ABSTRACT
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The discovery of insulin by Banting and Best is one of the miraculous achievements of twentieth century medicine. Eli lily and company although introduced the first commercial insulin preparation in 1923, the first rDNA human insulin was introduced nearly 70 years later and the first bioengineered commercial insulin analog was available during the 75th anniversary year for discovery of insulin. Awareness of immunological side effects of insulin therapy became apparent during the later forties. Later it was established that virtually all insulin treated diabetics have insulin antibodies. Severe insulin resistance developed in some patients due to the high level of antibodies. These side effects were thought to be inevitable until the introduction of highly purified insulins in the early seventies. Improvement in the insulin purity as regards character and quality of an insulin preparation was the first challenge and had been a goal since early seventies. Various advances have been made in increasing the purity of insulin preparations. Introduction of actrapid—the first neutral insulin solution, microcrystalline insulin—the first chromatographically purified insulin and recently human insulin have been the major success for the development of pure insulin preparations that does not induce production of antibodies. Although the availability of human insulin represents an important advancement from the immunological point of view, clinically the insulin resistance and episodes of hypoglycemia have remained a problem in the therapy. Trace elements have been reported to possess beneficial effects in diabetes. Among various trace elements vanadium and chromium have shown to have antidiabetic activity in various animal models and clinical studies. In the light of problems related to insulin resistance, hypoglycemia, fluctuations of glucose and insulin levels, it has been an endeavor in the present investigation with an
objective to develop insulin formulations with trace elements viz., chromium and vanadium. The developed formulations were optimized, standardized and studied for their stability. The developed insulin formulations were evaluated pharmacologically for antidiabetic activity and their effects on lipid profile, kidney function and liver function in STZ-induced type I diabetic rats were envisaged.

We developed three different formulations of insulin with trace elements of chromium and vanadium. While preparing these formulations adjustments with respect to concentration of insulin, metal ion concentration, buffer concentrations, pH optimization and methyl paraben were required.

The optimum pH for chromium porcine insulin formulation was found to be 4.5-4.6. Any variation in the ionic concentration beyond an optimum level leads the formulation unstable. The pH also plays an important role in the development of new drug formulations of insulin with trace elements. To prepare insulin formulations as suspension acetate buffer of different pH were tried to precipitate the insulin. At lower pH of acetate buffer (2.28) we found development of clear solution. Where as, at higher pH (10) and 0.4% w/v ionic concentration, hard cake was formed after 24 hours storage. With the acetate buffer [pH 12.2] and metal ion concentrations (0.02% w/v and 0.03% w/v) the formulation became a hazy suspension. Thus, the optimum ionic concentration of chromium picolinate solution was found to be 0.05 % w/v. Optimum pH for acetate buffer was found to be 12.2. The final pH of all the formulations was in the range of 4.4-4.6. Preservation of chromium and vanadium insulin suspensions with phenol is not possible without influencing the physical stability of the preparation, whereas methyl 4-hydroxy benzoate (methyl paraben) does not exhibit this effect, so methyl paraben was selected as preservative for all the formulations developed. The optimum
concentration of chromium and vanadium metal was worked out at 12.42 μg/ml and 15.6 μg/ml respectively to prepare the insulin formulations.

The formulations developed were reproduced by preparing another formulation of 100 ml batch size for stability and pharmacological studies. The purified porcine insulin (160 mg) was added to 20 ml of chromium picolinate (0.05% w/v) solution. Then acetate buffer (pH 12.2; 20 ml) was added to get the precipitate. To this, 20 ml of methyl paraben solution (0.17% w/v) was added and final volume was made upto 100 ml with sterile water for injection. The pH was adjusted to 4.53 with 0.1 M HCl. The same procedure was followed for the preparation of chromium human insulin except the use of human insulin in place of porcine insulin.

The vanadium porcine insulin formulation was prepared by following the method described above except that instead of chromium picolinate 10 ml of vanadium sulphate (0.05% w/v) solution was used.

The porcine insulin injection and human insulin injection were prepared to compare with chromium porcine insulin, vanadium porcine insulin and chromium human insulin in pharmacological point of view. Pharmacological evaluation was carried out for these formulations on STZ-induced type I diabetic rats.

