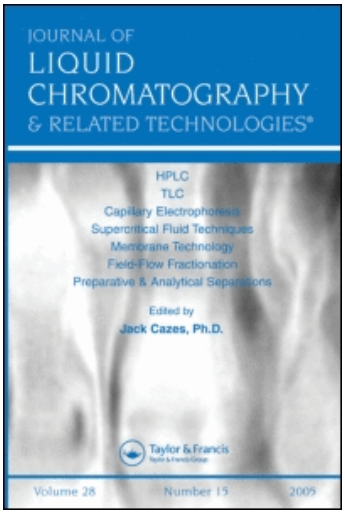

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Original Article

CHROMATOGRAPHY WITH METAL SPECIFIC DETECTION OF URINE SAMPLES FROM AN ARTHRITIS PATIENT ON AURANOFIN THERAPY

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ABSTRACT

Six sequential urine samples obtained from a patient on auranofin therapy were analyzed for total gold, zinc, copper and creatinine levels. They were separated by high performance liquid chromatography (HPLC) on a weak anion exchange column. The detector utilized was an inductively coupled plasma mass spectrometer (ICP-MS). Five, gold-containing metabolites as well as multiple zinc- and copper-containing materials were eluted. The distribution of all three metals changed with time on therapy.

INTRODUCTION

Gold drugs have been used in the treatment of rheumatoid arthritis for more than 60 years (1). Of the three drugs currently in use, two are administered by injection, myochrysine (gold sodium thiomalate) and solganol (gold thioglucose), and one is taken orally, auranofin (triethylphosphine gold (I) tetraacetyl thioglucose). Gold therapy is effective in causing remission in many of the patients with a high gold drug tolerance, but

frequently the therapy must be discontinued due to detrimental side effects which usually manifest themselves during the first few months of gold therapy (2). Little is known about the metabolism of these gold drugs or why they work for some patients and not for others. However, it has been shown that gold is necessary for efficacy (3). Total gold levels in blood or urine fail to predict drug efficacy (4). Our preliminary studies using an atomic absorption spectrophotometer (AAS) as a gold specific detector for high performance liquid chromatography (HPLC) indicate that there are multiple, gold-containing metabolites in the urine of patients on chrysotherapy. The qualitative distribution of metabolites varied from one patient to another, suggesting that the appearance of a particular metabolite might serve as a prognostic indicator of efficacy or toxicity.

Since we typically find that gold levels in urine are less than 1 ppm, and the detection limit is about 5 ppm for AAS when used as an HPLC detector either preconcentration or a more sensitive detector was needed. We chose an inductively coupled plasma mass spectrometer (ICP-MS) which is three to four orders of magnitude more sensitive than the AAS (5). The ICP-MS is capable of measuring most elements in the range of 3 a.m.u. to 300 a.m.u. with similar detection limits. It also can monitor up to sixteen different elements essentially simultaneously (6). This makes it possible to monitor the distributions of several different metals in the course of a single HPLC injection from a patient sample such as blood or urine.

MATERIALS AND METHODS

Urine Samples

The samples were donated by a white, 28 year-old, female outpatient suffering from severe psoriatic arthritis. The samples were obtained at the University Hospital Arthritis Clinic over a time span of 21 weeks. The patient was initially taking 6 mg of Auranofin daily, after 12 weeks the daily dosage was increased to 9 mg.

Reagents

The reagents used were concentrated doubly distilled (DD) HNO_3 (GFS Chemicals, Columbus, Ohio), 1000 ppm $AuCl_4$, 1000 ppm ZnO and 1000 ppm $Cu(NO_3)_2$ standard solutions (Fisher Scientific, Fairlawn, New Jersey), trisma base (Sigma Chemical Company, St. Louis, Missouri), DD water, Creatinine Procedure No. 555 (Sigma Diagnostics, St. Louis, Missouri).

