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Gold concentrations in blood fractions of patients with rheumatoid arthritis treated with Myocrisin

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Abstract: Gold levels in the plasma and blood cells of patients treated with the gold drug Myocrisin (sodium aurothiomalate) were determined by atomic absorption spectrometry. There is a correlation between whole blood gold and plasma gold concentrations which is different for smokers and non-smokers. Most cellular gold is associated with the membrane and is present in concentrations approximately equivalent to the number of reactive sulphydryl groups on the exofacial surface of the cell. Since gold would be expected to react with SH groups and since these groups are vital for cellular function, a possible role for gold in modifying cellular metabolism is indicated.

Keywords: *Gold; Myocrisin; sodium aurothiomalate; blood cells; plasma; atomic absorption spectrometry.*

Introduction

Gold compounds are among the few therapeutic agents which affect the course of aggressive rheumatoid arthritis. Despite a considerable research effort stimulated by the development of the oral compound Auranofin, no definite mechanism of action has been established [1–3]. Part of this effort has been concerned with the determination of plasma gold levels either to provide a marker of therapeutic action or to aid in studies of transport and *in vivo* chemistry. For example, Lorber [4] reported that a minimum gold level of 3 $\mu g\ ml^{-1}$ in plasma must be maintained for clinical efficacy. Other groups have not confirmed this finding [5, 6] and there have been claims that high plasma gold levels are related to the onset of skin rashes [7].

Although the active site of gold is not established, the general nature of the reactions of gold drugs *in vivo* have been predicted on the basis of *in vitro* studies with proteins and cells and studies of gold distribution in tissue compartments [8]. Gold in its common oxidation states of gold(I) and gold(III) will only exist in aqueous solutions complexed to a ligand. Most gold appears to be present *in vivo* as gold(I) although some may be present as gold(0) colloid. The compound most widely used as a therapeutic agent is Myocrisin (sodium aurothiomalate), a polymer of gold(I) with thiomalic acid in which the gold is bound to the sulphur of the ligand. This compound reacts readily with sulphydryl groups and more slowly with disulphides [9]. From the considerable body of circumstantial evidence available, it is believed that the gold is initially carried as the 'free drug', bound to plasma proteins, and as a new species created by the reaction of the drug with thiol groups in plasma proteins [1–3]. By far the largest concentration of reactive thiol is due to one cysteinyl residue at position 32 on albumin. This thiol group is believed to be the main initial target for gold reactions with Myocrisin. However, at clinical concentrations, the amount of gold present would bind less than 1% of the available albumin thiol and consequently studies of gold binding do not show any evidence of site saturation; in addition the distribution between plasma proteins does not vary with the plasma gold level in any individual [10]. In the search for a mechanism of action, no one enzyme system has appeared as a key participant, although many have altered activity [11].

Recently, it was suggested on the basis of *in vitro* studies that gold reacts with the sulphydryl groups available on the surface of cells [12]. These exofacial groups form the

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obvious point of exchange for gold on to cells. The alternative mechanism of absorption of Myocrisin into the lipid is less likely since Myocrisin is not lipophilic. The largest concentration of reactive thiol groups at the membrane surface are those associated with the hexose and anion transport proteins with smaller concentrations on other proteins and on specific antigen sites. Unlike the situation in plasma, the amount of gold present after 12 weeks in the average patient is greater than the amount required to react with all the exofacial cellular thiol groups. Consequently site saturation is possible. It is not clear whether the site of action of gold is at the surface or whether the membrane acts as a transporting medium into other sites but it is clear that the reaction on the cell membrane is one key step in the distribution of gold in the body. It is perhaps surprising that little effort has been made to quantify the gold concentration and distribution in cells. Perhaps the most obvious exceptions are: studies of lysate where a dose-dependent relationship between smoking and gold concentration in lysate has been noted [13]; some labelling studies [14]; and studies by electron microscopy of gold in leukocytes [15].

