAT deficiency, the risk of atherosclerosis is not increased.[39,40] The apoE-rich HDL seen in biliary obstruction might inhibit platelet aggregation, decrease thrombus formation and decrease the risk of coronary artery occlusion.

A recent study compared carotid intimal-medial thickness (c-IMT) and wall stiffness (c-WS) between children with AGS, progressive familial intrahepatic cholestasis (PFIC) and familial hypercholesterolaemia (FH).[41] c-IMT and c-WS values of patients with AGS were normal, even though their TC and LDL-C levels were higher than the children with FH, whose c-IMT levels were significantly higher than those of age-matched normal control subjects. The elevated HDL-C seen in AGS may explain this, but the presence of LpX in these patients may have an additional inhibitory effect on the development of atherosclerosis. In this study LDL-C was measured by an enzyme immunoassay, which does not recognize LpX to a significant degree.

2.3.3 Hyperviscosity syndrome

The hyperviscosity syndrome is a dangerous consequence of high LpX concentrations in plasma. Prophylactic lipid apheresis should be considered if viscosity is > 3 centipoise.[42,43] Symptoms suggestive of hyperviscosity include visual changes, vertigo, ataxia, and changes in mental state. Clinical signs of hyperviscosity include dilated, segmented, and tortuous retinal veins, resulting in a "sausage-link" appearance.[42] If not recognised, it could lead to seizures and coma, bleeding diathesis, and volume overload with congestive heart failure.[43]

2.3.4 Pseudohyponatraemia

Pseudohyponatraemia, defined as a spuriously low serum or plasma sodium measurement, occurs with dilutional methods of sodium analysis due to an altered fraction of total plasma volume occupied by high molecular weight substances or a lipid phase, most commonly with severe hypertriglyceridaemia or paraproteinaemia.[44] Less commonly, pseudohyponatraemia is found in severe hypercholesterolaemia. Routine

measurement of serum sodium concentration by indirect ion-selective electrode involves dilution of the sample and is therefore prone to inaccuracy in severe hypercholesterolaemia due to LpX. The discrepancy between the measured and physiological sodium concentration may lead to serious errors in patient management.

2.3.5 Xanthoma

In chronic cholestasis, for instance in PBC, LpX can cause extensive cutaneous xanthomata. The planar xanthomata found on the eyelids, referred to as xanthelasma, are regarded as a typical clinical association of PBC. Additionally, xanthomata of peripheral nerves can lead to a painful sensory neuropathy. Medical treatment to reduce LpX levels has been found to be unsatisfactory, and HMG CoA reductase inhibitors (statins) are contraindicated in the setting of liver disease.[45] LDL-apheresis to remove LpX has been reported as successful in a patient with PBC who had planar xanthomata of both hands, interfering with his ability to work.[46]

3. LABORATORY ANALYSIS

The discussion in this section will indicate that there is no simple, reliable method to isolate or measure LpX in a clinical chemistry laboratory. The isolation may introduce changes and the measurement may describe only a component of LpX.

3.1 Isolation of LpX

Seidel et al isolated LpX by ultracentrifugation (at 4°C) of citrated plasma obtained from blood of patients with cholestasis.[47,48] As plasma was layered under buffer solution with a density of 1.0055 g/ml, the VLDL fraction with a density less than 1.006 g/ml rose to the top. This was removed by a tube slicing technique. The fraction with density greater than 1.006 g/ml was further fractionated by precipitation with heparin and manganese. After the precipitated lipoprotein was reprecipitated twice with magnesium chloride, heparin was removed by precipitation as heparin-barium mixture by dialysing the dissolved lipoprotein precipitate against barium chloride. The supernatant fluid resulting from low-speed sedimentation of heparin-barium precipitate was fractionated with ethanol using Cohn's procedure. This fractionation resulted in precipitation of Cohn Fractions I and III (which normally contain LDL). The supernatant solution made up of Cohn Fractions IV to VI was used to isolate LpX. This was accomplished by adjusting the density of the solution to 1.063 g/ml with sodium chloride and prolonged ultracentrifugation. LpX was in the fraction that had a flotation of 1.006 to 1.06 g/ml. By other isolation procedures, the fraction containing LpX was further subjected to zonal ultracentrifugation or hydroxyapatite chromatography.

Generally-used isolation techniques entail precipitation of LpX with heparin and manganese chloride, and subsequent utilization of a combination of techniques, such as ethanol precipitation and sequential ultracentrifugation, zonal gradient ultracentrifugation with or without prior ethanol fractionation or hydroxyapatite chromatography.[48]

3.2 Assay Methods

In the 1960s, a system of **agar gel electrophoresis** was described by Seidel et al.[47] With this method, LpX moves toward the cathode by electroendosmosis. Electroendosmosis may be defined as preferential movement of water in one direction through an electrophoresis medium due to selective binding of one type of charge on the suface of the medium.[49] In **agarose gel** electrophoresis (AGE) less electroendosmosis takes place and LpX migrates very slowly towards the anode. Therefore it does not discriminate as effectively as agar gel electrophoresis.[50] Chylomicrons migrate similarly and distinction

from LpX is sometimes problematic. A typical greenish discolouration helps with the identification of LpX. Figure 1 illustrates the possible patterns seen on AGE.

Figure 1: AGE patterns
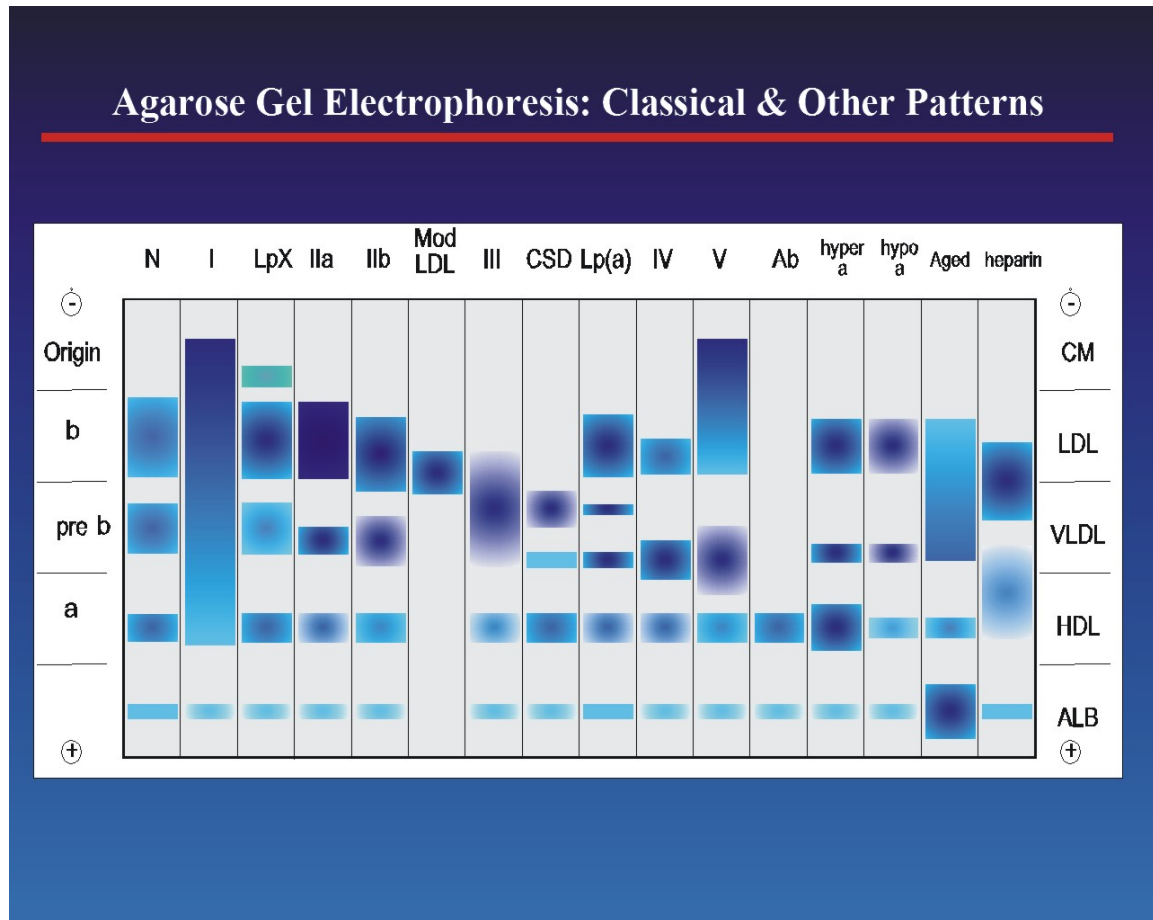
Agar gel electrophoretic demonstration of LpX is enhanced by the application of **polyanions** on the surface of the gel on the cathodal side of the well or by **immunodiffusion**, applying antiserum against apoC.[51] By densitometry of precipitation areas obtained after serum electrophoresis in agar gel followed by precipitation with polyanions, LpX can be quantified.[52]

A method for quantitative estimation of LpX based on the assay of **phospholipids** in precipitates of LpX has been described. This correlated well with results of agarose gel electrophoresis.[53] Other lipoproteins are first removed by precipitation with phosphotungstic acid solution. LpX is then precipitated from the supernatant fluid with an alkaline solution of magnesium ion, and phospholipid is determined in this precipitate.

A commonly used selective **immunoprecipitation method** was described by Poittevin et al.[38] Plasma is first treated with an anti-apoB serum. Alternatively, apoB-containing

lipoproteins can be precipitated by concanavalin A.[40] Supernatant containing HDL and LpX is then treated with phosphotungstate acid, pH 6.15, to precipitate the LpX. The LpX is then redissolved in sodium citrate (0.2 mol/L) and phospholipids and total cholesterol are then determined by conventional assays.

A **turbidimetric** method for determination of LpX is based on the separation of interfering lipoproteins by precipitation with **heparin** and **zinc acetate** followed by centrifugation.[54] In the supernatant the LpX, which is not precipitated under these conditions, is determined by measuring the turbidity after the addition of calcium chloride and heparin.

Another turbidimetric method involves the selective removal of apoB-containing lipoproteins by precipitation with specific **antibodies**.[55] To the supernatant, sodium-dodecyl sulphate (SDS) and $MgCl_2$ are added to precipitate LpX, and the turbidity is then measured at 360 nm.

Enzymatic determination of LpX has also been described.[56] Non-esterified cholesterol is determined enzymatically with the cholesterol oxidase-peroxidase reaction using phenol and 4-aminophenazone as chromogens. This assay is done with the supernatant containing LpX, after other serum lipoproteins have been removed by precipitation with heparin and zinc acetate followed by centrifugation. Although LpX consists of the subfractions LpX_1, LpX_2 and LpX_3, with slightly different cholesterol contents, this does not seriously affect the LpX determinations based on cholesterol content.

A **dye-binding method** for rapid colorimetric determination of LpX involves the staining of serum lipoproteins by the addition of Sudan Black B solution.[57] The normal lipoproteins and the excess of Sudan Black are removed with heparin and zinc acetate solution. LpX remains in the supernatant, and the blue colour is then measured. An empirically obtained constant is used for calculation.

Two **colorimetric procedures** for quantitative determination of LpX, based on ether-extractibility of phospholipids in LpX have been described.[58] Phospholipids are relatively loosely bound in LpX. The major part of them can be extracted with ether. Since the extraction is not quantitative, especially at low LpX concentration, it was necessary to add a detergent to complete the extraction. The cationic detergent cetyltrimethylammonium bromide at the appropriate concentration was found to meet these requirements.

Ritland described a method in which endogenous free cholesterol of the plasma lipoproteins is equilibrated with **exogenous labelled cholesterol** during an incubation at 37ºC for four hours.[59] After separation of LpX from the other lipoproteins by electrophoresis in agar, the percentage of the label found in the LpX fraction was measured by **liquid scintillation**. Based on this percentage, the plasma concentration of free cholesterol and the known composition of LpX, the concentration of LpX is calculated.

LpX has also been studied by ^{1}H and [31]P **nuclear magnetic resonance**.[60]

LDL fractions can be separated by **size exclusion column chromatography**, where LpX will be detected as an additional peak.[37]

A modified non-denaturing polyacrylamide **gradient gel electrophoresis** (GGE) system as described by Blom et al has proven useful in the diagnosis of dysbetalipoproteinaemia.[61] GGE separates particles according to size and is also a useful method to identify LDL and HDL subspecies. Its potential utility in the detection of LpX was investigated in this project. The experience of the lipid laboratory in the Lipidology Division of the Internal Medicine Department at Groote Schuur Hospital and the University of Cape Town suggested a typical band could identify LpX. Figure 2 depicts the patterns of dyslipidaemia that can be recognised on a GGE that separates lipoproteins containing apoB.

Figure 2: GGE patterns
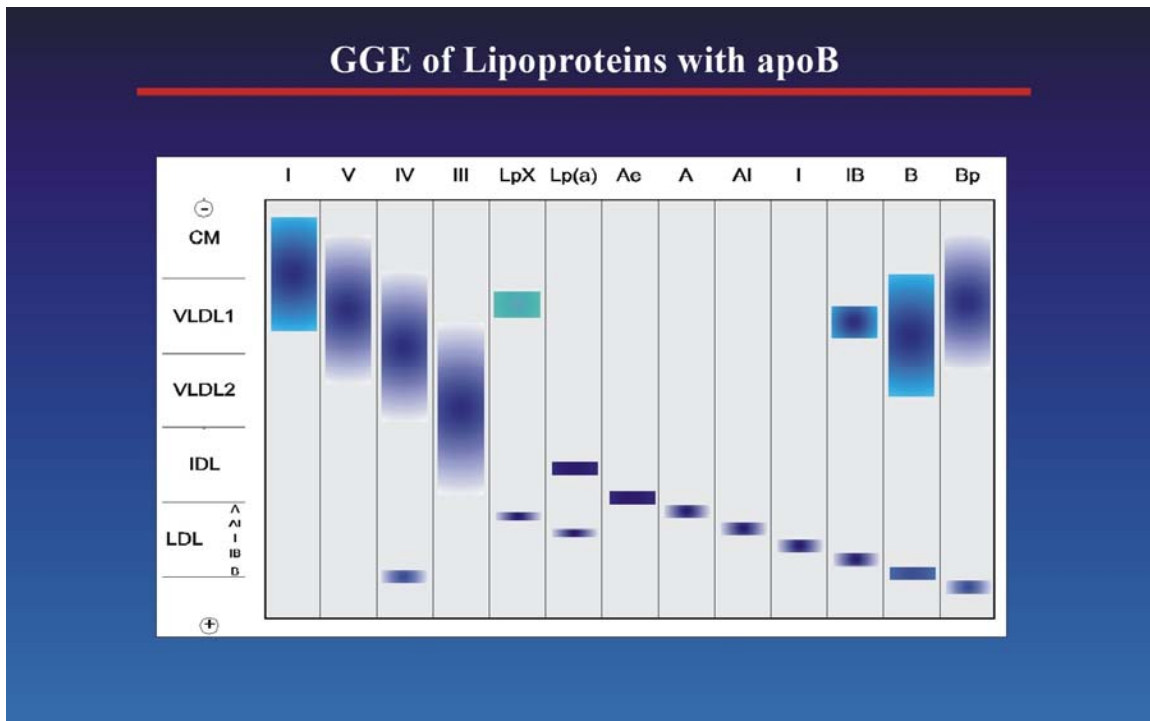
3.3 Analytical Considerations

LpX is destroyed by freezing.[57] It can be stored for 2 weeks at about 4**°**C.[57]

In cholestasis, high-molecular-mass ALP and ALP-LpX complex may complicate the interpretation of ALP isoenzyme electrophoresis.[62] High-molecular-mass ALP isoenzyme represents fragments of hepatic cell plasma membranes with various membrane-bound

enzymes localized on the surface.[63,64] Weijers and others demonstrated the existence of an ALP-LpX complex as an abnormally moving band (also called ultrafast liver isoenzyme) in the electrophoretic pattern of sera from patients with cholestasis.[65,66] This complex was purified and studied by gel filtration and affinity chromatography.[67] The position of the abnormally moving band in the alkaline phosphatase pattern and the percentage of alkaline phosphatase activity in the complex in relation to the total serum alkaline phosphatase activity depend on the serum LpX concentration. The presence of this complex is a reliable indicator of cholestasis, but is of limited value in the differentiation between intrahepatic and extrahepatic cholestasis.