Instrumentation

The HPLC system consisted of two Waters Model 510 pumps a Model 680 gradient controller and a Model 7125 sample injector (Rheodyne Inc., Cotati, CA). The HPLC procedure utilized a macrosphere 300 weak anion exchange chromatographic column (7 micron particles, 150 mm x 4.6 mm, WAX-diethylaminomethane packing material, Alltech Inc., Deerfield, Illinois). An ELAN inductively coupled plasma mass spectrometer (Model 250, Sciex, Thornhill, Ontario, Canada) was used as the element specific detector. A Parr Microwave Digestion Bomb (Parr Instrument Company, Moline, Illinois) was used for digesting the samples.

Sample Treatment

The samples were collected, refrigerated, then subsequently frozen at -20°C. Auranofin therapy was begun on 10/14/87. The dates of sample collection were as follows: 1, 11/18/87;22, 1/6/88; 3, 1/20/88; 4, 1/27/88; 5, 3/2/88; 6, 3/9/88. On 1/7/88 the dosage of auranofin was increased from 6 mg daily to 9 mg daily. By March 1988, the arthritis was responding very well to treatment. All samples were analyzed sequentially on the same day in order to afford direct comparison.

Digestion Procedure

The samples used for measurement of total metal concentration were digested in order to release all complexed metals to their aquated ion states. This procedure was followed since the complexed metals might require greater plasma power input to

fully atomize and ionize (5) and thus result in erroneously low results. A 10% solution of concentrated, doubly distilled (DD) HNO_3 was prepared. 2 ml of the 10% HNO_3 solution was placed in a Parr microwave digestion bomb. 1 ml of the urine sample was added to the container. The sample was heated in a microwave oven at 700 watts for 30 seconds. The digest was allowed to cool for at least 20 minutes and then analyzed. Three aliquots of each urine sample were digested.

Creatinine Level Determination

Creatinine levels were determined using the procedure described in the Sigma Diagnostic Creatinine Procedure No. 555 kit. The procedure is based on a colorimetric change from a modified Jaffe reaction with picric acid, measured at 500nm (7).

Metal Concentration Determination

Calibration curves were generated using serial dilutions of 1000 ppm Au, Zn and Cu standard solutions. The solutions were diluted using 1 part water to 2 parts 10% DD HNO_3. The concentrations of the standards were 0.010, 0.050, 0.100, 0.250 and 0.500 ppm Au, Zn and Cu. Using flow injection analysis (FIA) ICP-MS, the samples were analyzed. A 50 microliter stainless steel injection loop was utilized to introduce the standards as well as the samples into the DD water eluant stream flowing at 2.5 ml/min. The stream was connected to the ICP-MS via teflon tubing. The ICP-MS operating parameters were as follows: Ar cooling gas flow,14.0 L/min; nebulizer Ar, 0.45 L/min; auxiliary Ar 1.4 L/min, RF power, 1.2 KW; reflected power < 10 W; standard Sciex torch; Meinhard C-3 nebulizer (low flow type for use including organic solvents); sampler diameter 1.1 mm; skimmer diameter 0.9 mm; interface pressure 1.0 Torr; MS pressure 3.0 x 10^{-5} Torr; data acquired in multielement mode; measurement time, 0.2 sec; dwell time, 30.0 msec. Three 50 microliter injections of each standard and all digested urine samples were made. The concentrations of the metals in the urine samples were determined

from the calibration curves based on the areas of the standard peaks. The standard curves proved to be linear over the test range. The correlation coefficients for each of the calibration curves, i.e. Au, Zn, and Cu, were 0.9845, 0.9998 and 1.000 respectively.

