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THE INHIBITORY EFFECT OF BENZODIAZEPINE DERIVATIVES ON THE BOVINE LENS ALDOSE REDUCTASE ENZYME.

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Summary. The aldose reductase enzyme, involved in the sorbitol pathway which is an important mechanism in regulation of mammalian glucose metabolism, has been known to play a significant role in the initiation of diabetic complications. Numerous chemical substances have been prepared in order to improve the pharmacological profile of inhibition of aldose reductase enzyme. In this study, aldose reductase inhibitory activities of several benzodiazepine derivatives were investigated. The enzyme was obtained from bovine lenses via the ammonium sulphate-protein cut method with several steps. It was found that tetrazepam had a significant inhibitor potency among the other benzodiazepine derivatives showing very slight inhibitor activities that are indicated in terms of percent inhibitor potency at 10^{-4} M concentration.

Key words: Aldose reductase, Benzodiazepines, Lens

INTRODUCTION

The sorbitol pathway (1) consists of two dehydrogenases, aldose reductase and sorbitol dehydrogenase. In mammals, the conversion of glucose to sorbitol is slightly catalyzed by aldose reductase which has low substrate affinity to glucose. However, in diabetes mellitus certain tissues are subject to exposure to high levels of glucose. As a result of faster rate of sorbitol formation than its conversion to fructose and also the polarity of the sorbitol prevents its exit from the cell is

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associated with diabetic complications including those of a lenticular, retinal, neuronal and renal nature. These complications are generally known as diabetic cataract, retinopathy, neuropathy and nephropathy respectively (2,3).

Glycation of proteins is probably only one of the several factors involved in diabetic complications. During hyperglycemia of diabetes the enzyme aldose reductase is believed to play a key role in the loss of reduced glutathione (4). Aldose reductase is a NADPH-dependent enzyme which reduces glucose to sorbitol . High glucose levels results in the excess production of sorbitol thereby depleting the NADPH pool. This depletion of the NADPH influences another NADPH-dependent enzyme, glutathione reductase which is responsible for glutathione regeneration and thus maintaining a reduced environment within the tissue.

The fact that aldose reductase inhibitors can retard or prevent cataract formation suggest that these agents could retard the fall in glutathione levels (5) or could reduce tissue sorbitol accumulation in diabetes (6). Commercial aldose reductase inhibitors are under clinical trials and their value in the prevention of cataract, peripheral neuropathy and retinopathy is being studied. A number of inhibitors have been developed to the stage of clinical evaluation, some problems of lack of effect and adverse reactions have been encountered (7). Alrestatin (8), tolrestat (9) and sorbinil (10) were the first inhibitors to be clinically investigated. More recently a number of inhibitors such as benzothiazol-2-ylcarboxylic acids (11) and diterpenoids (12) were synthesized and evaluated for their ability to inhibit aldose reductase. In our recent study seeking for the inhibitors of aldose reductase enzyme we have reported the inhibitory pattern of aldose reductase enzyme, obtained by Computer Automated Structure Evaluation (CASE) program (13) and by 1-4-dihydro-4-oxoquinoline-3-acetic acid derivative which was synthesized and determined for the ability of inhibiting aldose reductase (14). It appeared that the effect of substituents on the phenyl ring was apparent among aldose reductase inhibitors which was consistent with Kador et al's observation (15). It has been thought that benzodiazepine derivatives containing two phenyl rings and carbonyl groups might interact with aldose reductase binding site.

The benzodiazepines widely used drugs that have been extensively studied, produce anxiolitic, muscle relaxant, hypnotic and anticonvulsive effects (16).The aim of the study was to investigate the effect of benzodiazepines on aldose

reductase enzyme activity. This is due to the effect of benzodiazepine usage of diabetics.