The developed formulations were characterized using various analytical techniques. The stability studies of the developed formulations were performed at 2-8 °C and the testing schedule followed for the above formulations was initial, 6 months and 12 months. During stability studies various critical parameters were evaluated viz. physical observation, crystal size and shape. Assay of insulin, insulin in supernatant and methyl paraben concentration was determined by using HPLC methods. The pH and metal ion concentration was determined. The stability results were found to be within acceptable limits for the developed formulations. The pH
of chromium porcine insulin injection was found to be 4.53 initially, after 6 and 12 months it was found to be 4.52 and 4.54 respectively. In case of vanadium porcine insulin injection the pH was found to be 4.53 initially, after 6 and 12 months it was found to be 4.55 and 4.59 respectively. The pH of chromium human insulin injection was found to be 4.50 initially, after 6 and 12 months it was found to be 4.52 and 4.58 respectively. The studies on the crystal structure revealed that there was no change in the crystal structure, size, and distribution. No clustering or agglomeration was observed in any of the insulin formulations. The crystal size and distribution remained well within the pharmacopoeial limits as specified for insulin preparations. From all these, it is inferred that the developed formulations are physically and chemically stable which may ensure good pharmacological response.

The assay of chromium porcine insulin injection was found to contain 40.31 IU/ml initially. After 6 and 12 months, the potency of insulin assay was found to be 39.95 IU/ml and 38.01 IU/ml respectively. The assay of vanadium porcine insulin was found to be 40.12 IU/ml initially and after 6 and 12 months it was found to be 39.85 IU/ml and 38.44 IU/ml respectively. Assay of chromium human insulin was found to be 39.8 IU/ml initially and after 6 and 12 months it was found to be 38.3 IU/ml and 38.24 IU/ml respectively. Insulin in the supernatant solution of chromium porcine insulin formulation was found to contain 68.95% initially. After 6 and 12 months, it was found to be 61.47% and 60.25% respectively. Insulin in the supernatant solution of vanadium porcine insulin suspension was found to be 65.33% initially. After 6 and 12 months, it was found to be 62.59% and 60.8% respectively. Insulin in the supernatant solution of chromium human insulin suspension was found to be 63% initially. After 6 and 12 months, it was found to be 60.5% and 58.5% respectively. Methyl paraben
content in chromium porcine insulin was found to be 0.31 mg/ml initially, and after 6 and 12 months it was found to be 0.32 mg/ml. In case of vanadium porcine insulin, it was 0.31 mg/ml initially and there was no change even after 6 and 12 months. In case of chromium human insulin it was 0.33 mg/ml initially and 0.32 mg/ml and 0.31 mg/ml after 6 and 12 months respectively. There was practically no change in methyl paraben concentration. The chromium and vanadium contents were determined by atomic absorption spectroscopy. The chromium content of chromium porcine insulin injection was found to contain 12.42 µg/ml initially, after 6 and 12 months it was found to be 12.40 and 12.38 µg/ml respectively. The vanadium content of vanadium porcine insulin injection was found to be 15.5 µg/ml initially. After 6 and 12 months, it was found to be 15.4 and 15.3 µg/ml respectively. The chromium content of chromium human insulin was found to contain 12.42 µg/ml initially, after 6 and 12 months it was found to be 12.40 and 12.39 µg/ml respectively.

The developed insulin formulations containing trace elements of chromium and vanadium along with the porcine insulin and human insulin formulations (without trace elements) were evaluated pharmacologically using STZ-induced diabetic rats and the effects were also compared with one of the marketed preparation i.e. mixtard insulin injection (highly purified biphasic human insulin).

