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Razvoj dvodimenzionalne gelske elektroforeze za določanje serumskih bioloških označevalcev pri poškodbah jeter podgan, povzročenih z ogljikovim tetrakloridom

Development of two-dimensional gel electrophoresis for detection of serum biomarkers of carbon tetrachloride-induced liver injuries in rats

Ljubljana, 2014

I performed experimental work of my thesis work on UCL School of Pharmacy, Department of Pharmaceutical and biological chemistry, London and at Faculty of pharmacy, University of Ljubljana under supervision of prof. dr. Irena Mlinarič Raščan and dr. Rosemary Smyth.

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DECLARATION

I declare that this thesis is my own work done under supervision of dr. Irena Mlinarič-Raščan, mag. farm. and dr. Rosemary Smyth.

Tasja Ceklin

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ABSTRACT

Hepatic biomarkers are proteins used as a measurable characteristic that indicates presence of liver injuries. Nowadays there is a huge tendency to discover novel biomarkers which will serve as diagnostic tool for detecting early stages of liver diseases. Liver diseases originate from liver injuries, acute or chronic depending on time duration exposed to hepatotoxin or extension of damage tissue in liver. To detect these specific biomarkers proteomic investigation needs to be performed. One of the widely used proteomic technique is two-dimensional gel electrophoresis (2-DGE), where proteins are separated based on two independent characteristics: pI and molecular weight. However, numerous parameters are critical for good performance of 2-DGE including effective sample preparation, first and second dimension of separation and at the end protein detection.

In thesis, rats as experimental animals were used in order to watch their response on oral administration of hepatotoxin carbon tetrachloride (CCl_4) in a single dose and tracked its impact on liver. To determine differences comparison to control group of rats were made. As well, rats serum samples were examined for any differences in protein expression by 2-DGE.

First aim of thesis was to evaluate CCl_4 influences on rat's body weight loss and to define correlation of CCl_4 to rat's liver weight. Rats were treated with CCl_4 in two different concentrations (1.2 and 2.0 ml/kg) and compared to control group. Results were statistically analysed with SPSS and showed loss of body weight in all groups but no statistical differences between the groups. Liver weights were after administration of 1.2 and 2.0 ml/kg of CCl_4 to rats significantly increased above the control group, 1.41-fold and 1.59-fold, respectively, indicating some alterations in structure of liver parenchyma.

In second part, two sample preparation methods were used in order to estimate their efficiency. After performing these methods, samples were analysed with SDS-PAGE or 2-DGE and stained with Blue Coomassie or silver. With depletion of albumin component (DAC) protocol, method based on salt precipitation, albumin was effectively removed from sample and other low abundant proteins present in serum were detected. As well, effective removal of salts and other interfering compounds were seen after usage of ReadyPrep 2-D Cleanup kit, which contributes to more focused spots on gel.

In third part, samples from control and from group treated with 1.2 ml/kg of CCl_4 were compared to find different expression of proteins serving as potential hepatic biomarkers. As well different immobilized pH gradients - IPG strips (4-7 and 5-6) were applied in first dimension of electrophoresis and use of different loading volumes (2.5 μl and 7.5 μl) were presented in order to estimate optimal condition for good figure resolution and well-focused proteins. On both samples DAC protocol and ReadyPrep 2-D Cleanup kit were performed and then analysed with 2-DGE and detected with silver stain. Results showed that using narrower IPG strip (5-6) contributes to better focused proteins helping to identify them easier. Using different loading volume of serum showed that the greater the volume of sample was, the more proteins could be detected on gel and they were more visible. However, proteins were not good focused because high abundant proteins overlapped the less abundant one.

POVZETEK

Dvodimenzionalna gelska elektroforeza (2-DGE) je osnovna metoda proteomike, katere namen je ločitev proteinov v dveh dimenzijah. Takšna kombinacija nam omogoča višjo ločljivost proteinov v gelu ter olajša njihovo identifikacijo. V prvi dimenziji ločba poteka v gelu z imobiliziranim pH gradientom, kjer se proteini ločijo glede na izoelektrično točko t.j. točko, kjer je neto naboj proteina enak nič. Ta postopek imenujemo izoelektrično fokusiranje (IEF). Proteini se nato v drugi dimenziji ločijo na osnovi velikosti, z NaDS elektroforezo. Ta tehnika je uporabna, ker NaDS denaturira proteinske molekule, s čimer povzroči enako (negativno) nabitost proteinov, njihova ločba pa poteka le na podlagi molekulske mase.

Poleg ločitve proteinov pa je za dobro resolucijo slike potrebna tudi ustrezna predpriprava vzorca pred 2-DGE ter barvanje vzorca z ustreznimi barvili po izvršitvi 2-DGE za ustrezno določitev proteinov na gelu. Vzorce navadno pridobimo iz različnih telesnih tekočin (npr. kri, urin) oz. z lizo celic. Med vsemi vzorci, je kri oz. serum najpogosteje odvzet in preiskan vzorec zaradi enostavnosti odvzema ter širokega spektra informacij o stanju organizma. Pri pripravi vzorca za analizo z 2-DGE je za boljše videnje proteinov potrebno odstraniti proteine, prisotne v visokih koncentracijah (npr. albumin, imunoglobulin). Prav tako je iz seruma potrebno odstraniti tudi soli, ki bi motile ločbo proteinov pri izoelektričnem fokusiranju. Vzorec seruma vsebuje široko področje različnih proteinov prisotnih v različnih koncentracijah, ki služijo kot biološki označevalci organizma. Po definiciji so to proteini, ki odražajo fiziološke in patofiziološke procese v organizmu ali pa služijo kot kazalci odziva organizma ali določenega organa na določeno (toksično) spojino. Ena izmed spojin, toksičnih predvsem za jetra je ogljikov tetraklorid (CCl_4). CCl_4 se uporablja kot napovedovalec toksičnih učinkov jeter, saj se v njih pod vplivom citokroma P-450 (CYP) pretvori v reaktivne radikale, kar v nadaljnji verižni reakciji privede do lipidne peroksidacije z vezavo na nenasičene maščobne kisline ali pa pride do kovalentne vezave reaktivnega radikala na biomolekule. Oba načina prispevata k izgubi integritete strukture in slabšega delovanja membrane hepatocitov, kar vodi v poškodbe jeter.

V okviru diplomske naloge smo želeli ugotoviti, kako 2-DGE skupaj z pripravo vzorca vpliva na končno resolucijo gela in na izostritev proteinov na njem. Pri izvedbi 2-DGE smo najprej vzorec ustrezno pripravili, nato ločili proteine z elektroforezo v dveh

dimenzijah ter na koncu barvali gel z ustreznim barvilom. Za izvedbo 2-DGE smo uporabili serumske vzorce podgan ter na podlagi končne slike ugotavljali prisotnost spremenjenih proteinov na gelih v kontrolni in testirani skupini.

V diplomski nalogi, smo kot testne živali uporabili podgane, katerim smo v enkratnem odmerku peroralno aplicirali CCl_4 ter opazovali njihov odziv. 30 podgan smo razdelili v 3 skupine (10 v vsaki) pri čemer je ena skupina služila kot kontrolna skupina, ostalima dvema skupinama pa smo aplicirali CCl_4 v dveh različnih koncentracijah (1,2 ml/kg oz. 2,0 ml CCl_4/kg telesne teže). Podgane smo stehtali v dveh časovnih točkah po aplikaciji CCl_4 – po 6 urah ter po 24 urah, da ugotovimo morebitne spremembe teže podgan. 24 ur po aplikaciji CCl_4 smo opravili obdukcijo. Pri tem smo odvzeli vzorce krvi ter odstranili jetra in jih stehtali. Ker smo želeli ugotoviti, kakšen vpliv ima CCl_4 na težo podgan in na težo njihovih jeter, smo rezultate tehtanj analizirali s SPSS metodo. Pri tem smo ugotovili, da aplikacija CCl_4 ni značilno vplivala na celokupno težo podgan. Pri vseh podganah (kontroli skupini in testiranima skupinama) smo ugotovili padec teže, ki pa je bil pri kontrolni skupini manjši (-14,4 g), kot v testnih skupinah (-15,0 g; -15,7 g). Pri analizi rezultatov absolutne teže jeter podgan pa smo ugotovili, da obstaja značilna razlika med kontrolno ter testirano skupino. Iz prejšnjih študij smo povezali, da naj bi šlo za kopičenje maščobnih kapljic v hepatocitih, s čimer se poveča teža jeter podgan. Za potrditev rezultatov opravljenih s SPSS metodo, bi morali opraviti še druge histopatološke ter klinične analize.

Priprava vzorca je ključnega pomena pri uspešni izvedbi 2-DGE. Neobdelan vzorec seruma vsebuje visoko koncentracijo albumina, približno 60 %, ki ga je potrebno odstraniti iz seruma za boljšo vidljivost ostalih proteinov v manjših koncentracijah. Pri tem smo uporabili metodo, ki temelji na obarjanju albumina v prisotnosti NaCl in EtOH (t.i. depletion of albumin component (DAC) protocol). Da preverimo učinkovitost metode, smo enemu vzorcu odstranili albumin, medtem ko smo drugega pustili nespremenjenega. Naredili smo NaDS elektroforezo ter jih barvali z barvilom Coomassie modrim in sliki gelov primerjali. Ugotovili smo, da ostranitev albumina pripomore, k boljši vidnosti ostalih proteinov na gelu. Pri neobdelanem vzorcu smo prav tako opazili številne navpične črte, ki nakazujejo na neuspešno ločbo proteinov. Iz tega smo sklepali, da je bila metoda učinkovita. Poleg albumina lahko izoelektrično fokusiranje v prvi dimenziji motijo tudi druge spojine (npr. soli, proteolitični encimi, polisaharidi, nukleinske kisline), ki jih je

potrebno odstraniti iz seruma. Pri tem smo uporabili očiščevalni komercialno dostopen set ReadyPrep™, ki je osnovan na obarjanju v kombinaciji trikloroocetene kisline in acetona. Ta set pripomore k boljši ločbi proteinov pri izoelektričnem fokusiranju. Enemu vzorcu smo odstranili samo albumin z zgoraj omenjeno metodo, medtem ko smo pri drugem uporabili tudi očiščevalni set ter opravili 2-DGE. Pri tem smo ugotovili, da je postopek bistveno izboljšal izostritev proteinov na gelu, kar bi pripomoglo k lažji identifikaciji proteinov. Hkrati smo opazili, da uporaba očiščevalnega seta vpliva tudi na proteine manjših koncentracij, saj po uporabi seta nekaj proteinov na gelu ni več prisotnih.

V nadaljevanju smo želeli doseči optimalne pogoje za dobro ločbo proteinov z 2-DGE, pri čemer smo uporabili različne volumne vzorca (2,5 µl in 7,5 µl) ter različne trakove dolžine 7 cm z imobiliziranim pH gradientom (4-7 in 5-6), potrebne za ločbo proteinov v prvi dimenziji. Pred samo ločbo smo iz vzorcev odstranili albumin ter druge moteče snovi z zgoraj opisanimi metodami, da bi dosegli optimalne pogoje analize. Pri tem smo uporabili en serumski vzorec podgane iz kontrolne skupine in enega iz testirane skupine, ju analizirali, barvali s srebrom ter ju primerjali. Prejšnje analize so pokazale podobno prisotnost proteinov na gelih obeh testiranih skupin (1,2 in 2,0 ml CCl_4/kg), zato smo v nadaljevanju za lažjo izvedbo in primerjavo uporabili le serumske vzorce podgan z nižjo koncentracijo CCl_4. Po ločbi proteinov z 2-DGE smo ugotovili, da se nekateri proteini na gelu po aplikaciji CCl_4 podganam zmanjšajo, nekateri pa popolnoma izginejo v primerjavi s proteini seruma iz kontrolne skupine podgan. Te proteine bi lahko v nadaljevanju analizirali z uporabo masne spektrometrije in bi služili kot biološki označevalci jetrnih poškodb.

Uporaba trakov z imobiliziranim pH gradientom pripomore k boljši ločbi proteinov ter večji ponovljivosti rezultatov. Primerjava gelov, kjer smo uporabili različne pH gradiente je pokazala, da se pri obeh analizah proteini ločijo, vendar so pri uporabi ožjega pH gradienta proteini bolj izostreni. Prav tako smo uporabili različne volumne vzorca, da potrdimo, da se koncentracija proteinov na gelu spreminja z različnimi volumni, predvsem pa kakšen volumen je primeren za uspešno ločbo proteinov. Analiza na 1 µl ni pokazala želenih rezultatov, zato smo opravili podobno analizo še z dvema volumnoma vzorca - 2,5 µl in 7,5 µl. Pri tem smo ugotovili, da se na gelu pri nižjem volumnu pojavijo številni proteini, ki pa so na določenih mestih slabo vidni. V primerjavi z uporabo višjega volumna pa postanejo ti proteini bolj opazni.

LIST OF ABBREVIATIONS

CCl_4	Carbon tetrachloride
CYP	Cytochrome P450
2-DGE	Two dimensional gel electrophoresis
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
SDH	Sorbitol dehydrogenase
GLDH	Glutamamte dehydrogenase
GGT	γ-glutamyl transferase
ALP	Alkaline phosphatase
$\bullet CCl_3$	Trichloromethyl radical
$\bullet CCl_3O_2$	Trichloromethyl peroxy radical
IEF	Isoelectric focusing
pI	Isoelectric point
CHAPS	3-(3 cholamidopropyl)dymethylammonio)-1-propanesulfonate
IPG	Immobilized pH gradient
DTT	Dithiothreitol
TFA	Trifluoroacetic acid
PBS	Phosphate buffered saline

Rpm	Revolutions per minute
TEMED	N, N, N’, N’–tetramethylethylenediamine
Tris-HCl	2-Amino-2-hydroxymethyl-propane-1,3-diol*HCl
DAC	Depletion of albumin component

INTRODUCTION

Liver

Anatomy

The liver is the largest organ in the body of all mammals. Its location is in the right upper quadrant of the abdomen on anterior side just below the diaphragm and it's protected by the arc of the rib cage. It is situated close by other organs such as right kidney, the stomach and other parts of gastrointestinal tract (1, 2). The normal adult liver weights from 1400 to 1600 g, representing 2.5 % of total body weight (3).
The colour of the liver is dark red due to the large volume of blood flowing through it. It has a dual blood supply: one third enters the liver through the hepatic artery as arterial blood and venous supply that passes from the gut to the liver through the hepatic portal vein providing two thirds of the blood supply (2).

Structure

The functional unit of an organ may be defined as the smallest, structurally distinct unit that is able to independently command all known functions of the organ (4). From previous research it was discovered that microscopic anatomy of the liver can be described from morphological and physiological point of view (2, 5, 6). Each way explains part of the processes in the liver, but none of them can explain them entirely. (4).

The classic lobule is a morphological unit of the liver described as hexagonal structure with portal triadas at the corners of the hexagon and a hepatic (central) vein at the centre. The portal triada or portal tract is defined by portal vein, hepatic artery and bile duct (4). Within classic lobule there are cords of liver cells - hepatocytes which extend from central vein to the periphery of the lobule, where they continuous with the hepatocytes of other lobules (7). Hepatocytes are the most numerous cells in the liver encompassing 80% of the volume of the liver (8).

Hepatocytes are the most richly perfused cells in the body because of mixed portal venous and hepatic arterial blood flowing from portal triadas towards the central vein through spaces called sinusoids.(3) The sinusoids are lined by endothelial cells and Kupffer cells (2). Between adjacent endothelial cells there are holes called fenestrae which allow free

access of plasma from sinusoids to space of Disse and, subsequently, to plasma membrane of hepatocytes (1, 2).