MATERIAL AND METHODS

Materials: NADPH (Sigma Chemical Company), DL-glyceraldehyde (Sigma Chemical Company), Ammonium sulfate (Merck), Tetrazepam (Myolastan, Clin-Byla, Paris), Clonazepam (Rivotril, Roche, Paris), Flunidazepam (Roipnol, Roche, Paris), Chlordiazepoxide (Librium, Roche, Paris), Temazepam (Levanxol, Roche, Paris), Lorazepam (Temesta, Am. Home prod., U.S.A.), Clorazepate (Clin-Byla, Paris), Oxazepam (Serax, Roche, Paris).

Methods:

Isolation of Aldose Reductase Enzyme:

The aldose reductase enzyme was isolated by a method described below. Pooled bovine lens were thawed on ice and homogenized with 3 volume of distilled water, followed by centrifugation at 10000 x g for 20 minutes. Saturated ammonium sulfate was added to the supernatant to 40 % saturation. The thick suspension had been stirred for 15 minutes, followed by centrifugation at 10000 x g for 20 minutes. The inert protein left in the supernatant was removed by increasing the ammonium sulfate concentration to 50 % saturation followed by centrifuging the mixture at 10000 x g for 20 minutes. The aldose reductase enzyme was precipitated from the 50 % saturated solution by adding powdered ammonium sulfate to 75 % saturation and was recovered by centrifugation at 10000x g for 20 minutes. Protein concentration was measured by the method of Lowry using bovine serum as the standard (17).

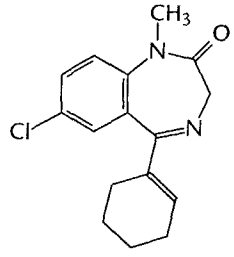
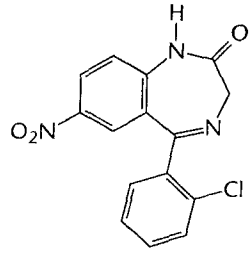
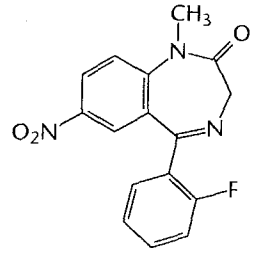
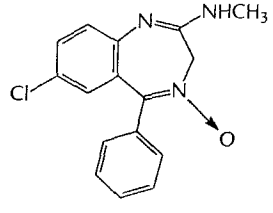
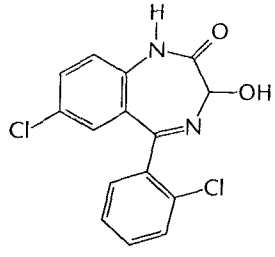
Determination of Aldose Reductase Activity:

Aldose reductase activity of the freshly prepared supernatant was assayed spectrophotometrically by determining the decrease in NADPH concentration at 340 nm by a SP8-100 Pye Unicam UV spectrophotometer. DL-glyceraldehyde was used as the substrate. The enzyme was dissolved in 10 ml 0.05 M NaCl solution and 0.1 ml was added to a quartz cuvette containing 0.2 ml phosphate buffer (0.067 M, pH 6.2), 0.5 ml NADPH (2×10^{-7} M final concentration), 0.1 ml of the test drug (each inhibitor was prepared as a stock solution and was diluted with distilled water to the desired concentration of 10^{-4} M) and 2 ml distilled water to obtain 2.9 ml solution. The reaction is started by the addition of 0.1 ml DL-glyceraldehyde (5×10^{-4} M, final concentration) to the cuvette and the decrease in NADPH concentration was recorded at 340 nm for 10 minutes at 37 $^{\circ}$ C. Readings were taken at intervals in the periods when the changes in absorbance were linear. The results are shown in Table 1 as percent inhibition values which were obtained by 10^{-4} M concentrations of each drug (18).

