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SHORT COMMUNICATION

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Sample handling for determination of free platinum in blood after cisplatin exposure

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Abstract Cisplatin is a commonly used cytostatic drug that can be pharmacokinetically monitored by measurement of non-protein-bound platinum (Pt) in plasma. The present report elucidates some practical aspects on blood sample preparation with the purpose of identifying methodological error sources and simplifying sample handling. Human blood was incubated in vitro with clinically relevant doses of cisplatin and then stored at different temperatures for various periods prior to deproteinization. Pt concentrations were analyzed by inductively coupled plasma mass spectrometry, which is a very sensitive technique that is well suited for determination of free Pt in biological samples. Free Pt concentration is usually determined after ultrafiltration of plasma. An alternative approach used to study the protein-free fraction is to precipitate the proteins with ice-cold ethanol and then analyze the aliquot. We compared the Pt concentrations in protein-free plasma obtained by these two methods and the levels were very similar. Thus, ethanol deproteinization may be an attractive alternative to ultrafiltration, since it is both simple to perform and very cost-effective. Storage of nondeproteinized whole blood or plasma at 4 °C or 20 °C did not affect the free Pt fraction significantly for the first 2 h, after which a gradual decrease was noted. This suggests that saving the blood sample for approximately 1 h before processing should not have any major impact on the result. On long-term storage of frozen blood samples prior to deproteinization the freezing temperature proved to be of critical importance. At −20 °C there was a gradual decrease in the free Pt fraction during the study period of 14 days, whereas the free Pt concentration remained unchanged at −70 °C for at least 3 months. Thus, samples may be stored unprocessed at −70 °C for several months, whereas −20 °C is not sufficiently cold.

Key words Cisplatin · Free platinum · Storage · Sample handling

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Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)] is one of the most widely used antineoplastic drugs for treatment of cancer patients with a variety of different malignancies. Several studies have shown correlations between clinical effects and pharmacokinetic parameters [3–6, 11, 12]. Thus, there are reasons to believe that the clinical use of cisplatin could be improved by concentration-guided dose adjustments. Before the introduction of individual dose adaptation into clinical practice, it is important that the processing of blood samples be simplified as much as possible and that the possible methodological error sources involved be identified.

After intravenous administration, cisplatin is rapidly and extensively bound to plasma proteins. The protein-bound drug is considered to be biologically inactive, whereas the active species are found in the pool of free platinum (Pt). The protein-free portion of plasma is usually obtained by ultrafiltration. This procedure requires special equipment for centrifugation, and the cost of disposable filters may become substantial when multiple samples are handled. An alternative approach for the removal of proteins from a biological sample is the addition of an ionic salt or an organic solvent [2], e.g., ethanol, which causes an instant precipitation of the proteins, leaving an aliquot of protein-free plasma. This method has been used to a limited extent to separate unbound from total Pt [7, 8] in plasma and cell cultures.

One of the objects of the present study was to compare the concentrations of unbound Pt in plasma as determined in ultrafiltered and ethanol-deproteinized plasma, respectively.

When blood is drawn for pharmacokinetic analysis from a patient treated with cisplatin the sample should ideally be processed immediately, but for practical reasons that may be difficult to achieve in the clinical routine setting. Storage of the samples prior to centrifugation and deproteinization may affect the relationship between free and protein-bound Pt, but the magnitude by which storage conditions affect the results is incompletely known. Therefore, the other main purpose was to study the importance of sample handling. What happens in the test tube after blood has been drawn from the patient and before deproteinization? How does the temperature and time of storage affect the results? Knowledge of these issues is of importance for proper evaluation of the results, especially when pharmacokinetics studies are to be performed in oncology units without special laboratory resources.

For monitoring of cisplatin pharmacokinetics, plasma (or serum) rather than whole blood is usually used. However, ethanol precipitation to obtain a protein-free fraction may also be performed in whole blood. A third aim was to compare the free Pt concentrations measured in whole blood and plasma. If they were similar, analysis of free Pt in whole blood might be better for routine sampling, since it can be performed very easily and rapidly after the blood has been drawn from the patient, without prior centrifugation to obtain plasma.

Materials and methods

In vitro incubation

A 70-ml sample of heparinized whole blood from a healthy volunteer was incubated with 175 μg of cisplatin (Bristol-Myers Squibb) and then divided into several portions. All the analyses described below were performed on the same batch of blood. The amount of cisplatin added gives a total Pt concentration that corresponds to the average peak plasma level found in patients after a 4-h infusion of 100 mg/m^2 of cisplatin [5].

