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Shubhangi Bhide Kshirsagar
Pharmaceutical Science
Career Point University
Kota, Rajasthan, India

Dr. Vikas Jain
Pharmaceutical Science, Career Point University Kota, Rajasthan, India

Corresponding Author:
Dr. Vikas Jain
Pharmaceutical Science, Career Point University Kota, Rajasthan, India

Bioassays of lipids: A review

Shubhangi Bhide Kshirsagar and Dr. Vikas Jain

Abstract
Many liver diseases are major health problems in worldwide. Nearly every acute as well as chronic liver injury can be experimentally induced; necrosis, hepatic injuries, steatosis, cholestasis and cirrhosis. The magnitude of hepatoprotective effect can be measured through survival rate, biochemical makers or histology of the liver. Hyperlipidemia is a condition which is characterized by cholesterol in the form of LDL (low density lipoprotein), VLDL (very low density lipoprotein) and chylomicrons, etc. It is necessary to discover new moieties that help to prevent liver diseases. Examinations aiming to discover natural or synthetic chemical compounds belonging hepatoprotective activity have been undertaken. Development of new moieties of antihyperlipidemia drugs is very challenging job for researchers, the reason behind this is that most of the screening techniques are time consuming. The development of particular new drug consists of number of steps starting from discovery of pharmacological effects in animal model to demonstrate their safety as well as efficacy in human beings. Various models for evaluation hepatoprotective activity include *in vivo* and *in vitro* models which can be found in medical literature. Ex vivo models are also part of evaluation techniques. These models are somewhat better co-related to clinical models as compared to *in vivo* or *in vitro* models. The goal of this review is to describe the purpose, features, disadvantages, advantages of the each model, biochemical parameters and hepatotoxic compounds most commonly used. Biochemical parameters are useful to evaluate liver damage in different models.

Keywords: Hepatoprotective, liver injury, biochemical makers, hyperlipidemia, hepatotoxicity etc.

Introduction
The liver is a key organ; it regulates different functions of the body, such as metabolism, secretion, storage and detoxification. Liver damage is usually associated with the distortion of some of these functions. The liver is continuously exposed to an elevated amount of toxic agents, because the portal vein supplies blood to this organ after intestinal absorption [1, 2]. The World Health Organization (WHO) determined that around 2.4 million deaths yearly are linked to some liver disorders and that around 800 thousand of these deaths are attributable to cirrhosis [3]. On the other hand, epidemiological studies conducted by the National Institute of Statistics and Geography (INEGI by its Spanish acronym) indicate that in 2013 in Mexico, over 600 thousand deaths were recorded. Metabolism of hepatotoxic agents is a detoxifying process where molecules are surgically modified into less toxic shapes by different enzymatic systems. The liver's ability to be able to carry out the different oxidative metabolisms is associated with the high cytochrome P450 cell content [4]. Due to the high metabolite biotransformation rate, free radicals can be generated continuously. Most hepatotoxic substances, mainly damage the liver because of the generated oxidative stress; oxygen reactive species induced arise in lipid peroxidation, a reduction of ATP and oxidative damage in the DNA and proteins [5-8]. Protecting the liver from the harmful effects of hepatotoxins which may be counteracting the alterations in the anti-radical defense mechanisms, is very important; the agents capable of doing this are called hepatoprotective [9]. Many allopathic hypolipidemic drugs like statins are available in the market, but they cause many side effects like hyperuricemia, diarrhea, myositis, hepatotoxicity etc. As they are mainly enzyme inhibitors, so they may be inhibit other grave enzymes in the body. Moreover, statins are intake on a long-term basis so it causes chronic toxic effects over a life time use. For this reason, researchers have been developed in the search of natural or synthetic compounds with hepatoprotective activity [10]. The development of new pharmaceuticals consists of a variety of steps, going from the discovery of pharmacological side effects in cellular and animal models, to finally prove its safety and efficacy in humans [1].
Hyperlipidemia is a disarray of lipid metabolism produced by elevation of plasma concentration of the diverse lipid and lipoprotein fractions, which are the source of cardiac disease. It is define as increase serum TC, TG, VLDL, LDL and HDL which are responsible for different complications like: heart attack, coronary artery syndrome, stroke, atherosclerosis,

myocardial infarction and pancreatitis. Hyperlipidemia can be either primary or secondary type, the primary syndrome may be treated by hypolipidemic drugs, but secondary induced by diabetes, hypothyroidism or renal lipid nephrosis which treated by treating the original disease moderately than hyperlipidemia.[11]Genetic disorders and lifestyle diet rich in calories, fat, and cholesterol play a vital role to cause dyslipidemia around the world.[12] The main factor which are responsible for hyperlipidemia includes changes in life style habits in which risk factor is mainly poor diet i.e. fat intake greater than 40 percent of total calories, saturated fat ingestion more than 10 percent of total calories; and cholesterol ingestion larger than 300 milligrams per day.[13] For hyperlipidemia large number of synthetic drugs available, not a bit is helpful for all lipoprotein disorders, and each drugs are linked with a number of adverse effects.

Classification of hyperlipidemia

Hyperlipidemia may be classified as either familial (also called primary) caused by definite genetic abnormalities, or acquired (also called secondary) that leads to change in plasma lipid and lipoprotein metabolism.

Familial (primary): -Familial hyperlipidemia is classified as:

Type I - Raised cholesterol with high triglyceride

Type II - High cholesterol with normal level of triglyceride

Type III - High cholesterol and triglycerides

Type IV - Raised triglycerides, and raised uric acid

Type V - Raised triglycerides

Acquired (secondary)

Acquired hyperlipidemia (also called secondary dyslipoproteinemias) in which increased risk of atherosclerosis, when associated with marked hypertriglyceridemia, may cause pancreatitis and various complications of the chylomicronemia disease.

Most ordinary causes of acquired hyperlipidemia are:

1. Diabetes Mellitus (Type 2)
2. Use of drugs such as diuretics, beta blockers, and estrogens etc.
3. The experiments usually employed for screening of antihyperlipidemic activity of new chemical entity could be classified under *in vitro* methods and *in vivo* methods.

Hepatotoxic agents

The molecules which are responsible for liver damage are called hepatotoxins. Now days it is possible to imitate any form of natural origin hepatic disease with different chemical substances. Hepatotoxins may be classified into two categories are as follows:-

Intrinsic

If agent's action is behavior is predictable; there is a period of constant latency between exposure and liver damage development or injure is dose dependent. Eg. - Carbon tetrachloride, ethanol, thioacetamide, acetaminophen.

Idiosyncratic

If agent's action is not predictable but generates liver damage in very small portion of exposed individuals, the injury is not related to the dosage, it occurs after a variable latent period and it is not reproducible in experimental models. Eg. - Isoniazid, halothane, sulfonamides. [14, 15]

Various chemical agents used to induce hepatotoxicity in experimental models for evaluation hepatoprotective agents are as follows:-

Acryl amide

Acryl amide or AA is a water-soluble vinyl monomer and is carcinogenic to humans. In the human body, AA is oxidized to the (epoxide glycidamide) 2, 3-epoxypro-pionamide through an enzymatic reaction involving cytochrome P4502E1. AA undergoes biotransformation by conjugation with glutathione and is probably being the major route of detoxification. Daily dose is 6 mg/kg; IP for 15 days is used for the production of hepatotoxicity in female Sprague-Dawley rats [16].

