Zinc(II) in Saliva: Determination of Concentrations Produced by Different Formulations of Zinc Gluconate Lozenges Containing Common Excipients

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Abstract □ A study of the pH of saliva produced by humans sucking hard candy lozenges containing zinc gluconate and citric acid demonstrated the probability that the formulation delivered an insignificant amount of the contained zinc as Zn^{2+}. This could account for the negative results of several clinical studies of this lozenge and similar formulations as treatment for the common cold. Direct measurement of unbound Zn^{2+} in saliva from this and other zinc gluconate formulations was required to substantiate the inference from the pH study. A specific-ion-electrode assay method was developed. Using this method, it was found that saliva completely suppresses the ionization of zinc to free Zn^{2+} in the presence of citric acid or a 30-fold molar excess of mannitol plus sorbitol. Under the same conditions, however, the presence of an excess of glycine does not interfere with ionization to produce Zn^{2+}. This finding supports the hypothesis that the positive clinical results of three studies were due to the use of formulations which release ionic zinc.

Interest in the possible utility of zinc gluconate as a treatment for the common cold was stimulated by a 1984 article from Eby et al.[1] In a double-blind clinical study, a >50% reduction in the duration of a cold, compared with placebo, and a corresponding decrease in symptom severity were reported when patients dissolved unflavored zinc gluconate tablets containing 23 mg of zinc in their mouths. Among four published attempts to obtain similar results with flavored zinc gluconate or acetate lozenges, three failed to show any effect of zinc gluconate[2,3] or acetate,[4] while one did show a similar, significant reduction of cold symptoms.[5] The latter study employed a simple combination of zinc gluconate and fruit flavor in a sucrose matrix tablet. The unsuccessful studies all used the zinc salt in a sweetened lozenge which contained, in addition, either citric acid,[2] mannitol and sorbitol,[3] or tartaric acid with sodium bicarbonate and mannitol.[4] One of the present authors had pointed out that, at the expected pH of the human mouth during dissolution of the formulations containing either citric or tartaric acids, the reported stability constants of zinc(II) plus citric acid or tartaric acid led to the conclusion that in those studies it was unlikely that any effective concentrations of zinc(II) had been delivered.[6] When the third unsuccessful study appeared in 1989,[3] the long history of mannitol as a complexing agent for transition metal ions, including zinc(II),[7] was revealed by a literature search. (The composition of the zinc gluconate lozenge used in the study of Smith et al.[3] was not given in the paper, but with the cooperation of the authors it was traced. The 1.5-g lozenges contained 90 mg of zinc gluconate trihydrate (11.5 mg of zinc), 14 molar equivalents of mannitol (relative to zinc), and 16 molar equivalents of sorbitol.) It was suggested[8] that the proposed mechanism of inactivation of zinc(II) by complexation with citric acid[6] was not tenable because of the anticipated low pH (near 2.3) of the mouth after dissolution of that formulation. Once the exact composition of the zinc gluconate plus citric acid lozenges was revealed in the Martin publication,[8] experimental verification was possible. It was of further interest to demonstrate directly the degree of binding of zinc(II) in complex ions by the development of an analytical method for unbound Zn^{2+} in naturally produced saliva. The presence of little or no directly measurable free Zn^{2+} in saliva from the flavored formulations employed by Farr et al.[2] and Smith et al.,[3] contrasted with greater amounts available in the unflavored formulation of Eby et al.,[1] would support the suggestion[6] that free zinc(II) is required for the desired biological activity. Furthermore, such findings would justify the search for a taste-acceptable formulation of zinc gluconate which would release free Zn^{2+}.

Such a formulation has now been developed and has been shown to have activity against the common cold which is similar to that reported by Eby et al.[1] The clinical study of this formulation, which consists of zinc gluconate and glycine in a hard candy base, will be reported elsewhere.[9]

Experimental Section

Chemicals and Reagents—Whatman buffers nos. 6200–0018 and 6200–0017 were used for pH 4.00 and 7.00, respectively, and USP pH 4.6 acetate buffer was also used. The "hard crack" candy base was composed of 360 g of sucrose, 40 g of fructose, 190 g of Light Karo (corn) syrup, and 160 g of water. This mixture was boiled to a final temperature of 149 °C, blended (while cooling to room temperature) in 12 mL of Ottens Flavors lot K1160 Orange Oil cold pressed. Anhydrous citric acid was from Chemical Dynamics Corp. (no. 22-8220-00). The copper-indicating solution was made by combining equal volumes of 0.0500 M Na_2EDTA solution and 0.0500 M cupric nitrate solution; the cupric nitrate was ACS Certified 98.4% $Cu(NO_3)_2 \cdot H_2O$. The following reagents and chemicals were used: equitransferrent filling solution (for Orion reference electrode, inner chamber; Orion Research Inc.; #900002); glycine (aminoacetic acid; FCC USP; Chemical Dynamics Corporation; no. 47-3200-00); ionic strength adjuster (ISA) solution (Orion; 5 M $NaNO_3$; #94001); mannitol (Aldrich Chemical Company; cat. no. M235-7); potassium nitrate (for Orion reference electrode, outer chamber; Orion; #900003), Na_2EDTA (USP Reference Standard; 0.0500 M); sorbitol (glucitol; Aldrich Chemical Company; cat. no. S375-5); and unflavored zinc gluconate tablets containing 23 mg of zinc plus excipients dicalcium phosphate, microcrystalline cellulose, sodium starch glycolate, magnesium stearate, FD&C Yellow #5, and Blue #1, (Truett Laboratories, Dallas, TX). Zinc gluconate lozenges with 10 molar equivalents (relative to zinc) of glycine were prepared by Godfrey Science & Design, Inc. (GSD), and by Simon Candy Company, Elizabethtown, PA. The lozenges contained 5.907% glycine and 4.003% zinc gluconate trihydrate in an orange-flavored hard candy base (vide supra). Zinc gluconate lozenges with citric acid were prepared by GSD and contained 2.000% anhydrous citric acid and 3.978% zinc gluconate trihydrate. Zinc sulfate was ACS Certified (98.3% $ZnSO_4 \cdot 7H_2O$).

