Binding of Gold to Bovine Serum Albumin Using Flameless Atomic Absorption

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Abstract □ A graphite furnace atomic absorption spectrophotometric assay capable of accurately determining nanogram amounts of gold in biological fluids was developed. The presence of bovine serum albumin and/or phosphate in the sample reduced the method sensitivity without affecting the linear response. Binding of gold was studied by ultrafiltration using cones with a molecular weight cutoff of 25,000. The binding of gold at various concentrations to 2 and 4% bovine serum albumin in 0.1 *M* phosphate buffer, pH 7.4, was independent of the gold and protein concentrations. In the 2–10-μg/ml range, the overall binding values (mean ± *SD*) of gold to 2 and 4% bovine serum albumin were 98 ± 1.6 (n = 35) and 99 ± 1.0% (n = 15), respectively. When ultrafiltration cones with a molecular weight cutoff of 50,000 were used, the extent of binding to 2% bovine serum albumin was 85.4 ± 1.6% (n = 11). This statistically significant difference ($p < 0.001$) was due to variations in the protein retention of the two cone types. Interaction studies showed that gold was not displaced from the binding sites by salicylic acid (200 μg/ml) or vice versa.

Keyphrases □ Antiarthritis agents—gold, effect of plasma protein and gold concentrations on gold binding, flameless atomic absorption determination of gold in biological fluids □ Aspirin—effect of salicylic acid on binding of gold to bovine serum albumin, flameless atomic absorption determination □ Protein binding—gold to bovine serum albumin, effect of plasma protein and gold concentration on gold binding, effect of salicylic acid on gold bound to albumin, flameless atomic absorption determination □ Salicylic acid—effect on gold bound to serum albumin, flameless atomic absorption determination

Gold salts are widely used in rheumatoid arthritis treatment (1). Although gold has a high affinity for plasma proteins (2–10), the specific protein to which it is bound most extensively is a matter of controversy. Some reports indicated that gold is bound exclusively to albumin (2, 3, 9), while others implicated fibrinogen (5) and α- and β-globulins (8). No data currently are available on the effect of protein concentration on the extent of gold binding. This aspect has important therapeutic implications since hypoalbuminemia often is encountered in arthritic patients (11). The recent observation (12) that aspirin hepatotoxicity increased in patients who were treated simultaneously with gold raises questions about competition for binding sites between salicylic acid and gold.

BACKGROUND

Several analytical methods have been used to determine gold in biological fluids. They include colorimetry (13), neutron activation analysis (14), polarography (15), and flame and carbon rod atomic absorption spectrophotometric techniques (4, 11, 16–21). Colorimetry and polarography require extensive sample manipulation, are time consuming, and suffer from low sensitivity. These disadvantages make these methods inapplicable to the routine determination of therapeutic gold concentrations (1–10 μg/ml). While neutron activation analysis has high sensitivity (0.01 μg/ml), the cost of instrumentation is prohibitive to most clinical laboratories.

Atomic absorption spectrophotometry has greatly facilitated the detection of minute concentrations of metals in biological fluids. Earlier procedures for gold estimation in biological fluids were conducted using flame atomic absorption spectrophotometric techniques (4, 6, 17). However, the required dilution, digestion, and extraction steps resulted in a loss of sensitivity and time. New atomic absorption spectrophotometric techniques using a carbon rod or a graphite furnace atomizer have overcome these problems (18–21). The advantages of these new techniques are high absolute sensitivity, microliter sample size, the potential for analyzing samples without pretreatment, speed of analysis, freedom from introduction of contaminants, and low background noise.

The first objective of this study was to develop a sensitive flameless atomic absorption spectrophotometric assay to determine gold in biological fluids. The second objective was to study the extent of the binding of gold to bovine serum albumin and the effect of salicylate on this interaction.

EXPERIMENTAL

Materials—Graphite tubes[1] for sample atomization, gold standards[2], ultrafiltration membrane cones[3], bovine serum albumin[4], and salicylic acid[5] were obtained commercially. Distilled, deionized water was prepared by passing distilled water through an ion-exchange system[6]. To avoid possible loss of gold by binding to glass, plastic laboratoryware was used.