The present work makes a preliminary assessment of the distribution of gold in blood. One reason more attention has not been focused on this target previously is that the methods of analysis are complex. In this study, membrane levels have been assessed by subtracting lysate gold from whole-cell gold. Analysis was by atomic absorption spectrometry with electrothermal atomization using an instrument with Zeeman-effect background correction.

Materials and Methods

Equipment

A Perkin–Elmer Model 5000 Zeeman atomic absorption spectrometer and a HGA-400 graphite furnace atomizer were used for all measurements. The spectrometer spectral band pass was 0.7 nm and a Perkin–Elmer gold hollow-cathode lamp, operated at 10 mA, was used to obtain peak-height and peak-area absorbance measurements at the wavelength for gold of 242.8 nm. A Perkin–Elmer AS-40 autosampler was used to deposit 20-μl volumes of the test solutions in the HGA-400 atomizer; manual sample introduction was used for some measurements. A Perkin–Elmer data system 10 was connected to the Perkin–Elmer Zeeman 5000. The atomizer was equipped with pyrolytic graphite-coated tubes.

Reagents

All chemicals used in this study were of AnalaR grade or equivalent and solutions were prepared with distilled water. The reagents were obtained from BDH (Merck, Poole, Dorset, England) unless otherwise stated.

Analyte solutions

A stock solution containing 1000 μg ml^{-1} of gold was prepared by dissolving 0.2020 g of $NaAuCl_4.2H_2O$ (Aldrich) in 100 ml of 1% (v/v) HNO_3. The solution was stored in a calibrated flask in a dark cupboard. Working standard solutions were prepared from this stock reagent by serial dilution with either distilled water or 0.01% (v/v) HNO_3.

Nickel chemical modifier solution

A stock solution containing 15 000 μg ml^{-1} of nickel was prepared by dissolving 7.4295 g of $Ni(NO_3)_2$ $6H_2O$ in 100 ml of distilled water. The modifier prepared from this stock solution contained 1000 μg ml^{-1} nickel. Nickel has been shown to have a beneficial effect in the determination of gold by allowing an increase in the maximum permissible char temperature from 800 (no modifier) to 1200°C [16]. This effect is thought to be due to formation of an alloy of nickel and gold in the graphite tube. The higher char temperature allows removal of interfering inorganic salts such as sodium chloride and magnesium chloride without loss of the analyte. Hence effects of chemical interference are reduced and the accuracy of the determination is enhanced. The usefulness of nickel modification for gold in chemical samples has been illustrated in a previous publication [17].

Patients

All patients were suffering from classic or definite rheumatoid arthritis, as defined by the criteria of the American Rheumatism Association [18]. All were undergoing a 12-week course of weekly injections of 50 μg of Myocrisin (sodium aurothiomalate). Since the patients attended an out-patient clinic for assessment, it was not possible to obtain a study over a regular time course and hence sequential results, given as first, second, third and fourth visit, do not represent any par-

ticular span of weeks but are in chronological order.

Collection of blood samples and separation into different fractions

Blood samples were provided by Glasgow Royal Infirmary and collected from patients undergoing treatment with Myocrisin. Although it was not possible to obtain samples from an out-patient clinic on a sufficiently regular basis to enable a true time course to be obtained, two or three samples were taken where possible to provide information on intra-patient variability during the time in which the gold concentration rises to the maintenance level of approximately 3 $\mu g\ ml^{-1}$. The heparinized whole blood samples were centrifuged at 3000 rev min^{-1} for about 10 min and the plasma supernatant was removed by suction. To the packed cells were added 3 ml of saline, with gentle mixing, and the suspension was centrifuged for 10 min at 3000 rev min^{-1}. The clear supernatant was removed. The treatment of packed cells with saline and subsequent removal of the clear supernatant was repeated twice. To 1 ml of packed cells was added 1 ml of distilled water and the mixture was centrifuged at 3000 rev min^{-1} for 10 min. The resulting supernatant is defined here as haemolysate.