Male Sprague Dawley rats weighing 200-225 g were used for the studies. The animals were housed under controlled conditions of temperature (22±2 °C), humidity (55±5%) and 12hr/12hr light-dark cycle. Animals had free access to standard diet and tap water ad libitum. Diabetes was induced with streptozotocin (STZ) (Sigma Ltd., USA) 45 mg/kg dissolved in 0.9% w/v NaCl solution, administered as a single intravenous (i.v.) tail-vein injection under mild ether anesthesia. The control animals were injected with an equivalent volume of 0.9% w/v NaCl solution. Animals were checked for the
extent of glucosuria 48 hrs after the injection of STZ using Diastix (Bayer Diagnostics, India). Animals showing glucosuria (>2%) were considered as diabetic. The control rats were randomly divided into two groups, namely control and control treated with test drug. Similarly, diabetic rats were also divided into two groups, namely IDDM control and IDDM treated with test drug. Study of effect of six weeks treatment of vanadium porcine insulin/chromium porcine insulin/chromium human insulin on type-1 diabetic rats involved different groups viz., group-I normal control, group-II diabetic control, group-III diabetic treated with porcine insulin formulation (5 IU/kg, twice daily, s.c), group-IV diabetic treated with human insulin formulation (5 IU/kg, twice daily, s.c), group-V diabetic treated with mixtard insulin (5 IU/kg, twice daily, s.c), group-VI diabetic treated with vanadium porcine insulin formulation (5 IU/kg, twice daily, s.c), group-VII diabetic treated with chromium porcine insulin formulation (5 IU/kg, twice daily, s.c) and group-VIII: diabetic treated with chromium human insulin formulation (5 IU/kg, twice daily, s.c).

Rats which received STZ, showed a significant increase in food intake and water intake and a significant reduction in body weight as compared to normal control rats. Treatment of rats with mixtard insulin, vanadium porcine insulin and chromium porcine insulin produced a significant increase in body weight as compared to diabetic control rats. However, rats treated with porcine insulin, human insulin and chromium human insulin failed to produce any significant change in body weight as compared to diabetic control rats. Rats which received mixtard insulin significantly reduced water intake, however rats treated with vanadium porcine insulin showed a significant increase in water intake as compared to diabetic control rats. Rats treated with human insulin, mixtard insulin, vanadium porcine insulin, and chromium human insulin
produced a significant reduction in food intake as compared to diabetic control rats. The diabetic control animals showed 33% mortality, but the animals treated with mixtard and porcine insulin and human insulin showed 50% of mortality, which may be due to hypoglycemia on insulin treatment. However the most significant observation in the study was that the animals treated with vanadium porcine insulin, chromium porcine insulin or chromium human insulin did not show any mortality.

At the end of the treatment period (6 weeks), animals were kept for overnight fasting and the blood samples were collected for biochemical analysis. Animals were anaesthetized under mild ether anesthesia and blood was collected by retroorbital puncturing. The blood samples were allowed to clot and the serum was separated by centrifugation at 5000 rpm for 15 min. Serum samples were analyzed for serum glucose, cholesterol, triglycerides HDL-cholesterol using respective diagnostic kits (Bayer Diagnostic, India). Serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were estimated spectrophotometrically by using respective diagnostic kits (Span Diagnostics, India). Serum insulin levels were estimated by radioimmunoassay method using the kit obtained from Bhabha Atomic Research Center, Mumbai, India. VLDL-cholesterol and LDL-cholesterol were calculated from the values obtained above as per Friedewald's equation given below.

Oral glucose tolerance test was performed after an overnight fast and the results were expressed as integrated area under the curve (AUC) for glucose and insulin which will be calculated by trapezoid rule \[ \text{AUC} = \frac{C_1+C_2}{2} \times (t_2-t_1) \] and changes in glucose and insulin concentrations over 120 min during OGTT and the results were expressed as AUC_{glucose} (mg/dl.120 min) and AUC_{insulin} (\mu U/ml.120 min) respectively.
Abstract

Histopathological studies of kidney, liver and heart was carried out to study the effects of treatment with insulin formulations on degenerative changes induced by diabetes and also to assess the cardiotoxic, nephrotoxic and hepatotoxic potential. The Hemotoxylin & Eosin stained sections of liver, kidney and heart from all the animals and were evaluated in 10x and 40x using Zeiss AxioLab microscope.