Between the liver cells are also lying the bile canaliculi which transport bile to the bile duct system (3). Bile flows in an opposite direction of the blood flow (7).

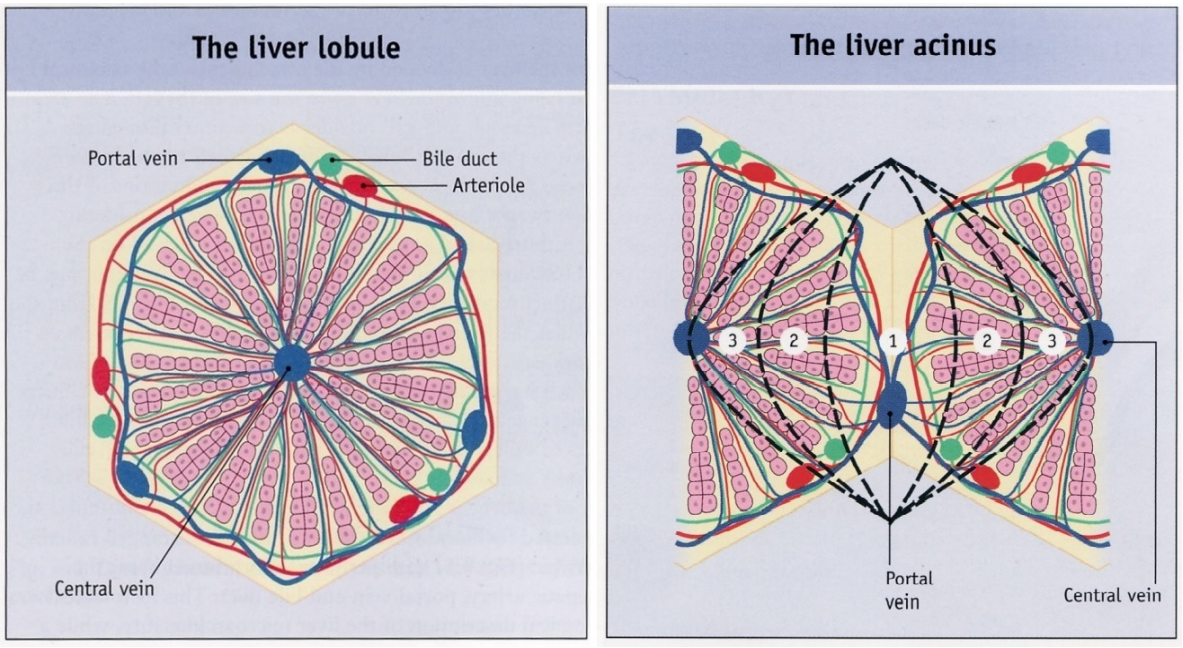


Figure 1: Liver lobule and liver acinus as fundamental units of the liver.

The liver lobule represents morphological unit of liver (left) and liver acinus is characterized as functional unit of liver (right). Liver lobule is according to histological appearance seen as hexagon, where venous and arterial blood flows from periphery to the centre of lobule where central vein is located. From physiological point of view, live acinus is characterized as area divided into 3 zones, where zone 1 is the closest to blood supplies and portal triada, with highest concentration of oxygen, zone 2 being intermediated and zone 3, most distant from afferent blood supply and with high concentration of enzymes involved in drug biotransformation. Figure adapted from (**9**).

The classic lobule described above is defined as an area surrounding the central vein because of the histologic appearance of the liver. However, from a functional point of view, the liver lobule can be thought of as an acinus with its centre in the portal triada (1). The simple acinus is divided into 3 zones: zone 1 being closest to the vascular supply and portal triada (periportal area), zone 3 including the central vein and most distant from the afferent blood supply (centrilobular area) and zone 2 being intermediate (mid-zonal area) (3). The zones of the acinus correspond to the following: zones of decreasing oxygenation, with oxygen concentration being highest in zone 1 and lowest in zone 3; distribution of

enzymes within liver cells where centrilobular area is the richest with cytochrome enzymes presenting the most common site of liver cell injury caused by toxin (7).

Physiology

The liver is structurally and functionally heterogeneous with multiple functions, most of which are vital for normal body metabolism (7, 8). Its function is synthesis of majority of proteins, and lipid metabolism compounds such as free fatty acids, cholesterol, phospholipids, and triglycerides. It is also the main site of amino acids metabolism, urea production and storage of some compounds such as glycogen (7).

Metabolism

In humans and other higher organisms the liver is the principal site for the metabolism of foreign substances because of its anatomical position between the gastro-intestinal tract and the systematic circulation (10, 11). It is responsible for metabolism/ detoxification of chemical substances from outside the organism i.e. xenobiotics or within the organism (10). It is not surprising, that the liver is also one of the major sites of toxicity, particularly from substances that enter the body through the gastrointestinal tract (2).

The major function of this metabolism is the conversion of lipophilic, water-insoluble, substances to water-soluble conjugates prior to the excretion. In phase I metabolism, compounds are modified with one of the following reaction – oxidation, reduction or hydroxylation while in phase II, called conjugation, a function group such as gluthatione, sulphate or addition of acetyl group are added to metabolites to facilitate their excretion from the body (2).

Most of the reactions in phase I are catalysed by the cytochrome P-450 (CYP) enzymes in mono-oxygenation reactions (2). CYP is known as membrane-bound haem proteins, bounded either on microsomal membrane or to the mitochondrial inner membrane. The highest expression of CYP enzymes is in the liver, mostly in the centrilobular part, but they are also expressed at a lower level in kidney, lung, intestine, brain and placenta (12).

Cytochrome P-450 proteins are arranged into families and subfamilies on the basis of percentage amino acid sequence identity. The CYP isoenzymes in families 1–3 are responsible for 70–80 % of metabolism of clinically used drugs and numerous of

xenobiotic chemicals (13). The majority of drug and xenobiotic metabolism is catalysed by the following six P450 isoenzymes: CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (14).

The levels and activity of each CYP-450 differ from one individual to the next due to environmental and/or genetic factors. It can be influenced by disease, medications, ageing, dietary or because of social habits such are alcohol consumption and cigarette smoking (14-16).

Liver injury

The phrase “liver injury” can be used in the broadest sense, and indicates any structural or functional liver injury resulting, directly or indirectly, from internal or external factors or their combinations. Liver injury can be induced by a number of factors including exposure to hepatotoxic compounds and radioactive materials, mechanical liver injuries, genetic predisposition, viral infections, ischemia, alcohol and drug abuse, autoimmune disease, or as a result of elevated in vivo levels of proteins (17).

The liver has a relatively limited repertoire of cellular and tissue responses to injurious events. Together with its injury and manifestations tend to follow characteristic morphologic and clinical patterns, regardless of cause (3, 18). Damage may result in liver regeneration, necrosis, degeneration and intracellular accumulation, inflammation, fibrosis or mixture of these processes, depending on the type and extent of injury and its location within the liver (17).

Liver injury encountered in clinical practice is divided into acute and chronic, based on the duration or persistence (19). Acute liver injury is generally a transient, self-limiting disease, regardless of its aetiology (20). The site of injury is according to the area between portal triada and central vein described as periportal, mid-zonal and centrilobular. Centrilobular injuries are by far the most common injuries, for two reasons: the predominant distribution of the CYP enzymes and relative anoxia (2). However, most of the acute liver injuries are caused due to exposure to potentially hepatotoxic agents (e.g. acetaminophen, carbon tetrachloride, alcohol). These injuries may result in liver steatosis or cell death (necrosis) depending on toxic agent the body was exposed to. In some

patients, the exposure to hepatotoxin might proceeds to acute liver failure, a severe condition with a high mortality rate (20).

Chronic liver injuries usually appear over some period of time being exposed to toxic compounds. The morphological manifestation of chronic injury in the liver is the result of two interlinked mechanisms: regeneration, where damaged cells are replaced and fibrosis, resulting in scarring of a tissue. Combination of these two events leads to the condition described as cirrhosis (2).

Fatty liver – liver steatosis

Fatty liver or hepatic steatosis is defined as an excessive accumulation of fat droplets in hepatocytes (21). In macroscopic appearance, the fatty liver are seen after chronical exposure, as a large sort organ that is yellow, greasy, and readily fractured (22).

Among the worldwide population, the prevalence of steatosis is very high, which is associated with several factors such as increased alcohol consumption, overweight and hyperlipidemia, leading to diabetes type II, and with administration of some drugs (23).

The liver has an important role in lipid metabolism, and triglyceride synthesis occurs particularly in zone 3 (24). The lipid accumulates within the hepatocyte is usually triglyceride, the result of imbalances in the uptake and synthesis of fatty acids, increased production of triglyceride and decreased release of triglyceride into the bloodstream (9). Fatty liver is as well a common response to toxicity, often the result of interference with protein synthesis, and may be response after exposure to drugs such as hydrazine, ethionine, and tetracycline and in combination with necrosis also response to carbon tetrachloride. It is normally a reversible process, which usually does not lead to cell death. However, repeated exposure to compounds, which cause fatty liver, may lead to fibrosis and cirrhosis (24).

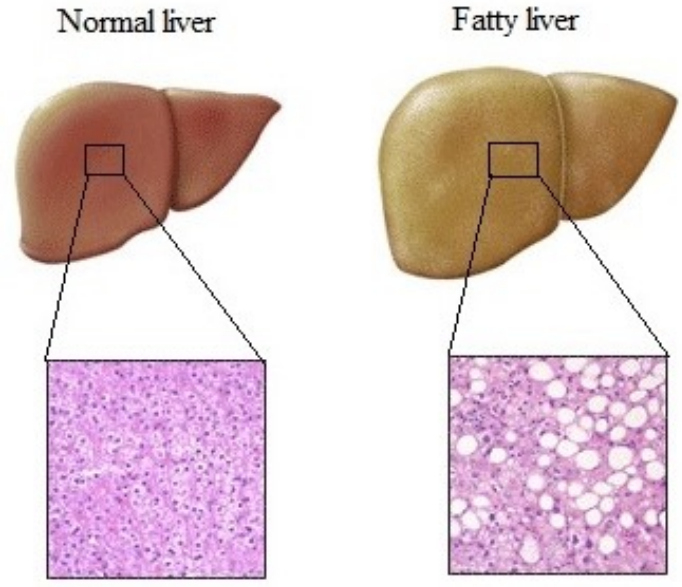


Figure 2: Comparison of normal and fatty liver.

Macroscopic appearance of normal and fatty liver and their histologic sample. Fatty liver are comparing to normal enlarged and in pale yellow due to accumulation of fat droplets within hepatocytes. The changes are in histological sample marked with arrows. Figure adapted from (25).

Steatosis is divided into 2 groups regarding on size of fat droplets. In microvesicular steatosis, in condition such as alcoholic fatty liver disease, multiple tiny droplets are present that do not displace the nucleus. In second one, a single large droplet that displaces the nucleus called macrovesicular steatosis which may be seen in the alcoholic liver or in the livers of obese or diabetic individuals (22).

Carbon tetrachloride

Despite the fact, that the use of many halogenated alkanes such as carbon tetrachloride (CCl_4), chloroform ($CHCl_3$) or iodoform (CHI_3) causes toxicity, CCl_4 is still used as a model substance to explain the mechanisms of action of hepatotoxic effects (26). A single dose of CCl_4 when administered to experimental animals produces centrilobular necrosis and fatty degeneration of the liver (fatty liver), and chronic low dose exposure is associated with liver fibrosis, cirrhosis and in final stages carcinoma (27). Also, the hepatic damage induced by CCl_4 causes significant increasing in clinical parameters alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (28).

The metabolism of CCl_4 begins with metabolic activation in the liver by CYP2E1 to the formation of the trichloromethyl free radical $\bullet CCl_3$ (26, 27). Besides CYP2E1, CYP2B1

and CYP2B2 are also capable of transforming CCl_4 (26). The reactive metabolite $\bullet CCl_3$ is capable of covalent binding to biologically important substances such as amino acids, lipids or proteins; or by abstracting a hydrogen from unsaturated fatty acids, to form chloroform (26, 27). This might results in destruction of cell membranes and also induces liver damage (26, 29).

In the presence of oxygen $\bullet CCl_3$ quickly reacts by formation of highly reactive trichloromethyl peroxy radical $\bullet CCl_3O_2$. In process of lipid peroxidation, a reactive oxidizing species $\bullet CCl_3O_2$ interacts with unsaturated fatty acids which initiates chain reactions terminating in disintegration of unsaturated fatty acids and formation of aldehyde and alkanes (26, 30). Consequently, this can result in increased membrane permeability leading to damaging the structure and compromising function of hepatic cell membrane (31).

Which of these radical-induced events plays the leading role in liver cell injury is controversial. However, at some point, both processes may join forces to execute the final result of cell death (26).

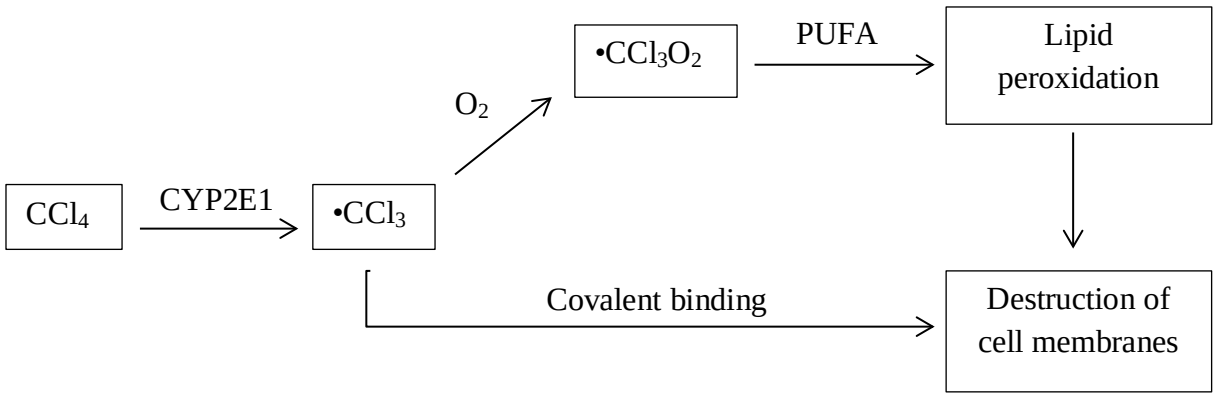


Figure 3: Biotransformation of CCl_4 in the liver.

In the liver CCl_4 is metabolized in the presence of isoenzyme CYP2E1 to reactive metabolite $\bullet CCl_3$ which in two initial pathways induce liver damage. In the presence of oxygen $\bullet CCl_3$ can react with it and form $\bullet CCl_3O_2$ which interacts with unsaturated fatty acids. This enables the peroxidation of the membrane lipids and causes increased membrane permeability. In second pathway, covalent binding of $\bullet CCl_3$ to hepatic microsomal lipids and proteins damage the integrity of the structure and function of hepatic cell membranes (31).* Abbreviations: CCl_4 - Carbon tetrachloride, $\bullet CCl_3$ - Trichloromethyl free radical, $\bullet CCl_3O_2$ - Trichloromethyl peroxy radical, PUFA - Polyunsaturated fatty acids.

Serum biomarker

Broadly speaking, a biomarker or biological marker can be any biomolecule or a specific characteristic, feature, and indicator of a change in any biological structure and function that is able to objectively measure the state of a living organism (32). More specifically, it serves as an indicator of normal biological processes, pathogenic processes, and pharmacological responses to a therapeutic intervention or as an adverse response to toxicants (33, 34). Ideal attributes of biomarker include organ specificity, strong correlation with well-defined hepatic histomorphologic changes and indication at early stages of an injury or disease. It should be also quantitative, sensitive, and easily measureable, sample should be accessible by non-invasive procedures and in the context of clinical transformation, all preclinical studies performed on experimental animals, should be successfully applied on humans (35, 36).

