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Ion-chromatographic study of the possible absorption of copper and zinc by the skin of *Rana pipiens*

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Abstract

Frogs are known to obtain some of their nutrients (e.g., glucose and sodium) through their skin. However, no studies have been made of the possible absorption of transition metals, which exist in most river water at low-ppb (w/w) levels. Therefore, this research was undertaken to evaluate the use of ion chromatography for such an investigation. Solutions of copper and zinc (20 ppb in each) were chosen for use in a small-scale screening study. Ten live frogs were each placed in individual baths for approximately 50 h. Of interest were the net changes in the concentrations of the metals. These differences were the result of any absorption and/or excretion processes that took place. A Dionex IonPac CS5 column was used to analyze this simulated river water, both before and after frogs had been placed in the solution. Included in this paper are: (1) methodology and calculation formulas; (2) precautions needed to ensure sample integrity; (3) statistical analyses, which indicated that ion chromatography is an accurate, precise technique for quantifying Cu and Zn in these samples; and (4) screening-study results, which were used to test the null hypothesis that frogs do not absorb copper and zinc either onto or through their skin.

Keywords: Statistical analysis; Calibration curves; Copper; Zinc; Metals

1. Introduction

It is known that frog skin plays an essential role in controlling sodium and water balance [1], as well as in obtaining glucose [2]. However, no studies have been found in the literature on the possible absorption of copper and zinc by this tissue. The concentrations of copper ions in river and coastal waters have been reported as 11 and 21 ppb (w/w), respectively, and of zinc ions, 31 and 164 ppb [3]. Absorption would be a reasonable means by which the individual could gain these trace metals, which are known to be present in the tissues of an intact frog.

The literature suggests that ion chromatography offers a sensitive, reliable technique for quantifying trace metals in biological samples. Ion-chromatographic studies to evaluate copper and/or zinc levels in human plasma [4], human hair [5,6], serum and blood [7] and coral skeletons [8] have been reported. This paper examines the instrumentation statistically for its applicability in such frog studies. The evaluation was accomplished by conducting a small-scale screening study on 10 live frogs.

In this research, a Dionex CS5 separator column was used with an eluent based on pyridine-2,6-dicarboxylic acid. Postcolumn complexation of the analytes with 4-(2-pyridylazo)resorcinol allowed

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UV–Vis detection. *Rana pipiens* were placed in simulated river water (metal concentrations, 20 ppb each) for about 50 h. To determine the net change in Cu and Zn levels, solutions were analyzed both before and after exposure to the animals. For each frog, the difference between these two values reflected any absorption and/or excretion that took place.

The initial bath concentrations were chosen for two reasons. First, such levels mirrored those found in natural waters, as noted above. Second, if absorption occurred, it was expected to take place at rates of only a few ng/h for a 50-g frog. This assumption was based on the fact that most animals need transition metals only in trace amounts. (For comparison, an important ion such as bicarbonate is excreted through the skin at around 100 μg/h for a 50-g frog [9].) Thus, low-ppb concentrations allowed reliable determinations of such small rates.

The primary goal of this paper is to present a statistical evaluation of ion chromatography for use in these types of balance studies; results indicated that this instrumentation is an accurate, precise quantitation technique for such investigations. Secondary goals are: (1) to discuss precautions necessary to ensure sample integrity; and (2) to report screening-study data, which were used to test the null hypothesis that frogs do not absorb transition metals either onto or through their skin.

2. Experimental

2.1. Materials

Pyridine-2,6-dicarboxylic acid (PDCA) and PAR [4-(2-pyridylazo)resorcinol] were obtained from Dionex (Sunnyvale, CA, USA), ULTREX trace-metal-grade glacial acetic acid (GAA) and trace metal-grade ammonium hydroxide (30%) from J.T. Baker (Phillipsburg, NJ, USA), sodium acetate from Fluka (Ronkonkoma, NY, USA) and trace-metal-grade ammonium hydroxide (30%) from Texas Instruments (Dallas, TX, USA). The eluent was prepared by dissolving 2.00 g PDCA and 8.20 g of sodium acetate in 1400 ml of deionized (DI) water, adding 5.8 ml of GAA and diluting to 2000 ml with DI water; final concentrations were 6 m*M* PDCA, 50 m*M* sodium acetate and 50 m*M* acetic acid. The postcolumn reagent was made by dissolving 0.077 g PAR in a mixture of 200 ml of ammonium hydroxide plus 400 ml of DI water, adding 57 ml of GAA and diluting to 1000 ml with DI water; final concentrations were 0.3 m*M* PAR, 1 *M* acetic acid and 3 *M* ammonium hydroxide.

