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A Physiological Solvent for Crystalline Insulin

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Summary. Insulin is insoluble in water at physiological pH, but dissolves relatively rapidly in plasma. To quantify the ability of various solutions to dissolve crystalline insulin, a simple assay measuring dissolution time was developed. At pH 7.5 and room temperature, distilled water, 0.154 mol/l NaCl, Ringer's lactate solution, and 5% albumin in 0.154 mol/l NaCl did not dissolve insulin crystals within 30 min. Normal postprandial human plasma and a protein-free cell culture medium dissolved insulin crystals within 3 to 8 min. This ability was inhibited by acid titration of the fluids to a stable pH of 6.30, at which point bicarbonate depletion could be implied. Repletion of bicarbonate did restore the ability of these solutions to dissolve insulin crystals, but back-titration to the initial pH with NaOH did not. The effect of sodium bicarbonate alone was strongly concentration dependent above 23 mmol/l. We suggest that the ability of physiological fluids to dissolve insulin crystals at normal pH depends on their bicarbonate content. The ability to dissolve insulin with a physiological solvent which prevents its reaggregation promises to facilitate its use in portable pumping systems.

Key words: Insulin, crystal, dissolution, bicarbonate, pH.

Aggregates of insulin occur under many conditions [1] and in this form have no biological effect [2]. We have recently reported that the addition of 1.5% (v/v) autologous serum markedly increased the biological activity of dilute insulin solutions [3]. Small quantitites of this substance prevented the aggregation of insulin which otherwise interfered with the flow of intravascularly pumped insulin and resulted in clinically unacceptable blood glucose control. To further explore this effect, we devised a simple in vitro assay to quantify the ability of various solutions to dissolve insulin crystals. The dissolution of crystalline insulin which does not occur in water at neutral pH, is accelerated by increased hydrogen ion concentration (pH 2–3). This process is used in the commercial preparation of insulin formulations which are subsequently back-titrated to physiological levels. However, solutions of this type have a tendency to reaggregate or precipitate. In this paper, we show that the bicarbonate ion greatly facilitates the dissolution of insulin crystals in the physiological pH range and that the ability of human plasma and other physiological solvents to dissolve insulin crystals is mainly determined by this ion.

Methods

Assay

Experimental equipment including a light microscope, a pH electrode, and a magnetic stirrer were placed in an Isolette infant incubator (Air Shields, Harboro, P. A., U.S.A.) at 24 °C. Sample aliquots of 5 ml were dispensed into a 10 ml beaker in the incubator and exposed when indicated to a humidified calibrated gas mixture of CO_2:O_2:N_2 (5:12:83). Gas equilibration took approximately one hour during which time the sample was gently stirred and the pH was monitored (pH meter PHM63 with a GK2421 electrode, Radiometer, Copenhagen, Denmark). A stable pH was indicative that CO_2/HCO_3^- equilibrium had been reached before the assay was commenced..

The dissolution time was measured as the elapsed time between the addition of a droplet of the test solution and the complete disappearance of a hexagonal insulin crystal viewed at 100× magnification. The initial step of the assay involved transferring pork insulin crystals (Connaught Laboratories, Toronto, Canada) to the grid of a clean Neubauer blood counting chamber. A single hexagonal crystal of 20–30 μm diameter was then isolated using the tip of a 23 gauge needle. A 10 μl aliquot of the test solution was finally pipetted onto the blood counting chamber (Micropipettor B, SMI Manufacturing Industries, Berkeley, Cal., U.S.A.) to form a small droplet covering the crystal.

Samples

Venous blood samples were drawn in heparinized syringes with no other additives from 7 healthy male and female subjects, aged 21 to 43 years. Immediately after sampling, the whole blood was centrifuged at 5000 g and 4° C for 15 min. The plasma was then separated and stored at 4° C until it was assayed. In addition, samples of a protein-free cell culture medium (alpha-MEM) (4) distilled water, dilute HCl (pH 3.0), 0.154 mol/l NaCl, Ringer's lactate solution, 5% human albumin in 0.154 mol/l NaCl, and solutions of 25 and 125 mmol/l $NaHCO_3$ were studied.