Separation Method

Chromatography on a weak anion exchange (WAX) column from Alltech was used to separate the various components present in the six urine samples. A linear gradient was formed in the mobile phase from 100% 20 mM Tris base (pH 5.5) to 100% 200 mM Tris base (pH 5.5) over a period of 15 minutes. The pH was adjusted with acetic acid. A 50 microliter injection loop was employed with a flow rate of 1 ml/min. The eluant flowed through teflon tubing into the ICP-MS which was programmed to detect Au, Zn and Cu. For each injection, data were collected for all three elements. Chromatograms for each element are displayed separately in Figure 1 to allow direct comparisons from one treatment time to another. The resulting chromatograms were smoothed using locally developed software on a microVAX II computer. Due to the gradient employed, peaks eluted at different times are in different solvent mixtures. This varying matrix affects the signal intensity slightly, depressing the signal as the amount of tris increases. In this system the total change is approximately 15%.

RESULTS

Concentration: Based on concentration calibration curves, the Au, Zn and Cu levels for all six samples were determined (Table 1). Since metal concentrations in the urine might be expected to be influenced by the amount of fluid intake, the levels of creatinine in each sample were measured as well. Creatinine is the major excretory form of creatine. The amount of creatinine excreted in the urine is directly related to the muscle mass of an individual. Since muscle mass varies little over time, creatinine levels in the urine basically remain constant for a given

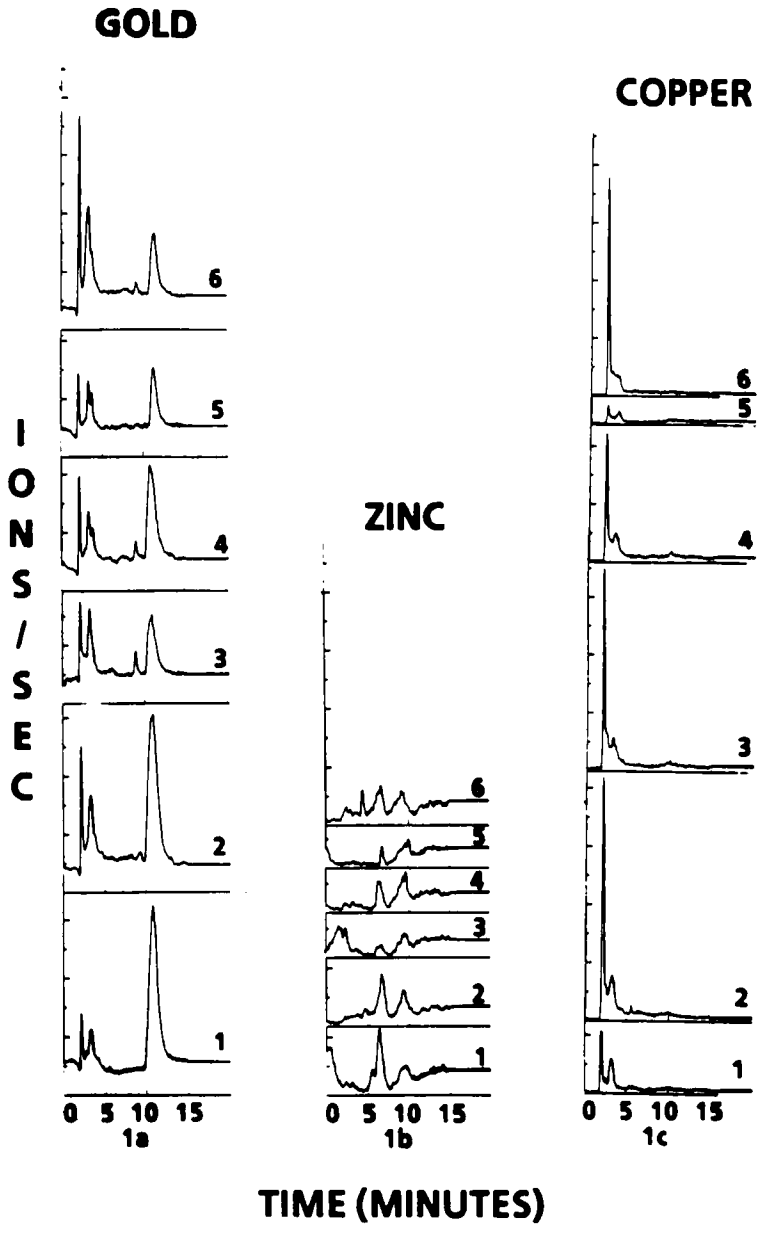

Figure 1, Element specific chromatograms of urine samples donated over a 21 week period. Those in 1a show gold containing species whereas 1b shows zinc and 1c copper. The chromatograms are arbitrarily displaced vertically for clarity with the earliest obtained sample at the bottom. For any given element all chromatograms have the same vertical scale; total element levels may be obtained from Table 1.