Instrumentation—An atomic absorption spectrophotometer[7] equipped with a graphite furnace atomizer[8], a deuterium-arc background corrector[9], and an automatic injector system[10] was used for gold determination. A gold hollow cathode lamp[11] was the light source. Atomization peak heights of gold were recorded on a strip-chart recorder[12] at a chart speed of 10 mm/min and a recorder current of 10 mamp.

Operating Conditions—The optimum parameters for gold determination in the test solutions were: drying cycle, 100° for 20 sec with a temperature ramp of 30 sec; charring cycle, 800° for 60 sec with a temperature ramp of 30 sec; and atomization cycle, 2200° for 5 sec. The ramp setting permits gradual rise in the furnace temperature during each run. The carrier gas was argon at a flow rate of 30 ml/min. Atomization peak heights were recorded at 242.8 nm.

Calibration Curves for Gold—A working standard, 100 μg/ml, was prepared by diluting commercially obtained gold solution with distilled, deionized water. This working standard was diluted further with 0.1 *M* phosphate buffer, pH 7.4, to give solutions containing 0.1, 0.2, 0.3, and 0.4 μg of gold/ml. Twenty-microliter aliquots of each solution were in-

[1] Part 290-1633, Perkin-Elmer Corp., Norwalk, Conn.
[2] Harelco, Gibbstown, N.J.
[3] Centriflo membrane cones, types CF25 and CF50A, Amicon Corp., Lexington, Mass.
[4] Sigma Chemical Corp., St. Louis, Mo.
[5] Fisher Scientific Corp., Springfield, N.J.
[6] Model D-0800 Barnstead demineralizer with D-0809 cartridge.
[7] Perkin-Elmer model 403.
[8] Perkin-Elmer model HGA-2100.
[9] Perkin-Elmer deuterium-arc power supply.
[10] Perkin-Elmer model AS-1
[11] Perkin-Elmer intensitron 303-6031.
[12] Perkin-Elmer model 56.

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Table I—Effect of Bovine Serum Albumin Concentration on Binding of Gold

Gold Concentration, μg/ml	Percent Bound[a]	
	2% Bovine Serum Albumin[b]	4% Bovine Serum Albumin[c]
2	100 ± 0.5	99 ± 1.4
4	98 ± 1.8	99 ± 0.5
6	99 ± 1.0	99 ± 1.1
8	97 ± 0.9	98 ± 0.9
10	99 ± 1.5	98 ± 1.1

[a] Expressed as mean ± *SD*. [b] Number of samples was seven. [c] Number of samples was three.

Table II—Effect of Salicylic Acid [a] on Binding of Gold to 2% Bovine Serum Albumin

Gold Concentration, μg/ml	Percent Bound[b]	
	Salicylic Acid Added 24 hr after Gold	Salicylic Acid Added 24 hr before Gold
2	100 ± 0.0	100 ± 0.3
4	99 ± 0.0	100 ± 0.5
6	99 ± 0.2	100 ± 0.3
8	98 ± 0.2	98 ± 0.8
10	98 ± 0.2	99 ± 0.3

[a] Concentration of 200 μg/ml. [b] Expressed as mean ± *SD*; $n = 3$.

Table III—Results from Linear Regression of Atomization Peak Heights [a] *versus* Gold Concentrations [b] in Various Media

Medium	Slope	Intercept	r[c]
Distilled, deionized water	193	1.5	0.997
0.1 *M* Phosphate buffer, pH 7.4	138	1.5	0.999
0.2% Bovine serum albumin in 0.1 *M* phosphate buffer, pH 7.4	77	1.0	0.996

[a] Expressed in arbitrary units. [b] Gold concentration ranged from 0.1 to 0.4 μg/ml. [c] Correlation coefficient.

jected into the atomic absorption spectrophotometer, and the atomization peak heights were recorded. Calibration plots were prepared on all days of sample analysis using the same graphite tube used for the samples.

Matrix Effects—Gold solutions of 0.1–0.4 μg/ml were prepared by diluting the working standard with (*a*) distilled, deionized water, (*b*) 0.1 *M* phosphate buffer, pH 7.4, or (*c*) 0.2% bovine serum albumin in 0.1 *M* phosphate buffer, pH 7.4. The atomization peak heights of 20-μl aliquots of these gold solutions were obtained as described earlier.