Insulin release studies was conducted using islets cultured from different groups of animals in individual culture flasks. They were hand picked under the microscope and 10 islet size matched cells were transferred into each of the wells in the 96-well cell culture plates containing insulin release media (RPMI 1640) containing 16.0 mM glucose and were incubated at 37 °C for one hour in a CO₂ incubator maintained with 5% carbon dioxide. After 1 hour, the release medium was collected and stored at -20°C until insulin determination was performed using insulin radioimmunoassay (RIA) kit from Bhabha Atomic Research Center, India.

Treatment of rats with STZ produced a significant hyperglycemia and hypoinsulinemia as compared to normal control rats. Treatment with all insulin formulations viz., porcine insulin, human insulin, mixtard insulin, vanadium porcine insulin, chromium porcine insulin and chromium human insulin produced a significant reduction in elevated glucose levels as compared to diabetic control rats. Reduction in serum glucose was comparatively less in rats treated with human insulin. Even the chromium human insulin showed comparatively less reduction in serum glucose levels. In both these groups, glucose levels were still higher as compared to non-diabetic control. Formulations containing trace elements (chromium or vanadium) produced greater reduction in glucose levels as compared to their respective insulin formulations not containing trace elements. However these changes were not significant statistically.
Rats which received STZ, showed reduced insulin levels as compared to non-diabetic control rats. Treatment of rats with porcine insulin and mixtard insulin produced a significant increase in insulin levels not only as compared to diabetic control but also as compared to non-diabetic control rats. Rats, which received human insulin did not produce any significant change in insulin levels as compared to diabetic control animals. There was a multifold increase in insulin levels in rats treated with vanadium porcine insulin as compared to its respective formulation porcine insulin. However, rats treated with chromium porcine insulin the decrease in insulin levels was not significantly different from porcine insulin.

In glucose tolerance test a significant increase in $AUC_{glucose}$ and a reduction in $AUC_{insulin}$ value was observed in STZ diabetic rats as compared to non-diabetic control rats. A significant decrease in $AUC_{glucose}$ was found in rats treated with various formulations viz., porcine insulin, human insulin, mixtard insulin, vanadium porcine insulin, chromium porcine insulin and chromium human insulin produced a significant increase in $AUC_{insulin}$ as compared to diabetic control rats. Maximum decrease in $AUC_{glucose}$ and increase in $AUC_{insulin}$ was observed in rats treated with vanadium porcine insulin. Significant elevation in fasting serum GOT and serum GPT levels were observed in STZ diabetic rats as compared to normal control rats. Treatment with all six insulin formulations produced a significant reduction in elevated SGOT and SGPT levels in diabetic rats. A significant increase in fasting creatinine was also observed in STZ diabetic rats as compared to normal control rats. However this rise was found to be preventing by porcine insulin, human insulin, chromium porcine insulin and chromium human insulin and not by mixtard insulin and vanadium porcine insulin.

Rats treated with STZ produced a significant increase in cholesterol, triglycerides, LDL cholesterol, VLDL cholesterol levels and a significant reduction in HDL cholesterol levels. STZ diabetic rats
also showed a significant increase in fasting levels of cholesterol, triglycerides, LDL cholesterol, VLDL cholesterol levels and a reduction in fasting HDL cholesterol levels as compared to non-diabetic control rats. Treatment of mixtard insulin caused a marked decrease in serum cholesterol, triglycerides, LDL cholesterol and VLDL cholesterol. Even HDL cholesterol was found to be decrease by mixtard insulin preparation. All these values were comparable or lower in non-diabetic control rats treated with mixtard insulin. Treatment of STZ diabetic rats with porcine insulin failed to decrease either triglycerides or VLDL cholesterol. There was a decrease in serum cholesterol but it was not significant. It was interesting to note that porcine insulin caused a significant decrease in LDL cholesterol and slight increase in HDL cholesterol. Treatment of STZ diabetic rats with chromium porcine insulin formulation produced not only a decrease in fasting cholesterol, triglycerides, VLDL cholesterol and LDL cholesterol but also an increase in HDL cholesterol. Increase in HDL cholesterol has been reported both in diabetic and non-diabetic subjects with chromium. In our investigation we found that HDL cholesterol levels are significantly elevated in diabetic rats when treated with chromium porcine insulin. Vanadium porcine insulin also produced effects similar to chromium porcine insulin formulation and rather produced significantly greater reduction in triglycerides, however it was found to cause decrease in HDL cholesterol.