Protocol

The initial dissolution tests for plasma and alpha-MEM were conducted in a 5% CO_2 environment. To remove bicarbonate from the sample, HCl (5 mol/l) was slowly added repeatedly to an aliquot which was kept in room air (0.03% CO_2) to establish and maintain a pH of 6.30–6.35. During this time room air was blown over the surface of the stirred sample which was also intermittently

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Table 1. Dissolution time of insulin crystals in plasma from healthy subjects (n = 7) and in alpha-MEM (a protein-free cell culture medium, n = 4) as a function of bicarbonate ion concentration, pH and pCO_2. Mean ± SEM

Sample + additive	% CO_2 in the equilibrated gas phase	Initial pH	HCO_3^- mmol/l	Dissolution time seconds	t-test on dissolution time
1. Plasma	5	7.52 ± 0.01	22.9 ± 1.4	441 ± 44	–
2. Plasma + HCl	0.03	6.26 ± 0.06	<5.0[a]	>1500[c]	[d]
3. Plasma ± HCl followed by NaOH	0.03	7.52 ± 0.01	<5.0[a]	692 ± 6[c]	[d]
4. Plasma + HCl followed by $NaHCO_3$	5	7.55 ± 0.01[b]	62.8 ± 7.5	77 ± 10	[d]
1. Alpha-MEM	5	7.50 ± 0.01	23.2 ± 0.4	210 ± 17	–
2. Alpha-MEM + HCl	0.03	6.34 ± 0.01	<5.0[a]	>1800[c]	[d]
3. Alpha-MEM + HCl followed by NaOH	0.05	7.51 ± 0.04	<5.0[a]	>1800[c]	[d]
4. Alpha-MEM + HCl followed by $NaHCO_3$	5	7.51 ± 0.03	19.5 ± 0.2	193 ± 19	NS

[a] Concentration below the lower detection limit in all samples observed
[b] Due to slow equilibration with the gas phase, these pH values increased slightly during the assay
[c] No indication of any crystal dissolution until time indicated in all samples observed
[d] $p < 0.01$ for the difference to assay No. 1
NS = denotes not significant for difference to assay No. 1

ultrasonicated (Ultrasonic Cleaner, Mettler Electronics Corp., Anaheim, Calif., U.S.A.) to accelerate the removal of CO_2. Insulin crystal dissolution time was measured when pH was stable at this level. Sequential assays were performed in the same sample after back-titration to the initial pH first using 10 mol/l NaOH in room air and then using 1 mol/l $NaHCO_3$ in the 5% CO_2 atmosphere. All titrations were performed using a thin stainless steel wire for transferring minute aliquots of the electrolytes. The bicarbonate content in other aliquots of the samples was measured using the Coulter Electrolyte System (Coulter Electronics, Hialea, Fla., U.S.A.) The individual dissolution times in plasma and alpha-MEM were the means of triplicates. Statistical significance was evaluated using the Student's t-test. Results are presented as mean ± SEM.

Insulin crystal dissolution times in the organic and inorganic solutions listed above were assayed in room air employing the same methodology. Except for the $NaHCO_3$ and HCl solutions, the pH was adusted to 7.50.

Methodologic Observations

The intra-assay coefficient of variation in the untreated plasma at a constant pH was 9% at low (mean 189 s) and 13% at medium (mean 345 s) dissolution times when the assay series was completed within 5 h of its start. Due to evaporation during the assay, the coefficient of variation could not be obtained for high (>1500 s) dissolution times. Sample storage for up to 12 h at 4 °C did not significantly influence dissolution time. However, haemolysis, freeze/thaw cycles, or simultaneous usage of several insulin crystals in one assay droplet led to inconsistent dissolution times and higher coefficients of variation. No protein precipitation occurred in plasma during acidification at pH 6.3 but was evident below values of 6.1. Aside from the results presented below, several assays were performed using pork insulin crystals from Nordisk Insulin Laboratorium (Copenhagen, Denmark) and several using serum instead of plasma. Dissolution times were unaffected by these conditions and no differences were observed between fasting and post-prandial plasma samples.

Results

In dilute HCl (pH 3.0), insulin crystal dissolution time was 242 ± 19 s (n = 5). In distilled water (n = 5), 0.154 mol/l NaCl (n = 5), Ringer's lactate solution (n = 5), and 5% albumin (n = 5) insulin crystals did not dissolve during an observation time of 30 min. In 25 mmol/l $NaHCO_3$, the insulin crystals became fragmented in 2–3 min but dissolution was incomplete at 30 min (n = 20). However, in 125 mmol/l $NaHCO_3$, the insulin crystal dissolved within 40–50 s (n = 10).

