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TOXICITY STUDIES IN PRECLINICAL DEVELOPMENT OF A NCE - FOCUSS ON FUROSEMIDE.

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INTRODUCTION

Toxicity testing is critical in the evaluation of novel medications before they are used on humans. The goal of toxicity testing is to identify any potential hazardous consequences that a test chemical may have, not merely to determine how safe it is. The main goals of toxicity testing are to determine how test compounds affect lab animals and whether they have any direct hazardous effects on humans. Secondly, high doses of test substances are given to lab animals in order to assess any potential risks to humans who receive much lower levels.

DIFFERENT EXPERIMENTAL ANIMALS IN TOXICITY STUDIES

Rat

Albino rat is one of the common laboratory animal suitable for experimental purpose because of its small size and greater sensitivity to most drugs.it is also the most standardised of all laboratory animals.

2. Mice

Mice are employed widely in acute toxicity studies. They are also used for the assay of insulin and analgesic, the later specially against chemically induced pain.

3. Guinea Pig

Guinea pigs are employed for the evaluation of bronchodilator compound against experimentally induced asthma (histamine or acetylcholine aerosols). They are widely used in immunology, particularly in the studies of delayed hypersensitivity.

4. Rabbit

rabbits are very docile animals employed for variety of studies. Rabbits are employed for the pyrogen testing in intravenous fluid. Insulin and other antidiabetic drug, curare and sex hormones are tested I n rabbit.

REGULATORY AGENCIES

1. ICH (international council for harmonization)
2. OECD (Organisation For Economic Corporation Development)
3. FDA (food and drug administration)
4. WHO (world health organisation)

ETHICAL CONSIDERATION

1. Ensuring The Safety of animal
2. Well, Being of animal
3. Rights Of Participants
4. Maintaining Transparency

FACILITIES AND DOCUMENTATION AND STATISTICS REQUIRED

1. Animal Care
2. Laboratory Equipment
3. Secure Storage
4. Waste Disposal
5. Quality Assurance
6. Documents Should Be Well Mainatained.

ACUTE TOXICITY STUDIES IN ANIMALS

Acute toxicity studies in animals are designed to evaluate the adverse effects that occur within a short period (usually 24 hours to 14 days) after a single dose or multiple doses of a chemical, drug, or substance. These studies help determine the *lethal dose* (LD_{50}), *target organs*, and potential symptoms of

toxicity, which are crucial for assessing the safety of chemicals and pharmaceuticals before human exposure.

Purpose of Acute Toxicity Studies

- Determine **LD_{50}** (the dose lethal to 50% of the test animals).
- Identify **clinical signs** of toxicity (e.g., convulsions, salivation, lethargy).
- Assess **target organs** affected by the substance.
- Provide information for:
    - **Classification and labelling** (e.g., under GHS).
    - **Dose selection** for subacute/sub chronic studies.
    - **Risk assessment** for human exposure.

Acute toxicity test methods measure the adverse effects that occur within a short time after administration of a single dose of a test substrate. This testing is performed principally in rodence and is usually done early in the development of a new chemical or product to provide information of its potential toxicity.[6] This information may also be extrapolated for use in the diagnosis and treatment of toxic reaction in humans.

The results from acute toxicity tests can provide information for comparison of toxicity and dose-response among members of chemical classes and help in the selection of candidate materials for further work. They are further used to standardize certain biological products such as vaccines.

PARAMETERS OBSERVED

1. Body temperature
2. Pulse rate
3. Respiratory rate
4. Arterial blood pressure
5. Volume of urine produced
6. Volume of tear produced

CALCULATION OF LD50

The LD50 is a standard measure used in toxicology to determine the median lethal dose of a substance. It is typically expressed in milligrams of substance per kilogram of body weight (mg/kg). For instance, if an LD50 value is 200 mg/kg, it means that 200 milligrams of the substance per kilogram of body weight is sufficient to kill half the population used in the test.

Subacute Toxicity Studies in Animals

Subacute toxicity studies (also called repeated dose 28-day studies) are used to evaluate the toxic effects of a substance after repeated exposure over a short period, typically 28 days. These studies help identify target organs, dose-response relationships, and No-Observed-Adverse-Effect Levels (NOAELs).[10]The animals used in subacute toxicity are bigger rodents such as rats. These animals are manipulated probably on a daily basis (according to designed protocol) and will have to resist test duration. For example, daily oral administration of test substance with an intubation needle or even IP (which are common exposure routes) for about 28 days may be stressful for mice [46–48]. Hence they are not very suitable.17 Even when the test product has to be mixed with feed or water for animals to freely consume orally or a product is meant for inhalation route, the use of mice should be strongly justified.Rodents (usually rats) preferred for oral and inhalation studies; rabbits for dermal studies; non rodents (Usually dogs) recommended as a second species for oral tests.