Currently, biomarkers are used in diagnostic tests in clinic, to evaluate the severity of a disease (37). A biomarker test is generally less expensive and invasive than other forms of testing (e.g. biopsy, ultrasound) facilitating a more efficient use of medical resources while improving health care (38).

Serum is derived from plasma with clotting factors removed and contains 60-80 mg of protein/ml. The major protein constituents of serum include albumin, immunoglobulins, transferrin, haptoglobin, and lipoproteins. There are also many other proteins, synthesized in liver and secreted through the body, present in serum at low abundance (39). Therefore, serum represents a wide spectrum of proteins, containing lots of information, which could serve for early detection of diseases (40). This makes it the most informative and accessible biofluids of the body for biomarker development in disease and toxicity (34)

Clinical parameters for liver injury

Most of liver injuries can be defined with measuring of basic clinical parameters corroborated with histopathological examinations. Basic clinical parameters, measured in blood, can be divided into 3 groups (41). First one represents hepatocyte’s leakage enzymes which are following hepatocellular injury released from cells into the blood. In systemic circulation levels of enzymes are measured and compared with those found in healthy individuals. The most important to assess the nature and degree of hepatic injury

are the following: alanine aminotransferase (ALT), aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH) and glutamate dehydrogenase (GLDH) (4, 36). However, in clinical practice, AST and ALT and their AST/ALT ratio are currently widely applied while SDH and GLDH are not commonly measured due to a lack of suitable standardized reagents (41).

Second group comprises parameters such as γ-glutamyl transferase (GGT), alkaline phosphatase (ALP) 5'-nucleotidase (5'-NT) and total bilirubin which belong to cholestatic-induction parameters. These are additional conventional biomarkers of liver function, applied to assess the function of biliary system (36). They show minimal activity in normal hepatic tissue and are elevated in the blood following impaired bile flow (cholestasis) or by treatment with glucocorticoids and corticosteroids (41).

The third group represents hepatic function tests, which assess the ability of the liver to synthetize variety of compounds and to test ability of metabolic processing and excretion function (41). Testing syntetic capacity parameters such as albumin, glucose, ammonia and urea nitrogen are included. Alterations in these parameters in the blood usually require significant loss of liver function since liver has tremendous reserve capacity for synthesis of these analytes. Measurements of total bilirubin and bile acids are applied to estimate metabolic and excretion function of the liver (41).

Table 1: Basic clinical parameters to evaluate liver and biliary injuries.

Membrane leakage enzymes	**Cholestatic-induction parameters**	**Liver function tests**	
		Synthetic capacity parameters	Metabolic and excretion parameters
ALT AST SDH GLDH	GGT ALP 5’-NT Total bilirubin	Albumin Glucose Coagulation factors Ammonia Urea nitrogen	Bile acids Bilirubin Triglycerides

ALT - alanine aminotransferase, AST - aspartate aminotransferase, SDH - sorbitol dehydrogenase, GLDH -glutamate dehydrogenase, GGT - γ-glutamyl transferase (GGT), ALP - alkaline phosphatase, 5'-NT - 5'-nucleotidase.

However, many of these clinical parameters are not organ specific and it is often necessary to measure a combination of them. Furthermore, in humans, in the case of some liver diseases, serum clinical parameters analysis may not detect any changes until the later stages of disease development (42). This leads to discovery of new biomarkers which will perform results that could be translated from preclinical to clinical studies.

Proteomics

The term “proteome”, composed from protein and genome, represents all proteins expressed in any biological organizational unit at a given time (43). As the cell and other higher levels of biological organization proceed through its cycle and responds to changing of the environment, the proteome changes with them (44).

Proteomics is the study of the proteome which purpose is to analysis gene expressions, via a combination of techniques for resolving and identifying proteins. Essential part of proteomic is also bioinformatics used for storing genomic and proteomic data and interlinking them. Although each technique can be applied independently, their impact can be increased when used associated for the study of complex of proteins (45, 46).

There is high interest in applying proteomics to identify new disease biomarkers. To define them comparative analysis of protein expression in healthy and disease individual needs to be performed. Proteomic approaches are also used to analysis secreted proteins in cell lines and to establish direct serum protein profiling (47).

The proteomics has impact also in drug discovery. The goal would be to identify measurable values of biomarkers in readily accessible body fluids, such as serum or urin, that could be used as surrogate markers and could help to predict the response of individuals and allow monitoring the therapy to achieve optimal efficacy (48).

Sample preparation

Effective sample preparation is absolutely essential for good 2-D figure to avoid problems with detection of proteins on gel. In ideal circumstances proteins should be completely soluble, disaggregated, denaturized and reduced for further identification (49). To achieve that, the procedure should be simplified to increase reproducibility, there should be minimum of protein modification during preparation of samples and all proteolytic

enzymes in the sample must be inactivated. So far, there is no single method of sample preparation that can be applied to all kinds of samples analysed by 2-DGE (50).

One of the main limitations of 2-DGE is its incapability to visualize and identify proteins present in low concentrations due to masking and precipitation of highly abundant proteins. One of the most abundant proteins in serum is albumin, presenting 60% of all serum proteins. Removing albumin from serum would improve resolution of final figure (51). On market, there are several techniques used for removing albumin from serum which rely on affinity-based methods using dyes or antibodies or on salt precipitation (52). Protein precipitation, followed by resuspension in sample solution, is widely used method for albumin depletion in serum sample. Precipitation is as well employed to selectively improve final figure of proteins in the sample from contaminated matter such as salts, detergents, nucleic acids, lipids, that would otherwise interfere with the 2-D gels.

Proteomic techniques

Proteomic techniques will separate and characterize proteins in biological samples in an attempt to acquire information about protein abundance, its location and modifications (53).

One-dimensional gel electrophoresis

By definition, electrophoresis consists of the separation of proteins as well as ions that are driven differently under an electric field (54).

One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is electrophoretic method allowing separation of proteins on the basis of their molecular weight. The use of anionic detergent SDS has two purposes in separation - it effectively solubilizes membrane proteins and coats the solubilized proteins such that the same negative charge is conferred across all proteins in the sample. This enables the separation of proteins only on molecular weight (55).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DGE) is a powerful and widely used method where proteins are separated regarding on 2 independent characteristics: in the first-dimension step, proteins are separated according to their isoelectric point. This process is

called isoelectric focusing. In second-dimension step, which is discreted from isoelectric focusing, proteins are separated according to their molecular weight with use of anionic detergent SDS (49). Isoelectric focusing as first step and SDS electrophoresis as second step of separation is used because of economical (second step which is larger, is better to be cheaper) and technical reasons (easier to stain SDS electrophoresis gels than isoelectric ones) (56).

On the final figure of 2-D gel, each spot corresponds to a protein in the sample (49). The aim of most 2-D gel applications is, however, to resolve as many proteins as possible within a particular pI/Mr range to facilitate comparison of two sets of data samples (57).

Immobilized pH gradient (IPG)

One of the improvements in 2-D electrophoresis was introduction of immobilized pH gradients or IPGs in isoelectric focusing (58). IPG strips are made with buffering acrylamide derivates with free amino group or carboxylic acid, which is copolymerized with acrylamide (59). These form a series of buffers with different pK values between pK 1 and 13 which indicated different pH ranges in strip: wide pH range (e.g. IPG 3-12), medium (e.g. IPG 4-7), narrow (e.g. IPG 4.5-5.5) and ultra-narrow (e.g. IPG 4.9-5.3). Besides different pH ranges, there are also strips of different lengths, usually from 7-24 cm. Copolymerization of reactive ends with acrylamide matrix allows truly steady-state isoelectric focusing with increased reproducibility. Other advantages of IPGs are increased resolution by the ability to generate narrow pH gradients, reproducible separation of alkaline proteins and increased loading capacity (50).

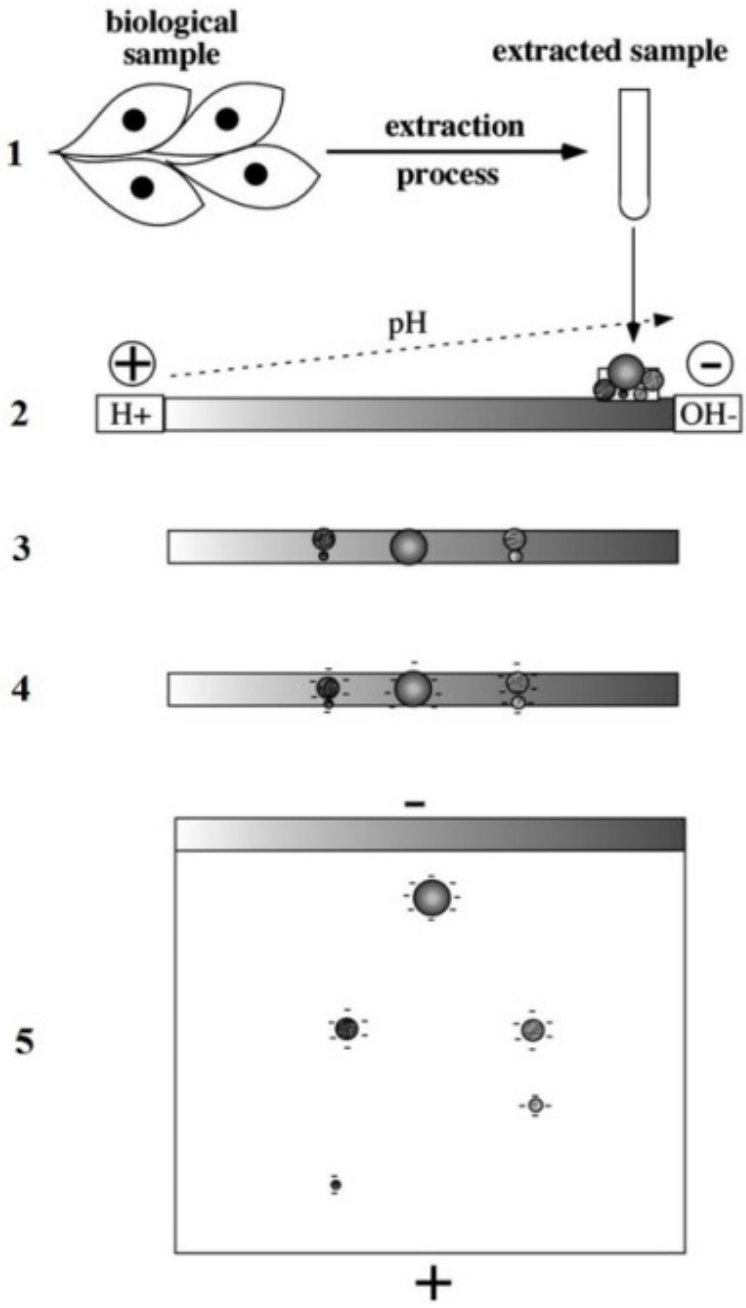


Figure 4: Scheme of 2-dimensional gel electrophoresis together with sample preparation

In first step sample needs to be extracted and treated with kits which will improve resolution of spots on the gel (1). A gradient of pH is applied to a gel and an electric potential is applied across the gel to start IEF (2). At the end of the IEF separation, proteins reached their pI, where their net charge is neutral (3). Addition of SDS is applied which cause that all proteins on gel become negatively charged (4). IEF gel is placed on the SDS-PAGE gel to separate proteins by their Mw. At the end of second dimension each protein has its own pI and Mw which enable their identification (5). Figure adapted from (56).

Staining methods

After performing 2-D electrophoresis, the separated proteins have to be visualized, either by universal or by specific staining methods. This step is essential in acquiring proteins, because only what will be detected on visualized gels can be further analysed with MS. As well, quantitative variations observed at this stage are the basis to select the few spots of interest (56). The most important qualities of visualization dyes are low detection limit, high linear dynamic range required for quantitative accuracy, reproducibility of result, and compatibility with procedures used for protein identification such as mass spectrometry (50). The visualization can be achieved by protein staining either with Coomassie Blue, silver or with fluorescent dyes.

Coomassie Blue dye is a reference standard for detection of 2-DGE proteins among all staining methods. It is widely used due to lower price, simplicity of use and compatibility with further identification methods. One draw-back is their inadequate sensitivity, which does not perceive proteins present in lower concentrations (50). On the other hand, silver staining is much more sensitive but less linear and homogeneous, because of its delicate mechanism, and its compatibility with mass spectrometry is problematic (56).

After staining the gels are scanned and the protein spots are quantified according their spot intensities (60).

AIM OF THE STUDY

Discovery of novel hepatic biomarkers will help to predict hepatic changes and serve as a diagnostics tool to detect early stages of liver diseases. To deliberately provoke liver injury, hepatotoxin will be applied to cause changes in liver. As experimental subjects, male Hanover–Wistar rats will be used due to similar physiology with human population. First aim of the thesis will be to discover how oral administration of hepatotoxin carbon tetrachloride (CCl_4) to rats influences on their total body weight. Rats will be treated with CCl_4 in two different concentrations and compared to control group of animals. As well, livers will be removed and weighted to determine correlation between application of CCl_4 to rats and their liver weights.

At autopsy serum samples from all groups of animals will be taken in an attempt to analysis serum for any alterations in protein expression. Proteins will be analysed with two-dimensional gel electrophoresis (2-DGE) technique used for protein investigation. However, to achieve adequate resolution of final figure – gel all steps of two-dimensional gel electrophoresis need to be optimized which will make well-focused proteins appropriate for further identification as potential hepatic biomarkers.

First step of 2-DGE is serum sample preparation which is essential for good focusing of proteins on gel. In second aim it will be examined if sample preparation methods - DAC protocol and ReadyPrep 2-D Cleanup kit, can effectively remove interfering compounds from serum and improve focusing of proteins on gel.

In third part of thesis, on serum samples different IPG strips in first dimension of 2-DGE will be applied and different loading volumes of serum will be used to determine which will give better focusing of proteins. During performing 2-DGE under variable conditions also comparison of control and treated serum samples will be presented. Any differences in pattern of expression of proteins between control and treated animals will be considered as potential biomarkers of liver injury caused by CCl_4.

MATERIALS

Rats were supplied by Harlan Laboratories Inc, UK; the metabolism cages were supplied by Techniplast, Kettering, Northants, UK. The animal diet came from SDS Ltd, Wiltham, Essex, UK. Carbon tetrachloride was supplied by Fluka Chemicals, New Road, Gillingham, Dorset, UK. Serum separator tubes came from Beckton and Dickinson and Co., Franklin Lakes, NJ, USA.

Euthatal (pentobarbital sodium) was supplied by Rhone Merieux Animal Health Ltd, Harlow, Essex, UK. Qiagen, Boundary Court, Gatwick Road, Crawley, West Sussex, UK provided the RNA Later tubes. Multistix® 10 SG reagent strips were supplied by Bayer plc, Newbury, Berks, UK.

Electrophoresis reagents, isolectric focussing equipment, 3-(3 cholamidopropyl)dymethylammonio)-1-propanesulfonate (CHAPS), electrode wicks and mineral oil were purchased from Bio-Rad laboratories, Hemel Hempstead, Hertfordshire, UK. IPG (immobilized pH gradient) strips and IPG buffer were supplied by GE Healthcare, Buckinghamsire, UK.