Binding Studies—The binding of gold to bovine serum albumin was studied using a previously reported ultrafiltration technique (22) with minor modifications. The membrane cones, after pretreatment as recommended by the manufacturer, were dried by centrifugation at 2000 rpm (484×*g*) for 25 min. A comparison of cone weights before and after soaking and centrifugation showed that <0.06 ml of distilled, deionized water remained in the cones.

Bovine serum albumin solutions, 2 and 4%, containing various concentrations of gold (2–10 μg/ml) were prepared in 0.1 *M* phosphate buffer, pH 7.4. Following equilibration for 16–24 hr (overnight), 5-ml aliquots were transferred to pretreated cones and centrifuged for 30 sec at 2000 rpm. The filtrate, ~0.1 ml, was discarded to minimize dilution errors that can be expected from the residual water in the cones. A second 0.4-ml aliquot of filtrate was collected, and a 20-μl portion was injected into the atomic absorption spectrophotometer. The atomization peaks at 242.8 nm were recorded and converted into concentrations using calibration plots prepared with standard gold solutions in 0.1 *M* phosphate buffer, pH 7.4.

To determine the nonspecific loss of gold due to binding to the ultrafiltration cones and/or the apparatus, gold solutions in phosphate buffer (2–4 μg/ml) were filtered through the cones in the absence of bovine serum albumin. Gold concentrations in the filtrates were compared to the corresponding concentrations prior to filtration.

Displacement Studies with Salicylic Acid—The procedure was identical to that described under *Binding Studies*, except for the addition of salicylic acid. These studies were conducted with the 2% bovine serum albumin solution. In one set of experiments, salicylic acid was added to bovine serum albumin 24 hr prior to the addition of gold. In the second set, salicylic acid was added 24 hr after the addition of gold to bovine serum albumin.

While gold concentrations varied from 2 to 10 μg/ml, the salicylic acid concentration was 200 μg/ml in all trials. Following the addition of gold in the first set of experiments and salicylic acid in the second set, the solutions were equilibrated overnight. Binding studies then were performed. Salicylate concentrations in the ultrafiltrates were determined by UV spectrophotometry at 295 nm.

RESULTS AND DISCUSSION

Analytical—Atomization peak heights for gold at 242.8 nm were linearly related to concentration in the 0.1–0.4-μg/ml range. Peak heights varied considerably among the graphite tubes. For instance, the slope of the peak height (arbitrary units) *versus* gold concentration (micrograms per milliliter) ranged from 139 to 240 during a 2-month period when most of the samples were analyzed. However, the correlation coefficients of calibration plots obtained during this period were better than 0.990, with very small intercept values that were not statistically significant.

Visual examination of the graphite tubes revealed differences in their thickness. The "thicker" tubes gave erratic results and were not used in the analyses. In view of these problems, the peak heights obtained with test solutions were converted to gold concentrations using calibration plots prepared using the same graphite tube. Under such controlled conditions, the overall coefficient of variation of the technique was <2% (Tables I and II). The life of a tube ranged from 50 to 60 determinations under the conditions used.

Matrix Effects—The medium or matrix in which the gold solutions were prepared also affected the method sensitivity (Table III). A decrease in the slope of the regression line corresponded to a decrease in the peak height. Based on the slope values in Table III, a gold solution prepared in 0.1 *M* phosphate buffer was expected to have a peak height that was ~25% lower than that obtained with an aqueous gold solution of equal concentration. The presence of even small amounts of bovine serum albumin in the buffer resulted in a further decrease in peak heights to ~40% of those obtained in water.

The reasons for these effects remain unclear. However, they emphasize the need to recognize that electrolytes and other normal constituents of biological fluids can interfere with atomic absorption measurements. The interference may be due to molecular absorption of electrolytes at the wavelength of gold absorption or to the physical occlusion of gold in a host of condensing interferent atoms.

While this work was in progress, Kamel *et al.* (11) reported a similar method to determine gold in blood. They reported that the organic matrix of blood reduced the sensitivity of the technique. It was speculated that a stable protein complex that was not destroyed during the charring cycle may have prevented the complete atomization of gold. However, such an explanation does not fit the interference observed with phosphate.