To another 1 ml of packed cells was added 1 ml of distilled water and the mixture was kept in a refrigerator for approximately 2 h; 0.8 ml of chloroform–ethanol (3:5, v/v) was added with gentle mixing on ice to obtain a precipitate of the haemoglobin. A few more drops of chloroform–ethanol were added to the ice-cooled precipitate and the mixture was centrifuged at 3000 rev min^{-1} for 10 min. The clear supernatant (clear lysate) was removed by suction. The blood fractions were stored in plastic vials and kept at 4°C in a refrigerator.

Direct determination of gold in whole blood, plasma, packed cells, haemolysate and clear lysate samples

The concentration of gold in the plasma of patients undergoing chrysotherapy is often in the range 0.1–3.0 $\mu g\ ml^{-1}$ Au [4] and gold levels in blood are similar [1–3]. Before analysis the whole blood and plasma samples were diluted 80-fold and 200-fold, respectively, with distilled water. Packed cells and haemolysate samples were diluted 10-fold and 5-fold, respectively, with distilled water; clear lysate samples were analysed directly or after 1 + 1 dilution with water.

A 20-μl volume of the diluted specimen was injected into the graphite furnace atomizer together with the same volume of 1000 $\mu g\ ml^{-1}$ nickel modifier. Calibration was achieved by injecting a series of 20-μl volumes of aqueous gold solutions with the same volume of the modifier solution. Peak-height and peak-area gold atomic absorption signals were obtained using the optimized atomizer programme given in Table 1 for wall atomization [17].

Interference by chloride salts on the determination of gold has been studied previously and the use of a nickel modifier has been shown to reduce the effect [16]. For the sample dilutions stated, recoveries of added gold (10–40 $\mu g\ l^{-1}$), using peak-area measurement, were in the range of 85–89% for whole blood and 100–113% for plasma, respectively. Although chloride salt interference will be less pronounced in the determination of gold in cells, haemolysate and clear lysate, the nickel modifier was also used for these analyses. A detection limit of 0.8 $\mu g\ l^{-1}$ Au was calculated for the diluted clinical solutions, on the basis of replicate ($n = 10$) blank measurements and application of 95% confidence limits. Replicate analysis ($n = 5$) of a single dilution of each specimen was performed and the results are quoted as the mean ± the standard deviation. All atomic absorption spectrometry measurements were made within the linear range of the instrument response for Au at 242.8 nm. The performance of the Zeeman background correction system was checked by making measurements at a wavelength close to the Au line (242.1 nm), obtained from a Sn hollow cathode lamp. The Sn line will not be absorbed by Au atoms but will be subject to the same molecular absorption as that at 242.8 nm. Lack of a signal at the 242.1 nm line

Table 1
HGA-400 atomizer programme for determination of gold in whole blood, plasma, packed cells, haemolysate and clear lysate

	Dry	Char	Atomize*	Clean
Temp (°C)	130	1200	2400	2700
Ramp (s)	5	5	0	1
Hold (s)	30	30	5	2
Argon internal gas flow ($ml\ min^{-1}$)	300	300	0	300

*The recorder and spectrometer read functions were activated at the start of the atomize stage.

confirmed accurate correction of molecular background and scatter for the solutions analysed.

Results and Discussion

The concentrations of gold are given in μg ml^{-1} for each sample in order to enable comparisons to be made between each of the biocompartments. Most gold is present in the plasma. There is no apparent correlation between plasma and cellular gold concentrations (Table 2). In the cellular fraction, most gold is in the membrane. The levels in the lysate are variable but where present are higher in the red lysate which contains more intracellular protein. Free Myocrisin would remain in solution even under the conditions used to prepare the clear lysate and thus the intracellular gold is in the main bound to the precipitated intracellular proteins and is not associated with the glutathione as suggested elsewhere [19]. There is no significant correlation between membrane and lysate gold levels.