According to one study, elevated ALP is the best predictor of the presence of LpX in liver disease.[68] In cholestasis, there is a mean increase in total cholesterol of 0.002 mmol/L per unit increase in ALP.[68]

Bile salts have been shown to act on LpX in vitro and cause a false-negative electrophoretic test. Frison et al have shown that bile salt levels more than five times the concentration that is usually seen in cholestasis are required for a change in electrophoretic pattern to occur.[69] No relationship between serum LpX and serum bile acids, either positive or negative, was found in 29 patients with cholestasis and detectable LpX.

LpX cannot be demonstrated by agar gel electrophoresis after incubation with lipoprotein lipase or postheparin plasma (which causes a release of lipoprotein lipase from endothelium).[70] This change in electrophoretic mobility may be induced by the release of free fatty acids. These impart a negative charge to the particle, resulting in migration towards the anode. Activity of lipoprotein lipase in postheparin plasma is low in patients with cholestasis. This may explain why in some patients with cholestasis, LpX may still be found by electrophoresis also after incubation of postheparin plasma.

LDL-cholesterol (LDL-C) is calculated in most routine clinical laboratories by the use of the Friedewald calculation. The formula is unreliable in non-fasting samples, if the triglyceride value exceeds 4,52 mmol/L, in dysbetalipoproteinaemia and in certain secondary dyslipidaemias. In such cases, direct measurement of LDL-C can be performed by ultracentrifugal single spin analysis, detergent assays or immunoprecipitation techniques. When the Friedewald formula is used, the estimated LDL-C concentration includes cholesterol contained in other lipoproteins, such as LpX. Direct methods for LDL-C determination recognise LpX to variable degrees.[71]

Although no direct link with LpX could be established, a study has demonstrated that homogeneous HDL-C assays significantly underestimate HDL-C compared with single vertical-spin ultracentrifugation in liver cirrhosis.[72] However, these investigators froze samples before analysis, and therefore no LpX would have been detected. This underestimation is probably due to LCAT deficiency, resulting in increased apoA-poor nascent HDL, which is not identified correctly by the homogeneous assays.

In immunonephelometric assays of lipoprotein(a), it has been shown that the presence of LpX may cause falsely elevated results.[73]

4. AIM(S) OF THIS PROJECT

A cheap, sensitive and specific method for determining LpX as a secondary cause of hypercholesterolaemia in cholestasis would be useful and could also suggest LpX due to LCAT deficiency and parenteral nutrition. The lipid laboratory at Groote Schuur Hospital and University of Cape Town observed a large particle staining with a lipid stain in some patients with hypercholesterolaemia and cholestatic liver disease. This was attributed to LpX. It was thus of interest to do a systematic evaluation in patients with jaundice for this phenomenon.

The aims of this study were to describe the prevalence of the putative LpX in icteric samples sent to the Chemical Pathology laboratory at Tygerberg Hospital in which LpX could be expected. Blood samples of patients with jaundice were run on lipoprotein B/LDL (LpB) polyacrylamide gradient gel electrophoresis (GGE) to detect LpX.

Apart from the GGE, various parameters and ratios known to associate with LpX were evaluated: total bilirubin (TB), conjugated bilirubin (CB), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), aspartate transaminase (AST), alanine transaminase (ALT), total cholesterol (TC), HDL-cholesterol (HDLC) (including subtypes HDL2C and HDL3C), triglyceride (TG), bile acid (BA), phospholipid (PL) and free cholesterol (FC) concentration. LpB GGE was compared with AGE in a subset of samples, as AGE was an affordable and available method for detection of LpX. As reported HDL may disappear or an abnormally large HDL subtype (HDL1) may be detected in cholestasis and thus the investigation was extended to include GGE for HDL or LpA particles.[74,75] The laboratory and clinical data in those patients with and without putative LpX were reviewed.

5. METHODS AND MATERIALS

This is a descriptive and prospective study, approved by the Ethics Committee of the University of Stellenbosch. The project was funded through profits from contract research undertaken by Prof A D Marais.

5.1 Samples

The samples were collected between 28/02/2005 and 16/03/2006. Icteric samples received at the NHLS Chemical Pathology laboratory Tygerberg Hospital for analysis of a liver profile were stored in the 4°C refrigerator and analyzed within a week by gradient gel electrophoresis in batches at the Lipidology Laboratory, University of Cape Town. Liver function tests were requested on these samples by physicians and the liver panel was performed in the NHLS Chemical Pathology laboratory, Tygerberg Hospital. In all patients a LpB GGE was performed and TB concentration measured. Depending on the volume of sample, in subgroups of the study population TC, HDL-C concentration (including subtypes HDL2C and HDL3C) and TG concentration, PL, BA concentration and FC concentration were measured, and AGE as well as LpA GGE was performed. The laboratory, demographic and clinical data in those patients with and without LpX was reviewed.

5.2 Liver function tests

5.2.1 Total bilirubin (TB)

Bilirubin reacts with **diazo sulfanilic acid** at a low pH to produce azobilirubin. The presence of caffeine permits the rapid reaction of both conjugated and unconjugated bilirubin. The absorbance of the azobilirubin complex is measured as an endpoint reaction at 545 nm. The assay was performed on the Bayer Advia 1650 Chemistry analyzer.

5.2.2 Conjugated bilirubin (CB)

Bilirubin reacts with the **diazo reagent** (sulfanilic acid) at a low pH to produce azobilirubin. In the absence of caffeine only a rapid reaction with conjugated ("direct") bilirubin occurs. The absorbance of the azobilirubin complex is measured as an endpoint

reaction at 545 nm. The assay was performed on the Bayer Advia 1650 Chemistry analyzer.

5.2.3 Alkaline phosphatase (ALP)

The sample is added to a *p*-nitrophenyl phosphate substrate. DEA buffer is used to maintain the reaction at pH 9,7 to 9,8. Magnesium ions are added to the DEA buffer to activate and stabilize the enzyme. During the reaction, alkaline phosphatase hydrolyzes the PNPP to form ***p*-nitrophenol**, which is yellow in alkaline solutions, and can be measured photometrically at 410 nm. The reaction rate follows zero-order kinetics. The units of activity can be calculated as micromoles of substrate hydrolyzed per minute, based on the molar absorptivity of *p*-nitrophenol. The kinetic reaction was performed on the Bayer Advia 1650 Chemistry analyzer.

5.2.4 Gamma-gutamyl transferase (GGT)

In the reaction with synthetic substrate (γ-glutamyl-*p*-nitroanilide), glycylglycine acts as an acceptor for the gamma-glutamyl residue and ***p*-nitroaniline** is liberated. The liberated product has an absorption maximum near 400 nm; the rate of formation is measured photometrically at 410 nm as a zero-order kinetic assay. The assay was performed on the Bayer Advia 1650 Chemistry analyzer.

5.2.5 Alanine aminotransaminase (ALT)

Alanine reacts with α-ketoglutarate under the catalytic influence of alanine aminotransferase (in sample), while the decrease in **NADH** is monitored spectrophotometrically at 340 nm. The rate of decrease in absorbance is directly proportional to the ALT activity. The kinetic reaction was performed on the Bayer Advia 1650 Chemistry analyzer.

5.2.6 Aspartate aminotransferase (AST)

Aspartate reacts with α-ketoglutarate under the catalytic influence of aspartate aminotransferase (in sample), while the decrease in **NADH** is monitored spectrophotometrically at 340 nm. The rate of decrease in absorbance is directly proportional to the AST activity. The kinetic reaction was performed on the Bayer Advia 1650 Chemistry analyzer.

5.3 Polyacrylamide gradient gel electrophoresis (GGE)

A modified polyacrylamide gradient gel electrophoresis method as described by Blom et al[63] was used. This technique allows separation of lipoproteins by size. For LpB (LDL) electrophoresis a 2-8% acrylamide gradient was used and for LpA (HDL) electrophoresis a 4-18% gradient. Lipoproteins are stained with Sudan Black before the procedure.

All LpB GGE electrophoreses were performed by Ms P Byrnes and all LpA electrophoreses were performed by Ms B Ratanjee. The gels were visually inspected. Previous experience was that VLDL and IDL gave broad bands in the mid (M) region between the origin and LDL bands on the LpB system. A sharp band in the M region was described as a focussed band: MF. This was previously found in hypercholesterolaemia ascribed to cholestasis. In the HDL gels, a band between "HDL2" and LDL suggested the presence of an unusual lipoprotein species. See Appendix A.1 for the complete methodology for LpB and LpA GGE. Figure 3 is a photograph of a GGE with MF bands.

Figure 3: Representative GGE of LpX
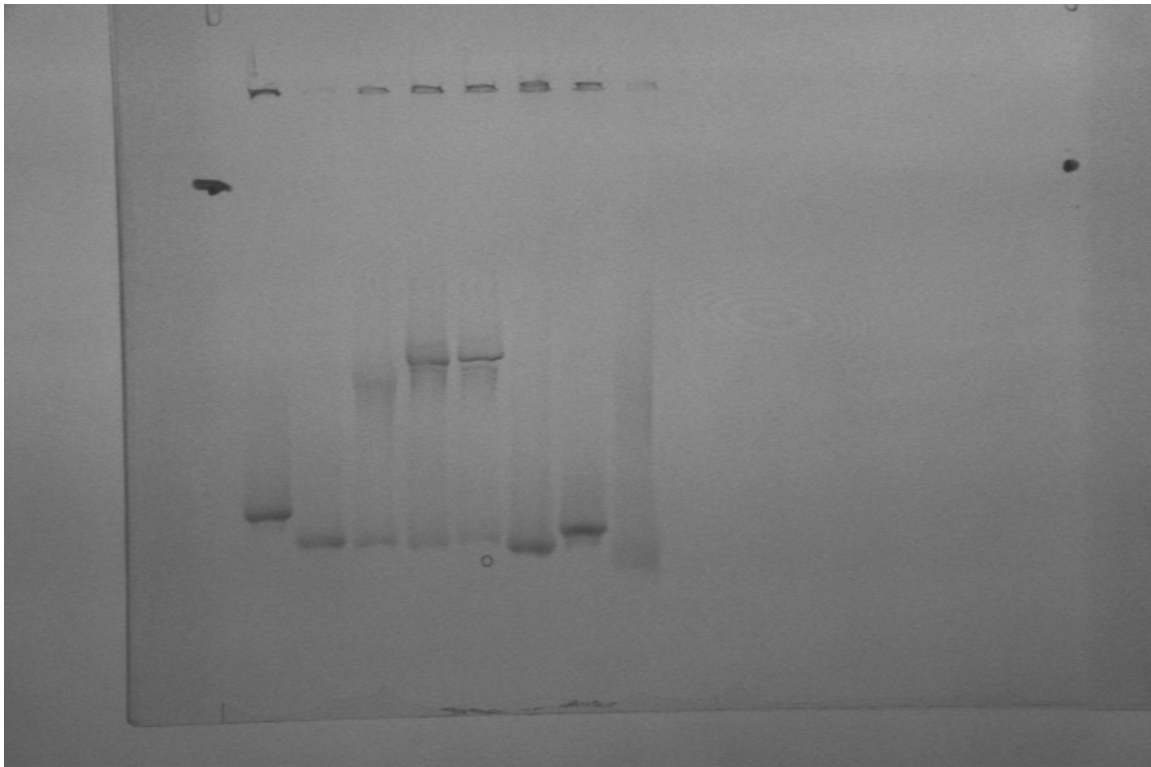
In lanes 3, 4 and 5 a MF band can be seen. The samples were from a patient with gallstones (lane 3) and a patient with head of pancreas carcinoma (lanes 4 and 5).

5.4 Cholesterol assay

TC and FC were measured by a colorimetric method from Wako Chemicals GmbH, Germany, utilising microtitre plates for rapid reading of large numbers of samples.

Cholesterol ester is hydrolysed by cholesterol esterase in the presence of water, to form cholesterol and a free fatty acid. All cholesterol is reacted with oxygen by cholesterol oxidase, to form cholest-4-en-3-one and hydrogen peroxide. Two molecules of hydrogen peroxide react with 4-aminoantipyrine and DAOS in the presence of peroxidase to form a blue compound that absorbs maximally at 600nm.

Free cholesterol is converted to cholest-4-ene-3-one by reacting with oxygen in the presence of cholesterol oxidase and this step produces hydrogen peroxide on a molar ratio with cholesterol. Next, 4-amino-antipyrene reacts with phenol and uses hydrogen peroxide in such a way that a red quinone pigment is formed along with water. See Appendix A.2 for the complete methodology.

5.5 Triglyceride assay

Lipase hydrolyses triacylglycerol to free fatty acids and glycerol. The latter is converted to glycerol-3-phosphate by glycerokinase and adenosine triphosphate (ATP). The glycerophosphate is reacted with oxygen by glycerophosphate oxidase yielding dihydroxy-acetone-phosphate and H_2O_2. Two molecules of the latter are used by peroxidase as well as 4-aminoantipyrine and 4-Cl-phenol to generate quinoneimine HCl and $4H_2O$, yielding a pink-red colour read at 500nm (or 546 in Hg) in a 1cm path. A GPO PAP kit was used. See Appendix A.3 for the complete methodology.

5.6 Bile acid assay

An enzymatic method was used in which, in the presence of nicotinamide-adenine dinucleotide (NAD), 3α hydroxysterol dehydrogenase from fungi is used to create 3-oxo-hydroxysterol and NADH. Then NADH and nitroblue tetrazolium (NBT) salt produce NAD and formazan. Formazan has a maximal absorption at 530 nm (still suitable at 540nm), and the intensity of the colour is directly proportional to the amount of bile acid. Adding phosphoric acid can stop the colour generation. The assay is actually for 3α-hydroxy-sterols and will thus reflect bile acid concentration as well as cholestanol concentration. An Adcock Ingram kit was used. See Appendix A.4 for the complete methodology.

5.7 HDL subtype assay

The method devised by Gidez *et al.* is a dual precipitation method, in which apoB-containing lipoproteins are first precipitated. HDL_2 is then precipitated from the clear HDL containing supernatant. Cholesterol is then analysed in the first (total HDL) and second (HDL_3) supernatants and HDL_2 cholesterol is calculated as the difference between the two. See Appendix A.5 for the complete methodology.

5.8 Agarose gel electrophoresis (AGE)

A Beckman Lipoprotein electrophoresis kit was used. Serum samples (less than three days old) were used. Lipoproteins are separated by electrophoresis based on charge in agarose medium. Sudan lipid stain was used. The presence of a cathodic band or smear suggested the presence of LpX. See Appendix A.6 for the complete methodology.

5.9 Phospholipid assay

This enzymatic method involves the hydrolysis of phospholipids by phospholipase D, releasing choline and phosphatidic acid. The choline, in the presence of 2 oxygen molecules and water, by means of choline oxidase, forms betaine and 2 molecules of hydrogen peroxide. Two hydrogen peroxide molecules react with 4-aminophenazone and phenol to yield 4(p-benzoquinone-mono-imino)phenazone and 4 water molecules. The enzyme is a peroxidase. The absorbance is measured at 500 to 505nm. See Appendix A.7 for the complete methodology.

5.10 Statistical analysis

Commercially available software packages were used. Spreadsheets were Excel (Microsoft) and Quatro-Pro (Corel). The database was Paradox 9 (Corel). Prism and GraphPad InStat were used for the statistical analyses.

