MATERIAL AND METHODS
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MATERIALS:

In the present investigation, effect of concurrent administration and repeated pro-treatment with some anti-inflammatory and beta-adrenoceptor blocking agents was studied on tolbutamide-induced hypoglycemia. In order to delineate the mechanism of interaction, serum tolbutamide concentration and tolbutamide half-life was estimated along with blood sugar level.

ANIMALS:

Healthy rabbits of either sex weighing between 1 and 1.6 kg were used in this study. Rabbits were divided into 43 groups of 6 each (as detailed in plan of study) to study drug interaction with tolbutamide. Drugs were administered as a single dose or once daily for 7 days, to see their effect on tolbutamide-induced hypoglycemia. The rabbits were fasted overnight but with easy access to water. On the following day drugs or drug-combinations under study were administered orally in the morning and blood samples were collected at 0, 2, 4, 7, 9, and 24 hours.
CHEMICALS

For estimation of blood glucose:
1. D-Glucose (GR - Sarabhai M. Chemicals).
4. Tartaric acid (Analar - BDH).
5. Copper sulphate (GR - Sarabhai M. Chemicals).
7. Sodium hydroxide (GR - Sarabhai M. Chemicals).
8. Phosphoric acid (GR - Sarabhai M. Chemicals).
10. Sulphuric acid (Analar - BDH).

For estimation of serum tolbutamide:
12. 2, 4-Dinitrofluorobenzene (1-Fluoro-2, 4-Dinitrobenzene - Puriss A.R. - K. L. England).
13. Hydrochloric acid (GR - Sarabhai M. Chemicals).

Other chemicals:
15. Xylene (LR - BDH).

METHODS:
1. GLUCOSE ESTIMATION
2. STANDARD SUGAR SOLUTIONS

Three standard solutions were prepared.
(a) A stock solution of 1 percent glucose was prepared with saturated benzoic acid solution and kept in a refrigerator.

(b) A solution containing 2 mg of glucose in 1 ml (20 ml of stock solution diluted to 100 ml with water) was prepared freshly before use.

(c) Solutions containing 0.05 and 0.1 mg of sugar in 2 ml made by dilution of (b) with distilled water. The dilute standards were prepared just before the experiment.

2. ALKALINE COPPER SOLUTION

40 g of pure anhydrous sodium carbonate was dissolved in about 400 ml of water and was transferred to a flask (1 l capacity). 7.5 g of tartaric acid was added and then the later was dissolved. 4.8 g of crystallized copper sulphate was added. It was properly mixed and volume was made up to 1 litre. If the chemicals used are not pure, a sediment of cuprous oxide may form in the course of one or two weeks. If this happens, the clear supernatant reagent was removed with a siphon, or filtered through a good quality filter paper. The reagent can be kept indefinitely.

PHOSPHOMOLYBDIC ACID SOLUTION

To 50 g of molybdic acid and 5 g of sodium tungstate, 300 ml of 10% sodium hydrosulphide and 200 ml.
of water were added. It was boiled vigorously for 20-40 minutes so as to remove nearly the whole of the ammoniac present in the molybdate acid. Then it was cooled, diluted to about 360 ml and to it 125 ml of concentrated (85%) phosphoric acid was added. The final volume was made up to 500 ml with distilled water.

**SODIUM TUNGSTATE SOLUTION:**

10 gm of sodium tungstate (Analar - BDH) was dissolved in 100 ml of distilled water and kept in glass stoppered bottle.

**STANDARD TOLBUTAMIDE (400 mg/ml):**

40 mg tolbutamide I.P. was dissolved in 10 ml of amyl acetate. From this concentrated (4000 mg/ml) tolbutamide solution in final standard solution was prepared by diluting 1 ml of concentrated standard with 9 ml of amyl acetate. This solution contains 400 mg tolbutamide per ml. It was stored in refrigerator.

**AMYL ACETATE:**

Amyl acetate is shaken with the same volume of water (Distilled water) and finally preserved over distilled water.
DMSO REAGENT

0.1 ml of 2, 4-Dinitrofluorobenzene (DNFB) was dissolved in 100 ml of amyl acetate and stored in refrigerator.

HYDROCHLORIC ACID

0.1 N Hydrochloric acid solution was prepared in distilled water and stored in glass stoppered bottle.

ALLOXAN MONOHYDRATE SOLUTION

Fresh solution of 100 mg/ml of alloxaan monohydrate was prepared in distilled water just before use.

DRUGS

Anti-inflammatory drugs under study are not soluble in distilled water but beta-adrenergic blockers are soluble. To maintain the homogeneity, all the following drugs were prepared in 2% gum acacia.

1. Acetosalol (30 mg/kg).
2. Aspirin (acetyl salicylic acid) IP (Viramak Pharma, Bombay).
3. Atensol (Giba - Bombay).
5. Propranolol (ASC I - Madras)
6. Tolbutamide IP (Hoechst - Bombay).
8. Troxaril (Unichem - Bombay).