Binding Studies—Small amounts of protein escaped through the ultrafiltration cones used in this study. Depending on this amount and the affinity of the drug for the macromolecule, the values obtained for the extent of binding may have been underestimated. The manufacturer specifies that cones with molecular weight cutoffs of 25,000 (Type 1)[13] and 50,000 (Type 2)[14] retain >95 and 90% of albumin, respectively. For five randomly chosen Type 2 cones, the mean protein leakage was determined to be 11% (range 9–12.5%); these values were obtained by expressing the protein concentration in the filtrate as a fraction of that placed in the cone (2%) prior to ultrafiltration. Protein concentrations were measured by the biuret method (23). In a similar study with Type 1 cones, four cones showed no detectable leak and one had a leak of 5.8%.

The extent of gold binding to 2% bovine serum albumin was 85 ± 1.6% ($n = 11$) with Type 2 cones. The corresponding value with Type 1 cones was 98 ± 1.0% ($n = 15$), which is significantly higher ($p < 0.001$) than the value obtained with the Type 2 cones. The extent of underestimation (13%) is in good agreement with the differences in protein leakage (11%) between these two cone varieties. Therefore, all binding studies used Type 1 cones. Although each cone was not tested for protein leakage, suspiciously low values for binding were always associated with cones that were found to leak, as indicated by the presence of protein in the ultrafiltrate. Such cases were repeated.

A paired *t* test showed that, in the absence of bovine serum albumin,

[13] Centriflo membrane cones, type CF25, Amicon Corp., Lexington, Mass.
[14] Centriflo membrane cones, type CF50, Amicon Corp., Lexington, Mass.

gold concentrations in the ultrafiltrates were not statistically different ($p > 0.05$) from the corresponding concentrations prior to filtration. Hence, no corrections for nonspecific binding were warranted. Gold was almost 100% bound to bovine serum albumin (Table I). These results indicate that gold may be bound exclusively to the albumin fraction of plasma, as was reported earlier (2, 3, 9). However, more definite conclusions can be made only after studies in the presence of other plasma proteins. The overall values for the percent of gold bound to 2 ($n = 35$) and 4% ($n = 15$) bovine serum albumin were 98 ± 1.6 and 99 ± 1.0% (mean ± *SD*), respectively.

Two factors that often determine the extent of binding are the concentrations of the drug and of the protein. The gold concentrations studied were selected to include the plasma concentrations of the drug encountered in chrysotherapy (2, 6, 16, 24, 25). While there is a paucity of data regarding the extent of hypoalbuminemia in arthritis patients, a recent study observed serum albumin levels as low as 2.47 g/100 ml (11). The results of the present work show that, in the clinically important range, the extent of gold binding was independent of gold and bovine serum albumin concentrations. The validity of these results in the *in vivo* situation in humans remains to be examined.

Interaction with Salicylic Acid—Salicylic acid did not compete with gold for binding sites on bovine serum albumin (Table II). Since the sequence of addition of salicylic acid had no significant effect on the affinity of gold for bovine serum albumin, inhibition of a noncompetitive nature also can be ruled out. Similarly, gold did not interfere with salicylate binding; salicylate was 62–70% bound to bovine serum albumin in the absence and presence of gold (2–10-μg/ml range). During aspirin therapy, plasma concentrations of salicylate range from 50 to 200 μg/ml (26). The *in vitro* results suggest that mutual displacement of gold and salicylic acid from plasma proteins may not be a serious drug interaction in arthritis management.

The results of this study show that clinically encountered plasma concentrations of gold, salicylate, and albumin are unlikely to cause statistically significant alterations in the extent of protein binding of these two drugs. However, for drugs that are bound extensively to plasma protein as is gold, minor binding alterations may be clinically significant due to the resulting dramatic changes in the free concentration. For instance, a 1% decrease in the binding of gold (assumed to be 99% bound) results in a twofold (1–2%) increase in the free concentration of gold. Such changes, although extremely difficult to document experimentally, could cause gold toxicity in arthritic patients.

REFERENCES

(1) "The Pharmacological Basis of Therapeutics," 5th ed., L. S. Goodman and A. Gilman, Eds., Macmillan, New York, N.Y., 1975, p. 931.

(2) B. R. Mascarenhas, J. L. Granda, and R. H. Freyberg, *Arthritis Rheum.*, **15**, 391 (1972).

(3) E. G. McQueen and P. W. Dykes, *Ann. Rheum. Dis.*, **28**, 437 (1969).