In the first experiment, whole blood was incubated at 37 °C in a shaking water bath for 1, 4, 24 and 48 h using one sample for each time point, respectively. After incubation, samples were immediately centrifuged for 10 min at 1,000 *g* to obtain plasma and each sample was then divided into three portions. One portion was frozen directly at −70 °C for later analysis of total Pt, one was ultrafiltered for 40 min at 4,000 *g* through a 10,000 MV filter (Centrisart C4, Sartorius AG, Göttingen, Germany), and the third portion was treated with ethanol as described below.

In the second experiment, whole blood was incubated with cisplatin at 37 °C in a shaking water bath for 4 h and then divided into multiple portions for elucidation of the effects of different storage conditions. Whole blood (16 samples) as well as plasma (16 samples) were stored at 20 °C for 15 min, 30 min, 1 h, and 2 h; at 4 °C for 30 min, 1 h, 2 h, and 24 h; at −20 °C for 24 h, 4 days, 8 days, and 15 days; and at −70 °C for 24 h, 8 days, 28 days, and 90 days. After storage, each sample was thawed and directly treated with ethanol as described below. Furthermore, one whole-blood sample and one plasma sample (from the first experiment) were deproteinized directly without prior storage.

Ethanol precipitation

Whole blood (0.5 ml) or plasma (0.5 ml) was mixed with 1 ml of ice-cold ethanol (99.5%) and then centrifuged for 5 min at 1,000 *g* at 4 °C and frozen at −70 °C until later Pt analysis.

Platinum determination

Pt was determined using inductively coupled plasma mass spectrometry (ICP-MS). Low-resolution equipment (VG PQ2+; Fisons Elemental, Winsford, Cheshire, UK) with a nickel sampling cone and a Gilson 222 autosampler (Gilson, Villiers, France) was used.

The samples (100 μl of ethanol-deproteinized samples and 30 μl of ultrafiltered samples) were diluted with a reagent (5 ml) containing ethylenediaminetetraacetic acid (EDTA, 0.5 g/l), Triton X-100 (0.5 g/l), and ammonia (5 ml/l) in Millipore water, and 100 μl of an internal standard solution containing 50 ng each of In and Tl was added. The diluted samples were introduced into the ICP-MS through a spray chamber (V-grove PTFE nebulizer) in a segmented-flow mode using the dilution reagent as a carrier/rinsing fluid. The sample-uptake time was 12 s and the rinse time was 20 s. The isotopes ^{115}In, ^{194}Pt, and ^{203}Tl were monitored in the peak jumping mode (3 points/peak, 10 ms dwell time/point). Spiked plasma samples were used for method calibration.

A total of 45 samples were analyzed. All ethanol-deproteinized samples ($n = 39$) were prepared in duplicate. The detection limit, calculated as 3 times the standard deviation for reagent blanks, was 1 μg/l, and the method precision, determined as the coefficient of variation for the sample duplicates, was below 2% in all concentration ranges.

Statistical analysis

The differences between the free Pt concentrations obtained by ultrafiltration and ethanol precipitation were analyzed by the Mann-Whitney *U*-test. The differences between the free Pt concentrations measured after storage at −20 °C versus −70 °C were also analyzed by the Mann-Whitney *U*-test. The correlation between storage time and free Pt level was calculated as Pearson's correlation coefficient, and the *P*-values given were obtained using the F-test for evaluation of significance of the correlation. The correlation between free Pt concentrations measured in whole blood versus plasma was calculated as Pearson's correlation coefficient.

Results and discussion

Determination of free Pt concentrations in plasma after the administration of clinically tolerable doses of cisplatin requires sensitive analytical methods. In the present study we used ICP-MS, which is a very sensitive technique for quantitation of Pt [10]. In comparison with flameless atomic absorption spectroscopy (FAAS), which is the most frequently used method for quantitation of Pt in biological samples, ICP-MS has considerably higher sensitivity [10]. A low detection limit was also found in the present study along with a very small variation between sample duplicates.

The concentration of total plasma Pt remained constant during cisplatin incubation for between 1 and 48 h at 37 °C (Fig. 1). A rapid decrease in free Pt levels was observed between 1 and 4 h of incubation, after which the fraction of free Pt was constant, amounting to approximately 10% of the total plasma Pt level.