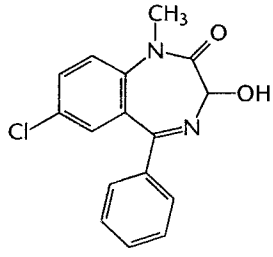
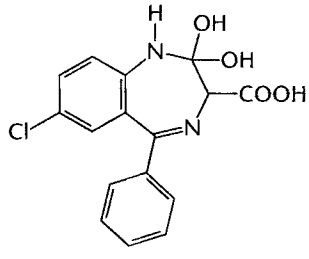
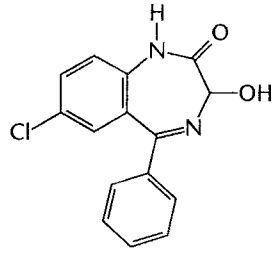
RESULTS

The inhibition obtained by benzodiazepine derivatives on the bovine aldose reductase enzyme were studied in vitro and the results are represented in Table 1. The inhibition study was performed merely by using a 10^{-4} M concentration in which no additional study seemed to be necessary to obtain pIC_{50} values. Within

Table 1: Aldose Reductase Inhibition by Benzodiazepine Derivatives

COMPOUNDS		INHIBITION (%)
Tetrazepam		**37.09 ± 2.09**
Clonazepam		**29.00 ± 3.00**
Flunidazepam		**23.00 ± 4.00**
Chlordiazepoxide		**11.45 ± 2.25**
Lorazepam		**8.39 ± 1.09**

continues

COMPOUNDS		INHIBITION (%)
Temazepam		**0.00 ± 0.00**
Clorazepate		**0.00 ± 0.00**
Oxazepam		**0.00 ± 0.00**

Values represent the mean ± S.D. of four individual experiments.

the benzodiazepines tetrazepam has shown the highest inhibitory effect. Clonazepam, flunidazepam and chlordiazepoxide have lower inhibitory activity than tetrazepam. Lorazepam has also shown lower inhibitory activity. 10^{-4} M concentrations of Temazepam, Clorazepate, Oxazepam have no inhibitory activity.

The inhibiton % of tetrazepam was 37.09, clonazepam was 29.00, flunidazepam was 23.00, chlordiazepoxide was 11.45 and lorazepam was 8.39 (Table 1).

DISCUSSION

In diabetes increased flux through the polyol pathway has been implicated in the development of diabetic complications in lens, retina, peripheral nerves (3). In lens, nerve, kidney and retina aldose reductase enzyme appears to be the key factor in the reduction of glucose to sorbitol (19). Accumulation of sorbitol in diabetes causes complications such as diabetic cataract, retinopathy, neuropathy and nephropathy (15). Aldose reductase enzyme inhibitors have been found to prevent cataract in the diabetic rat and to improve nerve conduction velocity (20) . They reduce sorbitol accumulation in tissues (21).

This study shows an inhibitory effect of benzodiazepine derivatives on aldose reductase activity. All compounds have a substituent which is mostly chloride at 7th position (Table 1). The nitro substitution occurred in two derivatives flunidazepam and clonazepam. The substituent change in 7th position seemed to affect the inhibitory potency of compounds to some extent. In fact, tetrazepam, the highest active derivative has chloride substitution on this position. The affinity of the inhibitor derivative on the aldose reductase enzyme may be affected by changes in the aromatic substitution pattern switching the chloride with nitro has been resulted in a decrease in the activity. Previous studies concerning the mechanism of aldose reductase enzyme inhibition have shown that certain electronic and steric requirements are necessary for biological activity. Most of these were based on computer modeling and molecular orbital calculations (11).

It has been proposed that the relevant structural features of aldose reductase enzyme inhibitors consist of at least one planar region with hydrogen binding substituents and a carbonyl or thiocarbonyl group which will be capable of undergoing reversible nucleophilic attack (22,23). The benzodiazepine derivatives containing two aromatic rings might be thought to interact with the aldose reductase binding sites. An aromatic ring with the capability of hydrogen-bonding ability may also be required to afford aldose reductase inhibitory activity. The results of the present study appeared to be consistent with the above hypothesis in which the planar features might have additionally substituent that should exhibit hydrogen bonding capability. If it was possible to replace the substutients with a convenient hydroxyl group on the aromatic ring, it seemed likely that the resulting compounds

might show more inhibitory activity. The result of the study shows the effect of benzodiazepine derivatives studied was less effective on aldose reductase enzyme activity. This study is still on progress.

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