TEMED, Protogel and Coomassie Blue R-250 were purchased from National Diagnostics, Itlings Lane, Hessle, Hull, UK. Acros Organics, Greel, Belgium, supplied silver nitrate and citric acid monohydrate 99.5%. Dithiothreitol (DTT) came from Melford Laboratories, Ipswich, Suffolk, UK. Methanol, ethanol 99.7 – 100% (v/v) and acetic acid were purchased from Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, Leicestershire, UK. Citric acid and trifluoroacetic acid (TFA) came from Avocado Research Chemicals Ltd, Heysham, Lanes, UK.

Iodoacetamide, Tween 20, copper/zinc superoxide dismutase, ammonium persulphate, urea, agarose, sodium lauryl sulphate, formaldehyde, phosphate buffered saline (PBS) and Brilliant Blue R were supplied by Sigma-Aldrich Co. Ltd, Gillingham, Dorset, UK.

METHODS

Experimental animals

Thirty Male Hanover – Wistar rats (Harlan Laboratories, UK) of 160-180 g were used throughout and caged in groups of 10 (Control group, Middle dose group of 1.2 ml of CCl_4/kg and High dose group of 2.0 ml of CCl_4/kg). All animals were labelled with pencil not to be mixed between each other. They were allowed to acclimatize for 4 days in communal cages before each experiment and were weighted and observed daily for signs of ill health before dosing and during the post-dosing period. Animals were bedded on wood shavings, with access to diet (Rat and Mouse No. 1 Maintenance, SDS, Witham, Essex, UK) and water *ad libitum*. A temperature of 19–22 °C was maintained with relative humidity of 45–65 % and a light–dark cycle every 12 hours (lights goes on at 7 am).

Administration of Carbon tetrachloride (CCl_4)

Carbon tetrachloride was dissolved in corn oil and administrated by gavage. The concentration of CCl_4 was adjusted according to the weight of the animals, such that a volume of 2 ml was administrated in each case. Control animals were dosed with corn oil alone. After dosing all animals were observed for signs of blood on the catheter.

After dosing period

After dosing, animals were individually placed into the metabolism cages to collect their urine overnight. In the metabolism cages, the animals have access to water *ad libitum*, but no diet to ensure that urine samples stay pure, with no dietary material. The water bottles were also weighted before and after placing animals in the metabolism cages, to estimate the water consumption. Urine was collected over the ice to avoid bacterial growth and stored at –80 °C.

Post mortem

Animals were killed via intraperitoneal injection of Euthatal (Sodium Pentobarbitone) and exsanguinated from the abdominal aorta and was placed into Microtainers (Becton Dickinson and Co. Rutherford, N.J., USA) for preparation of serum. After 4 hours at room temperature the Micotainers were centrifuged at 5000 rpm for 5 minutes and serum was

transferred to new eppendorf tubes and stored at -80 °C for further analysis. Both external and internal observations were made and recorded during the autopsies. Liver and kidneys was removed and weighted. Sections of liver lobes and both kidneys were placed in 10,5 % phosphate–buffered formalin. Also nasal epithelium was removed and stored in 10,5 % phosphate–buffered formalin for further analysis.

One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS gels were poured according to the method of Laemmli (61). Discontinuous gels were poured with a 12 % resolving gel and a 5 % stacking gel. After assembling the Bio-Rad casting equipment, 10 µL of N, N, N', N'–tetramethylethylenediamine (TEMED) and 100 µL of 10 % ammonium persulphate were added to the running gel solution (37.5 mM Tris–HCl, pH 8.8 at room temperature, 50 % protogel (30 % (w/v) acrylamide, 0.8 % (w/v) bis-Acrylamide), 0.1 % SDS). After gentle swirling the mixture was poured between two vertical glass plates to 5 cm in height. Water was poured to create a flat surface and to prevent oxygen reacting with the gel. Gels were allowed to polymerise at room temperature for at least 30 minutes after which water was poured off.

A 5 % stacking gel (12.5 mM Tris-HCl, pH 6.8 at room temperature, 1.7 % Protogel (30 % (w/v) Acrylamide, 0.8 % (w/v) Bis-Acrylamide), 0.1 % SDS), polymerized by the addition of 10 µL of N, N, N', N'–tetramethylethylenediamine (TEMED) and 100 µL of 10 % ammonium persulphate, was poured on top of the resolving gel and a comb inserted to create the sample wells. The stacking gel was allowed to polymerise for at least 30 minutes at room temperature after which the comb was carefully removed and the wells washed with reservoir buffer.

Prior to loading all samples were treated with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS, 0.05 mg/mL bromophenol blue, 1 M DTT) and heated to 80 ºC on the heat block for 5 minutes. Protein standards of known molecular weight were loaded in 1 lane of each gel and 5 µL of a blank solution containing SDS sample buffer was loaded into any unused well. Reservoir buffer was added to the upper and lower chambers of the gel electrophoresis apparatus and a constant voltage of 200 V was applied for one hour or until the dye front reached the bottom of the resolving gel. After running, the gels were removed from the plates and stained in Coomassie Blue stain and silver stain.

Sample preparation

Depletion of albumin content (DAC) protocol

Prior to one and two dimensional gel electrophoresis, albumin was depleted from serum samples using a modified version of an albumin depletion protocol (52). 1 M Sodium Chloride was added to total volume of 10 µl of serum sample and distilled water to yield a final concentration of 0.1 M and incubate at 4 °C for 60 minutes after which cold ethanol was added to a final concentration of 42 % and incubated for further 60 minutes at 4 °C. The sample was then centrifuge at 13.000 rpm for 45 minutes at 4 °C. The supernatant was discarded and the pellet resuspended in 100 µl of a solution of 7 M Urea, 2 M thiourea and 1 % CHAPS.

ReadyPrep 2-D Cleanup Kit Protocol

As a cleanup of samples prior to 2-DGE, was used ReadyPrep 2-D Cleanup kit from Bio-Rad. In the case of serum analysis the DAC protocol was carried out prior to the Cleanup kit. The structure of all used components in 2-D Cleanup kit is unknown. At the beginning 300 µl of precipitating agent 1 was added to the protein mixture, vortexed and left to incubate on ice for 15 minutes. 300 µl of precipitating agent 2 was added and vortexed before centrifuging at 13.000 rpm for 5 minutes at 4 °C to form a pellet. The supernatant was promptly discarded taking care not to disturb the pellet. 40 µl of wash reagent 1 was added to the top of the pellet and centrifuge at 13.000 rpm for 5 minutes at 4 °C and the supernatant discarded. 25 µl of distilled water was added and the tubes vortexed for 10–20 seconds, then 1 ml of wash reagent 2 (prechilled at -20 °C) and 5 µl of wash 2 additives were added. Tubes were vortexed for 1 minute and incubated at -20 °C for 30 minutes vortexing every 10 minutes during the incubation period. Finally, the tubes were centrifuged and the supernatant discarded. Pellets were allowed to air-dry for no more than 5 minutes before being resuspended in rehydration buffer.

Two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2-D SDS-PAGE)

Protein pellets from serum were re-suspended in rehydration buffer containing urea, non-ionic or zwitterionic detergents, carrier ampholytes and a reducing agent. Urea is the most commonly used chaotrope agent in sample preparation for 2-D PAGE and it will disrupt

hydrogen bonds. Detergents are also added to disrupt hydrophobic interactions and increase solubility of proteins, preventing protein aggregation. Detergents must be non-ionic or zwitterionic to allow proteins to migrate according to their net charge. Reducing agents such as dithiothreitol (DTT) are used to disrupt the disulfide bonds. Carrier ampholytes are a mixture of thousands of small ampholytes with different isoelectric points (pI). When an electric field is applied, the carrier ampholytes align themselves between the anode and the cathode according to their pI. In this way the ampholytes form a pH gradient during electrophoresis. Carrier ampholytes also enhance sample solubility and minimize protein aggregation.

Rehydration of IPG strips and sample loading

Protein pellets were resuspended in 125 µL of rehydration buffer (7 M urea, 2 M thiourea, 1 % CHAPS, 2 % IPG buffer pH 3-10, 20 mM DTT) and the sample pippeted along the whole length of the rehydration tray channel except for 1 cm at each end taking care not to introduce any air bubbles. The coversheet was peeled from the dried IPG strips using forceps, the IPG strip was then placed in tray with gel side facing down, on top of the sample making sure not to trap air bubbles between the strip and the sample. The IPG strips were 7 cm long. After one hour the strips were overlaid with 2-3 ml mineral oil to prevent evaporation of the sample. Passive rehydration of the IPG strips lasted 12–16 hours at 20 ºC overnight.

Isoelectric focussing (IEF)

The first dimension of 2-DGE is an isoelectric focussing (IEF) step. Proteins are separated on the basis of their pI, the pH at which a protein carries no net charge and therefore will not migrate in an electrical field. When a protein is placed in a medium with a pH gradient and subjected to an electrical field it will initially move toward the electrode with the opposite charge. Eventually the protein will arrive at the point in the pH gradient equal to its pI where it will be uncharged. At this point the protein will stop migrating and will become focussed into narrow zones. The first dimension is carried out using IPG strips which are made of acrylamide and have a broad or narrow pH range, depending of its use.

The isoelectric focussing step (IEF) was carried out in focussing trays that have an electrode at each end. Two paper wicks were wetted with 10 µL of de-ionised water and

were placed over the wire electrodes at each end of the IEF tray to prevent burning strips. The strips were removed from the rehydration tray, held vertically and the tips blotted onto a filter paper to remove as much mineral oil as possible. This step allows the removal of unabsorbed proteins and consequently reduces any horizontal streaking that could occur during the focussing step. The strips were then transferred to a channel in the focussing tray with the gel side facing down and were positioned so that the "+" sign on the strip was aligned with the "+" sign on the tray. Each IPG strip was covered with 2-3 ml of mineral oil. The lid was placed on the tray which was then transferred to the PROTEAN IEF cell. Isofocusing was carried out in 5 steps: step 1-150 volts for 1 hour (this is a low voltage step to help eliminate salts and other contaminants), step 2-1000 volts for 1 hour, step 3-2000 volts for 1 hour, step 4-3000 volts for 1 hour, step 5-4000 volts for 20,000 volt-hours. For all IPG strips the current did not exceed 50 µA/ strip and the temperature was set to 20 ºC. Following the focussing steps the strips were frozen at -80 ºC for further analysis.

Second dimension-SDS gel electrophoresis

The second-dimension is based on separation of proteins according to their molecular weight using SDS-PAGE. The IPG from the first dimension is applied to a SDS gel. The polyacrylamide matrix acts as a sieve separating proteins according to their molecular weight. Prior to SDS-PAGE the strips are equilibrated in a buffer solution containing SDS, DTT and iodoacetamide to ensure the proteins become linear and negatively charged.

Prior to running the second dimension the excess mineral oil was removed by placing the strips, gel face up, onto a dry piece of paper. Strips were then equilibrated in Buffer I (prepared immediately before use): 6 M urea, 2 % SDS, 0.375 M Tris-HCl pH 8.8, 20 % (v/v) glycerol, 130 mM DTT for approximately 30 minutes followed by 30 minutes in Buffer II (also prepared immediately before use): 6 M urea, 2 % SDS, 0.375 Tris-HCl pH 8.8, 20 % (v/v) glycerol, 135 mM iodoacetamide. Iodoacetamide alkylates residual DTT preventing streaking and re-oxidises proteins that were reduced by DTT.

For second dimension gels, there is not needed to prepare stacking gel for 2-D gels and therefore the running gel poured to within 1 cm of the top of the glass plates and was overlaid with water to create a flat surface. The gel was allowed to polymerise for at least 30 min and after that the water was poured off. The IPG strips were briefly dipped 3 times into reservoir buffer and then carefully inserted on top of the gel with gel side of the IPG

strip facing us. The strips were carefully pushed down so that there was perfect contact with the gel, ensuring no air bubbles were trapped beneath the strip. The strips were then overlaid with 1 % agarose in 0.5 M Tris-HCl pH 6.8, 0.05 mg/mL bromophenol blue. The agarose was allowed to solidify for 5 minutes and then electrophoresis was run at 100 V in cold room (4 °C) until the bromophenol blue dye front reached the bottom of the gel (~ 3 hours). After the second dimension the gels were stained in Coomassie Blue and silver.

Staining methods

Coomassie Blue staining

Gels were stained with Coomassie Blue (0.4 % Coomassie Blue R-250 in 50 % methanol and 10 % acetic acid) for at least 1 hour (usually overnight) after which they were destained in 30 % methanol and 10% acetic acid. Destaining solution was changed every 10-15 minutes for the first hour and then every 30 minutes until protein bands were visible on gel.

Silver staining

Gels were incubated in 50 % methanol solution for 10 minutes followed by 10 minute incubation in 5 % methanol solution. The next step was a reduction step consisting of 30 minute incubation in 4 % DTT. After 30 minutes the gels were rinsed with de-ionised water for 5 to 10 seconds to remove traces of DTT. Gels were then placed in a 0.2 % silver nitrate solution for 30 minutes and then briefly rinsed with de-ionised water for 10 seconds 3 times to remove silver residues. Gels were developed in 3 % sodium carbonate and 0.025 % formaldehyde until protein bands were visible. The developing process was stopped with 5 % acetic acid ad oculum until no more fizzing occurred.

Statistical analysis

Results of total body weights and absolute liver weights of experimental animals are expressed in each group as mean value ± SD, respectively. All statistical analyses were performed using IBM SPSS® Statistics version 20 for Windows. Comparisons among experimental groups were performed with one-way ANOVA followed by post-Hoc test. Differences were considered significant when the associated p value was <0.05 at comparison of body weights and <0.01 at comparison of liver weights.

RESULTS

Animal experimental design

Thirty male Hanover-Wistar rats were included in preliminary dose response study. Before the beginning of the study all animals were weighted (mean body weight 199.7 g ± 28.6 g) and were divided into 3 groups of 10 animals each and dosed with CCl_4 at 0 (controls), 1.2 ml/kg and 2.0 ml/kg. Rats were administered with vehicle or CCl_4 by gavage. Gavage is procedure where tube is introduced into the mouth and passed into the esophagus, where the test formulation is then administered (62). After administration they were returned to their communal cages (5 animals in each) for 6 hours. After 6 hours animals were placed individually in metabolism cages for 18 hours period where they had access to water but not diet. While animals were in metabolism cages urine samples were collected and afterwards stored at -80 ºC for further urine analysis. All animals were weighed before dosing, before being placed in metabolism cages and at 24 hours post-dosing at which time they were autopsied and a post-mortem carried out. During autopsy besides liver, right and left kidney and nasal cavity was as well removed, weighted and examined. Since the data were not relevant for my research they were not included in.

Rat body weight data

Table 2 shows the change in body weight over the 18 hour period while the rats were in metabolism cages for both control and CCl_4-treated animals. All animals lost weight over the 18 hour period – the greatest decrease was in the 2.0 ml/kg dose level group (-15.7 g), following the 1.2 ml/kg dose level group (-15.0 g) and the least decrease was in control group (-14.4 g).

Result were analysed with SPSS Statistics method, with analysis of variance (ANOVA). ANOVA was conducted to explore the impact of CCl_4 concentration on rat body weight. Animals were divided into three groups according to the concentration (0, 1.2 and 2.0 ml/kg of CCl_4). There was not statistically significant differences at the $p < 0.05$ level for the three groups ($p > 0.881$). Post-hoc comparison using the Tukey's HSD test indicated that the mean score for group treated with 2.0 and 1.2ml/kg were significantly different from control group.