6. RESULTS

6.1 Selection of samples

Not all analyses were possible on all samples as "left-over" serum from test tubes sent in to the laboratory for liver function tests were used. Patients were not approached for additional samples. 311 samples from 188 patients were analyzed. Samples with bilirubin > 40μmol/L were selected, comprising a group of 236 samples – called "entire cohort". A "final cohort" of 141 patients was used for the statistical analysis, since the group of 236 included repeat samples from some patients. These 141 samples were, according to sample size, selected for those with the most complete additional biochemical parameters for subsequent analysis for correlations.

6.2 Descriptive statistics of the final cohort of 141

The descriptive statistics of the final cohort is presented in Table 2. There was a wide range of ages. The age ranged from neonates to 85 years, with an average age of 43 years. The median was 46 years and standard deviation was 23 years.

There was a wide range of total bilirubin values, but the values were generally not very high. Total bilirubin (n=141) ranged from 40 to 616umol/l, mean 143μmol/L, median 92μmol/L, and standard deviation 121μmol/L.

The conjugated hyperbilirubinaemia comprised a wide range but not all were suggestive of obstructive jaundice or cholestasis. The mean was 107 μmol/L, with a standard deviation of 100 μmol/L and a median of 69 μmol/L.

ALP and GGT comprised a wide range but not all values were suggestive of canalicular induction. The mean ALP concentration was 353 U/L and the mean GGT was 310 U/L.

ALT and AST concentrations also spanned a wide range, with some suggesting significant parenchymal cell damage. The mean ALT concentration was 325 U/L and the mean AST concentration was 278 U/L.

Of the 188 patients in the original cohort serum cholesterol was only checked in 30 patients (16%) by the attending clinicians. Total cholesterol was measured in the Lipid laboratory in 98 patients of the final cohort of 141. The total cholesterol concentrations were very low in general, with only a few hypercholesterolaemic samples: TC > 7.5 mmol/L was present only in 12 patients. The mean TC concentration was 4.2mmol/L. Free cholesterol and phospholipid concentrations followed a similar trend.

The range of triglyceride concentration was wide but there were few subjects with significant hypertriglyceridaemia. None suggested the presence of chylomicrons. The mean concentration was 2.1 mmol/L.

Total HDLC, HDL2C and HDL3C concentrations were generally low. The mean HDLC concentration was 0.58 mmol/L.

The range of bile acid concentrations was wide with a few samples suggesting no hypercholanaemia. The mean concentration was 338 μmol/L.

6.3 MF detection on LpB GGE and abnormal LpA GGE bands

Of the "entire" cohort 80 (34%) LpB GGE patterns displayed MF and 75 (32%) LpA GGE patterns displayed an additional band that was larger in size than HDL2, while in the "final" cohort 44 (31%) LpB GGE patterns displayed MF and 33 (23%) LpA GGE patterns contained the additional band.

No MF was seen in the few neonatal samples (8) analysed; all had unconjugated hyperbilirubinaemia. The youngest subject with MF was a 3-month old with a choledochal cyst.

Comparison of the descriptive statistics, by 2-tailed Mann Whitney test, of the samples positive and negative for MF on LpB GGE showed several statistically significant differences – as shown in Table 3. In contrast, comparison of samples positive and negative for MF on LpA GGE showed no statistically significant differences – as shown in Table 4.

The classical indicators of cholestasis, namely TB, CB, ALP and GGT were strongly associated with MF, whilst parenchymal cell damage markers (ALT, AST) were not associated. Bile acid concentrations were practically all markedly raised, but higher values were found with MF. Hypercholesterolaemia and hypertriglyceridaemia, high FC and higher PL were associated with MF bands but not HDL. The ratios of FC/TC and TC/PL were higher with MF. As expected, higher CB/TB was associated with MF. The ALP/GGT ratio was a little lower with MF, and ALP dominated over ALT in MF-positive subjects.

6.4 Retardation factor for MF

The average retardation factor (Rf) of the MF seen on LpB GGE, relative to the B-particle of LDL, was 0.59. This differs from VLDL which is usually seen at Rf 0.2 – 0.45. The Rf of each individual patient was very reproducible, as shown in Table 5.

Table 2: Panel A. Descriptive statistics of the final cohort.

	Age	TB	CB	ALP	GGT	ALT	AST	TC
n	141	141	140	134	134	134	134	98
Minimum	0	40	3	36	9	6	20	0.68
25% Percentile	31	61	41	119	88	56	81	2.3
Median	46	92	69	212	210	122	161	3.46
75% Percentile	61	201	152	466	428	284	316	5.26
Maximum	85	616	491	1873	2163	2908	2280	16.5
Mean	43	143	107	353	310	325	278	4.2
SD	22.65	120.6	100.2	350.8	312.5	526.5	361.8	2.685

Table 2: Panel B. Descriptive statistics of the final cohort.

	TG	BA	HDLC	HDL3C	HDL2C	FC	PL	FC/TC
n	99	108	97	97	95	72	80	67
Minimum	0.43	8	0.08	0.07	0	0.17	0.85	0.12
25% Percentile	1.1	65	0.33	0.19	0.07	1.24	1.96	0.44
Median	1.6	186	0.48	0.31	0.14	1.87	2.75	0.6
75% Percentile	2.6	507	0.79	0.46	0.34	3.1	4	0.83
Maximum	7.4	1873	1.5	0.9	1.1	9.8	17.87	1.47
Mean	2.1	338	0.58	0.34	0.24	2.5	3.6	0.65
SD	1.377	370.4	0.3622	0.2064	0.2485	1.921	3.124	0.262

Table 2: Panel C. Descriptive statistics of the final cohort.

	FC/PL	TC/PL	CB/TB	ALP/GGT	ALP/ALT	TC/TB
n	60	79	141	135	134	95
Minimum	0.07	0.44	0	0.15	0.04	0.01
25% Percentile	0.58	0.92	0.68	0.73	0.79	0.02
Median	0.75	1.17	0.79	1.29	1.76	0.04
75% Percentile	0.95	1.61	0.87	2.2	5.1	0.07
Maximum	2.66	5.57	0.99	15.4	37	0.23
Mean	0.8	1.37	0.73	1.84	3.9	0.05
SD	0.4344	0.7912	0.2159	1.892	5.196	0.04156

The unit of age is years; TB, CB and BA in μmol/L; TC, TG, HDLC, HDL3C, HDL2C, FC and PL in mmol/L; ALP, GGT, ALT and AST in U/L. Abbreviations: n number, SD standard deviation.

Table 3. A comparison of MF-positive samples and MF-negative samples according to LpB GGE.

Parameter or ratio	P-value	MF positive: x±SD	MF negative: x±SD
Age	0.23	47±21.45	41.24±23.3
TB	0.0003	208.5±158.8	109.1±77.26
CB	< 0.0001	171.9±125.3	72.8±61.03
ALP	< 0.0001	545.5±430	248.8±248.7
GGT	< 0.0001	475.9±392.4	236.6±237.3
ALT	0.77	269.0±467.9	365.5±560.5
AST	0.34	292.3±398.5	280.3±349.0
TC	0.0044	5.348±3.36	3.635±2.082
TG	< 0.0001	3.058±1.348	1.631±1.15
BA	0.029	414.3±362.1	305.5±370.1
HDLC	0.55	0.5197±0.3164	0.596±0.3796
HDL3C	0.96	0.3431±0.2355	0.33±0.195
HDL2C	0.33	0.1868±0.1797	0.2654±0.2684
FC	< 0.0001	4.028±2.666	1.816±0.97
PL	< 0.0001	5.663±4.415	2.63±1.37
FC/TC	< 0.0001	0.8272±0.1445	0.58±0.26
FC/PL	0.161	0.9241±0.4887	0.756±0.41
TC/PL	0.012	1.15±0.6757	1.46±0.82
CB/TB	< 0.0001	0.8384±0.09405	0.68±0.24
ALP/GGT	0.035	1.416±1.242	2.07±2.14
ALP/ALT	0.007	5.05±5.173	3.19±5.06
TC/TB	0.149	0.047±0.05	0.05±0.037

Table 4. A comparison of abnormal-positive samples from abnormal-negative samples according to LpA GGE.

Parameter or ratio	P-value	MF positive: x±SD	MF negative: x±SD
Age	0.42	44.7±22.33	48.6±17.38
TB	0.63	131.2±123.4	138.4±114.8
CB	0.78	102.7±100.0	110.1±94.35
ALP	0.65	297.8±256.1	376.2±391.1
GGT	0.77	315.5±268.3	322.3±338.7
ALT	0.44	334.2±493.6	349.0±566.8
AST	0.88	288.0±341.6	299.4±391.5
TC	0.32	3.75±2.39	4.34±2.8
TG	0.053	1.63±0.85	2.3±1.5
BA	0.38	288.8±305.5	361.9±393.6
HDLC	0.101	0.64±0.34	0.53±0.36
HDL3C	0.76	0.39±0.24	0.308±0.19
HDL2C	0.87	0.25±0.26	0.23±0.24
FC	0.35	2.18±1.78	2.57±2
PL	0.99	3.33±2.2	3.76±3.4
FC/TC	0.9	0.66±0.33	0.65±0.24
FC/PL	0.27	0.76±0.59	0.82±0.38
TC/PL	0.76	1.2±0.77	1.39±0.79
CB/TB	0.74	0.77±0.12	0.77±0.16
ALP/GGT	0.87	1.98±1.85	1.9±2.09
ALP/ALT	0.55	3.3±4.4	4.06±5.6
TC/TB	0.4	0.04±0.03	0.05±0.45

Table 5: Rf of MF found relative to B-species of LDL.

Patient number	Observed Rfs of repeated GGEs	Average
12	0.6;0.61	0.61
18	0.42;0.42	0.42
30	0.6	0.6
50	0.67	0.67
51	0.63	0.63
52	0.63	0.63
53	0.59;0.55	0.57
61	0.6	0.6
62	0.71	0.71
65	0.56	0.56
66	0.5	0.5
69	0.52;0.71	0.615
73	0.6	0.6
74	0.58	0.58
79	0.5	0.5
81	0.53; 0.58; 0.44	0.517
95	0.64	0.64
96	0.7	0.7
97	0.66	0.66
101	0.68; 0.64	0.66
104	0.71	0.71
105	0.57; 0.56; 0.6	0.58
107	0.62	0.62
110	0.64; 0.73; 0.69	0.69
111	0.48; 0.48; 0.5	0.49
116	0.57	0.57
124	0.54	0.54
129	0.51	0.51
131	0.54	0.54
132	0.63	0.63
139	0.53	0.53
167	0.55	0.55
171	0.51	0.51
182	0.52	0.52
183	0.57	0.57
185	0.55	0.55
187	0.67	0.67
191	0.67	0.67
192	0.6	0.6
Total average ± SD		0.59 ± 0.07

6.5 Relationship of MF to biochemical parameters

In this unselected series of icteric samples, LpX can be expected to occur in increasing prevalence according to the hyperbilirubinaemia, hypercholanaemia, canalicular-enzyme derangement and hypercholesterolaemia. The relationship of MF on LpB GGE was evaluated in quartiles of TC, TB, CB/TB, ALP, GGT, ALP/ALT, FC, FC/PL, PL, FC/TC and BA, as depicted in Table 6. The approximate values of the quartiles can be found in Table 2. Reviewing the contingency table analysis results, the following conclusions can be made:

TC was not linked to MF directly, although a trend appears to be the case. Samples with MF had a range of TC of 1-11mmol/L, while samples without had a range of 0.8-16mmol/l. In the "final" cohort, where TC was > 7.5mmol/L eight out of twelve (66%) had MF, while where TC was < 7.5mmol/L 14 out of 86 (16%) had MF. Looking at the "entire" cohort, ten out of twelve with TC > 7.5mmol/L (83%) had MF. 2x2 Contingency comparison showed p<0.0006. Thus MF correlates well with severe hypercholesterolaemia.

TB had to be > 40μmol/L for inclusion in the cohort. The contingency table showed a graded increase, with ± 50% MF positive if TB > 321μmol/L. Cholestasis is said to occur when CB/TB exceeds 0.3,[76] but higher values suggest a greater impairment of CB excretion and are thus more suggestive of "obstruction". Looking at CB/TB, 60% had MF positive if the ratio was > 0.9. The median was 0.84; 31/50 (62%) had MF positive if > 0.84 and 8/50 (16%) MF positive if < 0.84.

Canalicular enzyme (ALP and GGT) induction is suggestive of cholestasis. ALP showed good predictive value for MF detection. Below the median (408U/L) 11/66 (17%) was positive and above the median 33/66 (50%) was positive. GGT showed similar results to ALP with 13/66 (20%) of results below the median MF positive and 30/66 (45%) above the median MF positive. The ALP/ALT ratio did not appear to govern MF positive samples.

Since LpX contains only FC, the correlation of MF with FC was tested. FC concentration showed a linear relationship with the prevalence of MF: below the median 3/36 (8%) had MF positive and above the median 18/36 (50%) had MF positive. The FC/PL ratio was not associated with changes in the prevalence of MF. PL concentration above the median was positive for MF in 22/40 (55%), while 5/40 (12.5%) below the median was positive for MF. FC/TC ratio strongly influenced the prevalence of MF; as shown in Table 6. For FC/TC below the median (0.6), 0/34 showed MF, while 17/34 (50%) above the median had MF.

BA was also correlated with MF prevalence. Below the median 13/54 (24%) were positive and above the median 21/54 (39%) were positive. If BA concentration was > 65μmol/L 32/34 (96%) had MF.

The conclusions are that the high ratio of FC/TC (>0.6) is a strong determinant of MF, together with markers of cholestasis: TB, CB/TB, BA and ALP.

Table 6: Contingency table of MF occurrence in quartiles of parameters.

Parameter (n)	Q1	Q2	Q3	Q4	$\chi2$
TC (98)	5	6	10	11	0.18
TB (141)	6	6	8	18	0.0025
CB/TB (141)	1	7	16	15	< 0.0001
ALP (97)	7	4	13	20	< 0.0001
GGT (97)	7	6	9	21	< 0.0002
ALP/ALT (97)	8	9	10	15	0.256
FC (69)	1	2	7	11	0.0006
FC/PL (80)	9	8	7	3	0.2
PL (80)	2	3	9	13	0.0004
FC/TC (58)	0	0	9	8	< 0.0001
BA (112)	2	11	9	12	0.008

Q1, Q2, Q3 and Q4 indicate the quartiles in ascending order as defined in Table 2. The number of subjects in each parameter is indicated in brackets.

6.6 Comparison of AGE and GGE

In 43 patients agarose gel electrophoresis (AGE) was performed to compare LpX with the LpB GGE findings. As shown in Table 7, LpX was recorded in 19/43 subjects on AGE and 14/43 on GGE. In 84% of cases there was agreement between the LpB GGE and AGE; in 16% of cases a discrepancy was found between the two. This prompted closer analysis of the AGE. Six reports of AGE are dubious. There had been a bias to find LpX and there is no objective way to validate the pattern on AGE.

Both patients 134 and 135 had true conjugated hyperbilirubinaemia (conjugated bilirubin > 30% of total), but with a relatively low ALP of 112 and 189 U/l respectively. In both the total cholesterol was extremely low, at 2.5 and 1.5 mmol/l, respectively. Patient number 136 had unconjugated hyperbilirubinaemia due to spherocytosis. It is very unlikely that LpX was indeed present in these patients. The AGE only showed the possibility of LpX − only a faint cathodic smear in each case, which could be due to differential background staining and staining is influenced by the wash-step. These factors do not affect GGE as staining takes place before the samples are loaded onto the gel. Likewise, in patients 173, 175 and 184, AGE showed a faint cathodic smear, but no convincing band of LpX. Accepting that in 6 of 19 cases there was a low likelihood of LpX, the agreement of LpX by AGE and MF by GGE is very good. It should be noted that the samples analysed were not necessarily obtained in the fasting state and that chylomicrons may thus have caused some ambiguity in some patients' samples. In only one patient (patient number 162) did the GGE show a MF band while the AGE did not suggest LpX. The patient had severe conjugated hyperbilirubinaemia and

hypercholanaemia. The discrepancy may be due to the fact that the total cholesterol was 1.9 mmol/l in this patient and AGE may not be sensitive enough to detect LpX if the concentration is that low.

