Advantages of the Hematocrit Method for Testing Isotonicity of Injectable Solutions

By IVO SETNIKAR and OLIMPIA TEMELCOU

Fifty solutions of substances of pharmaceutical interest, all found nonhemolytic at iso-osmotic concentration by Hammarlund and Pedersen-Bjergaard (11) on human blood, were tested for their hemolytic properties and isotonicity on rabbit red cells using the hematocrit method. It was demonstrated that several of these solutions showed some hemolytic properties on rabbit red cells because they were not isotonic at iso-osmotic concentration. Other solutions precipitated hemoglobin; this explains the absence of hemolysis when the hemolytic method was used. The reasons are given for preferring the hematocrit method to the hemolytic method for testing isotonicity and general compatibility with blood.

THE HEMOLYTIC METHOD (1–12) is probably among the simplest for testing the compatibility of injectable solutions with erythrocytes. However, it has the following drawbacks when the isotonicity of solutions has to be tested.

(*a*) It is not possible to calculate the isotonic concentration from a hemolysis-preventing concentration because the ratio between these two concentrations can vary considerably (14, 15). (*b*) The compatibility is tested by adding a very small quantity of blood to the solution. Thus the environment is very different from the actual conditions of injection. In particular, the pH depends on the pH of the solution and is not buffered by the blood (11). As a result, the information on the compatibility of the solution with erythrocytes may be misleading. (*c*) In absence of hemolysis it is difficult to distinguish between a real prevention of hemolysis and a precipitant action on hemoglobin (9, 15, 16). (*d*) The substances present in the solution may alter the colorimetric determination of hemoglobin in solution either by altering the absorbance of hemoglobin or by interfering with their own color with the colorimetric reading (11). For this reason the quantitative appreciation of hemolysis may be incorrect.

The hematocrit method (15, 16), which gives direct information on the isotonicity of a solution and which is performed in environmental conditions much closer to the actual conditions of injection, therefore seems better suited to test injectable solutions for isotonicty with erythrocytes and for general compatibility with blood.

In this paper a series of substances were tested for their isotonicity on rabbit red cells using the hematocrit method. All these substances were selected from those which, at iso-osmotic concentrations, were found not hemolytic on human blood by Hammarlund and Pedersen-Bjergaard (11).

Received January 4, 1963, from the Research Division, Recordati, Laboratorio Farmacologico S. p. A., Milan, Italy.
Accepted for publication March 5, 1963.

METHOD

The solutions to be tested were mixed 1 : 1 (v/v) with a suspension of red cells as previously described (16).

The hematocrit determinations were performed using tubes of 3-mm. diam. and a length of 100 mm. in duplicate on serial concentrations of the solution to be tested. In the concentration scale, the iso-osmotic and (if possible) the isotonic concentration, were included. Hemolysis on the supernatant was classified in the following way: absent (0); light (L), with a concentration of hemoglobin up to 0.5%; medium (M), with a hemoglobin concentration from 0.5 to 3%; heavy (H), with a hemoglobin concentration of more than 3%. The hemoglobin concentration was not determined on the supernatant of each sample but by comparing the sample with hematocrit tubes containing hemoglobin solutions in serial concentration. Complete hemolysis gave a hemoglobin concentration of 15%.

RESULTS

Rabbit Red Cells.—The substances tested for isotonicity and for hemolytic effects by the hematocrit method are listed in Table I. The iso-osmotic concentrations given in Table I are based on the experimental data obtained with human erythrocytes by Hammarlund and Pedersen-Bjergaard (17).

With regard to hemolysis, Table I shows that the results obtained on the carbohydrates and polyalcohols, all with six or more C atoms, were in agreement with those found by the hemolytic method by other investigators (8, 11). Moreover, the isotonic concentration found for these compounds was equal to the iso-osmotic concentration.

Iso-osmotic concentrations of calcium, magnesium, potassium, and sodium salts provoked in many instances a light or moderate hemolysis in disagreement with the results obtained by the hemolytic method. For 13 out of 32 of these solutions, the hematocrit method showed that the isotonic concentration for rabbit blood was different from the iso-osmotic concentration.