TABLE 1
Total metal concentrations

Sample	Date	Au(ppm)	Zn(ppm)	Cu(ppm)
1	11/18/87	0.26±0.08	0.42±0.10	0.017±0.002
2	1/6/88	0.49±0.04	0.36±0.17	0.040±0.001
3	1/20/88[a]	0.50±0.08	0.27±0.03	0.028±0.003
4	1/27/88	1.02±0.02	0.41±0.14	0.042±0.006
5	3/2/88	1.45±0.04	0.27±0.04	0.042±0.018
6	3/9/88	0.97±0.14	0.29±0.08	0.034±0.008

[a]2 weeks after increase of daily dosage (6 mg to 9 mg)

individual (8). Thus, dividing the metal concentrations by the creatinine levels may provide a normalization among the six samples. The levels of creatinine as well as the metal/creatinine ratios are given in Table 2.

All samples were chromatographed on a weak anion exchange column using a linear gradient beginning with 20 mM Tris buffer (pH 5.5) and ending with 200 mM Tris buffer (pH 5.5). The eluants were monitored for three different metals: gold, zinc and copper (Figures 1a, 1b and 1c respectively). Regarding the gold chromatograms, there are three major peaks eluting (2.2, 3.4 and 11.0 min respectively). There are two more minor components one appearing as a shoulder on the 3.4 min peak and the other clearly present in samples 3,4 and 6 at 9.1 min. As the therapy continued, the gold distribution changed. In Sample 1, the majority of the gold was found in the last peak. As the series continued, the majority of the gold was shifted from the last peak to the first two peaks.

Neither zinc nor copper chromatograms show much change as the patient underwent gold therapy. In the zinc samples, two rather ill defined peaks are observed in all chromatograms at retention times of 6.6 and 9.5 min. The copper chromatograms show

TABLE 2
Creatinine Levels and Ratios

Sample	Date Taken	Creatinine (ppm x10^3)	Au/Creat. (x10^{-6})	Zn/Creat. (x10^{-6})	Cu/Creat. (x10^{-6})
1	11/18/87	9.77±0.28	26.6± 8.2	43.0±10.7	2.0±0.2
2	01/06/88	9.70±0.04	50.5± 4.5	37.1±17.9	4.1±0.3
3	01/20/88[a]	5.80±0.12	86.2±14.0	46.6± 5.3	5.2±0.7
4	01/27/88	13.67±0.09	74.6± 1.5	30.0±10.7	2.9±0.4
5	03/02/88	13.68±0.38	106.0± 4.6	19.7± 3.3	2.9±1.3
6	03/09/88	9.43±0.05	102.9±15.7	30.8± 8.1	3.2±0.8

[a]2 weeks after increase of daily dosage (6 mg to 9 mg).

qualitatively similar results throughout the treatment period. Copper species elute with retention times of 2.1 and 3.3 min in all cases. Sample 5 is anomalous in that the overall copper levels seen in the chromatogram are extremely low. Since this sample shows normal Au and Zn chromatograms (measured on the same injected sample), the low levels of copper eluted must describe a real change and do not result from sample dilution or failure to inject properly.