DETAIL OF OBSERVATION

Sub acute and chronic toxicity studies are designed to characterize the toxic effects of drugs upon repeated daily administration for periods of time ranging from 2 weeks to 1 year and to determine no-toxic-effect dosage levels for short to long-term repeated dosing.

RECORDING AND ANALYSIS OF DATA

Data Recording

Accurate and detailed recording of all observations and data is crucial for a thorough analysis. This includes:

 Individual animal records: Detailed daily observations of clinical signs, including their onset, duration, and severity.

 Body weight: Measured and recorded at regular intervals (e.g., daily or every few days).

 Food and water consumption: Measured and recorded at regular intervals per cage. If animals are group-housed, individual consumption may not be possible.

Data Analysis

The collected data should be analysed using appropriate statistical methods to determine if there are any statistically significant differences between the treated

groups and the control group. Key aspects of data analysis include:

 Descriptive statistics: Calculation of means, standard deviations, and ranges

for all quantitative parameters.

 Inferential statistics: Use of appropriate statistical tests (e.g., ANOVA, t-

tests, chi-square tests) to compare treatment groups with the control group.

The choice of test depends on the type of data and the experimental design.

1. Purpose of Animal Toxicity Studies in NCE Development

When developing a new chemical entity (NCE), animal studies are performed to:

 Assess safety and toxicological profile.

 Identify target organs of toxicity.

 Determine no observed adverse effect level (NOAEL).

 Understand mechanism of action and off-target effects.

 Evaluate potential drug-drug interactions.

Hypertension

Hypertension commonly known as **high blood pressure**, is a chronic medical condition that affects millions of individuals worldwide. Often called the **"silent killer"**, it frequently presents with no symptoms while quietly damaging blood vessels and vital organs over time. As a leading risk factor for heart disease, stroke, kidney failure, and other serious conditions, understanding hypertension is crucial for effective prevention, early diagnosis, and appropriate management.

Purpose of Antihypertensive Drug Studies in Animals

- Animal studies are essential in understanding:
- The mechanisms of hypertension
- The efficacy of antihypertensive drugs
- Potential side effects
- Organ protection (heart, kidney, brain)

These studies help develop and refine drugs before human clinical trials.

Key Mechanisms in Hypertension Pathophysiology

1. Endothelial Dysfunction

A hallmark of hypertension is impaired endothelial function, characterized by:

- Reduced nitric oxide (NO) bioavailability.
- Increased production of reactive oxygen species (ROS).
- Elevated levels of endothelin-1 and angiotensin II.
- Inflammatory responses.

These factors collectively contribute to vascular stiffness, increased peripheral resistance, and target organ damage.

2. Vascular Re-Modelling

Chronic hypertension leads to structural changes in blood vessels, including:

- Inward eutrophic remodelling: Reduction in lumen diameter without changes in wall thickness, observed in models like spontaneously hypertensive rats (SHR) and angiotensin II infusion.
- Hypertrophic re-modelling: Thickening of the vessel wall, noted in renovascular hypertension and salt-sensitive models.
- Rarefaction: Loss of microvascular density, contributing to increased peripheral resistance.

3. Sympathetic Nervous System Activation

Elevated sympathetic tone leads to:

- Increased heart rate and vascular resistance.
- Enhanced renin release from the kidneys
- Elevated blood pressure

This activation is often observed in genetic and pharmacological hypertension models.

4. Renin-Angiotensin-Aldosterone System (RAAS) Dysregulation

Overactivation of RAAS results in:

- Vasoconstriction
- Sodium and water retention
- Increased blood pressure

This pathway is central in models like DOCA-salt and angiotensin II infusion.

Purpose of Toxicity Studies for Antihypertensive NCEs

1. **Identify toxic effects and target organs** (e.g., heart, kidneys, CNS).
2. Determine **NOAEL** (No Observed Adverse Effect Level) and **LOAEL** (Lowest Observed Adverse Effect Level).
3. Assess **dose-response relationships**.
4. Evaluate **species-specific responses**.
5. Support **regulatory filings** (IND, CTD Module 4).
6. Predict potential **human adverse effects**.