It is already known [13] that a different transport mechanism exists for gold in patients who smoke. The results for whole blood plasma given in Fig. 1 tend to substantiate this conclusion with the haemolysate of smokers

Table 2
Gold concentration in plasma, packed cells, haemolysate and clear lysate samples

Patients	Visits	Concentration (μg ml^{-1})			
		Blood plasma	Packed cells	Haemolysate	Clear lysate
1	1st	0.01 ± 0.003	0.003 ± 0.001	—	ND
	2nd	1.40 ± 0.02	0.78 ± 0.01	0.04 ± 0.001	ND
	3rd	1.90 ± 0.05	0.11 ± 0.01	0.045 ± 0.004	
2	1st	0.97 ± 0.07	0.040 ± 0.002	0.024 ± 0.01	ND
	2nd	2.20 ± 0.09	0.10 ± 0.01	0.032 ± 0.01	ND
	3rd	3.25 ± 0.15	0.053 ± 0.01	0.027 ± 0.01	ND
3	1st	2.30 ± 0.14	0.050 ± 0.01	—	0.01 ± 0.001
	2nd	3.20 ± 0.11	0.20 ± 0.05	0.053 ± 0.001	—
	3rd	2.86 ± 0.15	0.06 ± 0.001	0.050 ± 0.001	—
4	1st	1.49 ± 0.12	0.06 ± 0.003	—	ND
	2nd	1.86 ± 0.040	0.10 ± 0.01	0.04 ± 0.001	ND
5	1st	3.53 ± 0.06	0.17 ± 0.003	—	ND
	2nd	4.93 ± 0.10	3.37 ± 0.17	0.12 ± 0.01	0.045 ± 001
6	1st	0.34 ± 0.01	0.029 ± 0.01	0.01 ± 0.001	ND
	2nd	3.20 ± 0.12	0.08 ± 0.01	0.02 ± 0.001	ND
	3rd	1.63 ± 0.06	0.11 ± 0.01	—	ND
7	1st	1.22 ± 0.07	0.13 ± 0.01	0.04 ± 0.01	ND
	2nd	2.94 ± 0.12	0.90 ± 0.01	0.06 ± 0.01	ND
8	1st	ND	ND	ND	—
	2nd	0.24 ± 0.01	0.022 ± 0.01	ND	—
9	1st	ND	ND	ND	ND
	2nd	4.49 ± 0.22	0.09 ± 0.02	0.030 ± 0.01	—
	3rd	4.32 ± 0.20	1.47 ± 0.11	—	—
10	1st	ND	ND	ND	ND
	2nd	1.37 ± 0.12	0.10 ± 0.02	—	—
	3rd	1.82 ± 0.14	0.070 ± 0.01	—	—
	4th	1.79 ± 0.12	0.19 ± 0.01	0.06 ± 0.02	—
11	1st	2.95 ± 0.18	0.12 ± 0.01	0.02 ± 0.003	—
	2nd	1.97 ± 0.16	0.45 ± 0.02	—	—
12	1st	ND	ND	ND	ND
	2nd	2.26 ± 0.21	0.36 ± 0.01	0.13 ± 0.01	—

ND = Not detectable.

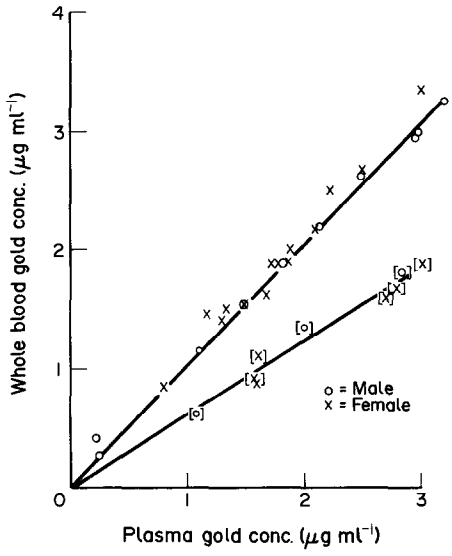

Figure 1
Plot of whole blood gold against plasma gold concentrations for Myocrisin-treated patients. ([ ] indicates non-smoking patients.)