Instrumentation—A Jenco Electronics Ltd. temperature-compensated pH meter (model no. 6209), with a Whatman pH

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electrode (no. H105) and matching reference electrode, was used for determination of pH in saliva samples. A Beckman Zeromatic pH meter, with an Orion cupric ion activity electrode (model 94-29) and a double junction reference electrode (model 90-02) with a magnetic stirrer, was used for determination of free Zn^{2+}. Electrodes were polished prior to standardization and determinations with Orion electrode polishing strips (cat. no. 948 201).

pH of Saliva from Zinc Gluconate and Citric Acid Lozenges—Each of 18 adult volunteers, who had fasted for at least 2 h, supplied an unstimulated saliva sample for individual baseline pH determination. Then, each volunteer dissolved in the mouth a 4.5-g lozenge of zinc gluconate and citric acid formulation and expectorated all saliva therefrom into a tared, coded plastic cup. The Jenco pH meter was standardized with buffers at pH 4.00 and 7.00, and the pH of the samples was read to the nearest 0.01 unit at room temperature. Because of the viscosity of saliva, vigorous hand-agitation of each sample was necessary while measuring, until a constant (final) reading was obtained. Results are shown in Table I.

Direct Measurement of Free Zn^{2+}—*Standardization*—Zinc gluconate · $3H_2O$ equivalent to 13–26 mg of Zn^{2+} (100–200 mg of zinc gluconate · $3H_2O$) was weighed and transferred to a 250-mL beaker. Then, 50 mL of deionized (DI) water was added, along with a stirring bar, and the mixture was stirred until dissolved. Then, 5.0 mL of pH 4.6 acetate buffer, 2.0 mL of ISA solution, and 3 drops of copper-indicating solution were added. Electrodes were then placed in the solution, the Beckman Zeromatic pH meter was set to read millivolts [mV], and the meter was adjusted to zero. Stirring was continued for 5–10 min, until sample and electrodes came to equilibrium, as shown by constant 0 mV readings. Increments of 0.25 mL of 0.0500 M Na_2EDTA titrant (standard titrant) were added, and 30 s later, an mV reading and standard titrant volume reading were taken, and the values were recorded. Addition of 0.25-mL increments of standard titrant was continued through the endpoint (i.e., the inflexion point at which the change in mV readings for each added 0.25 mL is maximum). Finally, mV readings versus standard titrant volume were plotted, and the endpoint was determined visually or by the second derivative (inflexion point) method. Each 1.00 mL of standard titrant is equivalent to 3.269 mg of Zn^{2+}.

Determination of Free Zn^{2+} in Zinc Gluconate Lozenges—One weighed lozenge was dissolved in 50 mL of DI water, and 5.0 mL of pH 4.6 acetate buffer, 2.0 mL of ISA solution, and 3 drops of copper-indicating solution were added. Then the procedure described in *Standardization* (see above) was used. The presence of insoluble matter (excipients) in the titration solution does not interfere with this determination; brisk stirring keeps electrodes free of insoluble, inactive substances.

Determination of Free Zn^{2+} in Lozenge-Stimulated Saliva—First, 60 mL of DI water was added to the sample (~30 mL from a 4.5-g lozenge), with stirring to disperse the viscous saliva. Then, 5.0 mL of pH 4.6 acetate buffer, 2.0 mL of ISA solution, and 3 drops of copper-indicating solution were added, and the procedure described in the *Standardization* section was followed. (N.B. Electrodes should be polished after each determination and during the titration, if necessitated by slow response of the mV meter.)