Blood Pressure Measurement Methods in Animals:

1.Indirect (Non-Invasive) Methods

- Tail-Cuff Method: Commonly used in rodents, this method involves wrapping a cuff around the tail and inflating it to occlude blood flow, then slowly deflating to detect the return of blood flow. While non-invasive, it can be influenced by animal stress and is less accurate for continuous monitoring.
- Doppler Ultrasonography: Utilizes an ultrasonic probe to detect blood flow velocity, allowing for the estimation of systolic BP. It's widely used in veterinary practice for companion animals.
- Oculometry: Measures oscillations in the arterial wall as the cuff deflates. This method is automated and suitable for high-throughput studies, though it may be less accurate in small or stressed animals.

2. Direct (Invasive) Methods: Catheterization: Involves surgically implanting a catheter connected to a pressure transducer to measure BP directly within the artery. This method provides continuous, real-time data and is considered the gold standard for BP measurement in animals.

- Radiotelemetry: Implants a small device under the skin that transmits BP data wirelessly. It allows for continuous, unrestrained monitoring of BP in conscious animals, making it ideal for long-term studies.

Common Antihypertensive Drugs in Animal Research

- **ACE Inhibitors**

Example-Enalapril, Captopril, Ramipril
Mechanism of Action-Inhibit angiotensin-converting enzyme (ACE), reducing Ang II and increasing bradykinin.
Common animal models-SHR, 2K1C, DOCA-salt rats.
Observation-↓ BP, ↓ cardiac hypertrophy, ↑ renal protection, ↓ inflammation.

- **ARBs (Angiotensin II Receptor Blockers)**

Example -Losartan, Valsartan .
Mechanism of Action -Block AT1 receptors → vasodilation and ↓ aldosterone.
Animal models --SHR, diabetic nephropathy rats.
Observation-↓ BP, ↓ proteinuria, ↓ cardiac and renal fibrosis.

- **Calcium Channel Blockers**

Example -Amlodipine, Nifedipine.
Mechanism Of Action -Block L-type calcium channels → vasodilation.
Animal used -SHR, stroke-prone SHR.
Observation - ↓ BP, ↓ vascular resistance, ↑ cerebral blood flow.

- **Beta-Blockers**

Example - Propranolol, Atenolol.
Mechanism of action -Block β-adrenergic receptors → ↓ heart rate and contractility.
Animal used -SHR, DOCA-salt rats.
Observation -↓ BP, ↓ cardiac remodelling, ↓ sympathetic activity

- **Diuretics**

Example -Hydrochlorothiazide, Furosemide
Mechanism of Action -Increase sodium and water excretion → ↓ plasma volume
Animal used - SHR, salt-sensitive rats.

Furosemide

A potent loop diuretic, has been extensively studied in animal models to assess its effects on efficacy.

Effects of Furosemide in Animal Models of Hypertension

1. Acute Antihypertensive Effects in Dahl Salt-Sensitive Rats

In Dahl salt-sensitive rats, administration of furosemide (4 mg/day) via bolus injection significantly lowered mean arterial pressure (MAP) within 6 hours, primarily through enhanced natriuresis and diuresis. However, continuous infusion of the same dose did not produce a significant reduction in MAP, suggesting that the acute blood pressure-lowering effect is closely tied to the transient diuretic response. Notably, these effects occurred without significant

changes in total body sodium or potassium levels, indicating that the antihypertensive action was independent of long-term electrolyte balance .

2. Acute Blood Pressure Reduction in Volume-Expanded Rats

In rats with surgically induced volume expansion (anephric or ureter-ligated models), intravenous administration of furosemide (1.25 mg/kg) led to a rapid and significant decrease in blood pressure. This effect was associated with a transient increase in heart rate. Importantly, the blood pressure reduction occurred independently of renal function, suggesting a direct vascular action of furosemide beyond its diuretic properties.

3. Dose-Dependent Diuretic and Cardiovascular Effects

In unanaesthetised rats, furosemide demonstrated a steep dose-response relationship for its diuretic effects. A dose of 5 mg/kg induced a clear diuretic effect accompanied by a 20–25% plasma volume deficit, but only a twofold increase in plasma renin activity (PRA). Higher doses (10 and 40 mg/kg) resulted in larger diuresis without further increases in PRA. Despite significant diuresis and plasma volume deficit, furosemide did not induce significant changes in blood pressure or heart rate, indicating that its cardiovascular effects may be independent of volume depletion.

4. Renin-Angiotensin-Aldosterone System (RAAS) Modulation

In a study involving Thoroughbred horses, administration of furosemide led to significant increases in plasma levels of angiotensin I, II, III, and IV, as well as aldosterone, at 4 hours

post-dosing. These findings suggest that furosemide may activate the classical RAAS pathway, potentially as a compensatory mechanism to counteract its diuretic effects.