Adriamycin

Adriamycin or Doxorubicin is a potent cytotoxic agent which has been shown to undergo redox cycling between semiquinone and quinone radicals during oxidative metabolism. A single dose of 10 mg/kg body weight of Adriamycin is given to rats for inducing hepatotoxicity [17].

Alcohol

Alcohol consumption causes fatty infiltration, cirrhosis and hepatitis of the liver. Fat infiltration is a reversible phenomenon which occurs when alcohol replaces fatty acids in the mitochondria. Hepatitis and cirrhosis occurs because of enhanced lipid per-oxidative reaction during the microsomal metabolism of ethanol. These effects of ethanol are a result of the enhanced generation of oxy free radicals during oxidation in liver. [18] The per-oxidation of membrane lipids leads to loss of membrane structure as well as integrity. These results in elevated levels of glutamyl transpeptidase, a membrane bound enzyme in serum. The liver is the most susceptible organ to the toxic effects of ethanol. The damage mechanism is due to the metabolism of ethanol by the CYP2E1 form of the cytochromeP450 producing oxidative stress with the generation of reactive species of oxygen and the increase of lipid per-oxidation, leading to the alteration of the compositions of phospholipids of the cellular membrane. [19, 20]Membrane lipid peroxidation results in the loss of its structure and integrity, elevating serum levels of glutamyl transpeptidase, a membrane-bonding enzyme. Ethanol inhibits glutathione peroxidase; it reduces the activity of catalyses and dismutase superoxide. [21]The decrease in the activity of antioxidant enzymes, dismutase superoxide and peroxidase glutathione is believed to come as a result of the harmful effects of free radicals produced after exposure to ethanol, or alternatively, they could be a direct effect of acetaldehyde, a product of ethanol oxidation. Also ethanol induces liposis to a different degree depending on its dose, route and time of administration such as:

1. A single dose of ethanol (1ml/kg) induces fatty degeneration.
2. Administration of 40%v/v ethanol (2 ml/100gm per day p.o.) for 21 days makes fatty liver.
3. Administration of country made liquor (3ml/100 gm per day p.o.) for 21 days makes liposis. Continuous administration of ethanol (7.9 g/kg body weight/d) for a period of six weeks causes liver damage in rats [22].

Antitubercular drugs

Isoniazid is metabolized to monoactyl hydrazine, which is further metabolized to a toxic product by cytochrome P450 leading to hepatotoxicity. Patients on concurrent rifampicin therapy have an increased risk of hepatitis [23].

Cadmium

Cadmium exposure causes testicular atrophy, anemia, renal dysfunction, hepatic damage, hypertension, and central nervous system injury. Cadmium induces oxidative damage in different tissues by enhancing per-oxidation of membrane lipids in tissues and altering the antioxidant systems of the cells. Cadmium is given orally (3 mg/kg body weight/d) as (cadmium chloride) $CdCl_2$for three weeks to induce hepatotoxicity in rats [24].

Carbon tetrachloride

Carbon tetrachloride (CCl_4) is metabolized by cytochrome P-450 in endoplasmic reticulum and mitochondria with the for-mation of CCl_3O^-, a reactive oxidative free radical, which initiates lipid peroxidation. Dose of CCl_4: 1 ml/kg body weight, IP, 1:1 v/v mixture of CCl_4 and olive oil induces hepatotoxicity. [25] CCl_4 toxicity depends on dosage and duration of exposure. In low dose, effects like loss of Ca^{+2} homeostasis, lipid peroxidation, and release of cytokines are produced; and apoptotic events may be generated, followed by cellular regeneration. In high doses, or if there is a longer exposure, the effects are more severe and the damage occurs during a longer period of time, the patient may develop fibrosis, cirrhosis, or even cancer. [26, 27, 28]$CCl_4$is metabolized by the cytochrome P450 dependent ofmono-oxygenases, mainly through the CYP2E1 is form in the endoplasmic reticulum and mitochondria. [29]Hepatotoxicity is produced by the formation of the trichloromethyl radical (CCl_3), which is highly reactive. These radicals may saturate the organism's antioxidant defense system, react with proteins, attack unsaturated fatty acids, generating lipid peroxidation, reduce the amount of cytochrome P450, which leads to a functional failure with the consequent lowering of protein and accumulation of triglycerides (fatty liver), and alter water and electrolyte equilibrium with an increase of hepatic enzymes in plasma.[30]Lipid peroxidation leads to a cascade of reactions, such as the destruction of membrane lipids, the generation of endogenous toxic substances, which originate more hepatic complications and functional anomalies. For this reason, lipid peroxidation is considered a critical factor in the pathogenesis of liver injuries induced by CCl_4 [31] the inhibition of the radical CCl_3generation is a key point in the protection against the damage generated. Because of this, this model is widely used for the evaluation of pharmaceuticals and natural products with hepatoprotective and antioxidant activity.[32,33]A number of carbon tetrachloride models are invented depending upon its dosage through different routes of administration is as follows:-

a) **Acute hepatic damage:** Acute liver damage, regarded as beside ischemia, hydropic deterioration and central necrosis is caused by oral or subcutaneous administration of CCl_4 (1.25ml/kg). The maximum elevation of biochemical parameters are found to be 24 hours after the CCl_4 administration normally administered as 50% v/v solution in liquid paraffin or olive oil

b) **Chronic reversible hepatic damage:** Administration of CCl_4 (1ml/kg S.C.) twice weekly for 8 weeks generates chronic, reversible liver injury.

c) **Chronic, irreversible hepatic damage:** Administration of CCl_4 (1ml/kg S.C.) twice weekly for 12 weeks produce chronic, irreversible liver injury.

Erythromycin

Erythromycin is a potent macrolide antibiotic which generates free radicals and induces liver toxicity. Erythromycin when given as erythromycin stearate (100 mg/kg body weight for 14 days) or erythromycin (800 mg/kg/d for 15 d) to albino rats produces hepatotoxicity [34].

Galactosamine

This hepatotoxin generates a similar damage to viral hepatitis regarding morphologic and functional characteristics. A single dose can cause hepato-cellular necrosis and fatty liver. It induces the exhaustion of the uracil nucleotide, resulting in the inhibition of RNA synthesis and consequently of proteins. The toxicity mechanism causes loss of the activity of ion pumps and an increase in cellular membrane permeability, leading to enzyme liberation and an increase in intracellular Ca^{+2}concentration, which is considered responsible for cellular death. Galactosamine decreases the bile flow and its content i.e. bile salts, cholic acid and deoxycholic acid. Also, it reduces the number of viable hepatocytes and rate of oxygen consumption. Hepatic injury is induced by intraperitoneal single dose injection of D-galactosamine (800 mg/kg) [35].

Lead

Lead-induced hepatic damage is mostly rooted in lipid peroxidation (LPO) and disturbance of the prooxidant-antioxidant balance by generation of reactive oxygen species (ROS). Hepatotoxicity can be induced by using lead acetate (550 ppm for 21 days in drinking water) [36] or lead nitrate (5 mg/kg body weight daily for 30 days) [37].

Microcystin

Microcystis aeruginosa is a potent hepatotoxin. Mice receiving sub lethal doses of microcystin (20 µg/kg) for 28 weeks developed neoplastic liver nodules [38].

