Chapter-3

Methodology
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Drugs and Chemicals

Oleamide

Oleamide, a selective CB1 cannabinoid receptor agonist ((Z)-Octa-9-decenamide), was purchased from Sigma Chemicals Co., St. Louis, USA and catalogue number is O2136. The molecular formula of oleamide is C\textsubscript{18}H\textsubscript{35}NO with molecular weight 281.48.

AM6545

AM-6545, a selective peripheral CB1 receptor antagonist (5-(4-[4-cyanobut1-ynyl]phenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-(1,1-dioxothiomorpholino)-1H-pyrazole-3-carboxamide) was purchased from Sigma Chemicals Co., St. Louis, USA and catalogue number is A1987. The molecular formula of AM6465 is C\textsubscript{26}H\textsubscript{23}Cl\textsubscript{2}N\textsubscript{5}O\textsubscript{3}S and molecular weight 556.46.

Insulin

Insulin mixtrad 10 was purchased from Novo Nordisk, Denmark.

DMSO

Dimethyl sulphoxide (DMSO) is an organosulfur compound with the formula \((\text{CH}_3)_2\text{SO}\). Was purchased from Sigma Chemicals Co., St. Louis, USA and catalogue number is D8418.

Thiopental Sodium

Sodium thiopental, also known as thiopental, thiopentone, or Trapanal is a rapid-onset short-acting barbiturate general anesthetic with molecular formula \(\text{C}_{11}\text{H}_{17}\text{N}_2\text{NaO}_2\text{S}\) and was purchased from Abbot Laboratories, North Chicago, Illinois.
Nephrectomy Procedure

Purpose of Uninephrectomy

Uninephrectomy results in the enlargement of the remaining kidney which is further aggravated by chronic hyperglycemic insult. Further, uninephrectomy has been shown to increase glomerular capillary pressure in rats which promotes diabetic glomerular injury. Uninephrectomy followed by hyperglycemia accelerates progression of diabetic nephropathy as in comparison to diabetic rats with intact kidney (Tesch and Allen, 2007).

Procedure

The procedure involves surgical removal of right kidney. The over night fasted rats were anaesthized with thiopental sodium (30 mg/kg., i.p.). The rats were laid on surgical bed and the hair was shaved with animal hair trimmer. A small incision of about 2 cm long was made using a surgical blade just below the rib cage on the right side. The skin was freed from the adjoining connective tissue that was attaching the skin and the body wall. A deeper cut was made along the body wall. The kidney was freed from the surrounding adipose and fat tissues. The right kidney was exposed and a thread was tied underneath the kidney. Now, the kidney was cut above the ligature and blood vessels that were tied with the thread were replaced safely into the body cavity. The body wall was carefully sutured with digestible catgut suture and the skin was sutured with silk thread using 10 size curved needle. The area of incision was swabbed regularly at each stage of surgery with antiseptic lotion. The area of suture was regularly treated with soframycin ointment to prevent infections for a period of one week. The rats were caged individually with food and water were given ad-libitum for a period of 15 days. Later they were marked and replaced back with other animals. The
uninephrectomised animals were allowed to recover for a period of 3 weeks for
the induction of diabetes-mellitus.

**Induction of Type 1 Diabetes Mellitus**

Streptozotocin-induced pancreatic injury is commonly used for creating
rodent models of type 1 diabetes mellitus. Streptozotocin (STZ, 2-deoxy-2-(3-
methyl-3-nitrosoureido)-D-glucopyranose) was discovered in a strain of the soil
microbe Streptomyces achromogenes. It was classified as an alkylating agent in
the nitrosourea class of anti-cancer drugs and was used to treat cancer of Islets of
Langerhans in the pancreas. STZ is toxic to the insulin-producing beta cells of the
Islets of Langerhans in the pancreas, and thus it is widely employed to induce
experimental diabetes in animals (Balakumar et al., 2008). Streptozotocin is
readily transported into pancreatic β-cells by GLUT-2 and causes β-cell toxicity,
resulting in insulin deficiency. STZ selectively inhibits the activity of β-cell O-
GlcNAcase, which is responsible for removing O-GlcNAc from protein. This
causes irreversible O-glycosylation of intracellular proteins and results in β-cell
apoptosis (Tesch and Allen, 2007).

