INTRODUCTION

In the normal erythrocyte, a redundancy of cell membrane gives the cell its characteristic discoid shape and provides it with abundant surface area. Decrease in surface area relative to cell volume resulting in their abnormal shape, (Gallagher *et al.*, 1998). Drug induced hemolysis is generally accompanied by the formation of Heinz bodies, particles of denatured hemoglobin and stromal protein. Exposure of red cell to drugs results in the formation of low levels of hydrogen peroxide; as the drug interacts with hemoglobin (Cohen *et al.*, 1964). Some drugs may also form free radicals that oxidize reduced glutathione (GSH) which through the action of peroxide or by the direct action of drugs may be followed either by oxidation of GSH to the diffuse form (Birchmeier *et al.*, 1973). Once such oxidation has occurred, hemoglobin is denatured irreversibly and will precipitate as Heinz bodies and lead to decreased red cell survival (Rifkind *et al.*, 1965).

The methods have been developed for measuring osmotic fragility (OF) by automatic procedures. The "Fragiligraph" (Danon, 1963) is an instrument in which red cells diluted in isotonic NaCl solution are contained in a microcuvette; made of two walls of dialyzing membrane, surrounded by distilled water. The cells lyse as the salt concentration of the medium decreases and the degree of lysis is recorded automatically in the form of fragility curve. Since, it reflects both hypotonicity and time of exposure; the curve is not exactly the same as that derived by the traditional method.

Therefore a design (Ahmad *et al.*, 2004; Zahidi and Nabavizadeh, 2002) adopted for this study is based upon the simple method of Parpart and Co-workers (1947).

MATERIALS AND METHODS

Choice of animals

A detailed examination of the literature indicates that reptiles have been mostly neglected as a research material. Large populations of the spiny tailed lizard exist in the desert and semi-desert regions of Karachi and Thatta Districts. The animals are easily available, cost less and are easily managed. Thus, for the present study animals were obtained from the local suppliers.

Temperature

One of the important factors in the physical environment is temperature (Ahmad *et al.*, 1980, 2002 and 2003). Poikilotherms are incapable of maintaining their body temperature and variation in the ambient temperature affects their body by altering their physiological state. Therefore, in order to obtain reproducible results the temperature was kept constant at 32 ± 1^{o}C during the experimental period.

Design of experiment

40 *Uromastix,* almost equal in body weight and size were sorted out from the stock to form four equal groups. One of the groups was kept as control and the others served as test.

Drug administration

Normally 2g of mefenamic acid (i.e. 8 tablets) is a dose prescribed for a human subject for 24 hrs. Therefore, normal dose of mefenamic acid for a *Uromastix* weighing 250 g was 7.1 mg/day. This dose of mefenamic acid was given orally everyday to each test animal. For this purpose the suspension was prepared in distilled water and diluted in such a way that 1 ml contained 7.1 mg of the drug. Thus, each test animal received 1 ml suspension per day for a period of 12 days; while control received 1ml of distilled water in a similar way for the same period. In addition, the test and control animals were fed 1ml 5 % glucose solution thrice a week. The OF of cell membrane was estimated for control and tests on the 6th and the 12th day (table 1).

In the second experiment 10 test animals were given 10.5 mg of mefenamic acid per day for a period of 12 days; and the OF was estimated in the same way as that of the first experiment (table 2).

In the third experiment 10 test animals were given 14 mg of mefenamic acid per day for a period of 12 days; and erythrocyte fragility was estimated in the same way (table 3).