ULTREX II hydrochloric acid (30–38%) (J.T. Baker) was used to prepare a 1 *M* solution (for acidifying samples and standards) and a 10 m*M* solution (for rinsing the injection loop between samples). Copper sulfate pentahydrate and zinc acetate dihydrate (J.T. Baker) were used to prepare the various calibration standards, as well as the 20-ppb baths for the frogs. Deionized water (18 MΩ cm) was provided by a point-of-use water purification system (Ahlfinger Water, Dallas, TX, USA).

Wide-mouth, 8-oz polypropylene bottles (Cole-Parmer, Vernon Hills, IL, USA; 1 oz=29.574 ml) were used to contain the frogs/baths during the 50-h experiment; an air hole was drilled in the top of each lid, using a titanium drill bit that did not contaminate the container. Borosilicate-glass culture tubes (16× 100 mm) (Baxter Scientific, McGaw Park, IL, USA) were used to centrifuge the post-frog solutions. Instrument-calibration standards were prepared by mass in 4-oz polypropylene specimen containers from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Apparatus and columns

Unless otherwise noted, instrumentation and reagents were from Dionex. All analyses were performed on a Series 4000i ion chromatograph equipped with: (l) a UV–Vis detector (520-nm filter); (2) a reagent delivery-module with a 375-μl knitted reaction coil (coil needed to mix eluent and postcolumn reagent); and (3) an IonPac CS5 analytical column. A 200-μl sample loop was used. Flow rates were 1.0 ml/min for the eluent and 0.5 ml/min for the postcolumn reagent. Samples and standards were delivered to the chromatograph via an automated sampler, using PolyVials (5 ml) and plain caps. Data collection and instrument control were accomplished with AI-450 software. Statistical analyses were conducted using JMP (SAS Institute, Cary, NC, USA) software.

A Sartorius R160P analytical balance (Sartorius,

Edgewood, NY, USA) was used to prepare all calibration standards and a Mettler H10T analytical balance (Mettler-Toledo, Worthington, OH, USA) was utilized to make the bath concentrates; masses were recorded to four decimal places. All weighings involving the frogs and their containers were performed on a Harvard triple-beam balance (Harvard Apparatus, Holliston, MA, USA) and were recorded to one decimal place.

2.3. Preparation of standards and baths

The ion chromatograph was calibrated and monitored by analyzing standards of copper and zinc. Separate stock solutions (1000 ppm each) were prepared from copper sulfate pentahydrate and zinc acetate dihydrate. Mixed working standards were prepared from the stocks and were acidified using 500 μl of 1.0 *M* HCl per 100 g of solution. Spiking solutions also were made from these two stocks. All such mixtures were prepared by pouring and were weighed accurately. Such a procedure prevented any possible contamination from transfer vehicles. Baths (20 ppb in each metal) were prepared as needed from separate concentrates (22.22 m*M* for Cu and 9.38 m*M* for Zn).

3. Results and discussion

3.1. Frog protocol

Rana pipiens of either sex (masses between 20 and 35 g) were used. The animals were kept in deionized water with tetracycline added to control bacteria growth, and were not fed. Since some functions of the frog show seasonal variations, it should be noted that these experiments were conducted during the course of one winter.

In January, 10 live frogs were used to evaluate absorption of the metals. Each animal was placed in a new 8-oz polypropylene container with 50 g of bath for a period of 50–53 h. The bath contained 20 ppb of copper as copper sulfate, and 20 ppb of zinc as zinc acetate, plus a small quantity of tetracycline. Room temperature was maintained at 18–22°C. At the end of the experiment, the bath was poured off and centrifuged; the supernatant was then poured into a container containing 400 μl of 1.0 *M* HCl, giving a pH of 2.0±0.4.