Chloroform

Chloroform produces hepatotoxicity with extensive central necrosis, hepatic cell degeneration, fatty metamorphosis and necrosis either by inhalation or by subcutaneous administration. (0.4 to 1.5ml/kg) [39].

Paracetamol

Paracetamol is a widely used analgesic and antipyretic drug which produces acute hepatotoxicity depending upon its dosage through different routes of administration. Paracetamol administration (800mg/kg i.p.) causes necrosis of the centrilobular hepatocytes characterized by nuclear pyknosis and eosinophilic cytoplasm followed by large excessive hepatic lesion without steatosis. Dose of Paracetamol that causes hepatotoxicity is 2 g/kg P.O. It gets 48 hours to provoke the toxicity [39].

Tamoxifen

Tamoxifen citrate (TAM) is a non-steroidal anti-estrogen drug used in the treatment and prevention of hormone dependent breast cancer. At high doses, it causes liver carcinogenicity in rats, due to oxygen radical over production and lipid peroxidation via formation of lipid peroxy radicals. An i.p. dose of 45 mg/kg/d of tamoxifen citrate in 0.1 ml dimethylsulfoxide and normal saline for 6 days induce hepatotoxicity in rats [40].

Thioacetamide

It is an organic compound containing sulfur, originally used as a fungicide and currently used for the treatment of leather, in labs and in the textile and paper industries.[28]It can (dose 100mg/kg S.C.) induce acute and chronic hepatic injuries after 48 hrs of administration by causing sinusoidal congestion and hydropic swelling with increased mitosis. It acts over the synthesis of protein, DNA, RNA and over glutamyl transpeptidase (GGT) activity. Thioacetamide is bio-activated by the CYP450 and/or by the mono-oxygenize system, which contains flavin, converting the compound into sulfine (a sulfoxide-type compound) and later into sulfone compounds. Sulfine is responsible for generating an increase in the nucleus volume, nucleoli enlargement, an increase in intracellular concentration of Ca^{+2}, generating changes in cellular permeability and mitochondrial dysfunction. On the other hand, Sulfone compounds are responsible for the liberation of nitric oxide synthase and the nuclear factor kappa B, protein denaturalization and lipid peroxidation.[41,42,43,]Thioacetamide is reported to interfere with the movement of RNA from the nucleus to cytoplasm which causes membrane injury. A metabolite of thioacetamide is responsible for hepatic injury. Thioacetamide reduces the number of viable hepatocytes and rate of oxygen consumption as well. It also decreases the volume of bile and its content i.e. bile salts, cholic acid and deoxycholic acid. I.P. dose of thioacetamide that causes hepatotoxicity is 200 mg/kg, thrice weekly for 8 weeks [44].

Acetaminophen

It is an analgesic antipyretic analgesic. In high doses, it produces acute liver damage, causing necrosis of the hepatocytes. It is a widely used experimental model of clinical importance as an example of drug-induced liver damage.[21]At therapeutic doses, it is mainly metabolized to glu-curonic or sulfated and excreted derivatives, the rest metabolizes to intermediate reactive, which are eliminated by conjugation with glutathione. At overdoses, the excess is oxidized by the cytochrome P450 (mainly theCYP2E1) [45] at N-acetyl-p-benzoquinone (NAPQI), which quickly attaches to glutathione. Under excessive conditions of NAPQI and glutathione depletion, a covalent bond of metabolite to proteins, adduct formation, mitochondrial dysfunction and oxidative stress occurs. The result is necrosis or hepatocellular death[46, 47].

Tert-Butyl hydro-peroxide (t-BuOOH)

Metabolized to free radicals by cytochrome P450 in hepatocytes generating lipid peroxidation, a decrease of glutathione, it reduces the potential of the mitochondrial membrane and cellular damage; generated damage is similar to oxidative stress, which occurs in cells and tissues. [48, 49] Alternatively, t-BuOOH can be converted by glutathione eperoxidase into tert-butyl alcohol and glutathione disulfide (GSSG). GSSG is converted into reduced glutathione (GSH)by the GSSG reductase, generating the oxidation of pyridinenucleotides (NAPD). All these events alter the homeostasis of Ca^{+2}which is considered a critical event to provide openings in the plasmatic membrane, and thus cellular damage [50].

Liver function markers

A decisive step when biological activity models are performed is the analysis of the activity of the tested analyte. Depending on the selected model and its characteristics, the survival rate and the damaged biochemical markers can be determined. Due to the wide variety of functions per-formed by the liver, there is a wide range of markers through which we are able to determine the functionality or damage generated by this organ or its cells. [27]Although there is no biochemical marker specific to liver damage, the combination of several of these, and knowing the correlation they have with the liver, will help to better interpret the results of the hepatoprotective models. Markers can be divided into tests related to the liver's excretory function (bilirubin), tests related to synthetic function (albumin and prothrombin time) and tests related to the integrity of hepatocytes (transaminases, alkaline phosphatase, GGT).

Transaminases or aminotransferases

Transaminases or aminotransferases are enzymes that transfer a group of amino from an amino acid to an acid alpha-acetate. This process is an important step in themetabolism of amino acids. The aspartate aminotransferase (AST) and the alanine aminotransferase (ALT) arewidely used enzymes; the increase in the liberation ofthese transaminases is linked to liver dysfunction. ALT catalyzes the amino group transference of the L-alanine to alpha-ketoglutarate to produce pyruvate and L-glutamate; it iselevated in hepatic and renal diseases, *i.e.* hepatitis, cirrhosis and mononucleosis. AST catalyzes the transferenceof the amino group of the L-aspartate to alpha-ketoglutarate toproduce oxaloacetate and L-glutamate; the heart, liver andskeletal muscle, are organs rich with this enzyme and theAST liberation is proportional to the damage generated. In amyocardial infarction it starts to increase between 3 and 9 hr after the event, reaching its peak on the second day; thelevels normalize between the fourth and the sixth day. Inhepatitis cases, observed elevations are between 7 and 12 times its normal concentrations, with increases of up to 100times [27].

Phosphatases

These enzymes belong to the hydrolases family and are known for their ability to hydrolyze a wide variety of organophosphate compounds with the formation of phosphate ions and alcohols. Clinically relevant phosphatases are acid phosphatase and alkaline phosphatase. Alkaline phosphatase (ALP) is produced mainly in the liver and bone; when there are no osteogenic diseases, ALP elevation is generally linked to hepatobiliary diseases. It is more specific in obstructive hepatic processes [27, 51].

Transpeptidase ϒ-glutamine (GGT)

This enzyme is bound to the plasmatic membrane, which catalyzes the transference of the ϒ-glutamine group of a peptide to itself or other peptides. It is located mainly in hepatocytes; however it can also be found in the proximal renal tubules, intestinal epithelial cells and the prostate. High GGTP levels usually indicate infection in the liver, pancreatic and biliary zones. The specificity of the test is relatively low, but since it is not linked to bone diseases, it is used to link high ALP levels to liver damage [51].

Bilirubin

Bilirubin is the most important metabolite of the hem group, found in hemoglobin, myoglobin and cytochromes.It is highly insoluble in water in its most common isomeric form, and most of it is transported by albumin. The liver is responsible for eliminating bilirubin by turning it to amore hydro-soluble compound, thus allowing its elimination of plasma for its

eventual excretion. It is the most importanttest of the hepatic metabolic function; however, it is only possible to determine it in in-vivo models [51].

