WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 6.041
Volume 5, Issue 3, 493-497 Research Article ISSN 2278 – 4357

MEMBRANE STABILIZATION ASSAY FOR ANTI-INFLAMMATORY ACTIVITY YIELDS FALSE POSITIVE RESULTS FOR SAMPLES CONTAINING TRACES OF ETHANOL AND METHANOL

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Article Received on
12 Jan 2016,

Revised on 01 Feb 2016,
Accepted on 22 Feb 2016

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ABSTRACT

There is an upsurge in the reports on erythrocyte haemolysis assay being used as a simple and rapid tool for screening of drugs and phytochemicals for anti-inflammatory activity. However, in case of hydroalcoholic extracts, there is a possibility of the trace amounts of ethanol or methanol retained even after drying. These traces are not accounted in the assay units representing the solvent control assay units. The present study was designed to substantiate the effects of low concentrations of ethanol and methanol on the heat induced haemolysis. It was observed that the presence of ethanol and methanol at concentrations as low as 0.1 µl/ml exert significant membrane stabilizing activity. Thus, this assay may give misleading results if the test drug solution contains even slightest amount of ethanol or methanol. Therefore, present study suggested that the heat-induced erythrocyte haemolysis assay is not suitable for testing membrane stabilizing and anti-inflammatory activity of the test samples containing ethanol or methanol.

KEYWORDS: Erythrocyte stabilization; Membrane stabilization; RBC haemolysis.

INTRODUCTION

The *in-vitro* erythrocyte haemolysis assay is generally used for screening anti-inflammatory activity of drugs.[1,2,3,4] Majority of the anti-inflammatory drugs stabilize the plasma

membrane of mammalian erythrocytes and thereby inhibit the heat-induced and the hypo tonicity-induced haemolysis.[5]

The plasma membrane of mammalian red blood cells (erythrocytes) has been particularly useful as a model for studies of membrane structure. Mammalian red blood cells do not contain nuclei or internal membranes, so they are used as a source from which pure plasma membranes can be easily isolated for biochemical analysis. The erythrocyte plasma membrane resemblances to the lysosomal membrane and hence the stabilizing effect of drugs on erythrocyte membrane may correlate with its lysosomal membrane stabilizing effect.[4] The lysosomal membrane stabilization leads to the inhibition of release of the inflammatory mediators and consequent inhibition of the process of inflammation.[6] In the membrane stabilization assay, the erythrocytes are challenged with different haemolytic stimuli like heat, osmotic shock and free radicals.[7] The heat-induced and hypotonicity induced haemolysis of erythrocytes is extensively used as a rapid, simple, economic and sensitive tool in determining the anti-inflammatory property of drugs. Due to this simplicity and economy, the researchers prefer to use this model in the preliminary screening of hydro-alcoholic extracts of medicinal plants. While standardizing this model in our laboratory, we came across a fact that ethanol and methanol have significant membrane stabilizing activity. There is dearth of reports on the evaluation of lower concentrations of ethanol and methanol on the erythrocyte membrane stabilization. In present work, we investigated the membrane stabilizing effects on ethanol and methanol against the-heat induced breakdown of human erythrocytes.

MATERIALS AND METHODS

All the solutions used in this study were of analytical grade. Ethanol was procured from Changshu Yangyuan Chemical, China. Methanol was purchased from Sigma Aldrich. Diclofenac (NSAID) marketed formulation in the form of Diclofenac sodium injection was used as reference drug for membrane stabilizing activity and was purchased from Troikaa Pharmaceuticals Ltd, India. All the dilutions were prepared in Phosphate buffer saline.

Erythrocyte suspension

To prepare the erythrocyte suspension, 12 mL of whole blood samples were collected through vein puncture from a healthy human volunteer. Each sample was mixed with equal amount of sterile phosphate buffered saline (PBS) containing 100 IU/ml of heparin. These samples were centrifuged at 2000 rpm for 10 minutes and the resultant erythrocyte pellet was washed thrice

with heparinized PBS and finally suspended in PBS to contain 40% v/v of RBCs in PBS.[8] We evaluated the effects of ethanol and methanol in concentrations ranging from 100µl/ml to 0.001 µl/ml in the final assay medium.