6.7 Clinical correlates of MF on GGE

The diagnoses of patients with MF on LpB GGE are shown in Table 8. Many died before a final diagnosis was made. Autopsies were not performed.

The sample of the one subject with cholangiocarcinoma and MF negative on GGE was taken after a stent had been placed in the common bile duct, so no LpX would be expected. On GGE, only patients with sepsis and severe cholestasis on biochemistry had an MF. Many patients with HIV/AIDS showed an MF. Possible causes include disseminated tuberculosis or other infiltrative parenchymal disease, drug-induced (TB-therapy or antiretroviral therapy) or tumour infiltration, including Kaposi sarcoma.

A clear MF was seen on GGE in all patients with undoubted extrahepatic obstructive jaundice, based on severe conjugated hyperbilirubinaemia, hypercholanaemia and clinical diagnosis. Causes were cholangiocarcinoma (8), common bile duct injury (1), choledochal cyst (1) and phlegmon obstructing the common bile duct (1). The other diagnostic entities most likely represent intrahepatic cholestasis. Of the 12 cases with TC > 7.5mmol/L, 8 had undoubted extrahepatic cholestasis according to diagnosis, although not all had MF on GGE.

Table 7: Comparison of GGE and AGE findings.

Patient Number	AGE: Suggestive of LpX?	LpB GGE: Suggestive of LpX?	Age and Clinical background
70	No	No	9 months old; Short bowel syndrome, TPN-induced cholestasis
121	Yes	Yes	73; PTB-treatment, ? underlying liver disease
130	No	No	62; Multiple complications after CABG, ? Fucidin-induced liver disease
134	Yes	No	47; Alcoholic liver cirrhosis
135	Yes	No	46; Alcoholic liver cirrhosis
136	Yes	No	14; Congenital spherocytosis
137	No	No	56; Chronic cholecystitis
144	No	No	61; Jaundice secondary to sepsis
145	No	No	39; ?PTB treatment-induced cholestasis
146	No	No	32; Unknown diagnosis
147	No	No	48; ? PTB treatment-induced hepatitis
148	Yes	Yes	70; Chronic active hepatitis B
149	No	No	53; Alcoholic liver disease
151	No	No	18; Hepatitis A
152	Yes	Yes	30; Hepatitis A and TB treatment
153	Yes	Yes	35; Disseminated TB, HIV
154	No	No	31; Unknown diagnosis
155	Yes	Yes	42; Cholangiocarcinoma
156	No	No	27; Chronic active hepatitis B
157	No	No	60; Hepatitis secondary to sepsis
158	No	No	48; Fatty liver
159	No	No	76; Gall stones
160	No	No	50; Alcoholic liver cirrhosis
161	No	No	79; Alcoholic liver cirrhosis
162	No	Yes	58; Sepsis, CRF
163	No	No	85; Acute cholangitis
164	No	No	67; CA breast, liver metastases
170	No	No	45; ? Drug induced liver failure, HIV
171	Yes	Yes	57;?PTB-treatment-induced hepatitis/cor pulmonale
172	Yes	Yes	9; Hepatitis A
173	Yes	No	15; Cryptogenic cirrhosis
174	No	No	36; Hepatitis A
175	Yes	No	48; Alcoholic liver disease
178	No	No	53; Metastatic cholangiocarcinoma
179	No	No	74; Alcoholic liver disease
180	Yes	Yes	46; Stomach carcinoma with liver metastases

181	No	No	70; Cholangitis
182	Yes	Yes	56; Cholangiocarcinoma, liver metastases
183	Yes	Yes	80; ? CA pancreas/cholangiocarcinoma
184	Yes	No	Myocardial infarction
185	Yes	Yes	Cholangiocarcinoma
186	Yes	No	Head of pancreas carcinoma
187	Yes	Yes	? TB treatment-induced hepatitis, HIV

Table 8: Most common diagnoses in MF positive samples.

Diagnosis	Patients MF positive	Other patients with same diagnosis but MF negative
Cholangiocarcinoma	8	1
Sepsis	7	7
HIV/AIDS	6	6
Liver metastases	4	1
Hepatitis A	3	6
Gall stones	3	3
Pancreas carcinoma	2	3
Hepatitis B	2	4
Common bile duct injury (MVA)	1	0
Hepatocellular carcinoma	1	0
Chronic pancreatitis, phlegmon mass obstructing common bile duct	1	0
Choledochal cyst	1	0
TB pericarditis	1	0
Acute alcoholic pancreatitis	1	0

7. DISCUSSION

7.1 Summary of project and justification of LpX

Although several methods to demonstrate and quantify LpX have been described over the past 50 years, few methods are easily performed in most routine laboratories. GGE may be a cheap, relatively easily performed test for laboratories to consider implementing. This study prospectively collected a series of icteric blood samples, documented parameters relating to cholestasis and LpX, and examined LpA and LpB GGE for patterns suggestive of LpX, creating the shorthand term of MF so as not to bias the interpretation. In the former gel system, unusual species of HDL-like lipoproteins did not correlate with any associations of MF, meaning these bands more likely represent altered HDL species rather than LpX. Since the MF in the LpB GGE had all the correlations expected for LpX and indicated particles in the size range for LpX, it can be concluded that the MF is indeed LpX.

Although there are many techniques to describe LpX, none is regarded as a "gold" standard. LpX becomes an important entity in severe hypercholesterolaemia where it may be necessary to distinguish primary from secondary hypercholesterolaemia. About a third of the serum samples collected from jaundiced patients showed the presence of LpX on GGE.

7.2 Detection of LpX

LpX should rather be called lipovesicle X as it consists of a lipid shell with an aqueous core – unlike other lipoproteins. The aqueous compartment contains albumin and bile salts. The particle also contains some surface (apo)proteins. Techniques to demonstrate the particle range from ultracentrifugation to differential precipitation, to antibody recognition and electrophoresis. The latter could utilise any centrifuent to demonstrate the particle. Apart from ultracentrifugation, alternative methods were not available for this study. Sample volumes were too small and staff were lacking to pursue this further as a way to prove that MF is LpX.

Lipid stains are generally sensitive and specific. In contrast to true lipoproteins, LpX has little neutral lipid and generally more polar lipids. This may limit its detection by a lipid stain. Protein stains are also sensitive and may be attractive provided that the background proteins have been cleared, as will be the case in GGE. Immunoassays, including Western Blot increase the complexity, duration and cost. If albumin is used for identification in Western Blots the assay may be less specific. If other epitopes on LpX are used they may not apply to all LpX and may still identify apoproteins. Owing to the

aqueous core, LpX is destroyed by freezing and may not reassemble on thawing. This means that samples with LpX cannot be frozen for demonstration of LpX by physical properties such as ultracentrifugation or electrophoresis.

In this project, electrophoretic assays for large (apoB-containing) and small (HDL-like) lipoproteins were utilised. This technique was originally devised for separation of LDL species but was later found to be useful in the diagnosis of dysbetalipoproteinaemia. During the development, an unusual, narrow band was observed in some samples coming from jaundiced patients. Since lipoproteins are pre-stained with Sudan Black, the patterns can be read without distortion and without delay. Additionally, the ingredients are inexpensive, the sample volume required is small and samples can be kept for several days at 4-8ºC. Although the staining by Sudan Black is likely more intense in AGE, by virtue of dehydration, there is greater time required after electrophoresis to demonstrate LpX. Additionally, different properties are employed for the migration of LpX. In AGE there could be misinterpretation with chylomicrons whereas in GGE there could be confusion with VLDL1 or VLDL2. The Rf of VLDL1 is larger than the Rf demonstrated for MF (LpX) in this study, but VLDL2 may be confused for MF. Apart from the narrowness of the MF band of MF relative to VLDL2, other features could be useful to discriminate MF. VLDL is generally visible, when the (fasting) TG is > 1.7 mmol/l and in this setting the LDL species is generally modulated by cholesterol ester transfer protein (CETP) and hepatic lipase (HL) to a smaller "B" species. Sample numbers were too small to investigate using these features in the discrimination between LpX and VLDL in dubious cases, of which there were none in the eyes of an experienced reporter of the GGE method.

7.3 The cohort of patients

There was no particular selection of patients in this series that examined jaundiced patients in general. The samples came from investigation of in-patients and comprised a wide variety of disorders and biochemical parameters, making this study very applicable to everyday practice. In general, these were very sick patients but exact clinical states were not investigated in detail and final clinical notes were accepted as reliable. Severe disease and/or malnutrition are suggested by the high frequency of very low cholesterol concentrations. The commonest diagnostic settings for extrahepatic obstruction were cholangiocarcinoma, gallstones and carcinoma of the head of the pancreas. In cases with convincing extrahepatic cholestasis, GGE was suggestive of LpX in 100% of cases. The severe hypercholesterolaemia cases were found in the following diagnoses: cholangiocarcinoma (3), head of pancreas carcinoma (2), chronic pancreatitis (2), gallstones (1), sepsis (1) and hepatocellular carcinoma (1).

7.4 Correlations of LpX

The exact mechanism(s) at play in the pathogenesis not being known, known correlates were examined in this study. CB, ALP, GGT, FC, PL, FC/TC and CB/TB are all good predictors of LpX. TC concentration was not statistically significantly correlated with the finding of LpX, presumably because of the high variability of the TC results. Samples suggestive of LpX had a range of TC of 1-11mmol/l. There were many patients with a very low TC, fitting in with the serious clinical diseases present in the cohort. It is not known whether significant or severe hypercholesterolaemia preceded the hypercholesterolaemia, permitting the formation of LpX, which persisted, or whether LpX can form even in the presence of low plasma cholesterol. At such low TC levels it would have been interesting to see if acanthocytosis, associated with chronic hypocholesterolaemia, was detected, but it was not checked for at the Haematology Laboratory. Where TC was > 7.5mmol/L eight out of twelve (66%) had LpX, while where TC was < 7.5mmol/L 14 out of 86 (16%) had LpX. FC/TC was the best predictor of LpX; unfortunately FC is not commonly available test. About 50% of samples had LpX if TB was > 321μmol/L, suggesting that severe impairment of biliary excretion could suffice for the formation of LpX. From this study it would appear that hypercholesterolaemia, hypercholanaemia of > 65 μmol/L and severe hyperbilirubinaemia are the appropriate constellation for finding LpX.

7.5 Clinical implications

Of the 188 patients in the original cohort serum cholesterol was only requested by the attending clinicians in 30 patients (16%), even though obstructive liver disease is a known cause of secondary hypercholesterolaemia. In cholestasis the secondary hypercholesterolaemia (due to LpX) may cause xanthoma, peripheral neuropathy, renal disease, pseudohyponatraemia, hyperviscosity syndrome and possibly atherosclerosis. Especially in patients with long-term cholestasis, it is therefore important to perform a fasting lipogram and to make physicians aware of the association with LpX and the potential complication of hyperviscosity, and to be cautious about interpreting sodium concentration. The most accessible diagnosis of LpX for the clinician is an electrophoretic procedure, with the agarose gel electrophoresis as this is conveniently commercially available as a kit. In high turnover laboratories GGE may be cost-effective.

7.6 Limitations of the project

Criticisms of this project can relate to the lack of validation of LpX by ultracentrifugation or other described methods, imperfect descriptions of the time course of LpX, deriving the best predictors for LpX by combinatorial techniques of biochemical and clinical

parameters, prognostic implications and alternative techniques of demonstrating LpX that could be more informative.

As previously stated, sample volumes were inadequate for ultracentrifugation. This procedure would also have been further limited by availability of staff. Similar constraints would apply to precipitation techniques. The small number of subjects with LpX and the limited follow-up period mitigated against detailed predictive and prognostic analyses.

In order that LpX can be derived by more conventional laboratory assays, it may have been worth following the approach of Blom et al[77] for dysbetalipoproteinaemia where the cholesterol/apolipoproteinB (apoB) ratio was informative of the kind of lipoprotein that had accumulated. The ratio of TC/apoB may distinguish hypercholesterolaemia due to high LDL-C from hypercholesterolaemia due to LpX. Since LpX does not carry apoB, a high TC:apoB ratio would suggest the presence of LpX.

7.7 Future studies

Several avenues of investigation are still of interest for LpX and this technique of demonstrating LpX.

Apart from comparing the MF with ultracentrifugal or differential precipitation for sensitivity of detection, the GGE method could be further investigated for better detection and/or discrimination from other lipoproteins. For example, proteins or phospholipids might be able to enhance the detection, as they are richer in LpX than similar-sized lipoproteins. A simple precipitation within the gel, of unstained plasma, could produce a precipitation of albumin in LpX (but not other lipoproteins) by using SSA or TCA. These procedures are quick. Whilst a protein dye such as Coomassie Blue may appear attractive, it requires a period of destaining, or, at low concentration, prolonged incubation. Using amido Schwartz in the electrophoresis buffer could be considered but is not likely to be sensitive. Victoria blue could be used to demonstrate phospholipids and could enhance the colour of phospholipid. But it will extend the time to making a decision. Fluorescent stains that integrate into phospholipids may be evaluated but they might not be practical. As glass does not transmit UV light, some dyes might require analysis outside the assembly. In any case, this approach would also require more sophisticated equipment than is generally available in routine clinical chemistry laboratories. The detection of bile salts may be limited by sensitivity or loss of the bile salts by diffusion.

Factors influencing the Rf of LpX might yield some insight into the pathophysiology of its formation. Furthermore, the clearance of LpX could be of interest: most likely the particle will retain its diameter whilst its concentration recedes after a curative intervention such as decompressing an obstructed biliary tree. Investigations should be done to determine the optimal storage conditions and time to analysis for LpX by GGE. It

is of interest to prevent disruption by freezing. Here dimethylsulphoxide or glycerol may be useful.

If possible, an examination for LpX should be performed in LCAT deficiency and parenteral nutrition to evaluate the LpX detection and characteristics in these conditions. The impact of cholestasis on lipoproteins in the LpA GGE is also of interest and may merit more detailed investigation.

8. CONCLUSION

Lipoprotein X is an abnormal "lipoprotein" that has been investigated since the early 1950s, but many questions still remain unanswered. The mechanism of hypercholesterolaemia in obstructive jaundice is not yet completely clear. The exact mechanism of formation of LpX is still unknown. Although LpX initially received much attention as a possible test to investigate the aetiology of cholestasis, the practical utility of this biomarker has been disappointing.

This study showed that the non-denaturing polyacrylamide gradient gel electrophoresis system (2-8%) is a useful test in demonstrating LpX. Although CB, ALP, GGT, FC, PL, FC/TC and CB/TB are all good predictors of LpX, and FC/TC is the best predictor of LpX a direct demonstration of LpX is still preferable. In the setting of obstructive liver disease LpX is seen in 66% of patients if TC is > 7.5 mmol/L and here it could be a useful way of discriminating an underlying condition such as familial hypercholesterolaemia from hypercholesterolaemia associated with cholestasis. The detection of LpX may also have application in suggesting LCAT deficiency.

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APPENDICES

Prof Marais kindly provided the standard operating procedures of the Lipid laboratory here attached for interest.

Appendix A.1

**NON-DENATURING GRADIENT GEL ELECTROPHORESIS
 (GGE) FOR LP**

GENERAL

The technique of non-denaturing gradient acrylamide gel electrophoresis allows separation of lipoproteins (LP) by size. The LP may be demonstrated by staining lipid or protein. Pre-staining the sample with a lipid stain allows specific visualisation of lipoproteins in the original glass sandwich while protein staining has to be done after removing the gel and is only specific for large, apolipoprotein B-containing lipoproteins. Separation of CM and VLDL is difficult because of the size of these LP and these triglyceride-rich lipoproteins are seen as broad size ranges. The IDL range is also not so distinctly separated. Gels may be selected to demonstrate LpB series (LDL) and LpA series (HDL). A 2-16% gradient is usually recommended for LDL and for HDL, 4-30%[1] but the Lipidology laboratory of the University of Cape Town uses a mini-gel of 2-8% acrylamide gradient for LpB, and a 4-18% for LpA.