Table 2: changes in mean (±SD) body weight for male Hanover-Wistar rats treated with vehicle (control) or carbon tetrachloride (CCl_4) at 1.2 and 2.0 ml/kg during an 18 hour period in metabolism cages[a]

Dose levels of CCl4 (ml/kg)	**Time post-dosing (h)**	**Mean (±SD) body weight (g)[b]**	**Change in mean body weight (g)[b]**
0 (control)	6	211.0 ± 7.1	-14.4
	24	196.6 ± 7.3	
1,2	6	231.1 ± 9.8	-15.0
	24	216.1 ± 6.6	
2,0	6	222.9 ± 10.1	-15.7
	24	207.2 ± 8.7	

[a]Animals (n=10) were dosed with vehicle or CCl_4 and placed individually in metabolism cages at 6 hours post-dosing when they were weighted. At 24 hours post-dosing animals were removed from the metabolism cages, weighed and autopsied.

[b]Body weight is expressed in grams.

Liver weights

At post-mortem all animals' liver was removed to determine their weights. As described above, they were divided in 3 groups and administrated vehicle or CCl_4 to estimate how much liver weight changes regarding to dose level.

Paragraph shows liver weights at each dose level. Weights at 1.2 and 2.0 ml/kg of CCl_4 were increased over controls, 9.3 g and 10.5 g, respectively. Furthermore, with increasing the dose level of CCl_4, greater the liver weight was. To confirm result SPSS analysis was used. ANOVA was conducted to discover if CCl_4 concentration influences on liver weight. As in previous SPSS analysis animals were divided into three groups according to dose level. There was a statistically significant difference at the $p < 0.01$ value. To estimate between which group of animals are differences, Post Hoc Tukey's HSD test was conducted.

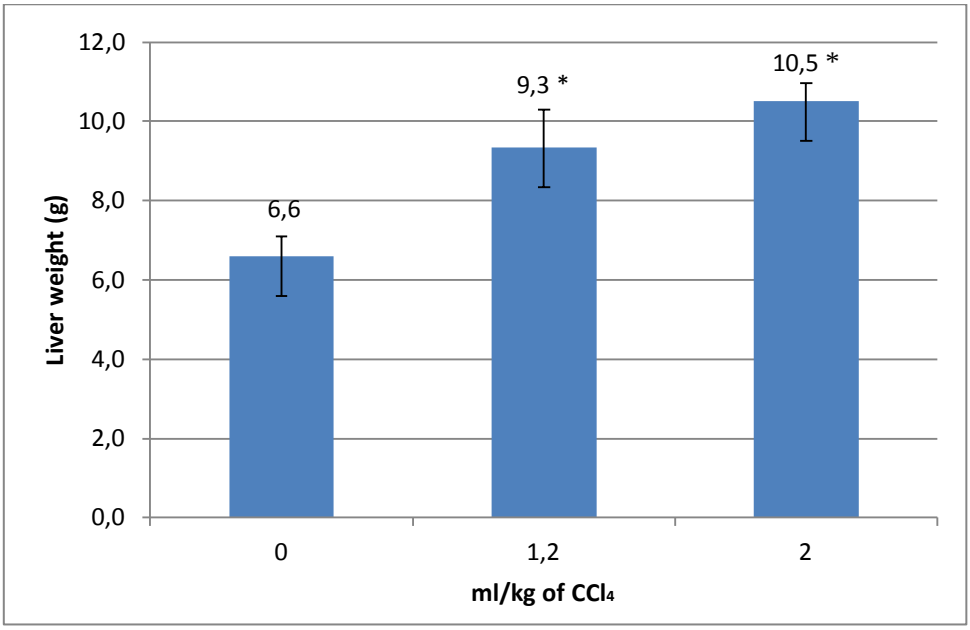


Figure 5: Mean (±SD) liver weigh (g) of rats treated with vehicle (control) or carbon tetrachloride (CCl_4) at 1.2 and 2.0 ml/kg

In each group 10 rats were included. Rats were treated with vehicle (control group) or with CCl4 in two concentrations, 1.2 and 2.0 ml/kg, respectively. All animals were autopsied at 24 hours post-dosing when their livers were removed and weighed. Liver weight results were shown as mean value of each dose level group. Results were analysed with SPSS and shown that liver weight at both dose levels (1.2 and 2.0 ml of CCl_4/kg) were statistically significant over liver weight from control group of rats. *P value < 0.01.

Sample preparation

Depletion of albumin component (DAC) protocol

Three serum samples from control group (labelled with 1, 2 and 3) were analysed by SDS-PAGE (1-DGE) without and with using DAC protocol in order to see how effectively albumin is removed from serum and consecutive confirmed presence of low abundant proteins in the sample. Equal loading volume i.e. 1µl was used. Resolved gels were visualized by Coomassie Blue staining.

In Figure 6 comparison between gels without using DAC protocol (labelled as »Undepleted serum samples«) and with usage of DAC protocol (labelled as »Depleted serum samples«) is shown. Without using DAC protocol no band is detected, only stain is seen in upper part in each lane. There is also some vertical and horizontal streaking present on gel and lots of spilling the sample over neighboring lane. When DAC protocol was used the resolution of spot is improved and also some bands are seen on gel. No streaking is present.

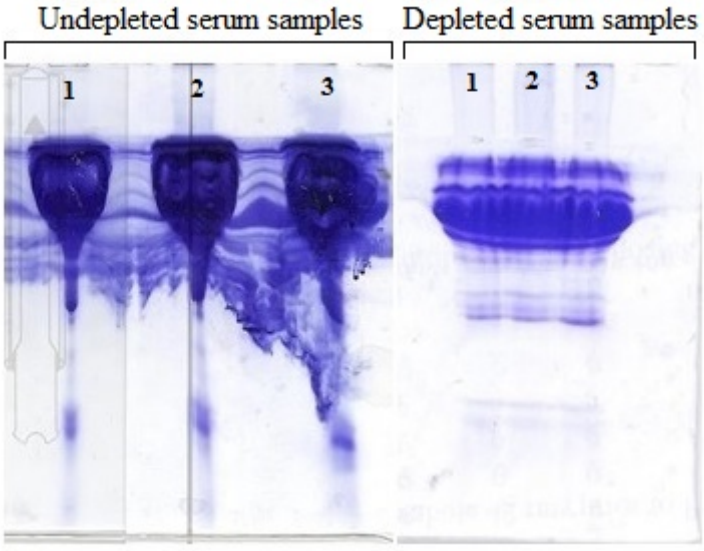


Figure 6: Optimization of DAC protocol using serum samples from control group of rats performed with SDS-PAGE (15%).

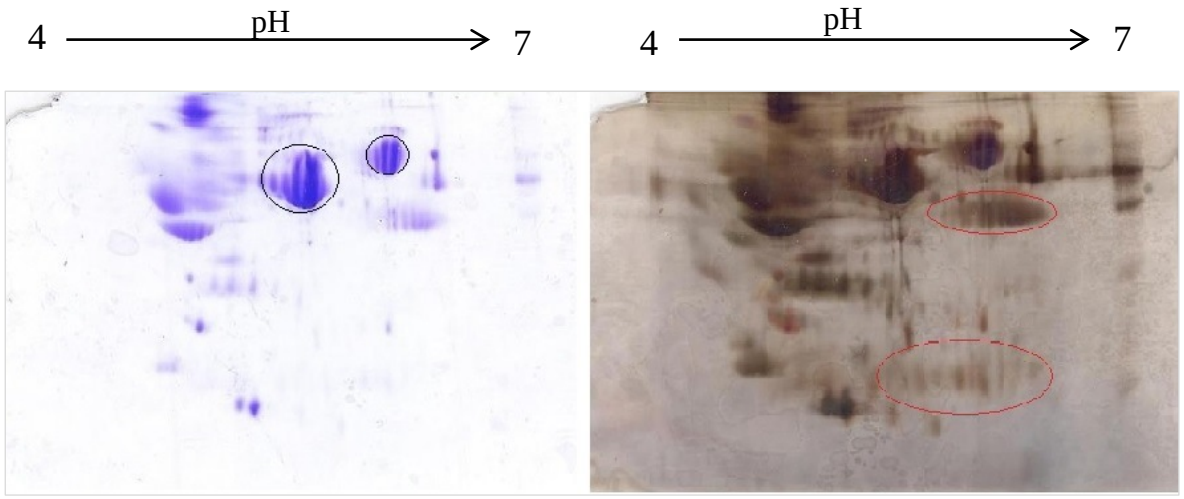
Three serum samples from control group of rats were divided into 2 groups where first group of serum samples stayed unaltered (»Undepleted serum samples«) while on the other group DAC protocol was used (»Depleted serum samples«). SDS-PAGE was run on 15 % running gel and 5 % stacking gel. Gels were Coomassie Blue stained. On unaltered samples stain is present in each lane, representing albumin, no other proteins is visible. When DAC protocol was used, a big stain was removed, other proteins are seen.

ReadyPrep 2-D Cleanup kit

Serum samples which were used in SDS-PAGE were carried out for 2-DGE for optimization of the clean-up kit protocol prior to isoelectric focusing. With clean-up kit samples can be concentrated and interfering compounds can be removed. Prior to first dimension of 2-DGE, DAC protocol was performed to remove albumin from serum samples. To determine differences before and after using 2-D clean-up kit protocol, 2-DGE was carried out where one sample was treated only with DAC protocol while on the other DAC protocol was performed followed by treatment with the ReadyPrep 2-D Cleanup kit. In both preparation of sample the same loading volume was used i.e. 2.5 µl. Following second dimension visualisation of gels was performed using Coomassie Blue and silver staining.

Figure 7A and B compares four 2-D gels from the same control animal using IPG strips 4-7 after Coomassie Blue and silver staining. On Figure 7A where gels were treated only with DAC protocol are shown some areas where proteins are not well focused. When both pre-treatment steps were performed i.e. DAC protocol and ReadyPrep 2-D Cleanup kit as is shown on Figure 7B, bands are better focused and also greater amount of albumin was depleted. On both, Coomassie Blue and silver stained gels differences before and after use of 2-D Cleanup kit can be seen. However, using ReadyPrep 2-D Cleanup kit might cause loss of some proteins, special in central area, seen on Coomassie Blue and silver stained gel. On silver stained gels before and after usage of 2-D Cleanup kit there is also some streaking present.

A



B

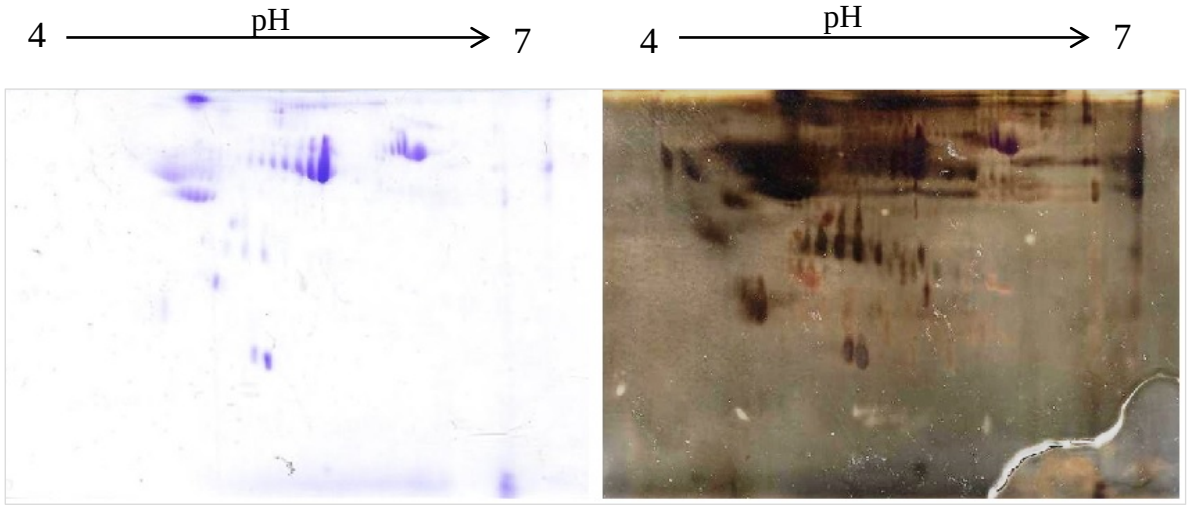


Figure 7: Optimization of ReadyPrep 2-D Cleanup kit using control's serum sample performed with 2-DGE.

2.5 µL of serum sample were used for performing 2-DGE in both gels. Prior to 2-DGE sample was separated into two samples where on one sample only DAC protocol was performed (A), while on the second one both procedures i.e. DAC protocol and ReadyPrep 2-D Cleanup kit were used (B). Samples were focused on IPG strips 4-7 and second dimension was run on 12 % SDS-PAGE. Gels were Coomassie Blue (up and bottom left) and silver stained (up and bottom right) to determine any differences in spot intensity. Black circled areas are showing increased focusing of proteins when clean up kit was used while red circled are pointing out eventual loss of proteins after use it.

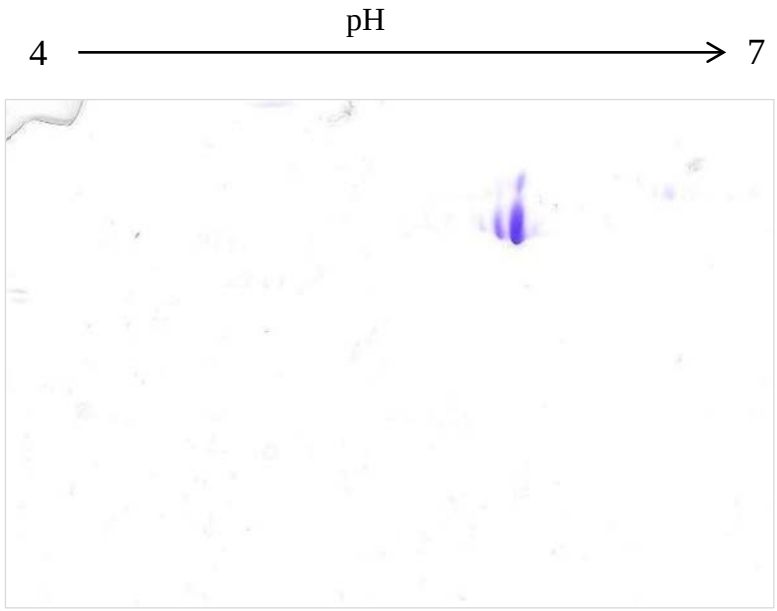
Sample loading and visualization

Prior to sample preparation and carrying out the 2-DGE there is need to define optimal loading volume of serum sample. To find adequate volume, initial loading volume i.e. 1 µl of serum sample was applied on IPG strips 4-7. Prior to first dimension of 2-DGE DAC protocol was performed in order to remove albumin from the sample, and ReadyPrep 2-D Cleanup kit was used to remove salts. After completing of second dimension the gels were visualized with Coomassie Blue and silver stain.

Different stains are used to detect proteins in gel after running 2-DGE. Most commonly used stains are Coomassie Blue or silver stain. In order to define where detection of bands is more sensitive and which stain is more compatible with identification method such as mass spectrometry both Coomassie Blue and silver stain were used.

Figure 8 shows two gels from the same serum sample with loading volume 1.0 µl using IPG strips 4-7. After performing 2-DGE gels were visualized with Coomassie Blue stain (Figure 8A) and silver stain (Figure 8B). On gel visualized with Coomassie Blue are shown only two bands that can be identified, while on gel visualized with silver stain more bands can be seen, although very poorly. However for identification of bands with mass spectrometry, it is suggested that bands are stained with Coomassie Blue because silver method is rather incompatible with mass spectrometry analysis.