Total proteins

The liver synthesizes most plasmatic proteins, and in most hepatic diseases the levels are reduced. Albumin, α-1 antitrypsin, ceruloplasmin, and α-fetoprotein are proteins linked to acute liver damages [39].

Lactato deshydrogenase (LDH)

Lactato dehydrogenase is an enzyme located in the cellular cytoplasm. It catalyzes the inter-conversion of the lactate and pyruvate. LDH liberation may be interpreted as the opening of the cellular membrane or cellular death. This enzyme is not specific to the liver and it is widely used in in-vitro models because it is expressed in most cellular lines.[52]AST, ALT and ALP are most commonly analyzed in all hepatoprotective models, while the quantification of total proteins and LDH are generally used as parameters of in-vitro cytotoxicitym [53].

Parameters

Parameters which reflect liver conditions are described as follows:-

Transaminases [54]

Transaminase is a process in which an amino group is converted into alpha-keto acid. It is an important step in the metabolism of amino acids. The enzymes responsible for transamination are called tranaminases, it also known as amino-transverses. Two diagnostically helpful transaminases are glutamate oxaloacetate transaminase (SGOT) and glutamate pyruvate transaminase (SGPT). These enzymes catalyze the following reaction.

GOT/AST

L- Aspartate +Oxoglutarate ⟹ Oxaloacetate + Glutamate (or ketoglutarate)

L- Alkaline + Oxoglutarate ⟹ Pyruvate + Glutamate (or ketoglutarate)

Clinical significance

Increased serum transaminase activity is seen in liver dysfunction. Greater activity of SGOT or AST over SGPT or ALT is typical of myocardial infarction.

Serum glutamate pyruvate transaminase (SGPT)

Principle

SGPT (ALT) catalyzes the transfer of amino group from L-alanine to alpha- ketoglutarate to yield L- glutamate and pyruvate. Then, lactates dehydrogenises convert pyruvate and NADH into NAD and lactate. The transfer taking place NADH to NAD decreases the fascination at 340 nm. The rate of reduces in absorbance is measured and is proportional to the SGPT activity.

GPT (ALT)

L-alanine + ketoglutarateL-Glutamate + pyruvate ⟹

LDH

Pyruvate +NADH + H^+ ⟹ Lactate + NAD^+

Clinical significance

Elevation of SGPT (ALT) activity is found in liver and kidney diseases like infectious or noxious hepatitis, mononucleosis and contagious cirrhosis. Extreme amplify is also originate in obtrusive jaundice, hepatic obstruction, metastasis carcinoma and myocardial infarction. SGPT levels may be decrease in patients undergoing long term hemodialysis without supplemental vitamin therapy.

Normal value: - SGOT (AST): 7 – 21 U/L; SGPT (ALT): 6 – 21 U/L

Serum glutamate oxaloacetate transaminase (SGOT)

Principle

SGOT or AST catalyzes the transfer of the amino group from L- aspartate to alpha – ketoglutarate to yield oxaloacetate and L- glutamate. Then, malate dehydrogenises (MDH) convert oxaloacetate and NADH in the direction of NAD and malate. The adaptation of NADH toward NAD decreases the absorbance on 340 nm, the time of which relative towards the SGOT movement.

GOT (AST)

L- Aspartate + ketoglutarate Oxaloacetate + L-glutamate ⟹

MDH

Oxaloacetate +NADH +H^+ ⟹ L-malate +NAD^+

Clinical significance

Organ rich in SGOT are heart, liver or skeletal strength, when a few of these organs are dented, serum GOT intensity rise in proportion to the severity of damage. In myocardial infarction SGOT starts increasing by 3 to 9 hours, peaks on second day return to normal on 4th-6th day. In hepatitis, SGOT peaks usually between 7 to 12 days and any increase up to 100 times. Increased levels SGOT are also found in pancreatitis mononucleosis, trauma of renal necrosis skeletal muscle, and cerebral necrosis.

Phosphatases [54]

Phosphates belong to the class of enzyme called as hydrolyses. They are characterized by their ability to hydrolyze a large variety of organic phosphate with the formation of an alcohol and phosphate ions. Phosphates of diagnostic significance are acid and alkaline phosphates. These are distinguishing through their reaction in acidic and alkaline medium. The PH for determining the alkaline phosphates' action is 10 and in favor of acid phosphates it is 5.

Alkaline Phosphatase (ALP)

Principle

The substrate, p-nitrophenyl phosphate (PNPP) is hydrolyzed by ALP to p- nitro-phenol and phosphoric acid. Some divalent ions like Mg^{++} are added to the system which acts like activators. PNPP is colorless into acidic medium or alkaline medium while PNP is yellow in color in the alkaline medium and colorless in the acid medium.

ALP/ACP

P-Nitrophenyl phosphate + H_2O ⟹ p- nitro-phenol + H_3PO_4

Clinical significance
Increased alkaline phosphatase activity may be related to hepatobiliary bone sickness. Very elevated alkaline phosphates' movement in serum is seen in patients with bone cancer and marked increased also occur in obstructive jaundice and biliuary cirrhosis. Moderate elevations have been noted in case of Hodgkin's sickness, infective congestive hepatitis, heart failure, and abdominal troubles.

Normal value: - Alkaline phosphatase (ALP**)**: 20 to 100 U/L

Gamma-Glutamyl Transpeptidase (GGTP) [39]:-
Principle
Gamma–glutamyl transpeptise catalyses transfer of gamma-glutamyl group from the substrate gamma- glutamyl p - nitroanilide to glycylglycine releasing free P- nitroaniline which absorbs illumination at 405 nm. Enzyme activity is comparative to the enhance absorbance at this wave length.

GGTP
G-glutamyl nitroanilide + glycylglycine ⇨P-nitroaniline + Glutamyl glucylglycine

Clinical significance
Elevated serum GGTP levels appear to be indicative of infection of liver, biliuary area and pancreas. Serum GGTP action is usually elevated in the cases of cholangitis, cholesystitis, cholelithiasis, viral hepatitis chronic hepatitis, and metastatic carcinoma. GGTP is particularly helpful in clinical assessment of alcoholic cirrhosis. While serum GGTP is not eminent in any appearance of bone disorder, it examine has been important in differentiating bone and liver disease in conjunction with alkaline phosphatase determination.

Normal values: Gamma-glutamyl transpeptidase (GGTP): 5 –24 IU/L

Serum Bilirubin
Bilirubin in serum would only react with daizo reagent in the existence of alcohol, after the proteins have been uninvolved by precipitation. Adding of alcohol just before the reaction give positive test for both conjugated and unconjugated bilirubin stain. Unconjugated bilirubin altitude is after estimate by subtracting direct bilirubin value from this total value. Normally, 0.25 mg/dlof conjugated bilirubin is present contained by the blood of a growing person. Bilirubin gets higher in disease of hepatocyte, obstacle to biliuary secretion into duodenum, within hemolytic and defect of hepatic uptake and conjugation of bilirubin treatment such as Gilbert's disease [55].