Sodium chloride solution series

No.	% NaCl	ml 1 % NaCl	ml dist. Water	No.	% NaCl	ml % water	ml dist. water
1	0.7	0.70	0.30	21	0.30	0.30	0.70
2	0.68	0.68	0.32	20	0.32	0.32	0.68
3	0.66	0.66	0.34	19	0.34	0.34	0.66
4	0.64	0.64	0.36	18	0.36	0.36	0.64
5	0.62	0.62	0.38	17	0.38	0.38	0.62
6	0.60	0.60	0.40	16	0.40	0.40	0.60
7	0.58	0.58	0.42	15	0.42	0.42	0.58
8	0.56	0.56	0.44	14	0.44	0.44	0.56
9	0.54	0.54	0.46	13	0.46	0.46	0.54
10	0.52	0.52	0.48	12	0.48	0.48	0.52
11	0.50	0.50	0.50	-	-	-	-

Table 1
Osmotic fragility of erythrocytes after 6 and 12 days following the oral dose of 7.1 mg/day mefenamic acid at 32 ± 1°C

Concentration of hypotonic solution (%)	Days after administration of drug			
	6		12	
	C	T	C	T
0.1	70	71	70	73
0.2	50	50	50	70
0.3	26	27	26	30
0.4	0	1	0	3
0.5	0	0	0	0
0.6	0	0	0	0
0.7	0	0	0	0
0.8	0	0	0	0
0.9	0	0	0	0
1.0	0	0	0	0

Each figure indicates the mean per cent lysis of 10 samples. C and T indicate the values for control and test respectively.

Table 2
Osmotic fragility of erythrocytes after 6 and 12 days following the oral dose of 10.5 mg/day mefenamic acid at 32 ± 1°C

Concentration of hypotonic solution (%)	Days after administration of drug			
	6		12	
	C	T	C	T
0.1	70	71	70	75
0.2	50	58	50	75
0.3	26	31	26	40
0.4	0	5	0	9
0.5	0	0	0	0
0.6	0	0	0	0
0.7	0	0	0	0
0.8	0	0	0	0
0.9	0	0	0	0
1.0	0	0	0	0

Each figure indicates the mean per cent lysis of 10 samples. C and T indicate the values for control and test respectively.

Collection of blood
Blood required for the determination of OF, was collected from the anterior abdominal vein of each individual of control and tests group on day 6 and day 12 (Ahmad *et al.*, 2001 a and b).

Osmotic fragility test (OFT)
The method basically devised by Parpart and Co-workers (1947) was adopted as a routine method by Zahidi and Nabavizadeh (2002) using concentration of 0.09 g/L NaCl. Whereas in the present study, hypotonic NaCl saline buffered to pH 7.4 was used, and the blood was added to the hypotonic solutions in the proportion of 1 to 100. The test was carried out at recommended room temperature of 25 – 27°C (Keele *et al.*, 1983) and hemolysis was read photoelectrically (Ahmad *et al.*, 2004).

A stock solution of buffered sodium chloride, osmotically equivalent to 10 % NaCl was prepared by dissolving 90g NaCl, 13.65g disodium hydrogen phosphate (Na_2HPO_4, anhydrous) + 2.43g sodium dihydrogen phosphate (NaH_2PO_4, $2H_2O$) in distilled water and the final volume was adjusted to 1 litre. This solution was kept for months without deterioration at 4°C in a well stoppered bottle. In preparing hypotonic solution for use, first a 1 % solution from the 10 % stock solution was prepared. This was again by dilution with distilled water was turned into 0.9 %, 0.8 %, 0.7 %, 0.6 %, 0.5 %, 0.4 %, 0.3 % and 0.1 %. Each of these dilutions were made upto a volume of 50 ml.

Principle and procedure of osmotic resistance
The resistance of erythrocytes against a hypotonic sodium chloride solution was tested by preparing a dilution series and recording the beginning of hemolysis (minimum resistance) and complete hemolysis (maximum resistance).
Sodium chloride solution 1%

Sodium chloride	1.0 g
Distilled water	to 100 ml

For this purpose 21 test tubes were prepared and each with 1 ml of a sodium chloride dilution series from 0.7 % to 0.3 % NaCl (concentration interval 0.02 %) by sucking up 1 ml of the 1 % sodium chloride solution into 1 ml measuring pipette and pipette 0.7 ml into test tube No.1 and remaining 0.3 ml into test tube No.21. Again sucked up 1 ml and pipette 0.68 ml into test tube No.2 and remaining 0.32 ml into test tube No.20 etc. Then all test tubes were filled with distilled water to 1 ml using the same pipetting technique in the reverse sequence (see table below).