A



B

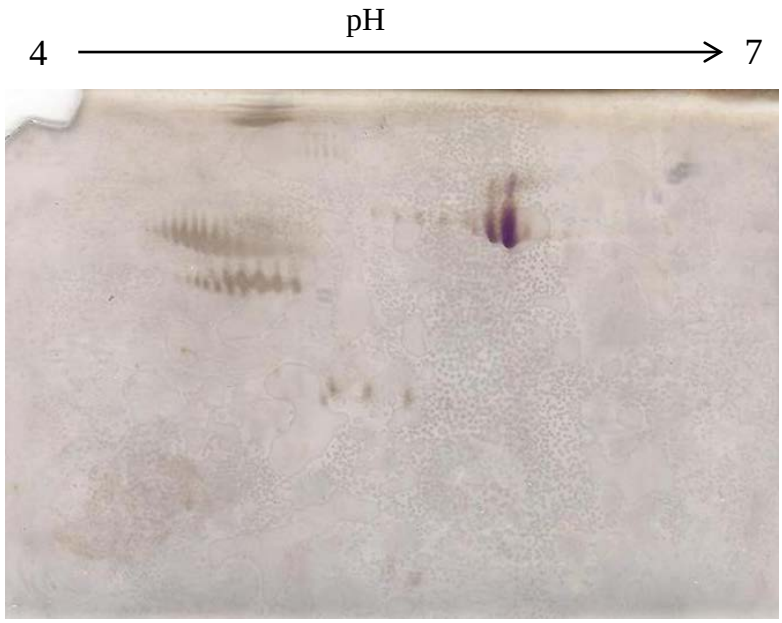


Figure 8: 2-DGE of serum samples of control group of animals Coomassie Blue (A) and Silver (B) stained.

Both samples were treated with DAC protocol and 2-D Cleanup kit prior to first dimension of 2-DGE. Equal loading volume was added i.e. 1 µl. After SDS-PAGE of 2-DGE was performed gels were stained with two different dyes. On silver stain gel numerous spots are seen, even though very poorly while on the Coomassie Blue strained gel only two spots are seen in upper part of gel.

Comparison of control and with CCl_4 treated serum samples using 2-DGE

After optimization of pre-treatment steps (DAC protocol and ReadyPrep 2-D Cleanup kit comparison between control and 1.2 ml/kg of CCl_4 treated animal was carried out with 2-DGE. The serum samples from animals treated with 2.0 ml/kg of CCl_4 were eliminated from further analysis because comparison to 1.2 ml/kg treated animals showed similar results on 2-DGE of serum samples (not shown).

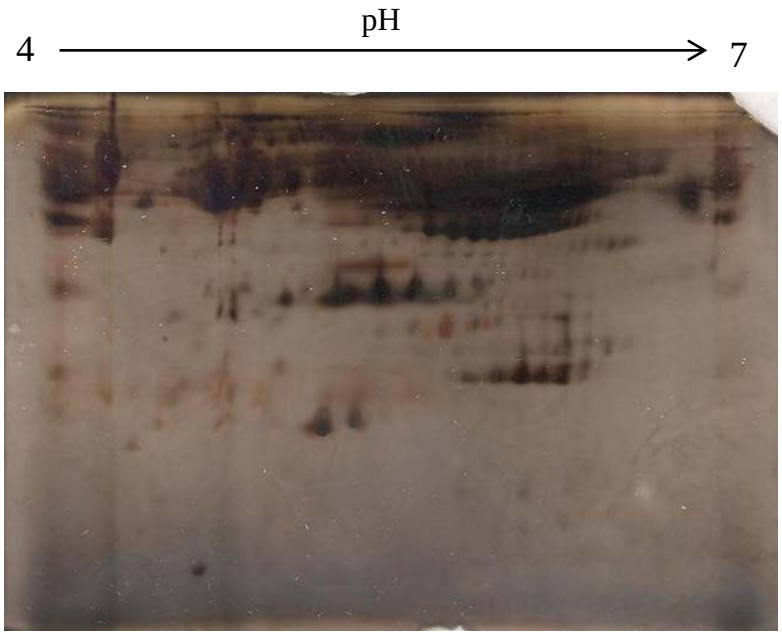
One serum sample from each group was analysed with 2-DGE. Prior to the isoelectric focusing, on all samples DAC protocol and ReadyPrep 2-D Cleanup kit was performed. After second dimension of 2-DGE, samples were stained with Coomassie Blue and silver. However, because more bands are shown on sliver stained gel regarding to Coomassie Blue stained gels (Figure 8B) in the future only silver stain gel will be shown in Results.

Figure 9 and 10 show comparison of 2-DGE gel of serum sample from animals treated with vehicle (Figure 9A and 10A) and with 1.2 ml/kg of CCl_4 (Figure 9B and 10B). In figures, different loading volume i.e. 2.5 µl and 7.5 µl, was used, respectively in order to determine if higher volume of sample will improve number of spots. Gels were focused on IPG strips 4-7 in first dimension of 2-DGE.

On Figure 9 there are not many alternations between gels from control and treated samples. Following Coomassie Blue staining spots, especially in central area of gel, have minor intensity on gel from treated rats (circled). This spots represent proteins which concentration decreased after administration of CCl_4. In upper part of both gels, there is some horizontal streaking present.

On Figure 10 where loading volume was 7.5 µl, there are differences in intensity and size of spots/proteins on gels from control and treated animals. Serum sample from the group treated with CCl_4, seen on Figure 10B, shows decreased intensity of spots in central area (black circled). On spot which were seen on gel from control animals (10B), disappear on treated one (red circled). As well as on Figure 9A and B some horizontal streaking is present on gels.

A



B

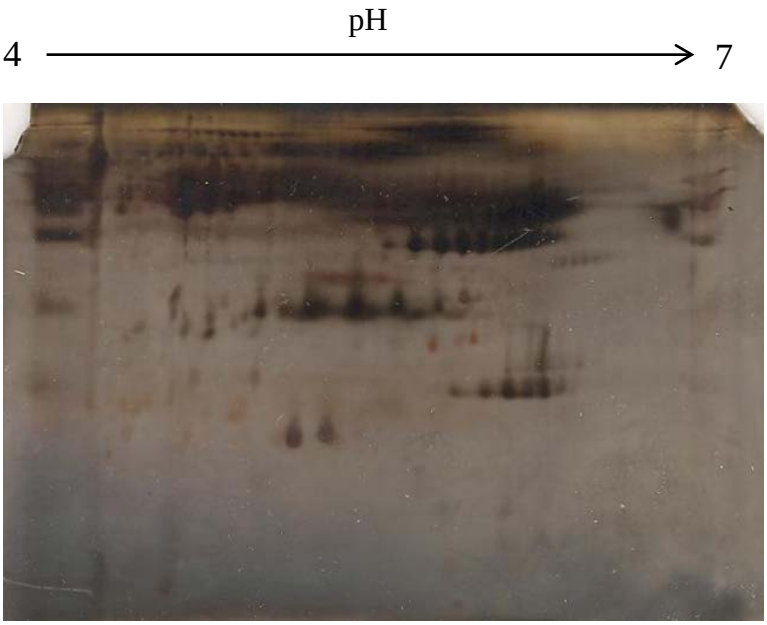
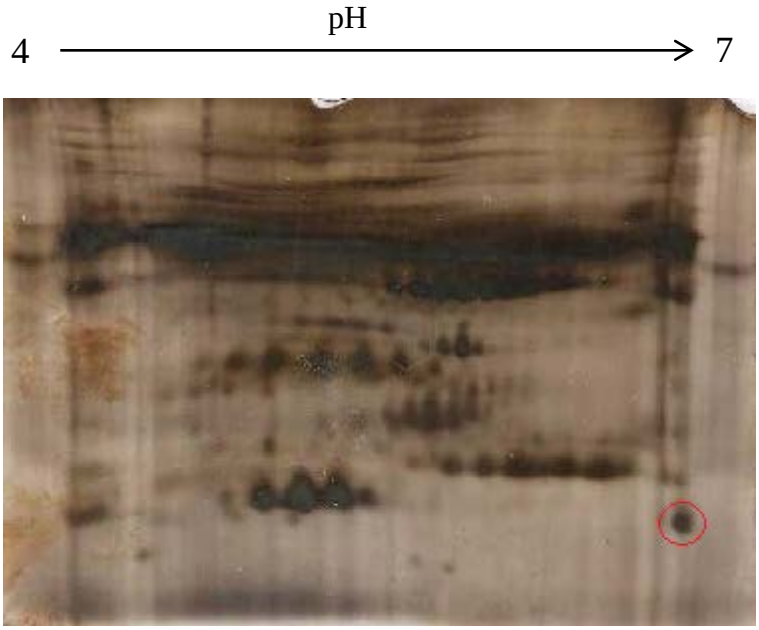


Figure 9: 2-DGE of serum samples from control (A) and with CCl_4 treated (B) group of animals using IPG strip 4-7 and loading volume 2.5 µl.

Serum sample from a control animal and a treated animal were pre-treated with the DAC protocol and ReadyPrep 2-D Cleanup kit as described in Methods. Samples were focused on IPG strips 4-7 and in second dimension run on 15 % SDS PAGE. Gels were silver stained. Both gels show similar size of proteins, however, the intensity of some proteins are minor on gels which rat samples were treated with CCl_4.

A



B

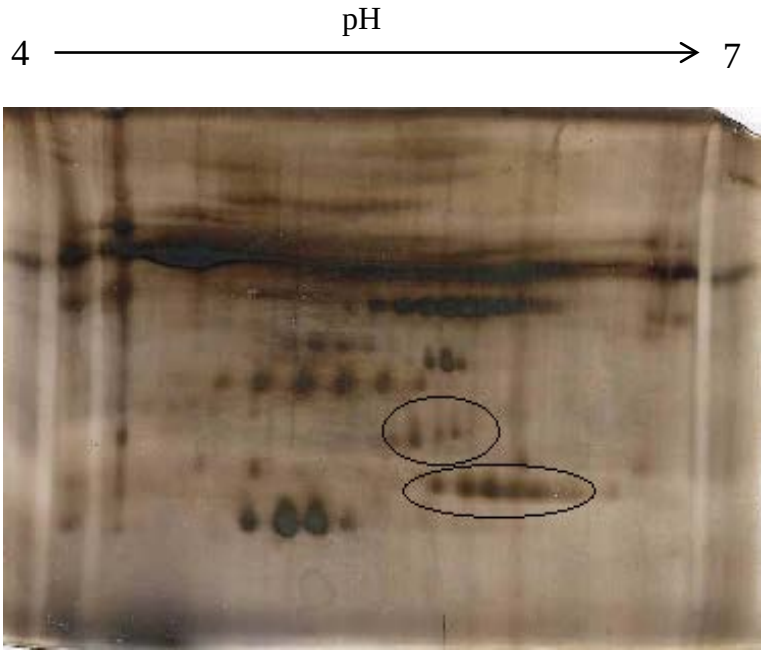


Figure 10: 2-DGE of serum samples from control (A) and with CCl_4 treated (B) group of animals using IPG strip 4-7 and loading volume 7.5 µl.

Serum samples were pre-treated with the DAC protocol and ReadyPrep 2-D Cleanup kit. Second dimension was run on 15 % SDS-PAGE. Gels were after performing 2-DGE silver stained. Using volume 7.5ul shows fewer proteins on gel from treated animal (black circled) and 1 protein, present on gel from control animal, has disappeared from gel of animal treated with CCl_4 (circled red).

To estimate how the usage of different IPG strips influences the resolution of spots on gels, narrow IPG strip was used. With narrow focusing we allow that proteins that were focused on IPG strips 4-7, will achieve better resolution and consequently will be better focused. However, the area for detection of proteins will be smaller. Instead of IPG strip 4-7, 5-6 was used.

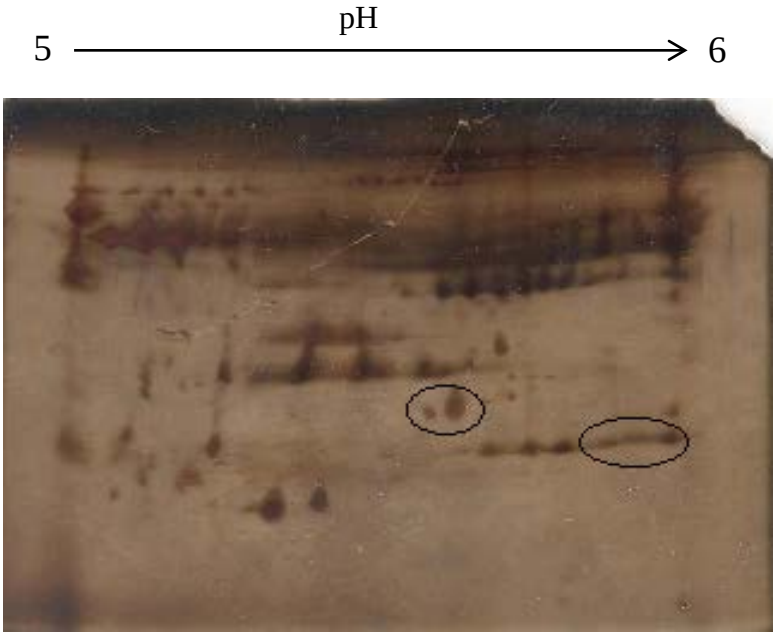
As mentioned above, the comparison between control and treated animals was made to evaluate if there are differences in serum protein concentration. Furthermore, with narrowing pH gradient better resolution of alterated spots will be seen between both groups. Different loading volume was used i.e. 2.5 µl and 7.5 µl, respectively, to estimate if volume also influences on resolution of spots.

All samples were pre-treated with DAC protocol and ReadyPrep 2-D Cleanup kit, as mentioned in Methods, respectively. Samples were run on 2-DGE were in first dimension for IEF strip 5-6 was used. Second dimension was run on 12 % SDS-PAGE. For visualization Coomassie Blue and silver were used.

On Figure 11 and 12 instead of IPG strip 4-7, narrow strip 5-6 was used in order to improve resolution of proteins that were gained on gels where IPG strips 4-7 were applied. As seen on Figure 11, were the loading volume was 2.5 µl, proteins in central area are rather good focused. There are some proteins missing or are decreased on gel which rat was treated with CCl_4 comparing to control (black circled) which could be isolated for further identification. There is some horizontal streaking present in upper part of the gel which might indicate to inadequate sample preparation.

On Figure 12, higher loading volume was applied. Some proteins seen on gel treated with CCl_4 are decreased in size and intensity (black circled). As well, proteins are better seen as in Figure 11. However, higher volume also caused poorer resolution of proteins. In upper right part (circled red) of the gel from treated rat sample was still a degree of horizontal streaking of protein spots possibly a result of incomplete focusing of the proteins in the sample.