Forty grams of the bath was placed in each of three plastic containers, kept there for the 50 h and likewise centrifuged. The solutions were then poured off into three containers, each containing 200 μl of 1.0 *M* HCl; final pH again was 2.0±0.4. These solutions were designated as control baths; their purpose was to account for any slight change in metal concentrations caused by any containers used during the research. Chromatographic analyses were completed within 6–20 h after collection of the final baths.

Samples for recovery studies were obtained by pouring spiking solution into 12 different frog-water baths and determining the mass of solution added. (These particular baths were obtained by placing 12 live frogs in separate containers. Each wide-mouth bottle contained 50 g of DI water; the frogs were left in the water for 50–53 h.) After spiking, the concentrations of the metals were increased by 20–45 ppb. These studies were performed in two sets: one in December and one in February.

3.2. Sample-handling precautions

Several precautions were found to be necessary in these experiments. First, the pH of all standards and samples had to be adjusted to between 1.6 and 2.4 before analysis. This range stabilized the metals, and matched eluent and postcolumn reagent buffers. Acidification was accomplished as soon as possible, to minimize absorption onto the container.

Second, contamination sources had to be minimized. New vinyl examining gloves were worn when working with samples. Polypropylene containers were used wherever possible. Syringes with rubber tips and polystyrene vessels were avoided, since both added metals to solutions. Deionized water (18.3 MΩ cm or better) was used for diluting all solutions and for rinsing.

Finally, to avoid carryover, the Dionex sample loop was rinsed with one vial of 10 m*M* HCl in between samples. This step was accomplished by switching back to ‘Load’ a few minutes after a sample had been injected.

3.3. Mass calculations

The initial mass of the bath (M_i) was 50 g. Calculation of the final mass (M_f) involved two mass changes. The container, containing the solution and frog, was weighed at the onset and at the end of the experimental periods. The difference in mass was considered to be due to evaporation from the container [$M_{evap} \equiv$(total mass, initial)−(total mass, final)]. Before being placed in the bath initially, the frog was blotted free of excess moisture and weighed; it was similarly weighed after completion of the experiment, to account for any net gain or loss of water by the animal. The change in frog mass was considered to have resulted in an equivalent gram change in the bath, but of opposite sign [$M_{\Delta frog} \equiv$ (frog mass, final)−(frog mass, initial)].

The final mass of the bath was then calculated, where:

$$M_f = M_i - M_{evap} - M_{\Delta frog}$$

3.4. Absorption calculations

Net copper absorption (Cu_{abs}) was first calculated in units of ng absorbed by the frog during the experimental period, by simply calculating the difference in copper content of the bath.

$$Cu_{abs} = (M_i[Cu]_i) - (M_f[Cu]_f)$$

A negative value, then, indicated net excretion during the period.

The absorption value was normalized for the slight difference in duration of the experiments and for the weight of the frog; the resulting units were ng/h·50-g frog, hereafter referred to as Units.

Net zinc absorption was similarly calculated.

3.5. Statistical results

3.5.1. Quantitative goals

The primary goal of this research was evaluating ion chromatography for quantifying copper and zinc in frog baths. Since animals may vary greatly in their responses to biological challenges, highly precise instrumental methods were not needed here. The primary analytical goal was determining the concentration *differences* between the before and the after baths; any bias in individual results would cancel out in the subtraction process and thus was not an issue. Since these compared samples were all analyzed on the same day, the main concern was instrument stability throughout that time period; within-day drift of ±10% was deemed acceptable for this study.

For quantitative research, a detailed calibration study typically is conducted and evaluated statistically [10]. However, since the variation among the frog specimens was expected to be high, such a calibration experiment was unnecessary. A small set of standards run two or three times on a given day was considered to be an acceptable design.

A related objective was determining how well the analytical technique measured all copper and zinc in the samples. This issue was assessed by conducting recovery studies.

The final goal was to make preliminary conclusions regarding the possible absorption of copper and zinc by frog skin. Student's *t*-tests [11] were performed on the data to evaluate this hypothesis.