When equilibrated with a pCO_2 corresponding to alveolar pCO_2 5% (v/v), normal human plasma containing 22.9 ± 1.4 mmol/l bicarbonate dissolved the insulin crystals in 441 ± 44 s (Table 1). Insulin crystals did not dissolve in plasma at pH 6.26. Back-titration of these bicarbonate depleted samples to pH 7.52 ± 0.01 using NaOH did not restore the initial dissolution time. However, the use of $NaHCO_3$ for back-titration to the initial pH, led to a highly significant reduction in the dissolution time to 77 ± 10 s, with bicarbonate significantly elevated at 62.8 ± 7.5 mmol/l.

In alpha-MEM the pH and HCO_3^- content with 5% CO_2 equilibration were 7.28 and 17 mmol/l respectively. To obtain initial conditions comparable to the plasma specimen, the pH was adusted to 7.50 ± 0.01 using $NaHCO_3$. Dissolution time in these samples was 210 ± 17 s when bicarbonate was 23.2 ± 0.4 mmol/l. As with plasma, dissolution was inhibited by acidification to pH 6.34 (Table 1). This inhibition persisted after back-titration with NaOH to the original pH. The restoration of the bicarbonate content, however, was followed by complete recovery of the ability to dissolve insulin crystals.

Discussion

We have devised a simple in vitro method to assess and quantify the ability of aqueous solutions to dis-

solve insulin crystals. Both the plasma of normal subjects and a protein-free but otherwise physiological solution dissolved the crystals at pH values which are normal for plasma [5].

The remarkable ability of these solutions to dissolve insulin was destroyed when bicarbonate was removed. It could not be restored by re-estabilishing the initial pH without restoring the bicarbonate content. The dissolution process was accelerated when the bicarbonate addition exceeded the initial concentration or if supraphysiological concentrations of $NaHCO_3$ alone were investigated. Insignificant volumes of the electrolytes were added and the entire titration process could be repeated in the same sample with similar results.

In the absence of detectable bicarbonate levels at pH 7.52 plasma and exhibited some dissolution of insulin which was not observed in the protein-free alpha-MEM. Both plasma and alpha-MEM contain mixtures of amino acids and the ability of these physiological solutions to dissolve insulin with only 23 mmol/l of bicarbonate exceeds that of bicarbonate alone. Thus it appears as if certain protein and/or amino acids enhance the effect of bicarbonate in dissolving insulin at physiological pH. The mechanisms of these effects remain to be elucidated.

The unusual action of bicarbonate on insulin dissolution might explain various pathophysiological phenomena. In this regard, bicarbonate is reported to play an "essential" role in insulin secretion [6] and islet respiration [7]. Whether this role is connected to its action on insulin granules following exocytosis remains to be established. To exert a first pass effect on the liver natural insulin granules must rapidly dissolve to a certain extent in the blood following their secretion from the B-cells of the healthy pancreas [8]. In the absence of a supraphysiological perislet bicarbonate concentration, it seems unlikely that physiologically secreted insulin granules dissolve completely during the few seconds it takes for them to reach the liver. It is tempting to speculate that pancreatic bicarbonate fulfills both an endocrine and an exocrine function. Furthermore, hepatic insulin extraction, which is reported to be 50% of the incoming insulin [9], may simply represent the removal of such partially dissolved granules. Previous observations in dogs support this hypothesis and indicate that 37% less insulin was required for glycaemic normalization when insulin was allowed ample time to dissolve in the presence of serum [3].

In the light of these observations the ability of plasma to dissolve insulin crystals may be altered in pathophysiological states with reduced bicarbonate concentrations such as metabolic acidosis. Accordingly, the well-known insulin resistance in diabetic ketoacidosis and its reversal by bicarbonate administration [10] may also be explained in part by changes in the ability of body fluids to dissolve insulin crystals.

The present need for stable, monomeric insulin solutions for insulin delivery devices may in part be satisfied by the addition of bicarbonate instead of serum to the insulin formulations. Unfortunately, bicarbonate containing solutions are difficult to stabilize with respect to pH. In the meantime, further experimental and clinical studies involving intravascular insulin infusions can now proceed unhampered by the uncertainties of variable insulin dissolution.

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