The miscellaneous substances, at iso-osmotic concentration, produced hemolysis with the exception of tetraethylammonium chloride. It was found also that the isotonic concentrations were

consistently higher than the iso-osmotic concentration.

Some of the solutions described in Table I under *Miscellaneous* were tested also by the hemolytic method on rabbit blood following the procedure described by Shaw and Husa (13). The compatibility of these solutions with rabbit hemoglobin was tested, adding to 10 ml. of these solutions 0.1 ml.

TABLE I.—ISOTONIC CONCENTRATIONS AND HEMOLYSIS OBSERVED WITH THE HEMATOCRIT METHOD ON RABBIT RED CELLS

	Iso-osmotic Concn., %	Isotonic Concn. Range, %	Hemolysis Observed at Iso-osmotic Concn.	Hemolysis Observed at Isotonic Concn.	Approximate Ratio between Isotonic and Iso-osmotic Concn.
Carbohydrates and Polyalcohols					
Dextrose U.S.P.	5.51	5.5 to 5.6	O	O	1
d-Fructose	5.05	5.0 to 5.1	O	O	1
Lactose U.S.P.	9.75	9.5 to 10.0	O	O	1
Mannitol U.S.P.	5.07	5.0 to 5.1	O	O	1
Sorbitol $^1/_2$ H_2O	5.48	5.4 to 5.5	O	O	1
Sucrose U.S.P.	9.25	9.0 to 9.5	O	O	1
Calcium and Magnesium Salts					
Calcium chloride·6 H_2O	2.50	2.4 to 2.5	O	O	1
Calcium lactate N.F.	4.50	4.4 to 4.6	O	O	1
Calcium pantothenate U.S.P.	5.50	5.4 to 5.6	O	O	1
Magnesium chloride	2.02	2.0 to 2.1	O	O	1
Magnesium sulfate·7 H_2O	6.30	5.0 to 5.1	O	O	0.8
Potassium Salts					
Chlorate N.F.	1.88	1.8 to 2.0	L	L	1
Chloride U.S.P.	1.19	1.1 to 1.2	O	O	1
Iodide U.S.P.	2.59	2.5 to 2.6	L	L	1
Nitrate N.F.	1.62	1.6 to 1.7	L	L	1
Penicillin G U.S.P.	5.48	9.5 to 10.0	H	M	1.8
Phosphate monobasic	2.18	2.7 to 2.8	O	O	1.3
Sulfate	2.11	1.7 to 1.8	L	O	0.8
Sodium Salts					
Acetate anhydrous	1.18	1.6 to 1.8	L	O	1.4
Ascorbate	3.00	3.0 to 3.1	O	O	1
Barbital	3.12	2.9 to 3.0	O	O	0.9
Benzoate U.S.P.	2.25	2.3 to 2.6	M	M	1
Bicarbonate	1.39	1.3 to 1.4	O	O	1
Biphosphate·2 H_2O	2.77	2.8 to 2.9	O	O	1
Bisulfite U.S.P.	1.50	2.0 to 2.1	O	O	1.4
Borate U.S.P.	2.60	2.6 to 2.7	M	M	1
Cacodylate N.F.	3.30	3.0 to 3.1	O	O	0.9
Chloride	0.90	0.91 to 0.93	O	O	1
Citrate U.S.P.	3.02	2.3 to 2.4	O	O	0.7
Fluorescein U.S.P.	3.34	4.5 to 4.6	?	?	1.3[a]
Iodide U.S.P.	2.37	2.2 to 2.3	M	M	1[b]
Nitrate	1.36	1.0 to 1.1	L	L	0.8[c]
Nitrite U.S.P.	1.08	1.0 to 1.1	O	O	1[d]
Phosphate dibasic·2 H_2O	2.23	1.6 to 1.7	O	O	0.7
Propionate N.F.	1.47	1.4 to 1.5	O	O	1
Salicylate U.S.P.	2.53	4.0 to 4.1	M	M	1.6
Sulfate anhydrous	1.61	1.5 to 1.6	O	O	1
Thiosulfate N.F.	2.98	2.6 to 2.7	O	O	0.9
Miscellaneous					
Ammonium phosphate dibasic	1.76	3.1 to 3.3	H	O	1.8[e]
Ammonium sulfate	1.68	5.0 to 5.2	H	L	3[f]
Amphetamine phosphate N.F.	3.47	>16	H	?	>5[f]
Amphetamine sulfate U.S.P.	4.23	9.0 to 9.2	M	H	2
Atropine sulfate U.S.P.	8.85	>15	L	?	>1.7[g]
Decamethonium bromide	5.00	6.2 to 6.4	H	H	1.3
Dextro-amphetamine sulfate U.S.P.	4.20	10.0 to 10.5	H	H	2.4[h]
Ephedrine sulfate U.S.P.	4.54	13.0 to 13.5	L	H	3
Hexamethonium chloride	3.30	...	...	...	[i]
Phenol U.S.P.	2.80	...	...	...	[j]
Silver Nitrate U.S.P.	2.74	...	...	...	[i]
Tetramethylammonium chloride	2.67	3.4 to 3.5	O	O	1.3