Pharmacological Actions

1. Cardiovascular Effects

Blood Pressure: Acute administration can lower blood pressure due to decreased plasma volume. In hypertensive rats (e.g., Dahl salt-sensitive rats), furosemide rapidly lowers mean arterial pressure.

Heart Rate: May increase reflexively due to volume depletion.

Cardiac Output: Decreases initially due to reduced preload.

Long-term use: Can activate compensatory mechanisms like the RAAS system.

2. Neurohumoral Effects

Renin-Angiotensin-Aldosterone System (RAAS):

Furosemide stimulates renin release due to reduced sodium delivery to the macula dens'. Increases in circulating renin, angiotensin II, and aldosterone have been documented in both rodents and larger animals (e.g., horses).

Sympathetic Nervous System: May increase due to volume depletion**.**

3.Effects on Electrolyte Balance

Potassium: Hypokalaemia is a frequent consequence.

Calcium and Magnesium: Increased urinary loss.

Acid-Base Balance: Can cause metabolic alkalosis due to hydrogen ion loss.

4. Renal Effects

GFR (Glomerular Filtration Rate): Often maintained or mildly decreased.

Renal blood flow: May increase initially.

Histopathological changes: Prolonged or high-dose exposure in some studies can induce tubular damage, especially in dehydrated animals.

5. CNS and Ototoxicity

Ototoxicity: High doses or rapid IV infusion have caused temporary or permanent hearing loss in rats and guinea pigs.

Mechanism: Possibly due to disruption of electrolyte balance in the inner ear.

DILTIAZEM

Diltiazem is a medication used to treat high blood pressure (hypertension), angina (chest pain), and certain arrhythmias (irregular heartbeats). It belongs to a class of drugs called calcium channel blockers (non-dihydropyridine type).

Pharmacological Effects in Animals

1. Cardiovascular System

Species studied: Rats, dogs, cats, rabbits, guinea pigs, monkeys.

Effects:

Decreased blood pressure (vasodilation of peripheral arteries).

Slowed heart rate (bradycardia) via effects on the sinoatrial (SA) and atrioventricular (AV) nodes. Reduced myocardial contractility (negative inotropic effect), though generally milder than other calcium channel blockers like verapamil. Coronary artery dilation, leading to increased blood flow to the myocardium.

2. Electrophysiological Effects

In dogs and rabbits, diltiazem prolonged AV node conduction and refractory period. It was effective in suppressing supraventricular arrhythmias in experimental models.

Toxicity and Safety in Animals

1. Acute and Chronic Toxicity

High doses led to hypotension, bradycardia, and AV block. In chronic toxicity studies (e.g., 6-month oral administration in rats and dogs),

high doses caused:

- Weight loss
- Liver enzyme elevation
- Cardiac conduction abnormalities at very high doses

2. Reproductive and Developmental Toxicity

Teratogenicity:

In rats and rabbits, diltiazem did not show major teratogenic effects, but high doses were associated with embryotoxicity (e.g., reduced foetal weight, increased resorptions).It crosses the placenta in animals.

3. Carcinogenicity & Mutagenicity

long-term animal studies (2 years) in rats and mice showed no evidence of carcinogenicity.

Diltiazem was not mutagenic in standard in vitro or in vivo tests.

DILTIAZEM IN NCE STUDIES

In new chemical entity (NCE) studies, researchers investigate a compound like diltiazem during its early preclinical development to establish its pharmacologic profile, toxicology, and mechanism of action before human trials. Here's a focused summary of how diltiazem performed as a new chemical entity in such studies:

Diltiazem as a New Chemical Entity: Key Findings

1. Pharmacodynamics

Primary target: L-type calcium channels in cardiac and smooth muscle.

Mechanism identified early:

Diltiazem inhibits calcium influx, which:

- Relaxes vascular smooth muscle → ↓ blood pressure
- Slows AV nodal conduction → used for arrhythmias
- Decreases myocardial oxygen demand → antianginal effect

In early animal and isolated tissue studies, diltiazem showed:

- Selective vasodilation of coronary and peripheral arteries
- Reduced afterload without significant reflex tachycardia (unlike dihydropyridines)

2. Pharmacokinetics

Absorption: Adequate oral absorption in rats and dogs

Distribution: High volume of distribution; penetrates cardiac tissue

Metabolism: Extensive first-pass metabolism in liver; multiple metabolites formed

Excretion: Primarily via bile and faeces; minor renal clearance[48]