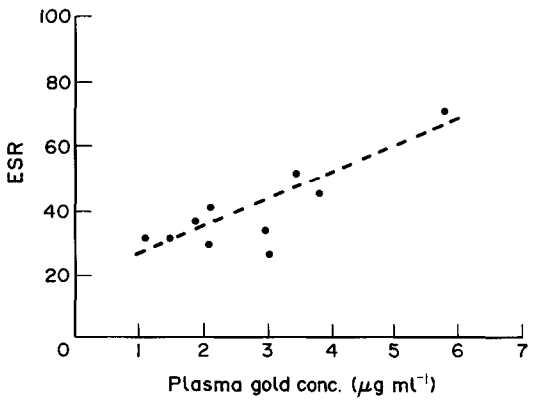

Figure 2
Plot of erythrocyte sedimentation rate (ESR) against plasma gold concentration for Myocrisin-treated cigarette smoking patients.

exhibiting higher concentrations of gold. Sex is not a significant factor. The likely explanation for this effect is that cyanide and thiocyanate are generated in the blood of smokers and they may form complexes *in vivo* which cross the cell membrane [13]. The blood from patients who smoke showed a correlation between plasma gold and erythrocyte sedimentation rate (ESR) which was not apparent in the non-smoking group (Fig. 2). This result may indicate a role for the haemolysate gold in affecting ESR; however, since ESR rises with gold concentration, a more likely explanation is that smoking affects both gold concentration and ESR and consequently no mechanistic conclusion is drawn from this observation.

The relatively large amounts of gold present in the membrane could be very significant for an understanding of the *in vivo* role of gold. Gold may react with the membrane by: association with adsorbed plasma proteins: complex formation at membrane sulphydryl group sites; complex formation with transmembrane protein or endofacial thiols and disulphides; or adsorption into the lipid bilayer.

In the experiments described here, the red cells were washed three times to effectively remove as much plasma protein as possible. It will always be difficult to assess the exact degree of separation between membrane and bound plasma proteins. However after three washes, the gold level had reached a constant value suggesting that three washes provide the best separation, since further washing will increase lysis. Therefore, the possibility of contamination from plasma proteins is not eliminated but is controlled as far as is possible. Some gold may be absorbed into the membrane but Myocrisin is not lipophilic and it is believed that this fraction is quite small [8]. However, the exofacial membrane thiol sites are very active and accessible to gold and *in vitro* experiments show that their reaction is affected and the cellular metabolism altered by gold compounds [12]. Consequently it seems likely that the initial site of action is probably with these groups and that further reactions occur by exchange with disulphides into deep-seated sites in the proteins.

The predominant cell type in the packed cells is the erythrocyte. The sulphydryl group concentration on the membrane of the erythrocyte is about 10^6 groups per cell. These arise from a thiol group associated with the hexose transport protein (approx. 60%), one associated with the anion transport system (approx. 18%) and others associated with other proteins and antigens (approx. 20%) [20]. The hexose transport protein uses sulphydryl groups in transport and gold interferes with glucose efflux from cells [21]. Comparisons between the literature values and the gold concentrations in the membrane are necessarily approximate. The variations in

numbers and cell size as well as the possibility of adhering proteins interfering with the analysis prevent a more accurate assessment. In addition, there is a wide variation in the membrane gold levels (Table 2). However, even at the lower values of membrane gold, there are sufficient gold atoms present to react with all the sulphydryl groups present on the surface. Thus, the situation at the membrane is very different from that in the plasma and there is a possibility that gold could saturate all of the exofacial sites and thus by a stoichiometric reaction alter hexose transport. However it is also possible that subsequent reactions at the membrane could lead to a redistribution of gold between the sulphydryl groups and the more deeply hidden disulphides.

The conclusion of this study is that gold is present at or on the membrane in sufficient quantity to react with most sulphydryl group sites at the exofacial membrane of the cells. It would be expected to alter sulphydryl group reactivity and consequently affect vital cellular functions including glucose transport and antigen response. The saturation of hexose sulphydryl sites certainly explains the otherwise rather curious observation that Myocrisin alters glucose transport [J. Campbell *et al.*, submitted]. Thus, membrane gold is a neglected target which could prove of value in the search for mechanisms of action, both therapeutic and toxic, of the gold drugs used in rheumatoid arthritis.

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