Qualitative differences in LDL may be important. The first relation between smaller denser LDL and heart disease was shown by Melissa Austin et al, who recognised two types of LDL.[2] Dormans suggested that 3 LDL subtypes could be identified by either ultracentrifugation or GGE, although the latter method could on occasion identify 5 bands.[3] Tashiro found a "midband", which is probably Lp(a), that predicts heart disease in FH subjects.[4] Similarly, HDL_3 and HDL_2 subtypes may influence atherosclerosis. The GGE and HDL ultracentrifuge subtypes have not been compared yet in this laboratory. In this system, small dense LDL can be demonstrated in the lowest quintiles of triglyceride, waist/hip ratio, body mass index and fasting glucose concentration. However, small dense LDL is seen usually at a triglyceride concentration of >1.7mmol/L, and almost always at TG >2.5mmol/L. Similarly, a waist/hip ratio of >1, BMI >30 or glucose >7mmol/L is highly associated or with small dense LDL. It has been shown that a TG/HDL-C ratio, in molar terms, of >1.33 is also strongly predictive of small dense LDL.[5] The system is also good for diagnosing dysbetalipoproteinaemia.[6]

Samples may be plasma or serum or isolated lipoproteins. Samples should immediately be placed on ice and processed as soon as possible, but have been satisfactory for LDL characterisation up to 1 week when stored at 4°C, either stained immediately or after a delay. The samples can be frozen at -20°C for a few weeks, and for several (>12) weeks at -80°C. There is a factor in the lipoprotein-deficient serum (LPDS) that can increase the size of LP on incubation. It is heat labile and dialysable but is neither CETP nor phospholipid transfer protein (PLTP).

Samples from animals with cloven hooves may carry foot and mouth disease virus. Samples can be sterilised by heating to 56°C for ≥30 minutes. This has been tested and shown not to affect human samples. After processing the same samples, they should be placed at pH <6 or >9.

MATERIALS

SUDAN BLACK STAIN. 1% Sudan Black is added to ethylene glycol. Filter the solution. It keeps for several weeks.
SATURATED SUCROSE with a spatula tip of bromophenol blue.
SOLUTIONS FOR SDS-PAGE as declared in the laboratory methods but omit SDS from all. Fresh acrylamide solution is better for clarity of separation. Depending on the brand and batch of acrylamide slight adjustments may need to be made for optimal perfomance, adjusting the denser solution to 7 to 8%.
NILE RED STAIN. Dissolve 1mg in 100ul of dimethylsulphoxide (DMSO) as a stock solution (50X), then dilute by adding 2ul to 98ul of DMSO for use on GGE. Add 10μl to 50μl of plasma. This stain with similar sensitivity to Sudan Black but requires UV light exposure for visualising the lipoproteins.

Store Minigel apparatus and powerpack in cold room or refridgerator.

PROCEDURE LDL FOR (LpB) GEL

Cast a 2-8% polyacrylamide gel and a 3% stacking gel with a minimal but definite layer of gel between the bottom of the wells and the separation gel. Mostly 2 gels are run in the system, with 15 lanes per gel. Label the glass plates. (The stacking gel may be coloured by a small amount of phenol red to make loading easier.)

To 100ul of plasma add 50ul of lipid stain, mix and incubate for a minimum of 1 hour at 4°C. Spin for 20 minutes at 10000G. Mix an equal volume of supernatent with saturated sucrose and load approximately 12ul per well.

Prefocus the gel at 20V for 30 minutes. This is ideal but not essential. Run the gel at 130V for 18-24 hours. The progress is visible! Remove the glass sandwich from the stand, dry with a paper tissue.

Recording of the gel: Write the gel identification on the dry glass plate. Mark the point at which the separation and stacking gels meet by placing a dot next to the spacer. The gel is now placed facedown on a photocopy machine and covered with white paper before a photocopy is taken. Afterwards, photograph the gel if necessary. The gel can also be scanned in the Hoefer densitometric scanner while in the glass plates, or it may be

recorded on the Biorad Geldoc videocamera. Hereafter it is dried on filter paper or cellophane.

PROCEDURE FOR HDL (LpA) GEL

Cast a 4-18% gradient gel and a 3% stacking gel. Prepare the plasma sample with Sudan Black in ethylene glycol as for LpB GGE but load directly, 16ul/lane.

Prefocus the gel at 20V for 30mins, run at 130V for 4 hours. The progress is visible! It is analysed in the same way as the LpB GGE.

INTERPRETATION OF LpB GGE

REPORTING

The gel is inspected without knowledge of clinical or biochemcial detail. Comment should be made about material in the stacking gel as occasionally there may be significant precipitate in the stacking gel, either from granules of Sudan Black that were not removed by the spin, or from lipoprotein aggregates. The origin (top or least dense part) of the separation gel reflects the largest particles. The minigel is about 60mm long. In the system the stain reflecting lipoproteins in the LDL density range, is from approximately the junction of the top 2/3 with the bottom 1/3. The chief LDL-related bands are in the bottom 1/6 of the stained area.

TERMINOLOGY

The gel is described by simple terminology avoiding terms inferring separation by density. Since the gel was developed to look at "LDL", the zone between the origin and the LDL being referred to as "mid" and staining in this region was consequently referred to as M, which was later modified according to an earlier or later observation to M-early, or M-late to describe particles that were respectively larger or smaller. Subsequently it became clear that chylomicron-like particles tended to remain at the origin of the separation gel, and hence were termed O. At the "LDL" range, the largest particle is designated "A" while some intermediate "I" bands may be discerned or the "B" band as the smallest (densest) LDL which has been associated with a higher risk of ischaemic heart disease in FCH and with hypertriglyceridaemia. These common species of A and B appear to agree with the prevalences of A and B reported in most studies although they have not been specifically prepared. Subsequently it became clear that even further size distinctions can be made, now abbreviated to A-early, A/I, I/B and B-post. Sometimes a

band between A and M-late could be discerned, and referred to as pre-A. This band specifically disappears on adding a reducing agent, proving it is Lp(a). A small letter is used to designate that the band stains faintly, and a capital letter is used to demonstrate dominant bands. A sharply focussed band in the M-early range is seen in cholestatic jaundice, and is referred to as M-focussed (MF).

Describing particles from large to small on this gel would thus be: O, M-early (Me), M-focus (MF, in Me range usually), M-late (Ml), pre-A, A-early (Ae), A, A/I, I, I/B, B, B-post (Bp). Chylomicrons correspond to O, VLDL1 (Sf 60-400) to Me, LpX to Mf, VLDL2 (Sf 20-60) to Ml, pre-A is Lp(a), Ae to Bp are all species of LDL.

RELATION TO DYSLIPIDAEMIAS AND FREDRICKSON TYPES

All **hypertriglyceridaemias** give rise to staining in the M region. In type I and IV and V there is intense staining of the M region, often with something visible near the origin and the LDL bands are often only slightly visible, if not absent. In type III dyslipoproteinaemia the band peaks in the M range, with minimal LDL banding and there may be some staining at the origin.

Hypercholesterolaemias will stain mainly the LDL range with some entry into the Ml region if IDL is present. The LDL-A pattern is seen in most normal persons and hypercholesterolaemias, including FH, in which there is no hypertriglyceridaemia. Thus in normal and Fredrickson IIa one expects large species of LDL. In type IIb it would appear that almost always there is the in mid-portions, particularly Me, indicating an excess of VLDL-like LP. The LDL particle size in X-linked icthyosis is large, so that agarose gel electrophoresis to demonstrate fast-migrating beta-lipoprotein is still preferable to prove this diagnosis.

The situation of Lp(a) is preA. LpX is seen in the M zone, usually as a sharp band and the distinct impression is that it is detected more sensitively than in agarose electrophoresis. LpX often gives the Sudan Black a greenish tinge.

The colour of the lipoproteins can vary considerably from brown to blue, and the sharpness of the bands is much better with fresh acrylamide. Lipid staining of isolated lipoproteins is also more intense than the staining in whole plasma.

CALIBRATION OF LpB GGE

Currently there is no standardisation for assays of small or dense LDL. Small, dense LDL can be demonstrated by density (ultracentrifugation), or size (non-denaturing polyacrylamide gradient gel electrophoresis (GGE) or size exclusion chromatography such as FPLC). The reported size of LDL varies, with a slightly broader range reported

between 21 and 29 nm.[7] The acrylamide gradients can vary.[8] Nuclear magnetic resonance can detect lipoproteins by unique emission signals according to their mass, allowing the discrimination of up to 15 lipoproteins with estimates for LDL at 19 to 22nm.[7] Ultracentrifugation uses a few ml of fresh plasma and permits compositional analysis but takes hours to days and gravitational forces may cause the loss of some apoproteins. Typically, three fractions are separated: LDL-I at 1.025-1.034g/ml, LDL-II at 1.034-1.044g/ml, and LDL-III 1.044 - 1.060g/ml.[9] The concentration can be reported as lipoprotein mass or protein or cholesterol.

It has not been possible to calibrate the system accurately so that the diameter of the particles can be properly calculated. Currently we readily identify A and B patterns amongst the 15 lanes and use one of each LDL type to carry across from gel to gel to bring some standardisation about. Lanes 7 and 8 contain an A and B species that are repeatedly used from gel to gel for a few weeks, but mixing them is not a good idea as some changes in size can occur. The observation is also that small LDL can enlarge with time. Of course gels that are prepared individually for each run are not exactly reproducible. However, it has been found that the classification is extremely reproducible on repeat samples.

Latex beads tended to clump and cause "ladders" and were thus not useful. We have generelly accepted that LDL particles will have the typically published diameters and that there is a broad size range for all other particles. There is even disagreement in diameters for LDL from different laboratories using size markers, and also with other methods of determining lipoprotein particle size. This makes accurate diameter determinations less useful. The gel is primarily used in this laboratory to describe patterns of major dyslipoproteinaemias involving apolipoprotein B-containing lipoproteins with particular reference to dysbetalipoproteinaemia and lipoprotein X, and to determine LDL particle size categories and whether there are significant changes in these as a result of interventions.

Retardation factors. The system has been standardised against ultracentrifugally prepared VLDL1 (Sf 60-400), VLDL2 (Sf 20-60), IDL (Sf 12-20) and LDL (Sf 0-12) . Taking the B particle as Rs=1.0, the following Rs values are typically found for V1: 0.2-0.45. For V2 the Rs is 0.45-0.7; for IDL it is 0.7-0.85 and for LDL A-B it will be 0.85-1.0 and about 1.05 for B-post.

DIAGNOSTIC ASPECTS OF LDL GGE

Hypertriglyceridaemias have M bands, and usually B or B-posts. In chylomicronaemia there is usually no LDL bands. Mixed hyperlipidaemias also have M bands. Dysbetalipoproteinaemia has a preponderance of Ml band with very little or no LDL bands, although in some instances the pattern may change to only a LDL band in this condition upon successful therapy. If there is a lapse of dietary and drug compliance, the lipoproteins may decrease in size. Most hypercholesterolaemias have A bands, sometimes

I bands and occasionally there is a B band; the B usually being associated with a M or m. This pattern is almost the norm when the fasting triglyceride concentration is >1.8mmol/L, or waist hip ratio is more than 0.95. It is also prevalent in diabetics, but more so in men. PreA is seen variably and generally correlates with an apo(a) concentration of >50mg/dl, and some size variation can be seen. There is a distinct impression that LDL particles distribution distribute more strongly to a single species compared with ultracentrifugally derived subfractions. In a population survey, it was found that about 2% of adults have no visible LDL bands, and about 2 % have 2 distinct bands, sometimes of equal intensity. Occasionally there may be more than 2 bands visible in the LDL zone. Interventions that modulate triglyceride concentration will usually alter LDL size at between 2 and 3 weeks. An interesting pattern of smearing through the preA region into the LDL region has not been explored.

INTERPRETATION OF THE LpA GGE

REPORTING

This is done blinded and after selecting from the many lanes, likely equivalents of HDL 2 and 3 species. The bands tend to be poorly staining and broad, and only occasionally can speciation be distinguished in the two regions. The LDL bands at the top may be classifiable as A or B but are often inadequately separated for comment.

TERMINOLOGY OF "HDL GEL"

Particles relating to HDL2b and HDL2a as well as HDL3a, HDL3b, HDL3c are described in the review by Silverman.[10] In the present system we find mostly 2 peaks, one with Mr of 135kD and another at about 165kD. The former appears to be HDL_3 and the latter HDL_2. In some patients there is a smaller size lipid-staining peak, at about 115kD. In some instances larger particles are seen in hyperalphalipoproteinaemia; in one patient discrete bands at 209, 229, 269, 300 and 365kD.

Current practice is to assume that the common species of smaller size is HDL3, the larger is assumed to be HDL2. The description is thus of the intensities being dominant in either one of the two bands or equivalent. This agrees remarkably well with the area under the curve and the peak intensities on the gel scanner. Occasionally there may be small species of lipoproteins, to which the lable HDL4 has been given, or larger species to which the lable HDL1 has been given. These can range to a size close to the LDL band. Typically the Me, B pattern of the Lp GGE has only a HDL3 band on the LpA GGE.

STANDARDISATION OF PARTICLE SIZE

This has not been satisfactorily performed yet. Usually the pattern can be described by comparison with the other 14 lanes on the same run. Latex beads of defined diameter do not provide a single neat band on the LDL or HDL system, while on the HDL system the rainbow markers do not give neat bands when undenatured. Protein staining on our system would be confusing especially in the HDL range as there are many proteins at these sizes.

The utility of using haemoglobin (Hb) is under investigation. Hb has a molecular mass of 64 458 and binds to haptoglobin (Hp) which has 2 binding sites for Hb. Hp is present in most individuals but rarely the Hp 0-0 status or anhaptoglobinaemia is found. The molecule is a tetrad of two a and b units. The a units can be a^1 or a^2 and slow and fast migrating forms of a^1 are known. Several phenotypes of Hp are known: Hp 1-1 ($a^1a^1b_2$) with mass of 80dk, Hp1-2 ($a^1a^2b_2$) with a mass of 120kd and ($a^2a^2b_2$) of 160kd. The Hp 1-2 and 2-2 phenotypes can dimerise to 200 and 400kd forms. Assuming one Hb to be bound per molecule, red bands should be seen on the GGE at 65, 145, 185, 225, 265 and 465kd. It is difficult to prepare suitable plasma samples to act as distinct markers, but sometimes haemolysis leads to these bands being visible in the HDL gels.

An unusual ladder of proteins has been observed between HDL and LDL on the HDL gels from some subjects in the coronary care unit. The calculated molecular mass seems to escalate approximately 300kd intervals up to 2.5 million molecular mass.

APPLICATIONS

FLUORESCENT STAINING. The preliminary observations suggest that pre-staining the plasma lipoproteins with Nile Red is as sensitive as Sudan Black staining for the LpB and LpA gels, but some optimisation is still required. There is intense staining at about 70kd on the LpA system which is not seen with a gel containing only albumin.

COOMASSIE STAINING. After the gel is run, it can be removed and placed in Coomassie staining solution according to laboratory protocols. If the plasma had not been pre-stained with Sudan Black, the LDL band is faint and possibly the preA band is more visible. Prestaining with Sudan Black enhances the Coomassie staining. Protein bands are visible below the LDL zone, at the edge of the gel and represent macroglobulins.