Unlike porcine insulin, treatment of STZ diabetic rats with human insulin failed to decrease fasting serum triglycerides and VLDL cholesterol levels but there was a significant decrease in serum cholesterol, LDL cholesterol as well as HDL cholesterol. Formulation of human insulin with chromium and porcine insulin with chromium were found to decrease not only serum cholesterol, triglycerides, LDL cholesterol and VLDL cholesterol but also the HDL cholesterol. Although the comparative effects of various insulin
formulations on lipid profiles appears to be complex. Mixtard, vanadium porcine insulin and chromium porcine insulin appear to have greater effects in controlling elevated lipid levels in conditions of diabetes.

Rats, which received STZ, showed a significant elevation in fasting serum LDH and serum CKMB levels as compared to normal control rats. Treatment with porcine insulin and chromium porcine insulin produced a significant reduction in elevated LDH levels without any significant change in CKMB levels as compared to diabetic control rats.

Rats which received STZ, showed a significant elevation in glycated haemoglobin levels as compared to normal control rats. Treatment with porcine insulin and chromium porcine insulin produced a significant reduction in elevated glycated haemoglobin levels as compared to diabetic control rats.

The cultured islets from diabetic control rats produced a significant reduction in glucose induced insulin secretion as compared to islets cultured from normal control rats. Islets cultured from mixtard treated diabetic animals produced a significant reduction in glucose induced insulin release as compared to islets cultured from diabetic control rats. Islets cultured from porcine insulin treated diabetic rats, vanadium porcine insulin treated diabetic rats and chromium porcine insulin treated diabetic rats produced no significant change in glucose induced insulin secretion as compared to glucose induced insulin secretion from islets cultured from diabetic control rats.

Results are presented as mean ± SEM. Statistical differences between the means of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test. Data was considered statistically significant at P value ≤ 0.05.
After 6 weeks of treatment all the animals were sacrificed and its kidney, liver and heart were subjected to histopathological studies. The histopathological observations of different groups of animals are explained as follows, Liver from the normal control rats showed normal histology. Liver from diabetic control rats showed damaged cell foci. Liver from porcine insulin treated groups showed portal lymphocytic changes. Liver from rats treated with human insulin showed congestion and dilatation of central artery. Liver from chromium porcine insulin treated animals showed no significant change in liver histology as compared to liver from normal control rats. Liver from chromium human insulin treated rats showed mild dilatation of central artery. Kidney from normal control rats was found to possess normal histology. Kidney from diabetic control rats showed urothelial hyperplasia, pyelonephritis and pelvis dilatation. Histological examination of kidneys from porcine insulin treated animals showed lymphocytic changes and pyelonephritis while kidneys from human insulin treated groups showed mild degeneration. Histological examinations of kidneys from chromium porcine insulin treated group showed normal urothelial hyperplasia and microlith, while kidneys from chromium human insulin treated group showed dilatation and degeneration. Histological examinations of heart from normal control rats were found to be normal. Heart from diabetic control rats showed degenerative changes in histological examinations. The histological examinations of the heart from porcine insulin and chromium porcine insulin treated groups showed normal histology. Hearts from human insulin and chromium human insulin treated rats showed normal histological features.

In conclusion, our results of HPLC chromatographic data and analytical data for metal ion estimation by atomic absorption spectroscopy revealed that all the formulations of insulin were stable at 2-8 °C for the period of 12 months. Among Porcine
insulin, mixtard (highly purified biphasic human insulin) and human insulin formulations, mixtard appears to produce not only more effective control of glucose levels but also prevents STZ induced dyslipidemia. Addition of chromium or vanadium in the insulin preparation appears to produce beneficial effects not only in terms of glycemic control but also with respect to prevention of dyslipidemia and decrease in mortality. Porcine insulin was found to increase HDL cholesterol and this effect was significantly enhanced when its chromium formulation was used. Porcine insulin and chromium porcine insulin produced significant reduction in LDH levels suggesting their beneficial effect in preventing cardiovascular complications.