3.5.2. Instrumental results

Typical chromatograms for an initial bath and a post-frog bath are shown in Fig. 1. The analysis time for both solutions was rapid (less than 10 min).

Within-day precision was evaluated by making replicate analyses. On separate days, a check standard and a post-frog bath were each analyzed 10 times. For the standard, which was 22.1 ppb in copper and 16.6 ppb in zinc, the RSDs were 4.3 and 7.2%, respectively; for the post-frog bath (27.6 ppb in Cu and 139.3 ppb in Zn), these values were 6.3 and 5.4%, respectively.

The chosen calibration design contained seven concentrations; each was replicated two to four times, all on one day (see Table 1). A straight-line model was proposed for each metal, with ordinary least squares (OLS) as the fitting technique. Residual plots did not reveal any unusual patterns and the *p*-values for the lack-of-fit test were 0.12 and 0.56, respectively. The plots of standard deviation (of responses) versus concentration did not have significant slopes or systematic residual patterns in either case, indicating OLS was appropriate. The resulting straight-line calibration curves had R^2_{adj} values of 0.9996 and 0.9980 for Cu and Zn, respectively;

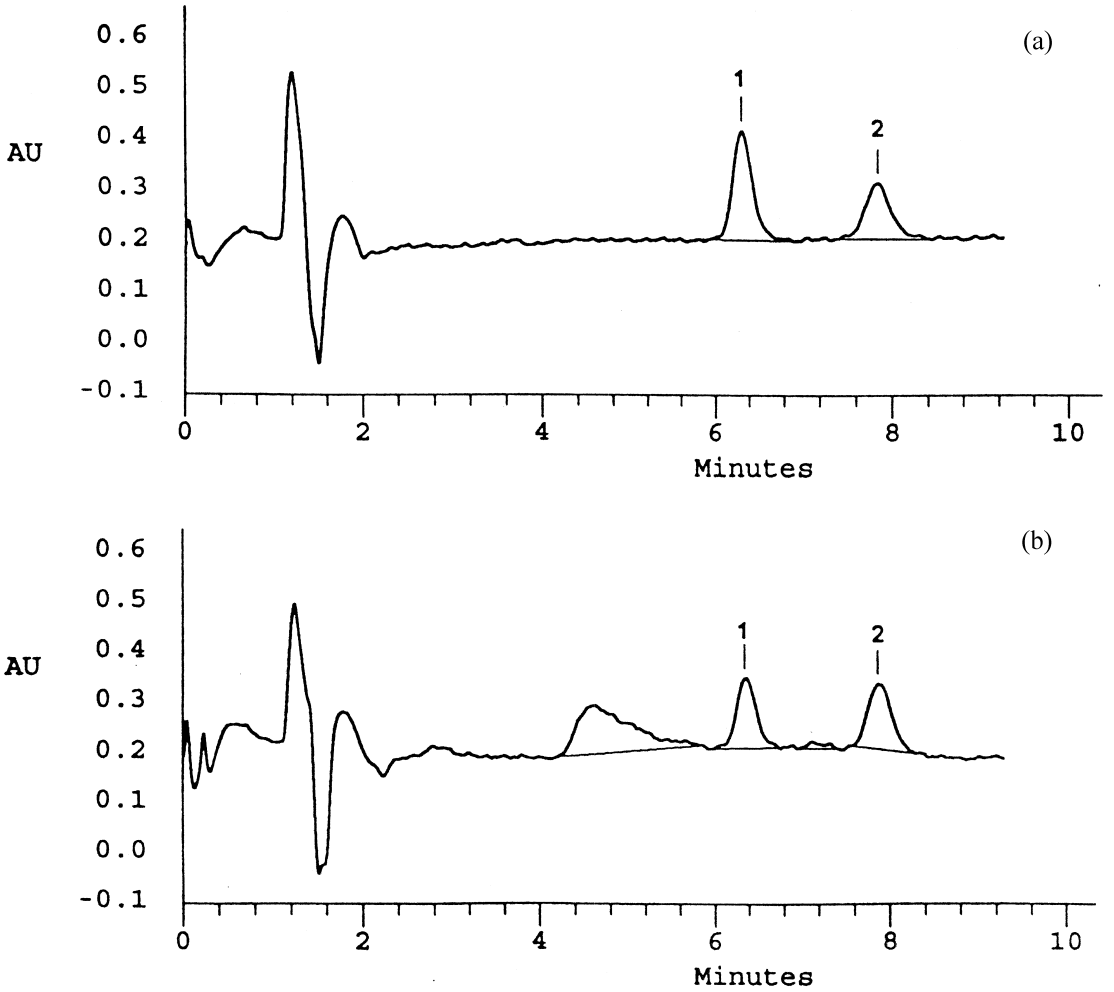

Fig. 1. (a) Chromatogram of an initial bath (20 ppb in each metal). (b) Chromatogram of a post-frog bath. Peak identities: (1) copper; (2) zinc.

Table 1
Calibration design

Level	No. replicates	ppb Cu	ppb Zn
1	4	4.4	3.9
2	3	7.8	6.9
3	2	11.8	10.5
4	3	19.6	17.3
5	2	50.6	44.8
6	2	98.3	87.1
7	2	147.8	130.9

p-values for the slope were significant at the 1% level. Therefore, a straight line with OLS fitting was an appropriate model for both calibrations. At 20 ppb ($\alpha = \beta = 0.025$), the prediction intervals were 2.3 and 4.3 ppb for Cu and Zn, respectively. Mean absolute deviations were 0.8 and 1.5 ppb, respectively. These widths and deviations were within the desired range for this project. Finally, g was <0.1 (and, therefore, acceptable) for both curves. These results are summarized in Table 2.