SILVER STAINING. This method becomes very sensitive for detecting protein and a decent band of LDL is seen with about 4μl of plasma in the lane. There is little protein visible in the lipoprotein range. Sudan Black enhances silver staining in a similar way that it enhances Coomassie staining and makes the entire range of lipoproteins from LDL to IDL stain very darkly. Acetone exposure of the gel for 30 minutes sensitises the unprestained LDL to silver.

WESTERN BLOT. This allows transfer to nitrocellulose and probing for apoproteins. The transfer of apoB is slow owing to its molecular mass and can be enhanced by adding SDS if lipids are not desired in further studies.

ANALYSIS ON GELDOC

The Biorad Geldoc apparatus allows convenient graphic recording of gels stained with visible dyes or of light emitted from fluorescent stains upon UV illumination. The system includes a personal computer, a videocamera mounted in a photographic black chamber with illumination facility, and a dedicated printer for images. The graphics files are saved in C: under directories created for the staff, and in file names that can trace the gel efficiently. The graphics file stored can be used to print images and to analyse selected portions of images directly on the screen or by transferring the data to other software as x,y coordinates. This latter process affords better analysis and interpretation, especially through Graphpad Prism. The University of Cape Town Information Technology has been unable to link the Windows NT system to the network and the files have to be manually transferred by stiffy disk from the Geldoc to other personal computers. This also poses problems for back-up.

CAPTURING THE GEL

Switch on the computer, the video camera and the printer. The computer will prompt signing on as the administrator and the password.

Select Multi-analyst Shortcut from the programme icons by a double click. The display will have toolbars available but no image will be seen until one is created or recalled from stored files. To create a new image, go to File and select New. The videocamera view will now be on the screen.

Dry the gel with a tissue and make certain there are no avoidable marks on the gel. It is a good idea to place a dot against the left and right spacers so that the junction of separation and stacking gels can be marked for reference during analysis.

Go to Set-up. Enter the appropriate light source (white light for GGE with Sudan Black). Prepare the photographic chamber. Note that a white transforming plate is placed over the UV source for analysing gels stained with dyes absorbing in visible wavelengths. Place the gels appropriately on the white plate or on the glass for UV analysis. Close the chamber. Adjust the aperture, focus, and zoom of the camera.

Make certain that the background density is uniform (best on analytical setting at the switch on the bottom front of the camera box). Once set up, the image needs to be

captured. Select Capture button at bottom left corner. This button may be masked by the lower edge of the screen, in which case click on the edge and drag it lower to display this button. Additionally click on View and remove the toolbar to allow the capture button to come into view. The capture process will prompt the file saving data: In the Save As windown, select the "Work" folder, double click on the appropriate "Name" folder and enter the appropriate Subfolder and file name. Enter the file name and save as a *.bif file.

Switch off the video camera (both the UV source and camera). To exit, select File, exit. To switch off the computer, go to Start, select shutdown and machine will close the programme and declare when it is safe to switch off manually.

PRINTING THE IMAGE

This can be done from the video camera while the gel is being recorded, or from a saved image. In both cases the picture will include the surrounds of the gels unless there is appropriate cropping.

With the programme activated, select the saved image: go to File, select Open, select Work, follow directory through Folders and then select the appropriate File. The image will be displayed, but should be maximised for better viewing. Now select from the toolbar the open square to perform cropping so that the gel only is displayed, by clicking in one corner and dragging the mouse to enclose the area of interest. Take the mouse's cursor to the Image button and select Crop. Now return to File, select Videoprint. Now press the button attached to the printer.

ANALYSING THE GEL

With the Multi-Analyst software activated, select the file from the appropriate subdirectory. The image will be displayed on the screen, best maximised. Select lanes to be analysed with care. The toolbar contains a button with a folded image for **single lane selection.** Click on this and then click the mouse to the appropriate point in the gel, typically at the left top of the lane to be analysed, and drag the mouse to the right and bottom of the portion of interest. Select the display in profile to view the lane as a densitometric scan, with optical density on the vertical axis and cm migration on the horizontal axis. If **several lanes** are to be analysed, use the "Selector" button which will place a "+"on the gel after which pressing "F" on the vertical toolbar will find lanes automatically. Select sensitivity required, usually at mid-point. The bands thus selected are demarcated in red. The profile can be viewed by pressing "View Profile"on the horizontal toolbar. To include all the selected lanes, go to "View"on main toolbar and select "Show all lanes".

With the scan profile image box on the screen the saving can proceed. From the File Menu select Export to Excel and select Active Window. Now open Excel and scroll far right across to columns until reaching the columns containing numerical values and labelled "cm" and "OD" for migration (x axis) and optical density (y axis). Select, copy and paste these 2 columns into a new Excel worksheet. This must now be saved as a text file (*.txt), onto a stiffy disk. On closing down the Multi-Analyst file, the process will request saving the changes (analyses and manipulations). Do not save these changes, as they will alter the original gel record.

Activate Graphpad Prism, go to Files and select Import. Select the *.txt file on the stiffy drive or directory and proceed to import. Prepare a profile tracing by making a graph from the data sheet, best displaying the image as a line without the approximately 250 data points. The baseline correction can be done according to a personally selected value by using the Analyze and then Transform selections, or by going to Remove Baseline. The data may also be pruned to the appropriate rows by selecting Analzye and Prune.

The lane is analysed with retardation standardised to the given B distance for the particular run from which it is derived. Retardation is standardised according to the B band, Rf(B) = 1.0. Peak particle size can be described as a Rf. The profile should be analysed for area under the curve with the baseline subtracted.

ANALYSIS ON HOEFER SCANNER

The first densitometric scanner for GGE scanned about a 1000 points as x,y coordinates at absorption of 595nm as it was originally designed for Coomassie stained SDSPAGE. It was found suitable for Sudan Black and thus was used for capturing data. Dr D J Blom developed the method below for capturing the data and processing it in Graphpad Prism. Locate the scan desired on the stiffy disks, bearing in mind that each scan is a single lane. Open the file using Notepad. Choose Edit and select all data. Select Edit again and do Copy command.

Open Prism and prepare a datasheet which will receive the y coordinates. Paste the data to the Y column. Create a serially numbered column in the X-axis starting at point 1 and using increments of 1 (column dialogue box is found under Change). This procedure will create a graph.

Manipulate the data via Analyse and Transform command to Prune Rows. Exclude the first 100 points according to judgement about the start of the separation gel. Optionally, prune the rows again, by averaging 4 consecutive points. Now copy the y-data to a new data sheet in which X starts at zero in a series.

To convert the data to retardation factors, create a data sheet that will span from 0 to 1.1. Compare the marker and other lanes in the photocopy of the gel for A and B markers and ascertain the Rf values from these lanes. The LDL_B is taken as Rf=1.0. Using the

transform function on all the x points, the function is X/K where K is the value identified to mark Rf=1.0.

FUTURE DEVELOPMENTS

The LpB system is useful for studying LP phenotypes in the common hyperlipidaemias and also in normal to moderately disturbed metabolism. It is a simple way of confirming dense species of LDL and for looking for unusually large species of HDL. It should be possible to reproduce it on larger gel systems but electrophoresis time may become impracticably long and may generate a lot of heat. The system should lend itself to transfer to membranes and immunodecoration to demonstrate qualitative differences in lipoproteins. In larger gels it may be possible to elute adequate amounts of material for sensitive methods to determine lipid (e.g. fluorimetric) and protein (e.g. immunoassays) contents. A combination of the two gels could be attempted as published from WV Brown's unit.[11]

The separation of HDL into preβ and α forms can be done in 2 dimensional electrophoresis in which the first dimension is in 0.1% agarose gels and the second dimension is in 2-30% non-denaturing acylamide gels.[12]

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Appendix A.2

TOTAL AND FREE CHOLESTEROL DETERMINATIONS WITH THE WAKO KITS

GENERAL REMARKS

Enzymatic assays for cholesterol are convenient and are commercially available until no longer viable or companies change policies. The lipid laboratory previously purchases the Boehringer Mannheim kit which was taken over by Roche and then discontinued. The advice from the Department of Pathological Biochemistry at the University of Glasgow was to purchase the kits now available from Wako, through orders placed in Germany. Wako Chemicals GmbH, Nissanstr 2, D-41468, Neuss, Deutschland (tel 02131311-0, facsimile 02131311100).

The kit was adapted for small-scale use in the lipid laboratory so that it may be used for assays starting with dry lipid or wet samples. This required extensive optimisation with Mrs M J Levey. The total cholesterol (TC) and free cholesterol (FC) assays made by Wako use different colour products. The red quinone of the free cholesterol assay absorbs best at 505nm, while the blue pigment of the total cholesterol assay absorbs maximally at 590nm. Bilirubin gives an enhanced absorbance, as does haemoglobin, in the free cholesterol assay.

The methods were designed to resuspend dried cholesterol into ethanol (or isopropanol) with a small aliquot of Triton X100, whereafter reagents are added. Attempts were made to commit the standards and sample preparation to about 125ul and then to proceed with the assay so that about 250ul are available for the microtitre plate reader.

FREE CHOLESTEROL ASSAY (274-47109E)

CHEMISTRY

Free cholesterol is converted to cholest-4-ene-3-one by reacting with oxygen in the presence of cholesterol oxidase and this step produces hydrogen peroxide on a molar ratio with cholesterol. Next, 4-amino-antipyrene reacts with phenol and uses hydrogen peroxide in such a way that a red quinone pigment is formed along with water and a phenol that now has =O and =N at para-positions relative to each other. The colour is stable for 90 minutes.

MATERIALS

Ethanol, absolute.
Triton X100 1% v/v in distilled water.
Saline.

Reagent kit containing buffer, colour reagent, standard solution, and diluent for the standard. solution. Store in refrigerator although the standard solution and diluent.
Buffer solution of 160ml contains 1% phenol and 0.1 molar phosphate at pH=7.0.
Colour reagent of 75ml contains cholesterol oxidase 0.04U/ml, peroxidase 0.8U/ml, 4-aminoantipyrine 0.015%. The colour reagent should be opened carefully as it is under negative pressure. It is stable at 2-4°C for two weeks after opening. Each bottle of colour reagent receives 75ml of buffer; prepared in either of two ways (1) first mix 10ml of the buffer with the powder by gentle inversion, transfer to a second container. Repeat this 2 or 3 times before making up to 75ml with more buffer and mixing or (2) grind the material to a fine powder with a clean dry glass rod and add to 75ml of buffer.
Standard solution (10ml) of cholesterol is at 100mg/dL and contains isopropanol, and the diluent (10ml) also contains isopropanol.

ORIGINAL PROCEDURE

This was designed for 50μl of sample and 3.0ml of reagent, with 50μl of standard being used for a concentration of 100mg/dL and 50μl of distilled water for a blank. The reaction is mixed and incubated at 37°C for 15 minutes. The absorbance is read at 505nm. The reaction is linear up to 400mg/dL but higher concentrations require a dilution, in distilled water is recommended but saline will also be fine.

The whole procedure can be scaled down to 10μl of standard or sample, and 300μl of reagent for microtitre plates.

PROCEDURE FOR DRIED LIPIDS

This procedure is designed for the use of dried standards and/or lipids but can incorporate aqueous samples. The dried lipid is dissolved in ethanol and triton and then admixed with saline whereas the aqueous samples are diluted with saline and then receive the triton and ethanol. The standards are aliquoted into microreaction vials and dried. The range is 0-100ug of cholesterol.

Use microreaction vials or 10x75mm glass tubes. Add 25ul of ethanol to the dried sample, vortex. Add 7.5ul 1% Triton and vortex. Add 92.5ul saline to bring the volume to 125ul. To aqueaous samples, add saline to make the volume 92.5ul and then add 7.5µl of 1% Triton and 25µl of ethanol.

Add 125µl of reagent and mix. Transfer to a microtitre plate and react for 60 minutes at 37°C. Read the absorbance at 500nm. Using the mass of the standards on the x-axis, the optical density of the standards on the y-axis, derive the unknowns by interpolation, linear regression or non-linear regression.

PROCEDURE FOR LIPIDS IN AQUEOUS SUSPENSION

This assay requires a plasma standard with a previously determined free cholesterol concentration. The standard curve ranges from 0 to 100µg in 5 to 7 steps.

The assay can be performed directly in a microtitre plate. The sample and balancing amount of saline should amount to 125µl. Add 125µl of reagent in such a way that the jet mixes the two components of the reaction. Tap the microtitre plate briefly to mix the reagent or shake in the microtitre plate reader.

Incubate at 60°C for 1 hour. Read the absorbance at 500nm. Using the mass of the standards on the x-axis, the optical density of the standards on the y-axis, derive the unknowns by interpolation, linear regression or non-linear regression.

TOTAL CHOLESTEROL ASSAY (L-type)

CHEMISTRY

Cholesterol ester is hydrolysed by cholesterol esterase in the presence of water, to form cholesterol and a free fatty acid. The cholesterol is reacted with oxygen by cholesterol oxidase, to form cholest-4-en-3-one and hydrogen peroxide. Two molecules of hydrogen peroxide reacts with 4-aminoantipyrine and N-ethyl-N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline Na (DAOS) in the presence of peroxidase to form a blue compound that absorbs maximally at 600nm.

Plasma samples are stable for 1 week at 4°C to 12°C, and for 6 months at -20°C. There is no interference from ascorbic acid (<50mg/dL), bilirubin (<684µmol/l), and haemoglobin (<500mg/dL). Lipaemic plasma is best diluted 4-fold before measuring. The reaction is said to be linear up to 1000mg/dL.
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MATERIALS

Isopropanol, absolute.
Triton X100 1% v/v in distilled water.
Saline.

The reagent kit contains 4 bottles of colour reagent A, 4 bottles of colour reagent B, cholesterol standard 4 bottles of 10ml each.
Reagent A: Good's buffer (pH=7.0) at 0.1mmol/L, DAOS, cholesterol esterase 1U/ml and ascorbic acid oxidase 1U/ml. 413-10085: 4x70ml, 41410095: 4x270ml, 410-10095: 4x540ml
Reagent B: Good's buffer (pH=6.5) at 50mmol/L, cholesterol oxidase 1.2U/ml, peroxidase 6U/ml, 4-aminoantipyrine 3.6mmol/L. 419-10185 4x24ml, 414-10195: 4x90ml, 410-10195 4x180ml.
Cholesterol Standard: 200mg/dL. 272-85009: 4x10ml.

ORIGINAL PROCEDURE

Sample or standard of 3μl is mixed with 270μl of reagent A and incubated at 37°C for 5 minutes. Then add 90μl of colour reagent B. Mix and react for 5 more minutes at 37°C, reading standards and samples at 600nm.

PROCEDURE FOR DRIED LIPIDS

To dried standards of cholesterol, 0-100μg in 5 to 7 steps, add 37.5μl of isopropanol and mix. Add 7.5μl of Triton X and mix. Add 80μl of saline and mix. Now add 120μl of reagent A and mix. Incubate at 37°C for 5 minutes and add 40μl of reagent B and mix. Incubate for 30 minutes at 37°C. Spin in high-speed centrifuge at 4°C to pellet insoluble material.

Transfer 230μl to a microtitre plate well and read absorbance at 600nm.

Use linear regression to obtain the μg of the unknown from the standard curve. The $r^2 =$ 0.998.

PROCEDURE FOR AQUEOUS SUSPENSIONS OF LIPIDS

The standard is a well-characterised serum/plasma sample that is placed in a standard curve representing 0-100μg in 5 to 7 steps.

In a microtitre plate well, bring the standards and samples to 125μl with saline. Mix by gently tapping the microtitre plate on the side or by placing on the shaker of the microtitre plate reader. Add 120μl of reagent A, incubate for 5 minutes at 37°C. Add 40μl of reagent B and incubate for 30 minutes at 37°C. Read the absorbance

Use linear regression to obtain the μg of the unknown from the standard curve. The r^2 = 0.9998.