To each test tube, 1 or 2 drops of freshly taken unaltered venous blood was added. Some authors prefer using defibrinated blood. Each test tube was gently shaked once more and left to stand in the rack for about 2 hours at a constant temperature (room temperature or at 37°C in an incubator).

Reading
Note the test tubes in which the NaCl solution showed the first reddish-yellow discoloration (beginning of hemolysis = minimum resistance) and that of the test tube in which no bottom sediment of erythrocytes was left (complete hemolysis = maximum resistance).

Normal value

Beginning of hemolysis	0.46 % to 0.42 % NaCl
Complete hemolysis	0.34 % to 0.30 % NaCl

The domain between these two points is called the resistance range.

Table 3
Osmotic fragility of erythrocytes after 6 and 12 days, following the oral dose of 14 mg/day mefenamic acid at 32 ± 1°C

Concentration of hypotonic solution (%)	Days after administration of drug			
	6		12	
	C	T	C	T
0.1	70	80	70	85
0.2	50	60	50	85
0.3	26	38	26	45
0.4	0	7	0	11
0.5	0	3	0	7
0.6	0	0	0	0
0.7	0	0	0	0
0.8	0	0	0	0
0.9	0	0	0	0
1.0	0	0	0	0

Each figure indicates the mean per cent lysis of 10 samples. C and T indicate the values for control and test respectively.

Care

Heparinized venous blood or defibrinated blood may be used. Oxalated or citrated blood should be avoided because of additional salts being added to the blood. The test should be carried out within 2 hours of collection, but can be delayed for 6 hours, if the blood is kept at 4°C. To test 0.05 ml volume of the blood was added to 5 ml volume of a suitable range of hypotonic solutions and immediately mixed by inverting several times. The tubes were allowed to stand at room temperature for 30 minutes and re-mixed and centrifuged for 5 minutes at 1,200 – 1,500 rpm. The amount of hemolysis in each tube was then compared with the tube containing 0.1% NaCl, and read in Beckman spectro-photometer at 540 nm. The supernatant from the 0.9% NaCl tube was used as the blank.

When a range of hypotonic solution has been used a "fragility curve" was drawn by plotting a graph of the percentage of hemolysis in each tube against corresponding concentration of salt solution. Normally a symmetrical sigmoid curve results (Dacie and Lewis, 1984).

RESULTS

A consideration of tables 1, 2 and 3 indicates that erythrocytes of control were completely lysed in 0.4% saline solution. Test groups administrated 7.1 mg/day and 10.5 mg/day mefenamic acid showed complete hemolysis in 0.5% saline solution. However the erythrocytes of test group given a high dose i.e. 14 mg / day mefenamic acid was more resistant to hypotonicity and 100 % lysis was observed in 0.6 % saline solution. In all experiments the percentage of hemolysis between control and test groups differ significantly ($p < 0.05$, t-test).

DISCUSSION

The factors that control red cells fragility are complex but of major importance is its shape; which in turn depends on its volume and surface area along the functional state of its surface membrane. Study of erythrocyte membrane has revealed quantitative abnormalities of several membrane proteins, (Pekrum *et al.*, 1993, Savoides *et al.*, 1993, Saad *et al.*, 1994). Erythrocyte membrane defect is found to be because of leakage to sodium and exhibit a loss of lipids leading to surface area deficiency. Abnormalities of protein of the erythrocyte membrane have been identified as the etiology of the defect (Cooper, 1980). The function of erythrocytes' spectrin is to maintain cellular shape, regulate the lateral mobility of integral membrane protein and provide structural support for the lipid bilayer, (Morrow *et al.*, 1997). Older erythrocytes are less resistant to lysis are destroyed early. Reticulocytes and young mature cells are more resistant to lysis (Ahmad *et al.*, 2004). An OFT is useful measurement in a patient suffering from hemolytic anemia.