Serum Protein
Liver cells synthesize albumin, prothrombin fibrinogen, hepatoglobin, alpha- 1antitrypsin, transferring alpha fetoproteins ceruloplasm, and acute stage proteins. The blood levels of these plasma proteins are decreased in extensive liver injured. A consistently predictable whole protein is in the ordinary vary of 5.5 to 8 gram/dl. Hypoalbuminanemia may possibly take place in liver disease having significant destruction of hepatocytic. Hyperglobulinaemia may possibly current in unceasing inflammatory disorders such as in cirrhosis and chronic hepatitis [55].

Liver function tests
Selection of tests is accessible to right and proper use of models. The variety of the test is subjective by its effortlessness, comparison, particular function and reliability. Some important liver function tests are given as follows [56]:-
Test for bilirubin metabolism
- Estimation of serum bilirubin
- Urinary bilirubin
- Urine urobilinogen

Test of protein synthesis and metabolism
- Estimation of plasma protein
- Albumin Globulin ratio (A/G ratio)
- Flocculation tests
- Plasma prothrombin and prothrombin time

Test based on excretory function serum alkaline phosphatase
- Bromsulphthalein (BSP) Excretion

Test to assess hepato-cellular damage
- Serum enzyme estimation

Screening methods for evaluation of antihyperlipidemic activity
Nearly every acute as well as chronic liver injury can be experimentally induced; necrosis, hepatic injuries, steatosis, cholestasis and cirrhosis. The magnitude of hepatoprotective effect can be measured through survival rate, biochemical makers or histology of the liver. Test methods may be *in vitro*, *in vivo* or ex vivo and each one of them can be evaluated to see if the new compound is hepatoprotective, depending on if the hepatocurative agent is administered before or after the hepatotoxin.

***In vitro* models**
Primary hepatocyte cultures, fresh hepatocytes and immortalized cell lines are used to measure the hepatocurative or hepatoprotective effect. These models represent the best option for selection and screening of potential hepatoprotective compounds. Also, it is possible to establish action mechanisms at cellular as well as molecular level. [21, 31]Primary hepatocyte cultures maintain the normal metabolic liver properties, but it is difficult to maintain them for longer time. On the other hand, cell lines maintain their properties for a longer time and can be cryopreserved, but carcinogenic or immortalized lines may differ in biochemical and metabolic aspects from normal cells. [1]In order to evaluate protection parameters like cell multiplication, morphology, transaminase liberation, oxygen consumption, macromolecular synthesis, etc., are measured [57, 58].

***In vitro* assay using Caco-2- cell lines**
This method is based on measurement of lipid which secreted from human intestinal epithelium cell from colon cancer cell line i.e. Caco-2.

Procedure: - Caco-2 cells are seeded in well plates which contains 10% fetal bovine serum penicillin and streptomycin for 2 days. The differentiations of Caco-2 cells are initiated by addition of sodium butyrate for 4 days. After an incubation period, Caco-2 cells will be converted into intestinal epithelium cells and plenty of microvilli can be observed on apical side of cell after observing under microscope. After that sodium oleate is added into the medium which contains culturing cells and then differentiated Caco-2 cells secrete lipoprotein to medium through the micro-porous membrane.

This secreted 4 class fractions such as chylomicron, high density lipoproteins, VLDL and LDL are determined. [59-62]

Inhibition of the isolated enzyme HMG-CoA reductase [63]
Purpose: HMG CoA reductase is an important regulating enzyme involved in the cholesterol biosynthesis from acetyl CoA. This enzyme reduces 3-hydroxy, 3-methyl glutacyl CoA (HMG CoA) to mevalonate. Due to inhibition of this enzyme, cholesterol is not synthesized. Hence, this *in vitro* model is issued to evaluate the hypolipidemic activity of a chemical moiety. The existing statin derivatives exhibit their hypolipidemic activity through this enzyme inhibiting mechanism. For screening purposes; studies on the inhibition of HMG-CoA reductase obtained from rat liver microsomal fraction can be used.

Requirement
Chemical: - Dithiothreitol
Animal: - Rats

Procedure: - The inhibitory activity of the test compound on HMG-CoA reductase is estimated with soluble enzyme preparations which are obtained from the microsomal fraction of rat liver. The enzyme reaction is carried out with 50 µl partially purified HMG-CoA reductase in buffer containing Tris, EDTA and dithiothreitol at pH 7.5, 20 µl of 910 HMG-CoA solution containing 100 nCi (3.7 KBq) of 14C-HMG-CoA and 20 µl of NADPH regenerating system (5.2 × 10–2 M glucose- 6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 5.3 × 10–3 M NADP), with the actual concentration of NADPH. The final incubation volume is 200µl. The main reaction is preceded by 20 min pre-incubation with the NADPH regenerating system at 37°C, followed by 20 min incubation at 37°C of the completed samples with the test compound or the standard and stopped by addition of 75 µl 2 N HClO4. After 60 min room temperature, the samples are cooled in anice-bath and neutralized by addition of 75 µl 3 N potassium acetate. Supplementing the volume with water to 500 µl, the precipitate is centrifuged and 250 µl of the clear supernatant are applied to a column (0.6 × 8.0 cm) of BIORAD AG1-X8 (100–200 mesh). Mevalonolactone is eluted with water discarding the first 750 µl and collecting the next 3 500 µl. Five hundred µl of the eluate are used for measurement in duplicate, mixed in vials with 10 ml Quickscint (Zinsser) and measured in a liquid scintillation counter (Beckman). The assay is generally performed in triplicate. Generally, Lovastatin sodium is used as standard.
Inhibition of HMG CoA reductase induces expression of LDL receptors in the liver, which lowers plasma concentration of cholesterol. This is an NADPH-dependent reaction. HMG-CoA assay kits are commercially available. This kit consists of HMG-CoA reductase assay buffer, HMG CoA reductase, NADPH, and an inhibitor (atorvastatin). Assay kit measures the utilization of NADPH, which can be measured by the decrease of absorbance at 340 nm. By using this type of kit, measurement of activity of purified enzyme is possible in addition to testing of HMG-CoA reductase inhibitors.[64, 65] And percentage inhibition is calculated which is ratio of absorbance of enzyme with inhibitor to absorbance of enzyme.

Evaluation: The mean values with and without inhibitors are compared for the calculation of inhibition and IC50 values are calculated.

Advantages of *in vitro* models
They are quick tests (between 2-3 testing days), they require small amounts of the test substances (milligram range) and the experimental conditions may be strictly controlled; different samples may be analyzed in the same test, they are cheap tests and there is little variability; therefore they are considered are producible test. In the case of primary cultures or fresh hepatocytes, they require few experimental animals in comparison to *in vivo* models.

Disadvantages of *in vitro* models
Cells do not function independently in the organism; on the contrary, they form close and complicated nets with each other and with the extracellular matrix; therefore, this should be taken into consideration when interpreting *in vitro* data and should experiments be verified with *in vivo* systems. Isolated cells and cell lines have an elevated cell differentiation rate due to the loss of natural environment. The substances tested do not go through the absorption and distribution processes, which occurs in the organism. There is little to no cell-to-cell interaction and there is no complexity proper of the organ.[46, 66, 67]

***In vivo* models**
This type of model has been widely used; through this model were able to determine the protection mechanism. The damage produced in experimental animals due to known dosage administration of different hepatotoxins and the magnitude of the damage and protection is determined by the different biochemical and metabolic markers, as well as histopathological determinations.