**Preperation and Storage of STZ**

STZ was pre-weighed into a plastic microfuge tube which was then
wrapped in aluminium foil (to protect against light sensitivity) and stored at -
20°C. Sodium citrate buffer (10 mmol/L, pH 4.5) was prepared by dissolving 147
mg of tri-sodium citrate in 49.5 mL of normal saline and adjusting the pH to 4.5
with approximately 0.5 mL of 1 mol/L citric acid. The citrate buffer should be
used fresh or frozen in 1 mL aliquots and stored at - 20°C. Required volume of
citrate buffer was pipetted into microfuge tubes, which were prefilled with STZ.
Induction of Type-1 Diabetes Mellitus to Rats

1. Wistar rats of either sex of 8 weeks of age were selected, marked, weighed (220-250 gms).
2. They were fasted over-night.
3. STZ was freshly prepared by dissolving in freshly prepared ice cold sodium citrate buffer of pH 4.5.
4. The STZ disintegrates within 15-20 minutes after dissolving into the buffer, hence should be administered immediately after preparation.
5. The rats were administered a single dose of STZ (55 mg/kg, i.p.,).
6. Following the STZ injection, rats were given drinking water supplemented with sucrose (15 g/L) for 48 h, to limit early mortality as stores of insulin were released from damaged pancreatic islets.
7. At 1 week after STZ, rats were assessed for hyperglycaemia and those with fasting blood glucose of above 280 mg/dL were included in the study.
8. To prevent subsequent development of ketonuria, diabetic rats were injected daily with long-acting insulin (2–4 U/rat), to maintain blood glucose levels in a desirable range 300–450 mg/dL, till the end of the experimental protocol.

Parameters Estimated

Blood Glucose

Principle

Glucose is oxidized by glucose oxidase (GOD) to produce gluconate and hydrogen peroxide. The hydrogen peroxide is then oxidatively coupled with 4 amino-antipyrine (4-AAP) and phenol in the presence of peroxidase (POD) to
yield a red quinoeimine dye that is measured at 505nm. The absorbance is directly proportional to concentration of glucose in the sample.

\[
\text{Glucose} + 2\text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{GOD}} \text{Gluconate} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O} + 4\text{-AAP} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoeimine Dye}
\]

**Sample Collection and Storage**

Unhaemolysed serum was used for the testing. Separated serum samples can be stored for 3 days at 2-8°C.

**Procedure**

- The test tubes were pipetted with reagents as per the table given below

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1</td>
<td>1000µl</td>
<td>1000µl</td>
<td>1000µl</td>
</tr>
<tr>
<td>Dist. Water</td>
<td>10µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>-</td>
<td>10µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10µl</td>
</tr>
</tbody>
</table>

- The test tube were mixed well and incubated for 15 min at room temperature.
- The absorbance of the standard and sample was measured against reagent blank at 505 nm.

**Calculation**

\[
\text{Glucose (mg/dl)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Conc of STD (mg/dl)}
\]

**Serum Creatinine**

Creatinine is the catabolic product of creatinine phosphate, which is used by the skeletal muscle. The daily production of creatinine depends on muscular mass and it is excreted out of the body entirely by the kidneys.
**Principle**

Picric acid in an alkaline medium reacts with creatinine to form an orange coloured complex with the alkaline picrate. Intensity of the colour formed is directly proportional to the amount of creatinine present in the sample.

\[
\text{Creatinine} + \text{Alkaline picrate} \rightarrow \text{Orange Coloured complex}
\]

**Sample**

Unhaemolysed serum was used for the testing.

**Deproteinization of serum:**

Serum samples were deprotenised by pipetting 2.0 ml of picric acid and 0.2 ml of serum sample into a clean dry test tube. The test tubes were mixed well and centrifuged at 2500-3000 rpm for 10 min. The clear supernatant thus obtained were transferred into sterile microfuge tubes.

**Colour Development**

- The clean dry test tubes labelled as Blank (B), Standard (S) and Test (T) and were pipetted with reagents according to the table given below.