DISCUSSION

We have demonstrated a relatively simple and extremely sensitive means to measure Au, Zn and Cu levels as they are excreted in the urine. Studies on urine may lead to the identification of specific gold containing metabolites with specific therapeutic results. The samples described here were analyzed for copper and zinc as well as gold because changes in these elements have been demonstrated (9). In the case of the patient studied here, the Au levels increased steadily with time. Sample 5 seems to have a significantly higher Au concentration (1.45 ppm) than the following sample 6 (0.97 ppm). However, when the data are normalized by dividing by the creatinine

concentration, the values for the two samples are quite similar. It should be noted that a recent study of urinary excretion patterns of patients on sodium aurothiomalate showed a correlation of gold levels with water excretion and not generally with creatinine (10). However, in that study for 6 of the 30 patients there was a correlation between gold levels and creatinine. Whether our case is following that pattern of those 6 patients or it is merely different because of the use of the oral drug auranofin, we can not say. Zinc and copper levels in the urine seem to vary randomly.

In comparing metal levels as in Tables 1 and 2, the gold values show smaller errors than the values for zinc or copper. There are several sources of error in these measurements. For these experiments, three samples were taken from each urine specimen. Each sample was then digested separately. For each digest, three injections were made into the ICP-MS. One source of variability comes from differences in either the injected amount or in the ion measurement. For these experiments, repeat injections of identical samples had an error of about 10%. Errors were not different for the different elements measured. Another source of variability comes from differences between independent samples of the same urine specimen. This includes both differences in handling and in sampling. The urine samples used had been frozen and thawed. They contained variable amounts of precipitable material. Sampling these inhomogeneous materials could result in increased variability. The fact that the gold samples were less variable than the zinc and copper suggests that the gold may have been more homogeneously distributed, i.e. in solution rather that associated with the precipitate. Even with these sources of variability, the data for gold excretion in this patient show a steady increase in gold levels as the patient proceeded through therapy. The data for zinc and copper show no clear trend.

Most importantly, we have developed a method with the necessary sensitivity to monitor gold as well as other metals in

biological fluids. These metals may either be present from exogenous, metal-containing drugs or toxicants or may represent normally-occuring, metal complexes present in ordinary or abnormal quantities.

The multielement capability of the Sciex Elan which permits the simultaneous monitoring of several elements provides "quality control" for chromatography. For example, in sample 5, the copper levels shown in the chromatogram are extremely low. Since in flow injection this sample exhibits normal copper levels, the question occurs whether the low copper levels shown in the chromatogram are the result of operator error, such as the failure to inject the standard amount of sample. Comparison of the gold-specific chromatogram 5 with others shows that the gold levels are in rough agreement with those of other samples, thus ruling out injection failure or other trivial explanations as the source of the low copper levels in the copper-specific chromatogram 5. These levels must therefore result from a difference in the chemical state of the copper in sample 5, which results in copper compounds being "permanently" retained on the column either as particulate material filtered out or as chemical species which bind extremely tightly to the column. The multielement capability also provides an opportunity to introduce a void-volume marker easily into the system with little or no affect on the rest of the chromatography. Thus in anion exchange chromatography addition of lithium chloride will result in a non-retained lithium peak which marks the void volume.

As we have pointed out in a recent review (11), a number of metal-based drugs are now in widespread use. These include in addition to the gold drugs studied here, technetium radiopharmaceutical imaging agents, a platinum anticancer agent used to cure testicular cancer, a rhenium therapeutic in testing for treatment of metastatic prostate and breast cancer as well as numerous other metal complexes in very early stages of testing as therapeutics. In most cases the metabolism of these drugs is understood poorly if at all. The analytical approach we have

demonstrated here should be easily extended to any of these other cases. Detection limits in the ppb range on a continuously flowing sample make the ICP-MS an ideal detector for HPLC. Since the Sciex Elan instrument can monitor multiple elements simultaneously, separations of several different metal species can be followed in the same chromatogram.

Using the ICP-MS, we have shown that multiple metabolites are present and that the quantities of the various materials vary with time. We anticipate two further extensions of this study, first, to determine whether any of the specific metabolites as characterized by retention time is also correlated with efficacy or toxicity and, second, to separate and collect the metabolites so that they may be chemically characterized.

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