Triton Wistar Rat 1339 Induced hyperlipidemia [68]
Purpose: -The systemic administration of the surfactant triton to rats results in a biphasic elevation of plasma cholesterol and triglycerides.

Requirement
Chemical: -Surfactant, Triton, momordicia diocia roxb
Animal: -Wistar strain male albino rats

Procedure
The animals are maintained in polypropylene cages in the well-ventilated room, at temperature of 25± 1°C with 12:12 h light/dark cycle. Standard pellet feed and filtered tap water should be provided throughout the experimentation period.
42 male wistar rats weighing 190gm to 230 gm were randomly divided into 7 groups. In each group contains 6 male rats and kept in their cages for 5 days prior dosing to allow for acclimization to laboratory condition. The animals were starved for 18hr and i.p. with 10% aqueous solution of triton at 400mg /kg body weight. The test drugs employed (or) the solvent for control was administered simultaneously with triton injection. Serum analyzed made on 24hr and 48 hr after triton injection. The drug was administered in the vehicle in the same volume orally. After administration of triton, in the 24hr, blood was collected by retro orbital puncture under ether anesthesia and subject to centrifugation to obtain serum. Again, 48hr, blood was collected by retro orbital puncture under ether anesthesia and subject to centrifugation to obtain serum with 2ml syringe [69].

Evaluation: Serum was analyzed for serum triglyceride, serum total cholesterol, serum high density lipoprotein cholesterol, serum low density lipoprotein cholesterol, serum

very low density lipoprotein cholesterol, serum glucose. The result is evaluated by ANOVA test and Dunnet Multiple comparison test.

Hypolipidemic activity in rats [70]

Purpose

Hyperlipoproteinemia with increased concentrations of cholesterol and triglyceride carrying lipoproteins is considered to be the cause of arteriosclerosis with its dual sequel of thrombosis and infarction. Lipoproteins are divided into 6 major classes: chylomicrons, chylomicron remnants, very low density lipoproteins, intermediate density lipoproteins, low density lipoproteins, and high density lipoproteins. High density lipoprotein promotes the removal of cholesterol from peripheral cells and facilitates its delivery back to the liver. Therefore, increased levels of high density lipoproteins are desirable. On the contrary, high levels of very low density lipoproteins and low density lipoproteins promote arteriosclerosis. Low density lipoproteins, especially in its oxidized form, is taken up by macrophages via a scavenger mechanism. Therefore, anti-arteriosclerotic drugs should reduce very low density lipoproteins and low density lipoproteins and/or elevate high density lipoprotein.

Requirement

Chemical

Methanol extract of trianthema portulacasstrum

Animal

Wistar albino male rats

Procedure

Groups of 10 male Wistar rats weighing 180–200 g are used. They are given once daily in the morning over a period of 8 days the test compounds or the standard in various doses ranging from 1 to 100 mg/kg via stomach tube in a volume of 5 ml/kg. The control group is given the solvent (e.g., PEG 400) only. Body weight of each animal is registered at the beginning and at the end of the experiment. Twenty hours prior to the experiment food but not water is withdrawn. On the morning of the first day, blood samples are taken under light ether anesthesia by retro orbital puncture. Then, the first dose is applied. During the whole period, the animals have free access to food and water. Twenty hours prior the end of the experiment, food is again withdrawn and blood samples are taken by retro orbital puncture. Immediately thereafter, the animals are sacrificed and the liver removed, blotted free from blood and weighed. Samples of liver are frozen analysis. The bloods samples are centrifuged for 2 min. Total cholesterol and total glycerin as a measure of triglycerides are determined in each blood sample. To estimate the serum lipoproteins, the serum of each rat group is pooled. The serum lipoproteins are separated by means of a preparative ultracentrifuge e.g., KONTRON TGA 65, Rotor TFT 456. The separation of fractions very low density lipoprotein, low density lipoprotein, high density lipoprotein and of the subnatant of high density lipoprotein is carried out as follows: very low density lipoprotein native density of the serum (1.006), 16 h at 40000 rpm, Low density lipoprotein density range from 1.006 to 1.04, 18 h at 40 000 rpm, High density lipoprotein density range from 1.04 to 1.21, 18 hr at 40 000 rpm, Subnatant of High density lipoprotein density > 1.21. The density is adjusted by addition of a calculated amount of NaBr solution. Cholesterol is determined using Boehringer Mannheim test combinations by the CHOD-PAP high performance method and triglycerides by means of an enzymatic assay.

Evaluation: Cholesterol is determined using Boehringer Mannheim test combinations by the CHOD-PAP high performance method and triglycerides by means of an enzymatic assay.

Fructose induced hypertriglyceridemia in rat [71]

Purpose: -Rats switched from a diet low in carbohydrates and high in protein to a high intake of fructose, develop an acute hypertriglyceridemia. Compounds are tested for inhibition of this phenomenon.

Requirement

Chemical: -Serratia Liquefaciens

Animal: Sprague Dawley Rat

Procedure

Male Sprague Dawley rats weighing 200–250 g are fed over a period of one week a diet enriched in protein with reduced carbohydrate content. Groups of 10 animals are treated for 3 days daily with the test compound or the standard or the vehicle (polyethylene glycol) by oral gavages. From the second to the third day water is with held for a period of 24 h. immediately afterwards, the animals are offered 20% fructose solution and libitum for a period of 20 h. After this time which is also 20 hr after the last application of the test compound, the animals are anesthetized with ether and 1.2 ml blood is withdrawn by retro orbital puncture. The blood is centrifuged for 2 min at 16 000 *g*. Total glycerol is determined in the serum according to Eggstein and Kreutz (1966) and total cholesterol according to Richterlich and Lauber (1962).

Evaluation: The average values of total glycerol and total cholesterol of the treated groups are compared with the control group using Student's *t*-test.

Effect of HMG-CoA-reductase inhibitors *in vivo* [63]

Purpose and Rational: A strain of rabbits with heritable hyperlipidemia, the WHHL strain, has been described by Watanabe. These animals develop digital xanthoma and aortic and coronary atherosclerosis already at an early age. This animal is considered to be a suitable model for the evaluation of preventive or even regressive effects of drugs on hyperlipidemia and atherosclerosis.

Requirement

Chemical: Glutathione, Lovastatin

Animal: Zucker obese rat, WHHL rabbits

Procedure

Male heterozygous WHHL rabbits weighing 1.8 to 2.5 kg at an age between 8 and 20 weeks are used. The animals are housed individually under standard conditions and are allowed to accommodate 2 weeks prior to treatment. The test compounds are suspended in 0.5% methylcellulose and are administered each day orally by gavages in the afternoon to insure an increased plasma level at night, since in man HMG-CoA reductase activity has been found to be higher at night than during daytime (Shapiro and Rodwell (1969); Shefer (1972) similar to the enzyme in rodents. The treatment is continued for 14 days. Blood samples are taken in the

morning without previous feeding. Two ml of blood are drawn from the outer ear vein 5 days prior to the beginning of treatment, on days 3 and 8 of treatment and 30 days after the end of treatment for the determination of biochemical parameters. In addition, 6 ml blood is drawn at the first and the last day of treatment and 10 days after the end of treatment for determination of biochemical parameters and lipoprotein profile.

Evaluation

The separation of serum lipoproteins by gel permeation chromatography is performed according to Ha and Barter. Student's paired *t*-test is used to calculate for each group the significance of difference between mean values.