[a] Hemolysis not appreciable because of the intense coloration of the sodium fluorescein solution. [b] An increase of concentration increases hemolysis and diminishes hematocrit values. [c] Denaturation and flocculation of hemoglobin. [d] Dark color of the red cell column. [e] Transparent red cell column. [f] Transparent red cell column with some floccules. [g] Transparent and brownish-red cell column. [h] The minimum hemolysis was observed at concentrations ranging from 6 to 8%. [i] Dark precipitate. [j] Precipitation.

TABLE II.—HEMOLYTIC AND HEMOGLOBIN PRECIPITATING ACTIVITIES OF SOME SOLUTIONS

	Iso-osmotic Concn., %	Hemolytic Method		Compatibility with Rabbit Hemoglobin	
		Hemolysis	Color Changes	Precipitation	Color Changes
Ammonium phosphate dibasic	1.76	O[a]		...	...
Ammonium sulfate	1.68	L[a]		...	...
Amphetamine phosphate N.F.	3.47	O[a]		...	...
Amphetamine sulfate U.S.P.	4.23	L[a]		...	...
Atropine sulfate U.S.P.	8.85	O[a]	dark	...	dark
Phenol U.S.P.	2.80	O	grey	complete	grey
Silver nitrate U.S.P.	2.74	O	dark	almost complete	dark

[a] A layer of hemoglobin in solution was noted on the surface of the red cell sediment.

of a 20% rabbit hemoglobin solution obtained from red cells washed three times with 0.9% NaCl and hemolyzed with repetitive freezing.

Table II shows that the results obtained on ammonium phosphate and sulfate, amphetamine phosphate and sulfate, and atropine sulfate roughly agree, in regard to hemolysis, with those described by Hammarlund and Pedersen-Bjergaard (11). However, a thin layer of hemoglobin in solution was always noted above the red cell sediment, showing that a partial hemolysis was present and explaining the hemolysis observed with the hematocrit method (*cf.* Table I). The hematocrit method seems therefore more sensitive for hemolysis, probably because comparatively about 100 times more red cells are added to the solution to be tested.

Phenol and silver nitrate provoked precipitation of hemoglobin; this explains the absence of hemolysis reported by Hammarlund and Pedersen-Bjergaard (11). The precipitation appeared very clearly in the hematocrit tubes showing the incompatibility of these substances with blood. Using the hemolytic method, a darkening and shrinking of the red cells with a milky appearance of the solution (for phenol) was reported (11)—phenomena which may be sometimes overlooked and not necessarily attributed to an incompatibility with blood.

Hemolysis—as shown in Table I for amphetamine sulfate, dextro-amphetamine sulfate, and ephedrine sulfate—is not necessarily correlated with osmotic pressure, since on increasing the concentration of these substances an increase of the osmotic pressure can be demonstrated together with an increase of hemolysis. An example of this behavior is given in Table III.