<table>
<thead>
<tr>
<th>Addition Sequence</th>
<th>B (ml)</th>
<th>S (ml)</th>
<th>T (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td></td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>Picric Acid Reagent (L1)</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine Standard (S)</td>
<td></td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Buffer Reagent (L2)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

- The test tubes were mixed well and incubated at room temperature for exactly 20 min.
- The absorbance of the standard (Abs. S), and test Sample (Abs.T) were measured against the blank at a wavelength of 520nm.
Calculaion

Creatinine (mg/dl) = \frac{Absorbance of Sample}{Absorbance of Standard} \times 2.0

Blood Urea Nitrogen

Urea is an end product of protein metabolism and is excreted by the kidney. Blood urea nitrogen (BUN) varies directly with protein intake and inversely with the rate of excretion of urea.

Principle

Urea is converted to ammonium by the use of urease. Ammonium ion thus reacts with a mixture of salicylate, sodium nitroprusside and hypochlorite to yield a blue-green chromophore. The intensity of the color formed is proportional to the urea concentration in the sample.

\[
\text{Urea} + \text{H}_2\text{O} \xrightarrow{\text{Urease}} \text{Ammonia} + \text{CO}_2
\]

\[
\text{NH}_3 + \text{Hypochlorite} + \text{Salicylate} \rightarrow 2,2\text{-Dicarboxyindophenol (Blue-green Chromophore)}
\]

Sample

Unhaemolysed serum was used for the testing.

Procedure

- BUN enzyme reagent was reconstituted according to the instructions.
- 1.5 ml of BUN enzyme reagent were pipetted into each of the labeled test tubes and were incubated at room temperature.
- To the above solution 10 µl of sample was added each of the sample labelled test tube respectively and mixed gently.
- Deionized water was used instead of the sample for the reagent blank and standard BUN solution was used instead of the sample for standard.
All the tubes were incubated for five minutes at 37 °C. Then, 1.5ml BUN color developer was added and mixed gently.

The absorbance of the standard (Abs. S) and test sample (Abs. T) were measured against the blank at a wavelength of 630nm.

**Calculation**

\[
\text{BUN (mg/dl)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Conc of STD (mg/dl)}
\]

**Urinary Microprotein**

**Principle**

Pyogallol Red is combines with molybdenum acid at a low pH. When the complex is combined with protein, a blue purple colour solution is formed. The increase in absorbance at 600nm is directly proportional to the protein concentration in the sample.

\[
\text{Proteins + Pyrogallol Red Molybdate} \rightarrow \text{Blue Purple Coloured Complex}
\]

**Sample**

Tests were performed on urine samples collected for 24-hours.

**Procedure**

- Test tubes (labelled as blank (B), standard (S) and test (T)) were pipetted with reagents as given in the table below:

<table>
<thead>
<tr>
<th>Dye Reagent</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Standard</td>
<td>1000 µl</td>
<td>20 µl</td>
<td>---</td>
</tr>
<tr>
<td>Urine/CSF Sample</td>
<td>---</td>
<td>---</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
The test tubes were mixed well and incubated at room temperature for 3 min.

The absorbance of the standard (Abs.S) and test sample (Abs.T) against the blank was measured at 620 nm within 30 minutes.

**Calculation**

\[
\text{Protein conc (mg/dl)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times 100
\]

Total microprotein excreted (mg/24 hours) = Urinary protein concentration (mg/dl) x 10 x total volume of urine (liters) excreted for 24 hrs.

**Urinary Microalbuminuria ELISA Assay**

**Principle**

In this assay, the albumin present in the sample reacts with the anti-Albumin antibody, which has been pre-adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-Alb antibody conjugated with horseradish peroxidase (HRP) is added. This HRP-conjugate antibody forms a complex with the previously bound albumin. Following another washing step, the enzyme bound to the immunosorbent was assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of albumin in the sample. The quantity of albumin in the test sample can be interpolated from the calibration curve constructed from the calibrators.
**Reagent Preparation**

**Diluent Concentrate**

The diluent solution supplied was at a 5X concentration and was diluted to 1X concentration with distilled or de-ionized water.

**Wash Solution Concentrate**

The Wash Solution supplied was at 20X concentrate and was diluted 1:20 with distilled or de-ionized water.

**Enzyme-Antibody Conjugate Concentrate**

Required amount of conjugate concentrate solution was calculated and diluted by adding 10 μL Enzyme-Antibody Conjugate to 990 μL of 1X diluent concentrate.

**Dilution of Samples**

Urine samples were collected for a period of 24 hrs and diluted at a dilution of 1:500.