Procedure for heat-induced haemolysis

Various concentrations of ethanol and methanol such as 100µl/ml, 10µl/ml, 1 µl/ml, 0.1 µl/ml, 0.01 µl/ml, 0.001 µl/ml were prepared in PBS. The experiments were carried out in triplicates. In separate eppendorf tubes, equal amounts of each of PBS, stock erythrocyte suspension and ethanol or methanol dilutions were taken. Negative controls in which ethanol/methanol dilutions were replaced with PBS were also maintained. Positive control contained diclofenac at concentration of 50µg/ml of assay medium.[9]

The eppendorf tubes were heated in a waterbath at 54˚C for 20 minutes. After heating, the reaction mixtures were centrifuged at 5400 rpm for 5 minutes and the absorbance (O.D.) of the supernatant was measured using microplate reader (Powerwave XS, Biotek) at 540 nm.[10]

RESULTS AND DISCUSSION

Heating the assay units at 54^0C induced haemolysis in the negative control samples. The Figure1 depicts the haemolysis in the negative control samples as 100 percent and the relative percentage of haemolysis in the samples treated with various concentrations of ethanol and methanol. The samples treated with diclofenac (at concentration of 50 □g/ml) were protected from heat induced haemolysis of erythrocytes.

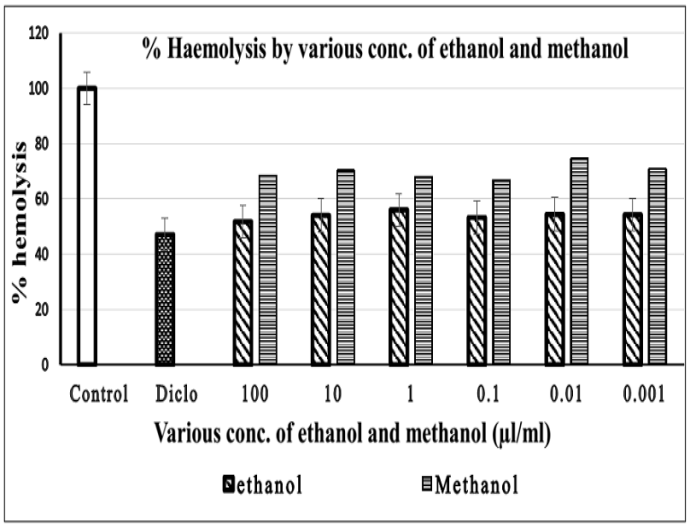

Figure 1. Ethanol and methanol protects the human erythrocytes against the heat induced haemolysis.

The data were analysed by the one-way ANOVA. $p < 0.01$ was considered as statistically significant. The assay units containing ethanol and methanol were also protected from the haemolysis and this protective effect was statistically significant even at alcohol content in assay medium as low as 0.01 µl/ml. Our results indicate that even trace amount of alcohol significantly protects the erythrocytes from heat induced haemolysis Erythrocyte membrane stabilization model is widely used model and it is very simple, easy to carry out. It includes incubation of erythrocytes with drug and then heating is used to induce haemolysis. Non-steroidal anti-inflammatory drugs protect proteins from heat denaturation.

Membrane stabilization involves the process in which the integrity of the erythrocyte membrane and lysosomal membrane is maintained by anti-inflammatory drugs by stabilizing the membrane. This stabilizing effect of anti-inflammatory drugs on erythrocyte membrane may be due to a stabilizing effect of the drugs on certain proteins in the membrane.[11] Lysosomes contain several enzymes which may be involved in the process of inflammation. During inflammation, lysosomal enzymes are released into the cytosol, causing damage to the surrounding tissues, thereby triggering inflammation. Most of the anti-inflammatory drugs stabilize lysosomal membrane and inhibit the inflammatory process by restricting the release of lysosomal enzymes.

Our interest in this topic was developed as many studies have reported the membrane stabilizing activity of alcoholic extracts of various plants. For the sake of our interest we designed this study using the plain ethanol and methanol at various concentrations. Because ethanol and methanol are widely used as solvent for extraction of active phyto-constituents from plants as well as it is used in preparation of various homeopathic remedies.

CONCLUSION

Hence, this assay may yield misleading results particularly for the alcoholic and hydroalcoholic extracts which may contain traces of alcohol even after sufficient drying and reconstitution. It is suggested that this assay should be used with caution for the extracts and drug solutions which are suspected to contain ethanol or methanol.

Conflict of interest

The research described in this manuscript was carried out at an educational Institute as a part of a dissertation. We did not receive any funds that could influence our work. We have not received any honoraria, consultancy fees.

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