A



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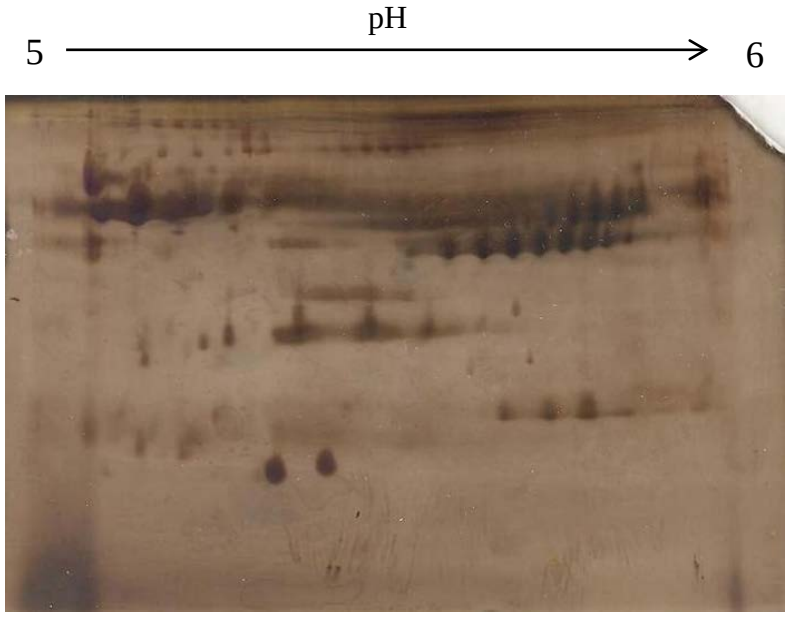
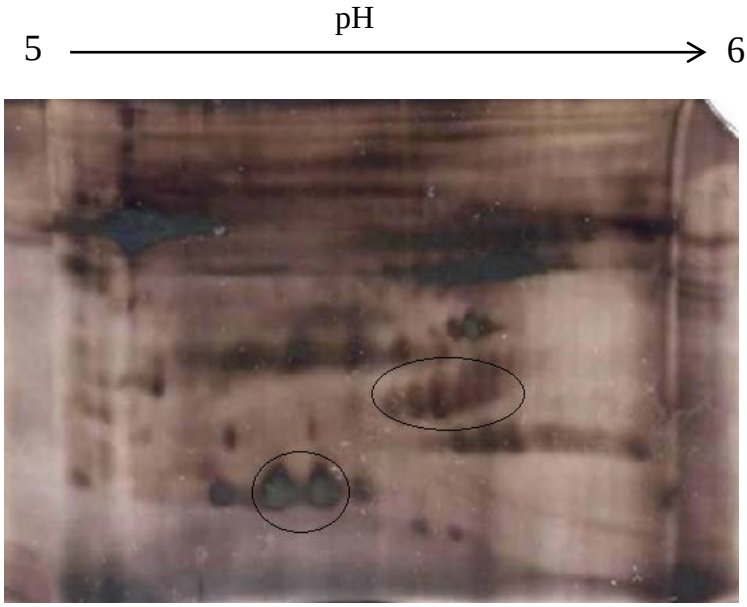


Figure 11: 2-DGE of serum samples from control (A) and with CCl4 treated (B) group of animals using IPG strip 5-6 and loading volume 2.5 µl.

Serum samples were treated with pre-treatment procedures prior to 2-DGE. For isoelectric focusing narrow IPG strip 5-6 were used. The second dimension was run on 15 % SDS PAGE and afterward gels were silver stained. Proteins are better focused when narrower IPG strip was used, especially in upper area. Circled proteins on gel from control rat serum are missing on gel from serum of treated rat.

A



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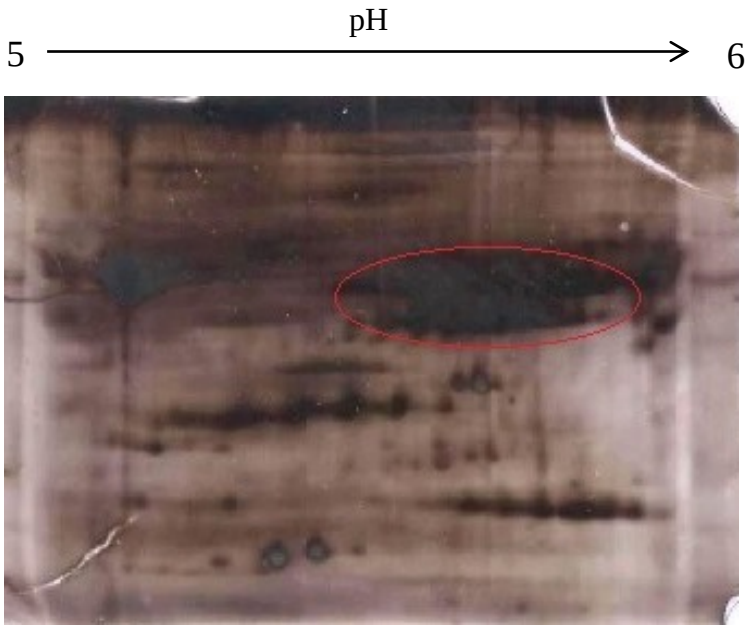

Figure 12: 2-DGE of serum samples from control (A) and with CCl4 treated (B) group of animals using IPG strip 5-6 and loading volume 7.5 µl.

Prior to 2-DGE serum samples were treated with DAC protocol and ReadyPrep 2-D Cleanup Kit was used as described in Methods. Second dimension was run on 15 % SDS PAGE. Gels were silver stained. Loading higher volume i.e. 7.5 µl, cause increased intensity of proteins, however, the focusing is not so well, especially in upper right part of gel (circled red). Some proteins seen on gel from control sample are almost totally disappeared on gel where CCl_4 was administered to rats (black circled).

DISCUSSION

Body weight and liver weight

One of the purposes of this preliminary dose response study was to determine if here is a connection between the oral administration of CCl_4 and body weight loss and if this is correlated to hepatic response in the experimental animals – rats.

CCl_4 was used since it has been widely used as an experimental hepatotoxin for years and it proves highly useful for the study of certain hepatotoxic effects. At lower or single doses CCl_4 produces centrilobular necrosis and fatty accumulation in the liver, while at higher or repeated administrations of CCl_4 liver fibrosis and cirrhosis might occur (26, 63). A preference of CCl_4 is that it consistently produces liver injury in many species, including non-human primates and man; and shows similar and comparable results (26). In the present study two different doses of CCl_4 were given to experimental animals – 1.2 ml/kg and 2.0 ml/kg of body weight. To the third group vehicle was administrated and presents control group to which treated groups were compared. All experimental animals followed the same procedure described in methods to avoid any abnormalities. After administration animals were placed back to communal cages. After 6 hours they were weighted and separately placed in metabolism cages for 18 hours allowing metabolizing. During this period animals had access to water but not diet. 24 hours after administration animals were weighted again to determine change in body weight before and after administration of vehicle or CCl_4. They were autopsied where blood samples were taken for the preparation of serum and livers were removed and weighted.

Total body weight of animals was decreased in all groups after administration of vehicle or CCl_4 but the least decrease was in control group of animals. After analysing with ANOVA it was shown that treatment with CCl_4 at both dose levels didn't show statistically significant decrease in the body weight compared to control rats at $p < 0.05$.

Liver weights of animals were recorded during the autopsy and expressed as absolute weights. Livers from CCl_4-treated animals at all dose levels i.e. 1.2 and 2.0ml/kg were significantly heavier than the livers from control animals, 1.41-fold and 1.59-fold, respectively at $p < 0.01$ value (Figure 5). Previous studies confirmed the increase in liver weight following a single administration of CCl_4 to female Hanover-Wistar rats at 0.4, 0.8,

1.2 and 1.6 ml/kg (64). Despite the fact that there was no histopathology analysis or any clinical chemistry test performed to confirm results from post mortem, some previous studies showed that administration of CCl_4 induces injuries in rats' liver. Studies by Smuckler et al. demonstrated necrosis of the central zone and fat accumulation shown by light microscopy (65). Elevation of relative liver weights, as a result of CCl4 administration, are generally considered to be related to hepatocyte cytoplasmic swelling as a result of cellular injury and degeneration, and the infiltration of inflammatory cells (66).

Sample preparation

Serum samples from experimental animals were collected post-mortem. To remove blood cells and fibrinogen, supplement of anticoagulants was added and centrifuged.

The second aim of the present study was to optimize procedures in serum sample preparation for further analysis with 2-DGE. Sample preparation for 2-DGE is a key component of successful two-dimensional gel electrophoresis of proteins which purpose is to convert the sample into a suitable physicochemical state for first dimension of IEF while preserving the charge and molecular weight of proteins (58, 57). One of procedure included in sample preparation is depletion of albumin component (DAC) protocol. It is used for effective removal of albumin from serum in order to detect other low abundant proteins which are smeared by albumin band. The removal of highly abundant protein is essential and will aid in the search for new proteins - biomarkers.

On serum samples from control group of animals comparison was made in order to determine if usage of DAC protocol was successful. Three serum samples were used where DAC protocol was applied (labelled as »Depleted serum samples«) and three samples were DAC protocol was not used (labelled as »Undepleted serum samples«). Equal loading volume was used. For analysing of serum samples 1-DGE was carried out and afterwards visualized with dye Coomassie Blue (Figure 1). DAC protocol as is described by Colantonio was performed with some modifications (52). In this procedure NaCl and EtOH are used as precipitating agents where albumin is precipitated and discarded together with supernatant (52).

On Figure 6 albumin band represents the big band in upper part of gel in each lane. Due to the abundance of albumin no other band is visible, when protocol without DAC is used. Due to high concentration of albumin in sample, the separation of proteins was not successful causing passing of sample over to neighbour lanes. When DAC protocol was performed the majority of albumin is depleted but it can still be seen on gel. However, with removal of albumin from sample also other bands were seen. For complete depletion of albumin band the DAC protocol should be repeated or performed fully as was described in study by Colantonio (52). However with repeated performing of DAC protocol also other low abundant proteins might disappear from sample.

Albumin is the most abundant protein in serum, representing approximately 60 % of total proteins and it causes dark protein smears on gel electrophoresis. By significantly depleting the amount of albumin present in serum sample, protein resolution can generally be improved and increased protein loads are possible without the problem of albumin smearing (49).

Another procedure which improves resolution of bands on 2-DGE is ReadyPrep™ 2-D Cleanup kit. With this cleanup kit interfering compounds are removed from serum in order not to interfere with isoelectric focusing in gel electrophoresis. As well, usage of cleanup kit reduces appearance of vertical or horizontal striking on gel.

Although the structure of reagent in 2-D Cleanup kit is not known, it is known that is based on trichloroacetic acid (TCA)-Acetone precipitation (67, 68). For explanation mode of action of cleanup kit on serum all reagent should be known.

Serum sample from control group of animals was used to define efficiency of 2-D Cleanup kit. One sample was treated only with DAC protocol in order to remove albumin from serum while on the other both DAC protocol and 2-D Cleanup kit were performed. Both samples were carried out on 2-DGE and afterwards stained with two dyes Coomassie Blue and silver.

On Figure 7A lots of spots are visible after staining but are badly focused while the usage of Cleanup kit, as it is seen on Figure 7B, improves resolution of spots on gel. With removing interfering compounds such as proteolytic enzymes, salts, nucleic acids, polysaccharides and/or highly abundant proteins, better resolution is achieved and less streaking occurred (50). It was shown that higher concentration of salts in serum impact on

good resolution of proteins on 2-D gel (69). Cleanup Kit also raises protein concentration allowing higher protein loads that can improve spot detection (70). Liu et.al. in his research showed how usage of ReadyPrep 2-D Cleanup kit improve resolution of bands and reduce streaking in rat samples (71).

The combination of DAC protocol and 2-D Cleanup kit gave much better spot resolution and there was greater depletion of albumin and other interfering compounds. However, the drawback was that some low abundant proteins were lost during both procedures which can be also seen on gels (central area on Figure 7B).

Comparison of control and with CCl_4 treated samples

The third aim of this study was to define novel biomarkers after acute liver injury induced with CCl_4 in serum samples of experimental animals. There is several studies were a proteomic approach was applied in detection and identification of novel biomarker of hepatotoxicity and liver injuries in laboratory animals after application of hepatotoxin.

In Figures 9-12 comparison between control and sample treated with 1.2 ml/kg of CCl_4 was made in order to detect any proteomic alterations. Serum samples were analysed by using 2-DGE and prior to first dimension on samples pre-treatment procedures i.e. DAC protocol and 2-D Cleanup kit were performed.

During the performances of 2-DGE of samples different loading volume was applied and different IPG strips were used in order to determine their impact on resolution and focusing of proteins on gel.

One of the key objectives of proteomics is to identify the differential expression between control and experimental samples run on a series of 2-D gels. That is the protein spots that have been inhibited (disappeared), induced (appeared) or have changed abundance (increased or decreased in size and intensity). Once these gels features have been found, the proteins of interest can be identified using mass spectrometry (50).

Defining adequate volume of serum sample to apply on IPG strip and run 2-DGE is needed to get optimal results on gels. On Figure 8, where loading volume was 1.0 µl, poorly visualization of spots on gel are seen. Initially loading volume 2.5 µl was applied on IPG strips 4-7 (Figure 9). On both gels similar position and concentration of proteins are shown, only few proteins are on gel which sample was treated with CCl_4 decreased

comparing to control one. In upper part of gels some horizontal streaking is present which might indicate on some problems with the sample preparation. With applying higher volume of sample more proteins can be seen on gel (Figure 10). Differences between control and with CCl_4 treated sample are showing that CCl_4 influences on protein concentration in serum.

Comparison of application different loading volume of sample but same IPG strip on 2-DGE is shown on Figure 9 and 10. To visualize lower abundance proteins, attempts have been made to increase the total protein loaded onto a 2D gel. With higher volume of sample, more proteins can be detected on gel and they would be easier visible. However, this approach results in decreased resolution of protein spots, where abundant proteins smear and overlap the less abundant proteins in the sample with a similar pI and size seen as vertical streaking (51, 69). The amount of protein which can be loaded onto a single IPG strip for optimum resolution, maximum spot number and minimum streaking/ background smearing depends on parameters such as the pH gradient used (wide or narrow), separation distance, and protein complexity of the sample (50). Berkelman et.al. showed in his study guidelines for sample loading on IPG strips to achieve good protein detection and visualization and to avoid excess of sample loading causing horizontal or vertical streaking (72).

One of the most important improvements in 2-D electrophoresis was introduction of immobilized pH gradients or IPGs (58). Instead of medium range IPG strip 4-7, narrow pH gradient 5-6 was used to determine how influences on resolution and separation of proteins.

On Figure 11 and 12, narrow IPG strips were used in comparison to medium range IPG strips (Figure 9 and 10). Results show better resolution of gels and as well better separation of proteins was achieved which would allow their further identification with MS. Many studies showed how does usage of narrow-over lapping pH gradients improves protein detection (73-75). Besides, there was less horizontal streaking present on gel when IPG strip 5-6 was applied. With increasing pH range there is higher chance to applied higher loading volume on IPG strip which might increase possibility of detection of low abundant proteins serving as potential biomarkers of liver injuries.

With higher loading volume there are some differences in spot intensity and size which indicates higher concentration of proteins. On gels were loading volume was 7.5 µl (Figure 12) more spots or detected proteins can be seen, while on gel with lower volume some proteins are decreased in concentration and some have totally disappeared. On Figure 12, especially in upper part, there can be seen some indistinct areas, where proteins are hard to detect. To acquire better resolution of spots in upper part, 2-DGE should be carried out again or smaller loading volume should be applied.

There are also some differences between control and treated samples seen on Figure 11 and 12. The most abundant serum proteins which had increased/decreased in concentration following the induction of liver injury should be excised and identify by mass spectrometry.

CONCLUSION

From results from current studies, it can be concluded that administration of single dose of CCl_4 in different concentrations have impact on liver weight. However there were no significant differences in body weight between the groups. Liver weights were after administration of 1.2 and 2.0 ml/kg of CCl_4 to rats significantly increased above the control group, 1.41-fold and 1.59-fold, respectively which indicates on some alterations in structure of liver parenchyma. Total body weight of rats was decreased in all groups after administration of vehicle or CCl_4 but the least decrease was in control group, -14.4 g, -15.0 g, -15.7 g, respectively. The higher decrease in treated groups was not statistically different which indicates on other reasons causing decrease of body weight. For the future work, histopathology examination should be performed and some serum enzymes (AST, ALT) should be measured to verify these results. As well, bigger number of samples should be acquired to make any reliable conclusion concerning liver and body weights.