**Procedure**

- All the reagents were brought to room temperature. 100μl of calibrator solutions, diluted positive control solution and diluted sample solution were pipetted into pre-designated wells of the micro-titter plate. The plates were covered and incubated at 22°C for 30 mins.
- The plate was washed with the diluted wash solution and aspirated. The plated was then inverted on a blotting paper to remove the residual washing solution. This process was repeated thrice.
- Then 100μl of diluted Enzyme-Antibody Conjugate was pipetted into each well, incubated at room temp and washed. 100 μL of TMB Substrate Solution was pipetted onto each well and incubated in dark for 10 mins,
followed by addition of 100 μL of stop solution and the absorbance of contents of each well was determined at 450 nm.

**Determination of collagen content**

Total collagen content was calculated as hydroxyproline concentration assuming that hydroxyproline constitutes 12.5% of total collagen (Edwards and O’Brien, 1980).

**Preparation of Renal Homogenate**

The kidney was dissected out and washed in ice cold isotonic saline and weighed. The kidney was then minced and a homogenate (10 g/mL) was prepared in chilled normal saline. The homogenate thus obtained was used for estimating hydroxyproline concentration.

**Determination of hydroxyproline concentration**

- Hydroxyproline was measured by the modified alkaline hydrolysis method (Reddy and Enwemeka, 1996). Briefly, to an aliquot of homogenate was added into NaOH (2 N final concentration) and was hydrolyzed by heating in a boiling water bath for about 3–4 h.
- About 900 μl of 56mM chloramine-T reagent was added to the hydrolyzed sample and oxidation was allowed to proceed at room temperature for 25 min.
- Then 1000 μl of 1 M Ehrlich’s reagent (p-dimethylaminobenzaldehyde) was added to the oxidized sample and the chromophore was developed by incubating the samples at 65°C for 20 min.
- The absorbance was read at 550 nm using an UV/visible spectrophotometer. The hydroxyproline concentration in the samples was calculated from the standard graph of hydroxyproline.
Serum Rat TNF-α ELISA Assay

**Principle**

The TNF-α (Rat) ELISA kit is a solid phase sandwich Enzyme Linked-Immunosorbent Assay (ELISA). A monoclonal antibody specific for rat TNF-α has been pre-coated onto the wells of the micro-titer strips provided. Samples including standards of known rat TNF-α content, control specimens and unknowns were pipetted into these wells, incubated for 15 mins and washed. During the incubation, the rat TNF-α antigen binds to the immobilized (capture) antibody on the site. After washing, a biotinylated monoclonal antibody specific for rat TNF-α was added and incubated for 15 mins and washed. During the second incubation, this antibody binds to the immobilized rat TNF-α captured during the first incubation. After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) was added. This binds to the biotinylated antibody to complete the four-member sandwich. A substrate solution was added, which acts upon by the bound enzyme to produce color. The intensity of this colour produced is directly proportional to the concentration of rat TNF-α present in the original specimen.

**Sample Preparation**

Unhaemolysed serum was separated from whole blood and 1:2 dilution of the sample was made with the incubation buffer. The rat specific TNF-Alpha ELISA kits were purchased from ALPCO Diagnostics, Salem with catalog no. 45-TNFRT-E01.1.
Serum Rat TGF-β ELISA Assay

**Principal**

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for TGF-β1 has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any TGF-β1 present in the sample bounds to the immobilized antibody. After washing away the unbound substances, an antibody specific for TGF-β1 was added to the wells. Following a wash to remove the unbound antibody reagent, Avidin-HRP Conjugate was added to each of the wells. After washing away any unbound enzyme, a substrate solution was added to each of the wells and colour developed was in proportion to the amount of TGF-β1 bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

**Sample**

Unhaemolysed serum was diluted with assay buffer in a ratio of 1:20 (190 μl Assay Buffer + 10 μl sample). Add 20 μl 1N HCl to 200 μl of pre-diluted sample, mix and incubate for 1 hour at room temperature. Neutralize by addition of 20 μl 1N NaOH.

The rat specific TGF-Beta ELISA kits were purchased from Cell Sciences Incorporation with catalog no. 670.020.096.

**Statistical Analysis**

All values obtained in the present study were expressed as mean ± S.E.M. The data obtained from various groups were statistically analyzed using one way ANOVA followed by Tukey's multiple comparison test. The p value of less than 0.05 was considered to be statistically significant.
Bibliography