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Appendix A.3

GPO PAP KIT FOR TG ASSAY

GENERAL

Lipases hydrolyse triacylglycerol to free fatty acids and glycerol, which is converted to glycero-3-phosphate by glycerokinase and ATP. The glycerophosphate is reacted with oxygen by glycerophosphate oxidase yielding dihydroxy- acetone-P and H_2O_2. Two molecules of the latter is used by peroxidase as well as 4-aminoantipyrine and 4-Cl-phenol to generate quinoneimine HCl and $4H_2O$, yielding a pink-red colour read at 500nm (or 546 in Hg) in a 1cm path. Kit no:10164.

MATERIALS

BUFFER solution contains Pipes (pH=7,5) 40mmol/l, Mg 5mmol/l and chlorophenol 5mmol/l and NaN3 0,1g%.
ENZYMES solution contains aminoantipyrine 0,4mmol/l, ATP 1 mmol/l, lipases >150U/ml, glycerokinase >0,4U/ml, glycero-3-P oxidase > 1,5U/ml, peroxidase >0,5U/ml. Reconstitute the enzyme lyophilisate with 15ml buffer. This will be stable at 4°C for 21 days or 20°C for 3 days.

CALIBRATION. Commercial calibration sera can be employed but it is cheaper to use gravimetric standards although now ethanol must be employed to dissolve the triacylglycerol. Use sunflower oil or triolein. Dissolve 10,0mg in 10ml benzene and mix for 1ug/ul (soln A) and take 200μl for bringing to 2ml for 0,1μg/μl (solution B). Prepare 2,5; 5 and 10μg standards from B and 25, 50, 75, 100, 125, 150, 175 and 200μg standards from A. Dry in fume cupboard.

PROCEDURE FOR MICRO-REACTION VIALS

This design is for a final volume of about 750μl and allows reading in a microcuvette in a spectrophotometer and also a transfer of some of the reacted solution to a microtitre plate for rapid reading.

PLASMA. A standard is provided with the kit. Blank has no plasma while 10ul of sample is reacted, or in turbid plasma a dilution. Add 250μl of saline, vortex. Add 500μl of reagent, vortex. Incubate at room temperature for 15 minutes and read within an hour.

EXTRACTS. Dry the lipid extract transferred in organic solvent (glass or other insoluble tube). Prepare blank and standards (2 to 200µg, similarly dried). Add 20µl ethanol, vortex. Add 250µl saline, vortex. Add 500µl reagent, vortex. Incubate 15 minutes and read absorption.

Analyse by linear regression, r=0,999. Ethanol affects neither blank nor standard readings and two assays can be combined, adding saline first to plasma samples to avoid ethanolic denaturation. Subtract 0,11mmol/l for free glycerol.

PROCEDURE FOR MICROTITRE PLATE

Performing this assay in a microtitre plate makes the assays rapid and cheap. It is designed for a standard or sample volume of 50µl and 200µl of reagent. However, the sample volume can be made smaller if only plasma is being analysed.

Using the calibrator with the kit (2µg/µl) a standard curve is set up from 0.5µg to 60µg so that there is a blank and 5 points for linear regression. Plasma samples should be between 2 and 10µl, depending on the triglyceride content. Very lipaemic samples have to be diluted in saline before use in the assay. Once the volumes have all been brought to 50µl, add 200µl of reagent. Stand for 15 minutes. Shake for 30 seconds before reading in the machine at 500nm.

Analyse by linear or non-linear regression.

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Appendix A.4

SIGMA 3-α-hydroxy BILE ACID ASSAY

GENERAL

BILE ACID METABOLISM

Bile acids are synthesised in the liver and concentrated in bile. They can be found in the blood only at low concentrations owing to a very efficient enterohepatic circulation. Their synthesis characteristically includes the switching of the position of the 3-OH group from beta to alpha orientation on the sterol, as well as the addition of other -OH moieties and the cleavage of the isooctyl side chain of the sterol. Thereafter follows conjugation to glycine and taurine.

QUANTIFICATION

Bile acids can be quantified by gas chromatography, fluorimetric or spectrophotometric methods, or by immunoassay. This method is an enzymatic assay in which, in the presence of NAD, 3α hydroxysterol dehydrogenase from fungi is used to create 3 oxo-hydroxysterol and NADH. Next NADH and nitroblue tetrazolium (NBT) salt produce NAD and formazan. Formazan has a maximal absorption at 530nm (still suitable at 540nm), and the intensity of the colour is directly proportional to the amount of bile acid. Adding phosphoric acid can stop the colour generation. The assay is strictly speaking for 3α-OH sterols and will thus reflect bile acid concentration as well as cholestanol concentration.

The assay takes into account the other plasma dehydrogenases of which the most important is lactate dehydrogenase (LDH). Heating plasma to 67°C for 30 minutes will inactivate all enzymes but is tedious. Since the bulk of the plasma or serum dehydrogenase activity resides in LDH, it is possible to block this activity with sodium pyruvate (200mmol/L), or oxamic acid. The stop reagent is phosphoric acid at 1.33mmol/L with 200mmol/L sodium pyruvate. The optimal NBT concentration is 0.2g/L. In order that formazan precipitation is avoided, a non-ionic detergent, polyoxyethylenolylether is added.

The enzymes used in the assay are sensitive to the bile salts in pure solutions that may be used as standards, especially when the enzymes are ageing. The result is an underestimation of the colour change leading to an exaggeration of the concentration is

the samples. This can be avoided by adding protein to the standards's solution, typically to a final concentration of 2g% in the standard.

The samples should be taken in plain tubes after an overnight fast and clot should be removed. Serum can be kept at 4°C for a day or frozen until the assay, at -20°C. The normal fasting range for bile acids is 0-8μmol/L. The assay is linear up to 200μmol//L. The intra-assay coefficient of variation (CV) is about 1-7% and the inter-assay CV is about 2-8%. An increase in the systemic concentration of bile acids means either significant porto-systemic shunting or poor hepatic extraction during enterohepatic circulation. A decrease indicates bile acid malabsorption.

The lipid laboratory has modified the assay to a microtitre plate. A solution of sodium deoxycholate is used as standard. Subjects with liver disease or who are expected to have a high concentration should have their samples diluted with saline, either 1 in 5 or 1 in 10.

The reference 95% confidence interval in a group of healthy adults was found to be 14-20μmol/L (n=14). The mean and standard deviation were 16.6 and 4.2 μmol/L respectively and the range was 8.5 to 25. This needs reconfirmation after discovering the matrix effects.

Sigma discontinued manufacture 2003 and a similar kit has been devised by Adcock Ingram for approx R6000 in 2004 for 40 samples. Samples should be provided as serum but the addition of calcium to at least 10mmol/L final concentration may enable analysis.

MATERIALS

SAMPLES. Fasted serum. No anticoagulants. It is possible to use EDTA plasma which has been enriched with Ca chloride to a final concentration of 20mmol/L.

BILE ACID REAGENT A. Cat No 450-1. Store at 4°C. Contains NAD at 2.5mmol/L, NBT 0.61mmol/L, diaphorase 625 U/L, buffered to pH=7.0. Reconstitute with distilled water. Invert but do not shake or vortex. Stable for at 4°C for 1 week, at room temperature for 24 hours.

BILE ACID REAGENT B. Cat No 450-2. Store at 4ºC. 3α Hydroxysterol dehydrogenase 1250 U/L with stabilisers. Reconstitute with distilled water. Invert to mix but do not shake or vortex. Stable for at 4ºC for 1 week, at room temperature for 24 hours.

The reagents should not be caked and should dissolve completely for proper reactions. The reconstituted solutions should have an optical density (OD) of <0.3 against distilled water.

BILE ACID STOP REAGENT. Cat No. 450-3.   Na-pyruvate (MW 110.05) at 200mmol/L in phosphoric acid 1.33mmol/L.
BILE ACID CALIBRATOR. Cat No. 450-100.
BILE ACID CONTROL SERUM. Cat No. 450-22.

ALBUMIN 4g% SOLUTION for dilution.
BILE ACID HOME STANDARD. Na Cholate has molecular mass of 430.6d. Note that the Sigma preparation is mixed conjugated bile salts and does not give molarity expected.

PROCEDURE

Prepare the reagent solutions A and B, and samples and warm them to 37°C. The **active test reagent is A+B** (4 volumes A + 1 volume B) while the **blank reagent** is 4 volumes of A plus water 1 volume.

In the original procedure the sample volume is 200µl, the reagent volume is 500µl and the stop solution volume is 100µl. The calibrator is used and the assay is assumed to be linear. The reaction time is said to be 5 minutes but the Lipid laboratory experiences better linearity, especially at larger amounts of standard, if the reaction runs for 45 minutes.

MICROCUVETTES

Set up tubes to reflect a range of standards and their blanks, a Calibrator, a Calibrator blank, a Control and a Control blank, a Sample and Sample blank. Prepare the standards in 100µl such that saline makes up 50µl and with 50µl being 4% albumin solution to avoid matrix effects. Pipette in 100µl of calibrator, control and sample into the relevant tubes. Pipette in 250µl of test reagent to all the active assays and blank reagent to all the blank assays. Take note of the timing, as the reaction times have to be identical.

Let the reaction proceed for 45 minutes and then add stop reagent, 50µl at the exact intervals for all tubes. The colour is stable for 1 hour.

Read the OD at 530nm and calculate the change between active and inactive assays. Derive the concentration of unknowns by regression curve analysis from the known values.

MICROTITRE PLATES

The same as above. The standard curve is made up from 1 to 50nmol of 3α hydroxysterol as cholic acid. The standards and samples are presented in 100μl, which should be balanced with 4g% albumin to avoid matrix effects when <100μL is used. Serum expected to have normal values should be 100μL. Add 250μl of reagent or reagent blank. Add 50μl of stop solution.

CALCULATIONS

The bile acid content in the assay is calculated by linear or non-linear regression, or by proportion to a known concentration. Noting that changes in absorbances for test and blank reagents are used to derive the Δabsorbance for the unknown (U) and calibrator (C), the serum bile acid concentration can be calculated from the umol/L of C.

uM/L unknown = (ΔU/ΔC)*([C])

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Appendix A.5

CHEMICAL PRECIPITATON OF LIPOPROTEINS

GENERAL

Polyanions (heparin, dextran) and cations (Mg, Ca, Mn) have a specificity of precipitating lipoproteins according to their concentration, the lipid/protein ratio of the lipoprotein, and environmental factors in the reaction (protein, solute & chelatant concentrations and pH). Precipitation is complete within 10 minutes and is stable for hours. The first to last in sequence are CM, VLDL, LDL, and HDL. It is probable that apo-E in HDL does not coprecipitate under conditions bringing apoB down when using heparin-Mn but will coprecipitate with dextran-Mg and phosphotungstate-Mg. Hypertriglyceridaemia (>3mmol/l) may interfere with complete precipitation and the VLDL may be first spun up or subsequently the sample should be spun harder to sediment the apoB containing lipoproteins. On ageing (>4days) apoB tends to precipitate less completely if low HDL, while more if high HDL. These effects are lessened by storing at -20°C or -70°C. Mn can interfere with subsequent enzymatic assays and should be removed by bicarbonate rather than EDTA since the latter also interferes.

Polyethylene glycol can also effect LP precipitation by altering the dielectric constant of the water, and its concentration (60-120g/l final) and pH affect the extent of precipitation and triglyceride has very little effect up to 15mmol/l. All the above methods agree very well with ultracentrifugation. The use of enzymic reagents for heparin-Mn has the added complication of incomplete LpB precipitation with EDTA, Mn-EDTA turbidity and phosphate-Mn turbidity unless bichromatic correction and reagent addition of EDTA to chelate Mn is made.

Turbid samples can be clarified by (i) dilution with saline before the procedure, (ii) dilution with saline after the procedure so that the density is lower for improved precipitation, (iii) ultrafiltration through Millipore 0,22um pore size but larger volumes are required and losses of HDL may occur. In general the least interference can be expected from polyethylene glycol (PEG) method which does not interfere with enzymic reagents, yields very good precipitations even on old specimens but can read 0,05mmol/l lower with EDTA. It also tolerates high triglycerides best.

MATERIALS

HEPARIN Na. Porcine intestinal mucosal (35mg= 5000 USPU). Keeps for months in fridge.

MnCl2+4H2O. 197,7g/M. Final conc 0,042 to 0,092mol/l. Keeps for months as concentrated stock solution.
DEXTRAN SO4 (50kd) Stock solution usually 2% at pH=7,0.
$MgCl_2+6H_2O$ at 1mol/l, pH=7,0.

PROCEDURE

Usually 100μl of 10X stock of mixed heparin/Mn (or dextran/Mn or dextran/Mg) is added to 1,0ml of plasma and the apo-B containing lipoproteins are precipitated for 15 minutes before being spun at 6000G for 10 minutes. The cholesterol concentration in the supernatant is determined and corrected by a 10% increase to allow for the dilution by the additives, yielding the "HDL-C".

HEPARIN-Mn. Final concentration for plasma is heparin 0,2% and Mn 0,092mol/l. Plasma diluted x10 with saline requires only 0,005%. Add 10X Mn-heparin stock to appropriate volume at room temperature, standing on ice (not essential) for 15 minutes before pelleting the lipoproteins by 6000G for 10 minutes. Note the white pellicle is Mn oxide and not floated lipoproteins. The supernatent "HDL" is removed by aspiration.

Heparin is removed by precipitation with 5% BaCl2 whereas Mn is removed by precipitation with 10% $NaHCO_3$. Spin for 3 minutes to remove cleared supernatent. Lipoproteins are redissolved by 1 to 5% NaCl (incubate 30 minutes at 37°C) or 10% $NaHCO_3$, or by adjusting pH to <4,0 or >8,8.

HEPARIN $MgCl_2$. Final concentration is 0,05% heparin and 0,1mol/l Mg. This concentration will precipitate all lipoproteins. Prior solution of 1g/ml sucrose in plasma will cause precipitation of only apoB-containing lipoproteins which will float on centrifugation so that HDL is retrieved by piercing the tube and aspirating the infranatant. More heparin is required as sucrose is decreased.

DEXTRAN $MgCl_2$ tends to precipitate more apoAi but more complete apoB precipitation. Final concentration is 0,91mg/ml and Mg 0,045mol/l.

DEXTRAN $MnCl_2$. Final concentration is 0,05% with Mn 0,05mol/l.

PEG Fluka/Sigma Mr=6000. Dissolve 45g in 60ml distilled water, bring to 100ml. Stores at room temperature for 2 months. To 1ml of serum add 200ul of solution and vortex. Keep at room temperature for 15 minutes. Spin in microfuge for 15 minutes at 2300G. Determine the cholesterol concentration in the supernatant and correct for dilution by 1,2.

HDL SUBFRACTIONATION (GIDEZ)

Variations in total HDL concentrations are primarily due to HDL_2 concentration variations. HDL_2 and HDL_3 levels were previously measured following ultracentrifugation. This method cannot be applied to large numbers of samples, therefore use precipitation methods to separate HDL_2 and HDL_3.The method devised by Gidez *et al.* is a dual precipitation method, in which apoB-containing lipoproteins are first precipitated. HDL_2 is then precipitated from the clear HDL containing supernatant. Cholesterol is then analysed in the first (total HDL) and second (HDL_3) supernatants and HDL_2 cholesterol is calculated as the difference between the two.

MATERIALS

$MnCl_2+4H_2O$ 1,12mol/l (22,166g% in distilled water).
Heparin 135mg/ml (2,025g Na heparin in 15ml, or 20000USP U/ml). Commercial source 168mg/ml.
Dextran (15000) 14,3 mg/ml.
MIX 6ml of heparin and 50ml of Mn for 14,5mg/ml heparin and 1mol/l MnCl2 which in reaction will become 1,3mg/ml and 0,091mol/l.

MICROSCALED PROCEDURE FOR LIPID LABORATORY

Transfer 600µl plasma to a 1.5ml Eppendorff tube. Add 60µl MIX and mix thoroughly but avoid foaming. Allow the sample to stand at room temperature for 20 minutes, then centrifuge at 1500g for 1hour at 4°C.Immediately remove an aliquot of clear heparin-$MnCl_2$ supernatant for the analysis of total HDL-cholesterol and another for HDL_2 precipitation.