Cholesterol-diet induced hyperlipidemia and atherosclerosis in rats [72]

The way, 2% cholesterol diet is given to rats for 6 weeks. After 6 weeks plasma cholesterol and triglyceride levels become increased. The essential molecular mechanisms of the direct effects of cholesterol diet induced hyperlipidemia reticence of mevalonate pathway, decrease in cGMP metabolism and bioavailability, increase in free radical and peroxynitrite formation, inhibition of heat shock response, and expression of oxidized low-density lipoprotein receptors which induces apoptosis have been shown to play role in the effects of hyperlipidemia.

Cyclosporin A-induced hyperlipidemia

Cyclosporin is injected to male Wistar rats intraperitonally 10mg/kg daily for 2 weeks. After 2 weeks, cholesterol, bilirubin, bile flow and biliuary secretion can be measured by insertion of fistula into rats. From blood or serum samples concentration of these parameters is firmed. [73]CsA (cyclosporine)is basically an immunosuppressant drug widely used in organ transplant recipients and patients with autoimmune disorders. Cyclosporine is allied with hyperlipidemia after long term deployment. [74]

Poloxomer 407 induced hyperlipidemia

Poloxomer 407 is injected intraperitoneally to rat at dose of 1g/kg. After 15 and 24 hours, blood samples are collected and examined for cholesterol parameters. Before this, for 26 days food and tested drug daily specified to animals. Poloxomer 407 is non-ionic hydrophobic surfactant. It causes hypercholesterolemia by targeting enzyme in chosterogenesis, HMG-CoA reductase which is rate limiting enzyme in cholesterol synthesis. [75]

PTU-induced Hyperlipidemic Rat Model

This method requires short time. Hyperlipidemia is induced by propyl-thiouracil (PTU). PTU is a drug used for hyperthyroidism. It produces hypothyroid state associated with increased total cholesterol, LDL, VDL, and triglycerides. Here, 32 rats are required and divided into 5 groups, and a high dose of cholesterol is injected to all groups 6 hrs before evaluation of total cholesterol, VLDL, and LDL. PTU at a dose of 10 mg/kg body weight of rat is given to all groups except control group for 7 days. PTU at a concentration of 0.01% is given for 7 days. Standard drug should be given to Groups 4 and 5. Measurement of total cholesterol in serum, feces, and liver extract and comparison with control group measurements is carried out finally [76].

High-Fat Diet (HFD)-Induced Hyperlipidemia (Chronic Model): High intake of cholesterol and fats diet leads to an increase in the triglycerides and fatty acid level in serum and induce the hyperlipidemia. This results in the generation of atherosclerosis and various cardiovascular diseases. [77, 78, 79] This response produced in rodent is very similar to symptoms produced in human during hyperlipidemia conditions. Hence, high-fat-induced rat model is widely used for screening hypolipidemic agents. This model mimics human hyperlipidemia. In this method, high amount of cholesterol is mixed with vegetable oil and treated with all group except the control group. After the chronic treatment with high fat, 2nd group receives standard drug, 3rd group receive test sample, and 4th group receives only normal diet as considered as control group. At the end of 30th day, collect the blood sample by a suitable method under slight anesthesia. Sacrifice the animals and isolate the organs such as heart, liver, aorta, pancreas, spleen, and kidney, weigh and are subjected to histopathological studies.

Other than this, following models are also used for evaluation of hyperlipidemic activity [80]:-

- Streptozotocin induced diabetic method
- Alloxan induced diabetic method
- Tylaxapol induced hyperlipidemic method
- Hydrocortisone induced hyperlipidemic method
- Atherogenic diet induced hyperlipidemic method

Advantages of *in vivo* models

It is the model with the highest degree of correlation with what occurs in humans and all biochemical and histopathological parameters can be measured. They let us take into account the possible effects of the immune and central nervous systems in the development tof hepatic diseases [81].

Disadvantages of *in vivo* models

They require a large number of animals, and usually the studies are developed for long periods of time, increasing ethical and financial aspects. There is an inter-individual variation, and even though models imitating the different hepatic diseases have been developed, there are relevant differences in the molecular pathogenesis between the model and human species. They require a larger sample size to perform the experiment which may be a limiting factor, especially when analyzing natural products [82].

Ex vivo models

In this type of model, after completion of pre-selected *in vivo* methods protocol hepatocytes are isolated and then percentage of viable cells as well as biochemical parameters are determined as liver function tests. These methods are somewhat better co-related to clinical models as compared to *in vivo* or *in vitro* methods.[83]PCLS (Precision cut liver slices) are an ex vivo tissue culture which resembles multi-cellular characteristics of *in vivo* organs. Spatial disposition and cellular interaction remain intact in this model, with the possibility of performing morphological studies. Liver slices have the characteristic of functionally maintaining metabolizing enzymes and biliary canaliculus [67] they have proven to be a valid ex vivo system to study metabolism and liver damage and function asa bridge between *in vivo* systems and cell cultures [84]. Isolated perfused livers represent model combining *in vitro* characteristics under *in vivo* circumstances. The first model was developed in porcine livers and later the livers of smaller animals such as rats, mice and rabbits. This model preserves the tridimensional structure and the cell-to-cell interactions with the possibility of collecting bile in real time. If blood is used as a perfusor liquid, then hemodynamic parameters may be observed and studied [85].

Advantages of ex vivo models: - They imitates vivo atmospheres, are low cost, reproducible models. In PCLS the number of experimental animals is reduced, and also the model can be developed with human organs.

Disadvantages of ex vivo models: - In PCLS the bile flow and functional parameters, like portal flow cannot be analyzed [1]. There is poor diffusion of oxygen nutrients to the more internal cells, and even with the development of new means of culture, the viability of the slices remains short (8-10 Days). [84]In small laboratory, because of space and budget, the best option is the development of perfused rat liver; however, there are significant differences in the size, geometry and function of the murine liver compared to the human [1].

Animal Models

As we know, there are many medicinal plants which might be source of new phytotherapeutic entities that can be developed into new form of hypolipidemic or antihyperlipidemic drugs. Therefore, to estimate the herbal plants various methods using array of animal species like mice, rats, rabbits are being utilized. Many textures of these models are stellar to human diseases which mainly contribute in disease state. Mostly all of animal models are developed from first to last by number of etiologic factors responsible for development of hyperlipidemia. Several new animal models for hyperlipidemia are being developed as new insights into the pathogenesis of hyperlipidemia and mainly to know how to treat cardiovascular risk factors. Various animal models are used for evaluation of antihyperlipidemic Activity of new drugs which are as follows [86]:-

1. Use of normal hyperlipidemic animal for primary screening

a) Leghorn
b) Rat

2. Use of hyperlipidemic Animals

a) Weanling Rats
b) Adult Rats
c) Rabbit
d) Birds

3. Intermediate animal models

a) Dog
b) Swine

4. Estrogen induced hyperlipidemia in birds

5. Triton induced hyperlipidemia

a) Mice
b) Rat

Use of normal hyperlipidemic animal for primary screening

Normocholesterolemic animals use for preliminary screening has the advantage of rapidity, simplicity and detects inhibitors of the absorption and synthesis of cholesterol.