To monitor the instrument's performance, check standards were chromatographed any day the instrument was in use. Predicted concentrations were

Table 2
Diagnostics for straight-line/OLS calibration curves (see Section 3.5.2 for details)

Statistic	Copper	Zinc
R^2_{adj}	0.9996	0.9980
Mean absolute deviation ±prediction intervals	0.8 ppb	1.5 ppb
at 20 ppb ($\alpha=\beta=0.025$)	2.3 ppb	4.3 ppb
Lack-of-fit p-value	0.12	0.56
g	<0.1	<0.1

calculated, using the calibration curves described in the preceding paragraph. Mean absolute deviations ($n=50$) were acceptable (2.5 and 3.9 for Cu and Zn, respectively).

3.5.3. Live-frog results

Results on all frogs are expressed in Units (ng absorbed/h·50-g frog). If there was net excretion, the resulting Units were negative. The mean of the series was calculated using all negative and positive values. Student's *t*-test was used to determine if the mean of each series was statistically different from zero. Since either absorption or excretion would be considered significant, a two-tailed *t*-test was used.

Analyses of copper concentrations in the baths of the 10 frogs showed a net loss of copper in nine of the 10 frogs. The mean loss of the 10 baths (and also the mean adsorption by the frogs) was 13.82±8.73 Units (mean±standard deviation). (Analysis for significance from zero, $\ll 0.01$.) See Table 3 for complete data.

Analyses of zinc concentrations showed a net decrease in seven frogs, with a slight increase in two frogs and a marked increase in one animal (Frog 8). The mean absorption was 10.16±17.15 Units ($p=0.094$). See Table 3 for complete data.

As discussed earlier, these experiments determined only the net total absorptions of the metals. Consequently, any excretion of Cu and Zn into the bath would decrease the calculated Units absorbed. To assess the magnitude of this effect, 12 frogs were placed in DI-water baths for approximately 50 h. (These post-frog solutions were subsequently used for the spiking/recovery studies described in Sections 3.1 and 3.5.4.) The net excretion was found to be from 1 to 190 Units for copper, and from 2 to 585 Units for zinc.