In the present study the observation of OF curve at different doses of mefenamic acid shows that at lower dose for short duration, OF curve follows the same sigmoid pattern that of control, while for longer duration the difference is much prominent (fig. 1).

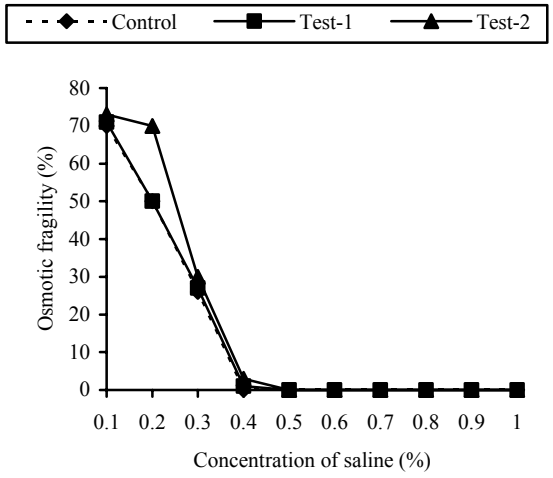

Fig. 1: Osmotic fragility of erythrocytes after administration of 7.1 mg/day mefenamic acid.

Figs. 1 and 2 indicate that higher doses of mefenamic acid greatly affect the osmotic resistance of erythrocytes, even given for 6 days only.

Furthermore, at very high dose of mefenamic acid administered for 12 days shows slight elevation in the lower part of curve indicating the entry of newly synthesized more resistant erythrocytes in circulation (fig. 3). This greater resistance indicates that newly synthesized cells substituting the older ones are comparatively more resistant to lysis.

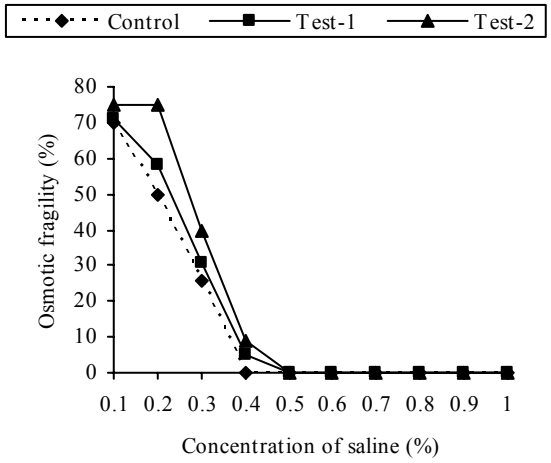

Fig. 2: Osmotic fragility of erythrocytes after administration of 10.5 mg/day mefenamic acid.

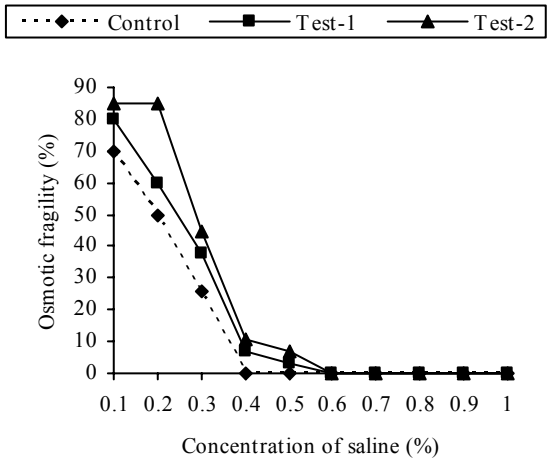

Fig. 3: Osmotic fragility of erythrocytes after administration of 14.0 mg/day mefenamic acid.

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