Table I—pH Changes and Saliva Volumes following Dissolution of Zinc Gluconate (4.5 g) and Citric Acid Lozenges

Subject Number	Baseline pH	Final pH	Difference (ΔpH)	Saliva Volume, mL
1	7.10	3.23	3.87	16.1
2	6.62	4.00	2.62	32.2
3	7.70	4.48	3.22	35.5
4	7.22	4.62	2.60	17.6
5	7.80	3.88	3.92	30.3
6	—[a]	4.11	—	11.5
7	7.63	3.81	3.82	14.7
8	7.43	4.03	3.40	29.9
9	7.14	3.38	3.76	20.1
10	7.60	3.53	4.07	18.3
11	8.54	4.11	4.43	25.5
12	7.14	4.04	3.10	22.9
13	7.80	4.79	3.01	43.2
14	6.79	4.99	1.80	46.5
15	6.92	3.82	3.10	25.6
16	7.21	4.51	2.70	31.9
17	6.70	4.47	2.23	21.6
18	7.43	4.77	2.66	30.4
Minimum	6.62	3.23	1.80	11.5
Maximum	8.54	4.99	4.43	46.5
Mean	7.34	4.14	3.20	26.3
SD	0.48	0.50	0.72	9.6

[a] Insufficient sample.

Results and Discussion

The determination of mean, standard deviation, and range of saliva pHs produced by sucking zinc gluconate lozenges containing 2% citric acid shows conclusively that the resulting mouth pH is not near 2.3 as suggested by Martin,[8] but is, in fact, nearly two units higher, with a standard deviation of one-half unit (Table I). The difference of 3.2 units between unstimulated saliva pH of 7.34 and the lozenge-stimulated pH of 4.14 indicates that stimulated saliva has somewhat greater buffer strength than that of unstimulated saliva (see ref 1 in Martin[8]). Using Martin's[8] mole fraction versus pH curves for the Zn^{2+}:citric acid system, at pH 4.14, 18% of zinc is present as Zn^{2+}, 29% as Zn citrate (no charge; i.e., the third carboxyl of the citric acid is not ionized), and 53% as Zn citrate$^-$. His suggestion that most of the zinc from the 2% citric acid lozenges would be present as Zn^{2+} is seen to be inaccurate.

Direct determination of Zn^{2+} in the presence of various amounts of citric acid by the method reported here yields the results shown in Table II. This method finds virtually all of the zinc as Zn^{2+}, even in the presence of large excesses of citric acid, as long as no saliva is present. The addition of even small amounts of unstimulated saliva to zinc gluconate samples containing small to large excesses of citric acid resulted in the finding of no detectable free Zn^{2+}. It was surprising to find that not even the pH-predicted small amount of Zn^{2+} (~25 mol%) at the ambient pHs of 3.80–3.92 could be detected under these conditions. It appears that under the catalytic influence of some of the 70 (or more) known components of human saliva,[10] rapid conversion of Zn^{2+} and citric acid to the thermodynamically stable equilibrium mixture of zinc citrate complexes occurs; these complexes appear to be more stable than in the "clean" system for which Martin[8] plotted the composition versus pH curves.

A similar situation exists with zinc gluconate in the presence of 14 molar equivalents of mannitol and 16 molar equivalents of sorbitol (the formulation employed in the Smith et al.[3] study). In the absence of saliva, all of the zinc was

Table II—Titration of Zinc Gluconate Samples Containing 23 mg of Zinc

Conditions	Citric Acid, mg	pH[a]	Zn^{2+} Recovered	
			mg	Percent
No saliva	none	5.61	22.25	96.7
No saliva	25.0	3.90	22.22	96.6
No saliva	50.0	3.90	21.92	95.3
No saliva	125	3.80	23.23	101.0
No saliva	125	3.80	22.90	99.6
No saliva	150	3.90	23.66	102.9
No saliva	150	3.90	21.64	94.1
No saliva	200	3.82	24.21	105.3
+ Saliva[b]	100	3.92	none	0.0
+ Saliva	150	3.86	none	0.0
+ Saliva	210	3.80	none	0.0
+ Saliva	210	3.80	none	0.0

[a] Before addition of acetate buffer. [b] 10 mL of unstimulated saliva was added to each sample.

found to be present as Zn^{2+}, but when fresh saliva was added to the solution, no Zn^{2+} could be measured.

Nine samples generated by sucking the unflavored lozenges used in the study of Eby et al.[1] were found to have a mean pH of 5.06 ± 0.57. Recovery of Zn^{2+} from these samples was 90.7 ± 13%. Eight samples generated by sucking the zinc gluconate lozenges containing 10 molar equivalents of glycine in the hard candy base had a mean pH of 5.08 ± 0.53, and 92.6 ± 6.5% of the zinc was recovered as Zn^{2+}.

In summary, the method developed for this study showed that in human saliva the zinc gluconate is present entirely in the form of complexes and/or complex ions when excesses of citric acid or mannitol (and sorbitol) are also present. In the absence of these known complexing agents, zinc gluconate is 90% ionized at mouth pH. The interaction between zinc and glycine was also found to be weak in these circumstances, with >90% of the zinc from zinc gluconate being found as free Zn^{2+}, even in the presence of a large excess of glycine. Prasad and Oberleas[11] have also reported a weak interaction between zinc and glycine at physiological concentrations.

This work supports the rationale for the investigation of the zinc gluconate plus glycine formulation as a treatment for the common cold and provides an explanation of the negative results in previous clinical studies of zinc gluconate and acetate.[2–4]

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