Human Red Cells.—Since Hammarlund and Pedersen-Bjergaard obtained their results on human red cells and since a difference between human and rabbit red cells is demonstrated in osmotic behavior (3, 15, 16), the hematocrit method was performed for some solutions on human red cells to establish whether the disagreement found between the results obtained by the hematocrit and the hemolytic method might be attributed to species differences.

TABLE III.—HEMATOCRIT VALUE AND HEMOLYSIS OBSERVED ADDING ONE VOLUME OF RABBIT RED CELLS TO ONE VOLUME OF DEXTRO-AMPHETAMINE SULFATE IN DIFFERENT CONCENTRATIONS

Dextro-amphetamine Sulfate Concn., %	Hematocrit Value	Hemoglobin in the Supernatant, %	Estimated Percentage of Hemolyzed Cells
4.0	0.87	5.5	4.3
5.0	0.76	2.2	3.5
6.0	0.67	0.3	0.7
7.0	0.63	0.2	0.4
8.0	0.56	0.3	0.9
9.0	0.54	2.5	7.3
10.0	0.50	5.1	17.0
Plasma	0.50	0	0

TABLE IV.—HEMATOCRIT VALUES AND HEMOLYSIS OBSERVED ON HUMAN RED CELLS WITH ISO-OSMOTIC CONCENTRATIONS OF DIFFERENT SUBSTANCES

Substance	Iso-osmotic Concn., %	Hematocrit Value	Hemolysis
Amphetamine sulfate U.S.P.	4.23		total
Atropine sulfate U.S.P.	8.85	0.65	light
Decamethonium bromide	5.00		total
Dextro-amphetamine sulfate U.S.P.	4.20	0.80	high
Ephedrine sulfate U.S.P.	4.54	0.75	high
Sodium acetate anhydrous	1.18	0.50	0
Sodium benzoate U.S.P.	2.25	0.50	light
Sodium citrate U.S.P.	3.02	0.48	0
Sodium phosphate dibasic $\cdot 2H_2O$	2.23	0.50	0
Plasma	..	0.50	0

In Table IV a hematocrit value higher than 0.50 indicates hypotonicity—a hematocrit value lower than 0.50, hypertonicity. Table IV shows that the results obtained on human red cells reproduce those obtained on rabbit red cells, with very slight differences for sodium acetate and dibasic sodium phosphate, the first of which showed a little higher osmotic pressure on human red cells and the second a little higher osmotic pressure on rabbit red cells compared to the red cells of the other species.

DISCUSSION

The results presented in this paper and those of the literature (1, 16) show that relatively few molecules or ions are unable to penetrate the red cells and therefore (18) to exert an osmotic pressure through the cell membrane. For this reason it must again be emphasized that isotonicity can be tested only by putting the solutions directly in contact with cells and cannot be calculated on the basis of the molecular weight and probable micellar behavior, or on the basis of physical methods as is suggested

also in recent literature (20). The danger involved in these procedures was demonstrated for iso-osmotic urea solutions by Setnikar and Temelcou (16).

Using the hematocrit method it can be demonstrated that, among the substances which are of interest for pharmaceutical formulation, some carbohydrates and polyalcohols exert an osmotic pressure through the erythrocyte membrane which can be predicted on the basis of the colligative physical properties of these solutions. For these solutes the red cell membrane appears therefore impermeable.

The same can be said for salts with calcium, magnesium, potassium, and sodium cations. In these cases the osmotic pressure exerted through the cell membrane is the same as if the total amount of ions in solutions was prevented from passing through the cell membrane. This confirms the view that it is enough for the membrane to be impermeable to the cation or to the anion component of a salt to be impermeable to the whole salt of which the ion is a part (19).