Vehicles:

25 Gum acacia IP (Vikash Pharma - Bombay).

METHODS:

COLLECTION OF BLOOD

The marginal ear vein was selected for collection of blood in rabbits. Xylene was not used to make the vessels prominent because it caused haemolysis and affected collection of serum in preliminary experiments. Therefore blood vessels were made prominent by applying heat with the help of an electric lamp to the pinna of the rabbit. Then a cut was made with the help of sharp edged blade, on marginal ear vein. Blood was collected in two different vials (1) Fluoride vials (for blood sugar), (ii) plain well dried vials(for serum tolbutamide).

ESTIMATION OF BLOOD GLUCOSE

Blood glucose was estimated by Felin & Wu(1980) method.
PRINCIPLES:

When the protein-free blood filtrate is heated with alkaline copper solution, cuprous oxide is formed (glucose reduces cupric oxide to cuprous oxide). Cuprous oxide thus formed when treated with phosphomolybdic acid solution forms a blue colour which is compared with that of a standard with the help of colorimeter.

PROCEDURE:

3.5 ml of distilled water was taken in a centrifuge tube and to it 0.1 ml of blood was added. 0.2 ml of 10% sodium tungstate and 0.8 ml of 0.07N sulphuric acid were added subsequently to precipitate the blood proteins. After mixing vigorously it was allowed to settle for sometime and then centrifuged for 10 minutes at 1,000 rpm. 2 ml of supernatant fluid was pipetted into a Folin's sugar tube. If blood sugar levels are expected to be too high the supernatant was diluted with same amount of distilled water. 2 ml of distilled water (blank) and 2 ml of standard sugar solution containing 0.25 and 0.1 mg of glucose (standards) were taken in similar tubes. 2 ml of the alkaline copper solution was added. Then the tubes were kept in a boiling water bath for 2 minutes and then cooled in running water without shaking. Then to each tube 2 ml of phosphomolybdic acid
Reagent was added. After about 1 minute distilled water was added to the mark (12.5 ml) and mixed thoroughly. It is essential that adequate attention be given to this mixing because the greater part of the blue colour is formed in the bulb of the tube. Since the colour is not stable for long time the colorimetric readings were taken in 30 minutes. The optical density (O.D.) was determined at 420 ml setting the photometer to 100 % transmittance with the blank.

**Calculation**

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\frac{0.9 \text{ of unknown}}{0.9 \text{ of standard}} \times \frac{\text{glucose (mg) in standard}}{0.85} = \text{Blood glucose in mg per 100 ml}.
\]

**Estimation of Serum Tolbutamide**

Serum tolbutamide was estimated by the method of Spingler (1967).

**Principle**

Serum tolbutamide is dissolved in acetylacetone (forms a distinct separate layer on serum) which is separated with the help of centrifuge. 3,6-Dinitro-fluorescencene forms yellow colour after reacting with tolbutamide which is estimated colorimetrically at 420 nm.
PROCEDURE

1 ml of clear serum was shaken with 5 ml of anhyd acetate for one minute in an ordinary test tube. Then 0.2 ml 1 N hydrochloric acid was added and shaken thoroughly for 3 minutes and then transferred to a centrifuge tube. After centrifuging for 2 minutes at 1000 r.p.m., 4 ml of the clear supernatant anhyd acetate solution was pipetted into a graduated test tube. 1 ml 4NFS (8,4-dinitrofluorescein) reagent solution was added. After mixing well, the test tube was placed in an oil bath maintained at 160 ± 1°C (ground nut oil was used to make oil bath) and left for 5 minutes. Then it was cooled in a cold water bath at room temperature. For preparing the blank, 1 ml of distilled water and for standard, 1 ml standard thylbutamide solution were used instead of serum. The O.D. of standard and samples were measured in an Elico Spectro photometer at 350 mλ by setting the instrument at 100% transmission with the blank.

CALCULATION

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\text{Optical density of sample} \times 400 = \text{serum thylbutamide in mg/ml}.
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INDUCTION OF DIABETES BY ALLOXAN

Alloxan monohydrate (C₄H₁₃N₂O₅, H₂O) was used to produce experimental diabetes in rabbits. Ten healthy rabbits of either sex weighing between 1 and 1.5 kg were selected and kept on fast overnight.

In the next morning, a fresh solution of alloxan monohydrate (180 mg/ml) was prepared in distilled water. Alloxan solution was injected into the marginal ear vein at a dose of 2ml/kg. Severe hypoglycemia occurs within 1 to 4 hours of alloxan injection (causing convulsions and death), which may last upto 48 hours (Barar, 1970). 3 gm of glucose was given 4 hourly with the help of feeding cannula to each alloxan treated rabbit.

METHOD OF DETERMINATION OF SERUM HALF-LIFE BY TOLERANCE:

The biological half-life (t₁/₂) is defined as the time required for blood level to decrease from the peak level by 50% (Shaw and Beeson, 1971). Serum tolbutamide concentration versus time was plotted on semilogarithmic scale. The plasma t₁/₂ was determined by interpolating the 50% of plasma peak level.
STATISTICAL ANALYSIS:

The data obtained in the study were analysed by Student's 't' test. The per se effects of talbutamide and other drugs under study for interaction were compared against the effect of the treatment with the vehicle (2% gum acacia) whereas the effect of combinations were compared against talbutamide.