During performing 2-DGE several steps were taken under observation. Firstly, serum samples were prepared for further analysis with 2-DGE. On serum samples DAC protocol and 2-D Cleanup kit was performed. DAC protocol successfully remove highly abundant albumin from serum causing better resolution and less smearing of other low abundant proteins. It also reduces vertical and horizontal streaking. With 2-D Cleanup kit interfering compounds were removed and better resolution of gel and focusing of spots was achieved. A drawback is that with these procedures other low abundant proteins were lost. For future work other procedures for removing albumin and interfering compounds should be tested and compare with these for any differences in expression of proteins on gel.

Different immobilized pH gradients - IPG strips (4-7 and 5-6) were applied in first dimension of gel electrophoresis. It showed that using narrower IPG strip causes better focusing of proteins which is easier for their further identification with MS. Using different loading volume of serum also bring promising results. The higher volume of sample is, more proteins can be detected on gel and they are more visible. However, higher volume causes decreased focusing of proteins and as well the resolution of gel, where abundant proteins overlap the less abundant one.

While using variable conditions for 2-DGE comparison between control and treated group of animals was made. From these result it can be concluded that administration of CCl_4

causes different pattern of expression of proteins on control and treated samples. Any decreased or missing proteins should be taken under observation as potential hepatic biomarkers that could help to discover early stages of liver diseases.

In the future, proteins that were well-focused and indicated different size and intensity between control and with CCl_4 treated rat serum samples should be identified with mass spectrometry to determine how and why they are changed during liver injury.

LITERATURE

1. Rubin R, Strayer D S: Rubin's Pathology: Clinicopathologic foundations of medicine, 5th ed, Lippincott Williams & Wilkins, Philadelphia, 2008: 617-674.
2. Turton J, Hooson J: Target organ pathology A basic text, Taylor & Francis, London, 1998: 61- 94.
3. Kumar V, Abbas A K, Fausto N, Aster J C: Robbins and Cotran Pathologic Basis of Disease, 8th ed, Saunders Elsevier, Philadelphia, 2010: 833-890.
4. Saxena R, Theise N D, Craword J M: Concise Review: Microanatomy of the human liver – Exploring the hidden interfaces. Hepatology 1999; 30: 1339-1346.
5. Rappaport A M, Borowy Z J, Loughhead W M, Lotto W N: Subdivision of hexagonal liver lobules into a structural and functional unit. The anatomical record 1954; 119: 11- 33.
6. Kiernan F: The anatomy and physiology of the liver. Philosophical transactions of the royal society of London 1863; 123: 711-770.
7. Cawson R A, McCracekn A W, Marcus P B, Zaatari G S: Pathology, The mechanisms of disease, 2th ed, C.V. Mosby Company, St. Louis, 1989: 348-359.
8. Malarkey D E, Johnson K, Ryan L, Boorman G, Maronpot R R: New insights into functional aspects of liver morphology. Toxicologic pathology 2005; 33: 27- 34.
9. Aspinall R J, Taylor-Robinson S D: Mosby's Color Atlas and text of Gastroenterology and liver disease, Mosby, Edinburgh, 2002: 162-163.
10. Boyer T D, Manns M P, Sanyal A J: Zakim&Boyer's Hepatology A text book of liver disease; 6th ed; Elsevier Saunders; Philadelphia; 2012: 417-461
11. Groneberg D A, Grosse-Siestrup C, Fischer A: In vitro models to study hepatotoxicity. Toxicologic pathology 2002; 30: 394- 396.
12. Oinonen T, Lindros K O: Zonation of hepatic cytochrome P-450 expression and regulation. Biochem J 1998; 329: 17-35.
13. Wijnen P A H M, Op Den Buijsch R A M, Drent M, Kuipers P M J C, Neef S, Bast A, Bekers, Koek G H: Review article: The prevalence and clinical relevance of cytochrome P450 polymorphisms. Aliment Pharmacol Ther 2007; 26: 211- 219.
14. Ishizaki T, Horai Y: Review article: Cytochrome P450 and the metabolism of proton pump inhibitors – emphasis on rabeprazole. Aliment Pharmacol Ther 1999; 13: 27-36.

15. Coon M J, Ding X X, Pernecky S J, Vaz A D: Cytochrome P450: progress and prediction. The FASEB Journal 1992; 6: 669-73.
16. Klaassen C D: Casarett & Doud's Toxicology: The Basic Science of Poisons, 7th ed, 2007, McGrawHill Medical, New York, 2008.
17. Svetlov S, Hayes R L, Wang K, Oli M: Biomarkers of liver injury. United States Patent, Patent number 8,048,638, date of patent Nov. 1, 2011.
18. Kumar V, Abbas A K, Fausto N, Mitchell R N: Robbins basic pathology, 8th ed, Saunders Elsevier, Philadelphia, 2007: 631-673.
19. Malhi H, Gore G J: Cellular and molecular mechanisms of liver injury. Gastroenterology 2008; 134: 1641-1654.
20. Miyazaki M, Kato M, Tanaka M, Tanaka K, Takao S, Kohjima M, Ito T, Enjoji M, Nakamuta M, Kotoh K, Takayanagi R: Contrast-enhanced ultrasonography using Sonazoid to evaluate changes in hepatic hemodynamics in acute liver injury. Journal of Gastroenterology and Hepatology 2011; 26: 1749-1756.
21. Timbrell J A: Principles of Biochemical Toxicology, 4th ed, Informa Healthcare USA, New York, 2009: 198.
22. McCullough A J: The clinical features, diagnosis and natural history of non-alcoholic fatty liver disease. Cln Liver Dis 2004; 8: 521-533.
23. Poynard T, Ratziu V, Naveau S, Thabut D, Charlotte F, Messous D, Capron D, Abella A, Massard J, Ngo Y, Munteanu M, Mercadier A, Manns M, Albrecht J: The diagnostic value of biomarkers (SteatoTest) for prediction of liver steatosis. Comparative Hepatology 2005; 4:10.
24. http://www.fatty-liver.com/what-is-a-fatty-liver/. Last view: 23 01 2014.
25. Cotran R S, Kumar V, Collins T: Robbins pathologic basis of diseases, 6th ed., WB Saunders Company, Philadelphia, 1999: 845-901.
26. Weber L W D, Boll M, Stampfl A: Hepatotoxicity and mechanism of action of haloalkanes: Carbon tetrachloride as a toxicological model. Critical reviews in toxicology 2003; 33: 105-136.
27. Uetrecht J: Adverse drug reaction, Springer Heidelberg Dordrecht, London, New York, 2010: 165-194.
28. Jia N, Liu X, Wen J, Qian L, Qian X, Wu Y, Fan G: A proteomic method for analysis of CYP450s protein expression changes in carbon tetrachloride induced male rat liver microsomes. Toxicology 2007; 237: 1-11.

29. Sheweita S A, Abd El-Gabar M, Bastawy M: Carbon tetrachloride changes the activity of cytochrome P450 in the liver of male rats: role of antioxidants. Toxicology 2001; 169: 83-92.
30. Slater T F, Cheeseman K H, Ingold K U: Carbon tetrachloride toxicity as a model for studying free-radical mediated liver injury. Phil. Trans. R. Soc. Lond. B 1985; 311: 633 – 645.
31. Wang D-H, Wang Y-N, Ge J-Y, Liu H-Y, Zhang H-J, Qi Y, Liu Z-H, Ciu X-L: Role of activin A in carbon tetrachloride-induced acute liver injury. World Journal of Gastroenterology 2013; 19: 3802 – 3809.
32. Silberring J, Ciborowski P: Biomarker discovery and clinical proteomics. Trends in Analytical Chemistry 2010; 29: 128 – 140.
33. Puntmann V O: How to guide on biomarkers: biomarkers definitions, validation and applications with examples from cardiovascular disease. Postgrad Med J 2009; 85: 538 – 545.
34. Wetmore B A, Merrick B A: Toxicoproteomics: Proteomics applied to toxicology and pathology. Toxicologic pathology 2004; 32: 619 – 642.
35. Timbrell J A: Biomarkers in toxicology. Toxicology 1998; 129: 1 – 12.
36. Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S: The current state of serum biomarkers of hepatotoxicity. Toxicology 2008; 245: 194 – 205.
37. Lewin D A, Weiner M P: Molecular biomarkers in drug development. DDT 2004; 9: 976 – 983.
38. Vekey K, Telekes A, Vertes A: Medicinal Application of Mass Spectrometry, 1st ed, Elsevier, Amsterdam, 2008: 505 – 532.
39. J N Adkins, S M Varnum, K J Auberry, R J Moore, N H Angell, R D Smith, D L Springer, J G Pounds: Toward a human blood serum proteome. Mol. Cell. Proteomics 2002; 1.12: 947 – 957.
40. Zhang A, Sun H, Yan G, Han Y, Wang X: Serum proteomics in biomedical research: a systematic review. Appl Biochem Biotechnol 2013; 170: 774 – 786.
41. Ramaiah S K: A toxicologist guide to the diagnostic interpretation of hepatic biochemical parameters. Food and Chemical Toxicology 2007; 45: 1551 – 1557.
42. Smyth R, Munday M R, York M J, Clarke C J, Dare T, Turton J A: Dose response and time course studies on superoxide dismutase as a urinary biomarker of carbon

tetrachloride-induced hepatic injury in the Hanover Wistar rat. Int. J. Exp. Path. 2009; 90: 500-511.

43. Wilkins M R, Sanchez J C, Gooley A A, Appel R D, Humphery-Smith I, Hochstrasser D F, Williams K L: Progress with proteome projects: Why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Eng 1996; 13: 19–50.
44. Righetti P G, Campostrini N, Pascali J, Hamdan M, Astner H: Quantitative proteomics: a review of different methodologies. Eur. J. Mass Spectrom 2004; 10: 335-348.
45. Hannivoort R A, Hernandez-Gea V, Friedman S L: Genomics and proteomics in liver fibrosis and cirrhosis. Fibrogenesis & Tissue repair 2012; 5: 1-14.
46. Herbert B, Righetti P G: A turning point in proteome analysis: Sample prefractionation via multicompartment electrolyzers with isoelectric membranes. Electrophoresis 2000; 21: 3639-3648.
47. Hanash S: Disease proteomics. Nature 2002; 422: 226-232.
48. Meneses-Lorente G, Guest P C, Lawrance J, Muniappa N, Knowles M R, Skynner H A, Salim K, Cristea I, Mortishire-Smith R, Gaskell S J, Watt A: A proteomic investigation of Drug-induced steatosis in rat liver. Chem. Res. 2004; 17: 605-612.
49. Berkelman T and Stenstedt T: 2-D Electrophoresis: using immobilized pH gradients, Principles and Methods, Amersham Biosciences.
50. Görg A, Weiss W, Dunn M J: Current two-dimensional electrophoresis technology for proteomics. Proteomics 2004; 4: 3665-3685.
51. Rothemund D, Locke V L, Thomas T M, Rylatt D B: A general method for depletion of albumin from plasma under denatured conditions. Gradipore, 2003.
52. Colantonio D A, Dunkinson c, Bovenkamp D E, Van Eyk J E: Effective removal of albumin from serum. Proteomics 2005; 5: 3831-3835.
53. Ilyin S E, Belkowski S M, Plata-Salaman C R: Biomarker discovery and validation: technologies and integrative approaches. Trends in Biotechnology 2004; 22: 411-416.
54. Rabilloud T, Vaezzadeh A R, Potier N, Lelong C, Leize-Wagner E, Chavallet M: Power and limitations of electrophoretic separations in proteomics strategies. Mass spectrometry Reviews 2009; 28: 816 -843.

55. Yates III J R, Gilchrist A, Howell K E, Bergeron J J M: Proteomics of organelles and large cellular structures. Molecular Cell Biology 2005; 6: 702-714.
56. Rabilloud T, Lelong C: Two-dimensional gel electrophoresis in proteomics: A tutorial. Journal of Proteomics 2011; 74: 1829-1841.
57. Shaw M. M., Riederer B. M.: Sample preparation for two-dimensional gel electrophoresis. Proteomics 2003; 3: 1408-1417.
58. Remy A, Imam-Sghiouar N, Poirier F, Joubert-Caron R: Focusing strategy and influence of conductivity on isoelectric focusing in immobilized pH gradient. Bio-rad laboratories; Bio-rad bulletin 2778.
59. ReadyStrip™ IPG Strip, Instruction Manual. Bio-Rad Laboratories, Inc.
60. Van Summeren A, Renes J, van Delft J H, Kleinjans J C S: Proteomics in the search for mechanisms and biomarkers of drug-induced hepatotoxicity. Toxicology in vitro 2012; 26: 373-385.
61. Laemmli U K: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680–685.
62. Eichenbaum G, Damsch S, Looszova A, Vandenberghe J, Van den Bulck K, Roels K, Megens A, Knight E, Hillsamer V, Feyen B, Kelley M F, Tonelli A, Lammens L: Impact of gavage dosing procedure and gastric content on adverse respiratory effects and mortality in rat toxicity studies. J. Appl. Toxicol. 2011; 31: 342–354.
63. Wong C K, Ooi V E C, Ang P O: Protective effects of seaweeds against liver injury caused by carbon tetrachloride in rats. Chemosphere 2000; 41: 173–176.
64. Smyth R, Turton J A, Clarke C J, York M J, Dare T O, Lane C S, Munday M R: Identification of superoxide dismutase as a potential urinary marker of carbon tetrachloride-induced hepatic toxicity. Food Chem Toxicol 2008; 46: 2972-2983.
65. Smuckler E A, Iseri O A, Benditt E P: An intracellular defect in protein synthesis induced by Carbon tetrachloride. The Journal of Experimental Medicine 1962; 116: 65-72.
66. Smyth R, Munday M R, York M J, Clarke C J, Dare T, Turton J A: Comprehensive characterization of serum clnical chemistry parameters and the indentification or urinary superoxide dismuatese in a carbon tetrachloride-induced model of hepatic fibrosis in the female Hanover Wistar rat. Int. J. Exp. Path. 2007; 88: 361-376.
67. http://www.bio-rad.com/en-si/applications-technologies/protein-extraction-cleanup; Last view: 9.12.2013.

68. Méchin V, Damerval C, Zivy M: Methods in Molecular Biology: Total protein extraction with TCA-Acetone. Humana Press Inc.; Totowa; 2007.
69. Schuchard M, Mehigh R, Cockrill S, Wildsmith J, Kappel B: Identification of human serum proteins by 2DE and MALDI Mass spectrometry otherwise masked by albumin using the ProteoPrep™ Blue albumin depletion kit. Sigma-Aldrich Biotechnology; 2004.
70. ReadyPrep™ 2-D Cleanup Kit, Instructual Manual. Bio-Rad Laboratories, Inc. Catalog #163-2130.
71. Liu S, Zhang Y, Xie X, Hu W, Cai R, Kang J, Yang H: Application of Two-dimensional electrophoresis in the research of retinal proteins of diabetic rat. Cellular & Molecular Immunology 2007; 4: 65-70.
72. Berkelman T, Brubacher M G, Chang H, Cross T, Strong W: Tips to prevent streaking on 2-D gels. Bio-Rad Bulletin 3110; 2000.
73. Wildgruber R, Harder A, Obermaier C, Boguth G, Weiss W, Fey S J, Larsen P M, Görg A: Towards higher resolution: Two-dimensional electrophoresis of Saccharomyces cerevisiae proteins using overlapping narrow immobilized pH gradient. Electrophoresis 200; 21: 2610-2616.
74. Westbrook J A, Yan J X, Wait R, Welson S Y, Dunn M J: Zooming-in on the proteome: Very narrow-range immobilised pH gradient reveal more protein species and isoforms. Electrophoresis 2001; 22: 2865-2871.
75. Rabilloud T, Chevallet M, Luche S, Lelong C: Two-dimensional gel electrophoresis in proteomics: Past, present and future. Journal of proteomics 2010; 73: 2064-2077.