Leghorn

In this test take white leghorn, 9-11 weeks of age, for which drug was administered in the diet for four consecutive days. Then on 5th day heart is puncture and collects the blood for analyses. Compounds that lowered serum cholesterol by at least 20% at a dose of 400mg/kg were finding vigorous.

RAT

The test drug given orally to the normocholesterolemic rat for four consecutive days and on 5th day serum cholesterol analysis.

Use of hyperlipidemic animals [39]

Hypercholesterolemia is produced in several animal species by including fats and cholesterol in their diets. Drugs that are active against the diet induced hyperlipidema by one or more mechanisms involving inhibition of intestinal absorption of cholesterol, enhancement of cholesterol degradation interference with lipoprotein production and acceleration of the removal of lipoproteins.

Weanling rats

The serum cholesterol levels of weanling rats are usually 2-3 times greater than adult levels. This hypercholesterolemia is sensitive to certain types of drugs like thyroxin and may be insensitive to certain inhibitors of cholesterol biosynthesis. In this model take weanling rats, weighing 60-95 gm, maintained on a normocholesterolemic diet supplemented with the test drug clofibrate for a 2-week interval. Serum level is depressed to 75 mg%. The use of weanling rats can provide a rather simple, rapid, and inexpensive means of testing potential hypocholesterolemic agents.

Rabbit

a. Cholesterol containing diets: - In this method feeding a diet with a high level of cholesterol and fat progressively elevated the serum cholesterol level so that at the end of a 2-week period the mean serum cholesterol has increased to 600 mg%. The test drugs are given in diet and administered to the animals.
b. Cholesterol free semi-synthetic diet: - In this method investigators have reported that feeding rabbits with saturated fat, without cholesterol, induces a hypercholesterolemia more closely related to the human type. This method is use for the screening of drugs affecting lipid metabolism since plasms cholesterol is endogenously synthesized, and its level is limits of that normally encountered in human hypercholesterolemia.

Adult Rats

Diet induced hyperlipidemia is useful only for detecting agents which interfering with the absorption degradation and excretion of cholesterol. In this model take male Sprague–Dawley rats weight about 200- 250 gm, were divided into 5 groups of 6 animals each. The animals of all the groups except normal group are given a lipid diet consisting of 2% cholesterol, 1% cholic acid and 2 ml coconut oil with standard pellet diet for 30 days. The first group (Normal control) is receive normal saline orally for 30 days and second group (High cholesterol diet (HCD)- positive control) is given High cholesterol diet while the third and fourth groups are treated with hydro-alcoholic extract of herbal formulation (200 mg/kg and 400mg/kg, p.o.) once a day for 30 days. The fifth group is treated with Atorvastatin suspension prepared with 0.5% CMC (10mg/kg; p.o.), once a day for 30 days. After 30 days blood is collected by retro orbital sinus puncture, under mild ether an aesthesia. The collected samples are centrifuged for 10 minutes at 2000 r.p.m. and this sample is used for various biochemical tests. The animals are then sacrificed and the liver collected. Biochemical Analysis of Serum samples are analyzed for TG, TC, LDL and HDL levels using standard enzymatic assay kits.

Birds

In this test take white leghorn, 9-11 weeks of age, for which drug was administered in the diet for four consecutive days. Then on 5th day heart is puncture and collects the blood for analyses. Compounds that lowered serum cholesterol by at least 20% at a dose of 400mg/kg were considered active.

Intermediate animal models

Dog

Hypercholesterolemia, which leads to the development of atherosclerosis, is produced in dogs by feeding diets containing cholesterol, bile acids, and possibly the thyroid inhibitor thiouracil. We produced hypercholesterolemia in four-month old dogs, maintain on a diet containing cholesterol (5%) and thiouracil (0.6 gm/day). At the end of one month marked hypercholesterolemia (1000 mg%, 5 times higher than the control value is developed.

Swine and primates

Hypercholesterolemia is produced in swine by feeding diets supplemented with butter, egg yolk, and saturated fats. It is shown that the combination of unsaturated fat with cholesterol in the diet produced the greatest degree of typical atheromatosis and the highest levels of cholesterol in the tissues. Similarly, hypercholesterolemia has been produced in primates by feeding experimental diet containing cholesterol and butter. Total serum- cholesterol levels of 300-600 mg% developed in 1-3 months. The animal models can be employed for assessment of hypocholesterolemic agents and their effects on the atherosclerotic process.

Estrogen induced hypercholesterolemia in birds

The administration of depot estrogens to hens and cockerels induces a hyperlipidemia which development of atherosclerotic. Blood cholesterol levels rise tenfold over control levels (908 mg% vs. 133 mg %) within 1 week following a single injection of depot β-estradiol in the chicken. This hyperlipidemia is endogenous and not dependent on the presence of dietary fat. Thus it seems that cholesterol biosynthesis inhibitors could be detected using this system. Thyroxin, an agent that can accelerate metabolic degradation of cholesterol and that promotes the removal of serum phospholipids, is the most active agent in reversing the estrogen hypercholesterolemia. Agents who inhibit intestinal cholesterol absorption, such as citosterol and amide derivatives of linoleic acid are inactive. The method is simple and rapid and is suitable for hypocholesterolemic screening of thyromimetic agents.

Triton wr 1339 (ISO octyl poly oxy ethylene phenol) induced hyper lipidemia model [39]

This method is used for detecting compounds which interfering the synthesis and excretion of cholesterol**.** The use of Triton WR 1339 induced hyperlipidaemia through accelerated hepatic cholesterol synthesis is suggested for test. In this model take Male Prague–Dawley rats with weight about 200- 250 gm, are divided into 5 groups of 6 animals each. The first group (Normal control) is received normal saline orally for one week. The second group (Triton positive control) is received Triton WR-1339 dissolved in 0.9% saline (400 mg/kg bodyweight) by i.p. route. The third and fourth groups are treated with hydro-alcoholic extract of poly-herbal formulation (200 mg/kg and 400mg/kg, p.o.) once a day for one week. The fifth group is treated with Atorvastatin suspension prepared with 0.5% CMC (10mg/kg, p.o.), once a day for one week. On the 7th day, the animals are fasted for 18 hrs (had only access to water) and 400 mg/kg Triton WR 1339 is injected (i.p), to all the four groups of rats immediately after drug administration. On the 8th day, blood is collected by retro-orbital sinus puncture, under mild ether anesthesia. The collected blood samples are centrifuged for 10 minutes at 2000 r.p.m. and serum samples are used for various biochemical tests [87].

Conclusion

Hyperlipidemia is a critical condition of elevated lipid levels in the body that ultimately lead to development of various cardio-vascular diseases. Development of a hypolipidemic or hyperlipidemic agent is a much desirable and important in the present scenario. However, the major hurdle towards this goal is the requirement of suitable screening methods for hyperlipidemic activity. The present evaluation methods for antihyperlipidemic activity classified as *In vivo* and *in vitro* methods. Animal models are use to find new chemical molecule of drug for treatment of respective disease and to find out ADRs, side effects of drug. Animal screening is very important for developing new molecule of drug. But, there is no perfect animal model which completely replicates all stages of human hyperlipidemic conditions, yet these small animal models as well as screening methods promising entity in exploring the etiopathogenesis and regression of antihyperlipidemic activity. Further studies should be conducted towards the purification and characterization of biologically active compounds that might be serving as novel lead molecules for the synthesis of new and safe therapeutic agents for hyperlipidemia.

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