Particular mention should be given to sulfate and phosphate ions, which, according to Davson (19), impede the penetration of the cation component into the red cells so that the whole salt can exert an osmotic pressure. The results of this paper show, however, that the iso-osmotic concentration of the phosphate and sulfate salts (*cf.* the ammonium, amphetamine, atropine, and ephedrine salts reported in Table I) is always lower than the isotonic concentration, that iso-osmotic concentrations always produce partial hemolysis and that often these substances affect the red cells in a way which makes the red cell column almost transparent. Therefore, these anions can not be considered equal (as Hammarlund and Pedersen-Bjergaard (11) suggested) to the cations already mentioned, which are unable to cross the cell membrane.

Among the really impermeable ions tetraethylammonium must finally be mentioned (*cf.* Table I).

Norlander and Sandell (21) and Setnikar and Paterlini (22) pointed out that for hypodermic use, strict isotonicity is not critical for good tolerance. The same was demonstrated by Hammarlund and Pedersen-Bjergaard (11) for intravenous solutions when small quantities are injected. This seems to diminish the importance of an accurate method as the hematocrit one for testing isotonicity of injectable solutions. Such a method becomes, however, indispensable for solutions which must be injected in large quantities into the blood stream or in some particular cases, *e.g.*, for solutions designed for rachidean administration. In this case the small quantity of cerebrospinal fluid and its slow replacement (23) can not buffer liquids which are not isotonic to a tolerable osmotic pressure, so that the very delicate nervous cells may be damaged.

In any case, it is very important to recognize damaging solutions, *i.e.*, strongly hemolytic (of the saponin type) or with a precipitating or denaturing action on proteins (as zinc sulfate, phenol, etc.). The former are easily detected both with the hemolytic and the hematocrit method; the latter are more immediately detected with the hematocrit method because a precipitation on the red cell column is immediately noted.

REFERENCES

(1) Husa, W. J., and Adams, J. R., This Journal, **33**, 329(1944).
(2) Grosicki, T. S., and Husa, W. J., *ibid.*, **43**, 632(1954).
(3) Easterly, W. D., and Husa, W. J., *ibid.*, **43**, 750 (1954).
(4) Hartman, C. W., and Husa, W. J., *ibid.*, **46**, 430 (1957).
(5) Cadwallader, D. E., and Husa, W. J., *ibid.*, **47**, 705 (1958).
(6) Thomasson, L. C., and Husa, W. J., *ibid.*, **47**, 711 (1958).
(7) Ansel, H. C., and Husa, W. J., *ibid.*, **48**, 516(1959).
(8) Zanowiak, P., and Husa, W. J., *ibid.*, **48**, 565(1959).
(9) Marcus, D., and Husa, W. J., *ibid.*, **48**, 569(1959).
(10) Winters, E. P., and Husa, W. J., *ibid.*, **49**, 709(1960).
(11) Hammarlund, E. R., and Pedersen-Bjergaard, K., *ibid.*, **50**, 24(1961).
(12) Schnell, L. A., and Husa, W. J., *ibid.*, **51**, 904(1962).
(13) Shaw, M. A., and Husa, W. J., *ibid.*, **51**, 929(1962).
(14) Wokes, F., *Quart. J. Pharm. Pharmacol.*, **9**, 455(1936).
(15) Setnikar, I., *Boll. Chim. Farm.*, **98**, 386(1959).
(16) Setnikar, I., and Temelcou, O., This Journal, **48**, 628(1959).
(17) Hammarlund, E. R., and Pedersen-Bjergaard, K., *ibid.*, **47**, 107(1958).
(18) Meschia, G., and Setnikar, I., *J. Gen. Physiol.*, **42**, 429 (1958).
(19) Davson, H., *Biochem. J.*, **34**, 917(1940).
(20) Goyan, F. M., Tuck, L. D., and Taylor, P. L., This Journal, **50**, 684(1961).
(21) Norlander, M., and Sandell, E., *Pharm. Acta Helv.*, **27**, 179(1952).
(22) Setnikar, I., and Paterlini, M. R., This Journal, **49**, 5(1960).
(23) Grundy, H. F., *J. Physiol.*, **163**, 457(1962).