(4) M. Harth, D. S. M. Haines, and D. C. Bondy, *Am. J. Clin. Pathol.*, **59**, 423 (1973).

(5) J. S. Lawrence, *Ann. Rheum. Dis.*, **20**, 341 (1961).

(6) A. Lorber, R. A. Bovy, and C. C. Chang, *Nature (New Biol.)*, **236**, 250 (1972).

(7) P. M. Smith, E. M. Smith, and N. L. Gottilieb, *J. Lab. Clin. Med.*, **82**, 930 (1973).

(8) N. Simon, *Science*, **119**, 95 (1954).

(9) D. S. Campion, R. Olsen, A. Bohan, and R. Bluestone, *J. Rheumatol. (Suppl. 1)*, **1**, 112 (1974).

(10) R. W. Mason, *Pharmacology*, **15**, 536 (1974).

(11) H. Kamel, D. H. Brown, J. M. Ottaway, and W. E. Smith, *Analyst*, **101**, 790 (1976).

(12) J. D. Davis, R. A. Turner, R. L. Collins, I. R. Ruchte, and J. S. Kaufmann, *Clin. Pharm. Ther.*, **21**, 52 (1977).

(13) J. F. Goodwin and A. J. Bollet, *J. Lab. Clin. Med.*, **55**, 965 (1960).

(14) A. A. Dietz and H. M. Rubinstein, *Ann. Rheum. Dis.*, **32**, 124 (1973).

(15) G. D. Christian, *Clin. Chem.*, **11**, 459 (1965).

(16) A. Lorber, R. L. Cohen, C. C. Chang, and H. E. Anderson, *Arthritis Rheum.*, **11**, 170 (1968).

(17) J. V. Dunckley, *Clin. Chem.*, **17**, 992 (1971).

(18) F. J. M. J. Maessen, F. D. Posma, and J. Balke, *Anal. Chem.*, **46**, 1445 (1974).

(19) J. Agget and T. S. West, *Anal. Chim. Acta*, **55**, 349 (1971).

(20) J. Agget, *ibid.*, **63**, 473 (1973).

(21) J. P. Matousek and B. J. Stevens, *Clin. Chem.*, **17**, 636 (1971).

(22) S. M. Singhvi, A. F. Heald, H. H. Gadenbusch, M. E. Resnick, L. T. Difazio, and M. A. Leitz, *J. Lab. Clin. Med.*, **89**, 414 (1977).

(23) D. T. Plummer, "An Introduction to Practical Biochemistry," McGraw-Hill, Berkshire, England, 1971, p. 156.

(24) A. Lorber, *Clin. Pharmacokinet.*, **2**, 127 (1977).

(25) J. T. Sharp, M. D. Lidsky, J. Duffy, H. K. Thompson, Jr., B. D. Person, A. F. Masri, and A. A. Andrianakos, *Arthritis Rheum.*, **20**, 1179 (1977).

(26) M. Mayersohn, *J. Am. Pharm. Assoc.*, **NS 17**, 107 (1977).

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GLC Analysis of Poison Ivy and Poison Oak Urushiol Components in Vegetable Oil Preparations

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Abstract □ A procedure is described for the analysis of urushiol content of pharmaceutical preparations containing extracts of poison ivy (*Toxicodendron radicans*) and poison oak (*T. diversilobum*) in vegetable oils. The procedure involves extraction of the urushiols from the oily solutions using 90% methanol in water followed by GLC analysis of the extracts. Recoveries of both poison ivy and poison oak urushiols from solutions in corn oil, olive oil, sesame seed oil, and cottonseed oil were calculated. Correlation coefficients (r^2) ranged from 0.97 to 1.00, and the coefficients of variations ranged from 3.08 to 7.90%.

Keyphrases □ Urushiols—GLC analysis, pharmaceutical preparations containing poison ivy and poison oak extracts in vegetable oils □ GLC—analysis, urushiols in pharmaceutical preparations containing poison ivy and poison oak extracts in vegetable oils □ Poison ivy—urushiol components in vegetable oil preparations, GLC analysis

Contact dermatitis due to poison ivy (*Toxicodendron radicans*) and other related *Anacardiaceae* species [*e.g.*, poison oak (*T. diversilobum*) and poison sumac (*T. vernix*)] is a major problem among outdoor workers in the

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