HDL_2 precipitation: Transfer 300µl clear heparin-$MnCl_2$ supernatant to an Eppendorff tube. Add 30µl dextran sulphate solution to the sample and mix well. Let the sample stand at room temperature for 20 minutes, then centrifuge at 1500g for 30 minutes at 4°C. The clear soluble fraction represents HDL_3.

The measured value for total HDL-cholesterol is multiplied by 1.10, and that for HDL_3 is multiplied by 1.21 to correct for dilution by the reagents. HDL_2 is then calculated as the difference between total HDL-cholesterol and HDL_3-cholesterol.

LARGE SCALE PROCEDURE

Lower density lipoproteins are first precipitated by adding 300μl of the MIX to 3,0ml plasma, vortexing, and standing at room temperature for 20 minutes. Spin 1 hour at 1500G at 4°C. Supernatant contains whole HDL.

Partial HDL precipitation is done by adding 200μl dextran solution to 2,0ml of above supernatant, vortexing and standing for 20 minutes at room temperature. Spin 30 minutes at 4°C. Measure the HDL3 concentration in the supernatant.

Calculate the whole HDL by multiplying the first supernatant concentration by 1,10 and the HDL3 by multiplying the concentration in the second supernatant by 1,21. The HDL_2 concentration is the difference.

OTHER METHODS

Wilson has described a method whereby VLDL-C, LDL-C and HDL-C can be derived by various precipitation techniques without involving the Friedewald equation, but on a fasted sample.[1] VLDL is aggregated by SDS between 0.6 and 0.75% but not at concentrations higher than 1.5%. Under these conditions some SDS already associates with LDL and HDL as they now migrate faster on electrophoresis. This step allows the estimation of LDL-C and HDL-C. HDL-C alone is estimated by Ca-dextran sulphate or heparin-Mn precipitation of LpB (latter method is more quantitative). Wilson described the method with EDTA plasma and found it suitable at least in some hypertriglyceridaemias, provided that the infranate of the VLDL aggregate is clear.

Firstly, total plasma cholesterol is determined, as well as triglyceride. One limb of procedures involves the VLDL aggregation. To 2 ml of plasma add 0.1ml 22.2% CaCl and 0.08ml Na dextran sulphate (MW 2E6, Sigma), incubate for 24 hours at 4°C. Centrifuge at 4°C for 20 minutes at 2500 rpm (no G force given). Determine the HDL-C in the supernate. The other limb is the aggregation of VLDL. To 2 ml of plasma add 0.15ml of 10% SDS in saline pH=9.0. Incubate for 2 hours at 35°C. Spin at 10 000G at ambient temperature for 10 minutes. The pellicel is VLDL. Aspirate the infranate to determine HDL-C and LDL-C.

The different fractions are now calculated: TC - (HDL-C + LDL-C) = VLDL-C, (HDL-C+LDL-C)-HDL-C = LDL-C.

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Appendix A.6

AGAROSE GEL LIPOPROTEIN (LP) ELECTROPHORESIS

GENERAL

An inexpensive, simple procedure by which a few µl of plasma can be analysed for its lipid content in lipoproteins, and diagnostic of type III dyslipoproteinaemia. Usually performed in an alkaline buffer made up of barbituric acid, and agarose gels while cellulose acetate or paper electrophoresis are also feasible. The chylomicrons remain at the (cathodic) origin while the other LP are separated from cathode to anode as beta- (LDL), pre-beta- (VLDL) and alpha-LP (HDL) with the fats in albumin often giving a faint stain nearest the anode. Densitometric scanning aids the interpretation by giving areas under relevant parts of the curve. Knowledge of the plasma lipids is useful in the interpretation as well.

A 10-14 hours fasting plasma (EDTA) or serum sample should be analysed immediately or could be stored at 4°C for up to 72 hours. Do not use heparinised samples.

MATERIALS

Most convenient is the LIPO kit with the Beckman Paragon system. This has sealed, pre-prepacked gels which must be kept horizontal in storage. The buffer contains, when reconstitiuted, 50mmol/l 5-diethylbarbituric acid, while the gel is made in this containing also 0,1% Na azide in 0,5% agarose. Never freeze the gels; store at room temperature. Use absolute ethyl alcohol for making up solutions.

BUFFER is made up by dissolving the bottle's contents in 1,5L distilled water and pH=8,6. Stores at room temperature for 60 days.
FIXATIVE SOLUTION is 180ml alcohol with 90ml distilled water and 30ml glacial acetic acid.
STAIN comes prepacked as 7% Sudan Black B, made up to to 0,07%. To 165ml of alcohol add 3ml of stain kit and add 135ml distilled water before stirring for 10 minutes. Stable at room temperature for 7days.
DESTAIN SOLUTION is 450ml of alcohol with 550ml distilled water. Set up 3 flasks of 300ml each.

PROCEDURE

An application template is used to apply 3-5μl of serum or plasma for 5 minutes. A blotting strip is now used to remove excess plasma.

The gel is placed in the correct orientation in the dish containing 45ml of buffer. The machine is switched on for the appropiate number of dishes and for 100V, for 30 to 40 minutes.

Upon completion remove the gel and place in frame. Place in fixative for 5 minutes, remove and wipe moisture off its back. Dry the gel at 50°C. Now place in working stain for 5 minutes. Dip in destain flask 1 three times, then in flask 2 thrice and in flask 3 for 5 minutes. Wipe off excess liquid and dry.

INTERPRETATION

The pattern may be interpreted visually or be analysed by densitometer at 600nm. In either case a normal control serum of known LP constitution is useful and may even be used as a standard in the scanning which should also yield areas under the curve as a fraction of total area. The CV is <5% according to the package insert.

Another method of quantifying the electrophoretogram is to elute Sudan Black with acetic acid/ethanol/water 11:5:4 and to read the absorbance. The lipid in a given fraction is then expressed by $L_F=TXD_F/D_T$ where T is absorbance and D_F and D_T are the dye in the fraction and total dye. The staining is however not equal for all lipids; if CE=1, TG=0,8 and PL 0,1 and FA 0,1 and free cholesterol is 0,02. Because of the differential lipid staining the corrected fraction that should be used for D_F is 1 for alpha, 0,75 for beta and 0,75 for prebeta and 0,63 for CM. For Fat Red 7B the corresponding corrections are 1,75; 1.0 and 0,82 and 0,82.[1]

A NORMAL pattern has <1% of lipid stain in CM, 40-70% in beta, 0-30% in pre-beta and 10-45% in the alpha band.

FREDRICKSEN CLASSIFICATION

TYPE I shows a definite dark band at the origin and frequently a smearing across the other bands to the alpha band. TYPE II has a more intense than normal beta. IIa has only this change while IIb has more than normal intensity of pre-beta as well and will have TG values slightly to definitely elevated. In TYPE III there is an abnormally broad band starting anodic to the usual beta site and ending at towards the anodic end of the pre-beta

band; sometimes some CM may be seen as well and alpha band may be feint. TYPE IV has increased pre-beta staining and often also diminished beta and alpha intensity. TYPE V has CM stain at origin, often smears as well, and in addition also has intenser staining of pre-beta while alpha staining often reduced.

In abetalipoproteinaemia there is no visible beta or pre-beta band. In hypoalphalipoproteinaemia the band is poorly staining while in hyperalphalipoproteinaemia it stains intensely.

UNUSUAL FINDINGS

A sinking pre-beta band due to larger quantities of Lp(a) can be seen at the cathodic end of the pre-beta band. The Lp(a) cholesterol can be calculated by the use of absorbance of Lp(a) (Aa) and beta band (Ab) and the total cholesterol (T): Lp(a) C = TX(Aa/Aa+Ab). Another diagnostic clue can be a very rapidly migrating beta band (LDL) in X-linked ichthyosis where steroid sulfatase is deficient and this results in the accumulation of cholesterol sulfate and thus more (negatively) charged LDL.

Heparinised samples present charged lipoproteins which migrate aberrantly in a fast, or streaking fashion. Aged samples may undergo lipolysis and the negatively charged fatty acids will permit fast migration of the beta band, and at 3umol/L will create a pre-beta migrating beta band and may aslo stain up on albumin. This phenomenon of accelerated beta migration can be cancelled by adding fat-free albumin to adsorb the fatty acids.

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Appendix A.7

PHOSPHOLIPID BY CHOLINE ASSAY KIT

GENERAL

The indications for measuring phospholipids are less commonly encountered than for measuring plasma cholesterol and triglyceride but phospholipids have a special role in the detection and following of dyslipidaemia in obstructive jaundice because LpX is so rich in phospholipid. Furthermore, maternal amniotic fluid is examined for fetal lung maturity, reflected in phospholipid concentration.

The assay methods for phospholipid classically were to extract the lipid by a procedure such as that of Folch, to mineralise the sample to inorganic phosphorus and then to demonstrate phosphorus through a colour development.[1] The oxidation to mineralise the sample is done with perchloric acid (± hydrogen peroxide) at 200°C to 225°C for 15 minutes, or with a mixture of sulphuric and perchloric acid and vanadium pentoxide as catalyst.

The assay meaures phosphatidylcholine (lecitihin), the bulk of phospholipids in blood and cell membranes.

This enzymatic method involves the hydrolysis of phospholipids by phospholipase D, releasing choline and phosphatidic acid. The choline, in the presence of 2 oxygen molecules and water, by means of choline oxidase, forms betaine and 2 molecules of hydrogen peroxide. Two hydrogen peroxide molecules react with 4-aminophenazone and phenol to yield 4(p-benzoquinone-mono-imino)phenazone and 4 water molecules. The enzyme is a peroxidase. The absorbance is measured at 500 to 505nm.

The assay is for moles of phosphatidylcholine, and not necessarily all phospholipid. The average molecular mass of phospholipid is 774d. The reference range is 158-284 mg/dL or 2.04-3.66 mmol/L. The assay behaves linearly and only one reference point is required. Serum or plasma (citrate, heparin) can be used, but not plasma containing EDTA for the Boehringer Mannheim kit. The Wako kit is compatible with serum, and plasma using EDTA, heparin, oxalate, and citrate. The samples are stable for 7 days at 4°C, and 2 days at room temperature but can be frozen for years. Ascorbic acid can interfere with colour production when present in high concentration, and haemoglobin and bilirubin can yield increase in absorbance when unusually high in concentration.

BOEHRINGER MANNHEIM KIT

Boehringer Mannheim kit: 691844. 5x40ml vials. Discontinued in 2001. Once prepared, the reagents from the kit are stable for 2 weeks at 4°C and 2 days at room temperature. Wako kit. 990-54009E. Caters for 50 test units: 190mL buffer, 4 vials colour reagent, standard is the same as for Boehringer Mannheim kit.

Standards. Choline 54.1mg% equivalent to 300mg% PC. The formula for choline is $C_5H_{15}O_2N$ and mass is 121.2d. Hence each ul contains 0.541ug or 4.46nmol/l.
Buffer. Set at pH=8.0. Tris-hydroxymethylaminomethane 50mmol/l, phenol 20mmol/l.
Reagents. PLase D >10E4U/L, choline oxidase >800U/L, 4-aminophenazone 8mmol/l.

PROCEDURE FOR MICROREACTION VIAL

Set up a reagent blank with water in equivalent volume to plasma, a calibration standard, and the samples for analysis.

ORIGINAL. Use 20μl of sample or calibrator, add 3ml reagent, mix. Incubate at room temperature for 20 minutes or at 37°C for 10 minutes. Read the optical density at 500nm.

MODIFIED. Use the appropriate pipetteman or positive displacement pippete to aliquot 7μl of sample or calibrator. Add 1ml of reagent. Mix, incubate as above, and determine optical density.

CALCULATE. Derive the change in absorbance by subtracting the value of the reagent blank from that for the sample(s) or calibrator. The sample concentration is 300x(dODsample/dODcalibr) in mg/dL. For mmol/L the calculation is 3.88x(dODsample/dODcalibr). The reaction is linear up to 13mmol/l or 1000mg/dL.

PROCEDURE FOR MICROTITRE PLATES

Scaling the assay down for a microtitre plate makes it both cheap and rapid. The design is for 50μl up to 100μl of sample and 150μl up to 250μl of reagent.

The standard curve is set up with the choline solution, having diluted the solution for the lower part of the curve. The standard curve should be from 0.0541μg to 5.415μg. The plasma (5-15μl) or other samples should also be brought to 100μl. Add 150μl of reagent. Stand for 30 minutes at room temperature, shake briefly before reading in the machine at

500nm. Alternatively, incubate at 37°C in an incubator for 10 minutes, stand at room temperature for 5 minutes and read absorbance.

Analyse against the standards by linear or non-linear regression, depending on the curve and the optical density of the samples.

WAKO PHOSPHOLIPIDS B KIT

The chemical basis is the same as for Boehringer Mannheim kit that was discontinued in 2001.

Code No. 990-54009E. Store at 2-10°C (do not freeze) A 50-test unit comprises:
Buffer 190ml. Tris 0.05 Molar, pH=8.0, Ca chloride 5mg/dL, phenol 0.05%.
Colour reagent in 4 vials each 45 when reconstituted. Per vial: Phospholipase D 20U, choline oxidase 90U, Peroxidase 240U, 4-amino-antipyrine 0.015% when reconstituted.

Standard solution. 10mL corresponds to 300mg/dL (3g/L or 3.88mmol/L) of phospholipid and contains 54mg/dL (0.54g/L or 5.2mmol/L) of choline, and phenol 1%.

ORIGINAL ASSAY

20µL of sample or standard or distilled water for the blank, is mixed with 3.0mL of reagent. Place at 37°C for 10 minutes. Colour develops in 5 minutes but the reaction can proceed at this temperature for another 15 minutes. The colour is stable for 2 hours. Read at 500 to 505nm.

Beer's Law is obeyed at 0-1000mg/dL of phosphatidylcholine. Thus, within the range of 3 times the corrected absorbance for the standard sample, the unknown concentration can be derived:

Unknown mg/dL = (A_u /A_s) x (300mg/dL)
Unknown mmol/L = (A_u /A_s) x (3.88 mmol/L)

PROCEDURE FOR DIRECT ASSAY IN MICROTITRE PLATES

Scaling the assay down for a microtitre plate makes it both cheap and rapid. The design is for 50µl (up to 100µl of sample) and (150µl up to) 250µl of reagent.

The standard curve is set up with the choline solution (0.54μg/μL), having diluted the solution for the lower part of the curve. The standard curve should be from 0.0541μg to 5.415μg choline, corresponding to 0.3 to 30μg phosphatidylcholine.

The plasma (5-15μl) or other samples should also be brought to 100μl. Add 150μl of reagent. Stand for 30 minutes at room temperature; shake briefly before reading in the machine at 500nm. Alternatively, incubate at 37°C in an incubator for 10 minutes, stand at room temperature for 5 minutes and read absorbance.

Analyse against the standards by linear or non-linear regression, depending on the curve and the optical density of the samples.

PROCEDURE FOR ASSAY OF DRIED LIPIDS FOR MICROTITRE PLATES

The dried lipid material is first resuspended. To the dried sample, add 50μL of ethanol, vortex. Add 15μL of 1% Triton X-100, vortex. Add 185μL of water, vortex. The standard curve uses the same standard curve as for the microtitre plate. The standard curve and blank are prepared similarly with ethanol and Triton.

Now add 250μL of reagent. Incubate at room temperature for 30 minutes or 37°C for 10 minutes. Transfer 250 to 300μL to the microtitre plate. Read absorbance at 500 to 505nm.

Using linear or nonlinear regression, the standard curve data allow the calculation of mass of unknown phospholipid.

Note, to limit costs, 125μL of suspension can be transferred to a microtitre plate well and reacted with 125μL of reagent.

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