Table 3
Net change in metals in baths of live frogs (see Section 3.5.3 for discussion)

Frog	Mass (g)	Hours	Cu units[a]	Zn units
1	27.1	50.8	−1.9[b]	−6.6
2	28.0	50.8	6.1	12.3
3	24.8	50.9	12.6	−5.5
4	31.0	51.0	22.5	26.5
5	24.0	51.0	26.8	27.0
6	31.3	51.6	20.4	19.5
7	23.9	51.7	15.3	5.9
8	25.1	51.9	10.8	−23.4
9	22.0	52.0	6.6	24.6
10	22.4	52.7	19.1	21.4
n			10	10
Mean			13.82	10.16
SD[c]			8.73	17.15
P[d]			$\ll 0.01$	0.094

[a] Units = ng/h·50-g frog.
[b] Negative values indicate a gain in total metal in the bath.
[c] SD = standard deviation.
[d] Student's *t*-test was used to determine if the mean was statistically different from zero. Since either absorption or excretion was considered significant, a two-tailed *t*-test was chosen.

Clearly, then, excretion by the frog could be high enough to mask any absorption that occurred. However, in these experiments, net absorption was great enough to give an extremely low *p*-value for copper. This evidence resulted in the rejection of the null hypothesis and the acceptance of the alternative (that frogs do absorb Cu either onto or through their skin). The zinc data did not cause the null to be rejected. (It is possible that the value for Frog 8 was an outlier, although no physical reason existed to support the idea. If Frog 8 were omitted, the mean absorption would be 13.89±13.20, with $p=0.013$).

3.5.4. Recovery results

Spiking studies on the 12 frog baths showed the mean recoveries to be 94.8% for copper and 102.3% for zinc; standard deviations were 11.8 and 5.3%, respectively. (It should be noted that two of the 12 recoveries for copper were noticeably below the others; these two values were 77.4 and 68.7%. Acid digestion most likely would have freed any bound copper. However, any metal attached to, e.g., exfoliated skin would be considered absorption in this study. Therefore, such pre-treatment of samples was not warranted or desirable.) When the Units for the

10 live frogs were adjusted for these mean recoveries, the statistical conclusions remained the same for both metals.

4. Conclusions

In this screening study, the copper data from the live frogs caused acceptance of the alternative hypothesis (that frogs do absorb Cu either onto or through their skin). However, for zinc, insufficient evidence existed to reject the null. Data from live frogs in DI-water baths indicated that urinary excretion of these two metals was high (and variable) enough to mask absorption effects.

More importantly, the conclusions regarding the applicability of ion chromatography for these types of studies were positive. The instrumentation was found to be a precise, sensitive technique for quantifying copper and zinc in frog balance studies. Seven-level calibration curves (straight-line with OLS fitting) were found to have high precision; prediction intervals at 20 ppb ($\alpha=\beta=0.025$) were 2.3 and 4.3 ppb for copper and zinc, respectively. Response stability remained high throughout the research. The technology, then, is a rapid, reproducible vehicle for quantitating these two transition metals in balance studies such as these.

5. Nomenclature

Mathematical symbols

α	average probability of false positives
β	average probability of false negatives
g	similar to a 'significance test' for the slope of the calibration line. Equals $[(\mathrm{RMSE})^2\cdot(t_{n-2,\ 1-[\alpha/2]})^2]/[b^2S_{xx}]$ for OLS fits. Should be less than 0.1 for each curve, or the curve should not be used for predictions.
OLS	ordinary least squares. A fitting technique that minimizes the sum of squares of the residuals
R^2_{adj}	R^2 for each independent variable used in the regression (R^2 measures the amount of total variation in the response 'explained' by the dependent variable)
RSD	standard deviation divided by the mean, expressed as a percentage

Terms used

Lack-of-fit (LOF) test	a test of the statistical significance of the residual variation that is above and beyond that attributable to pure error.
Mean absolute deviation	the mean of the absolute values of the quantities 'true minus predicted'.
Null hypothesis	usually, the hypothesis that the investigator is attempting to disprove, at a specified level of confidence. Note that lack of rejection does *not* prove the hypothesis.
p-value	the probability value associated with a statistical test, representing the likelihood that a test statistic would assume or exceed a certain value, if the null hypothesis is true.
Prediction interval	a pair of limits that bracket the uncertainty in one future measurement.
Residual	the actual (measured) value minus the predicted value.
Statistically significant	causing a null hypothesis to be rejected at some accepted confidence level.
Units	ng absorbed/h·50-g frog. A negative value indicates net excretion.

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