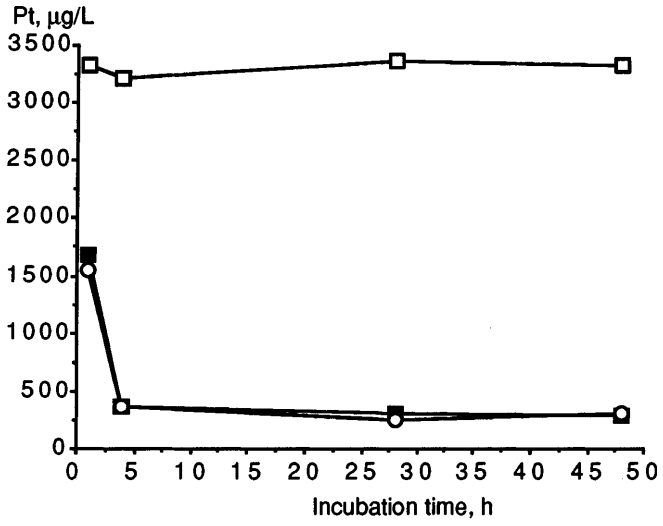

Fig. 1 Platinum concentrations measured in plasma samples after different periods of incubation with 2.5 µg cisplatin/ml blood. *Symbols* indicate total plasma (□), ultrafiltrated plasma (■), and ethanol-precipitated plasma (○)

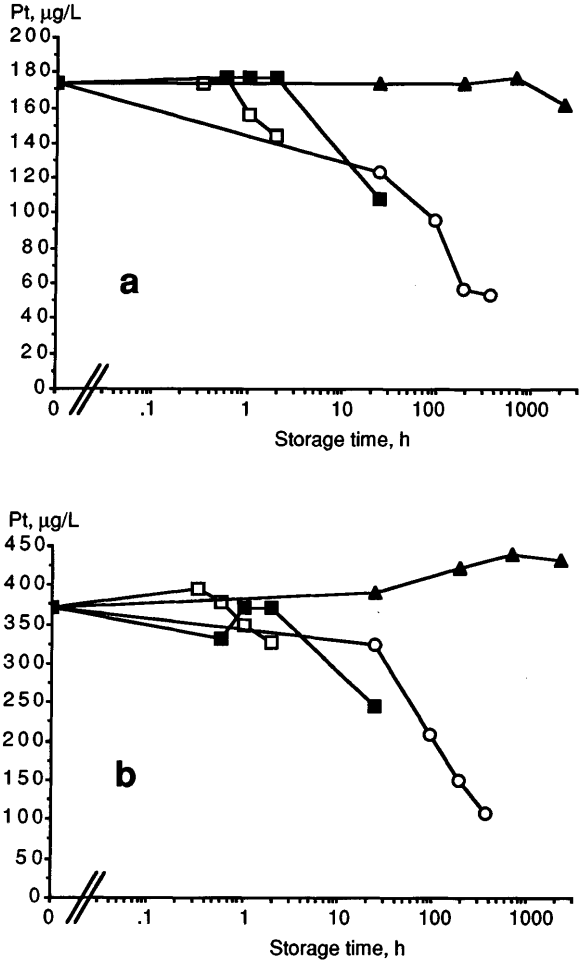

Fig. 2a,b Free platinum concentrations determined in **a** whole blood and **b** plasma. Blood was incubated with 2.5 µg cisplatin/ml. Samples were stored for different periods at 20 °C (□), 4 °C (■), −20 °C (○), or −70 °C (▲)

There was an excellent agreement in free Pt concentrations measured in plasma that had been ultrafiltered or deproteinized with ethanol (Fig. 1). Pt levels were slightly higher in ultrafiltered plasma than in ethanol-precipitated plasma, the average difference being 5%. This difference was not statistically significant ($P = 0.88$). Even though no firm conclusion should be drawn solely from the scarce data obtained in this study, our observation is well in accordance with the results of a recent study by Ma et al. [9], showing no significant difference between ethanol precipitation and ultrafiltration for analysis of unbound plasma Pt. It is also well established that proteins may be effectively precipitated from a biological sample by organic solvents. Blanchard [2] observed a 98.3% protein precipitation rate after the addition of 2 parts of ethanol to 1 part of plasma. Deproteinization with ethanol has previously been used for preparation of free Pt [7–9], but only to a limited extent. Thus, ethanol precipitation appears to be an attractive alternative to ultrafiltration to obtain the unbound fraction of Pt in plasma since the former is faster, simpler, and less expensive.

Storage of whole blood or plasma at 4° or 20 °C prior to deproteinization did not have any substantial impact on the free Pt concentration for the first 2 h, after which there was a gradual decrease (Fig. 2). This observation is in accordance with a previous study [1] showing that a patient's blood sample may be stored on ice for up to 1 h prior to processing.

On long-term storage of frozen samples prior to deproteinization the freezing temperature proved to be of critical importance. At −20 °C there was a gradual decrease in the free Pt fraction during the study period of 14 days, whereas the free Pt concentration remained unchanged at −70 °C for at least 3 months (Fig. 2). No significant correlation was found between time and storage at −70 °C of either blood ($P > 0.05$) or plasma ($P > 0.1$). The concentration of free Pt was found to be significantly lower after storage at −20 °C as opposed to −70 °C ($P = 0.02$) for both whole blood and plasma. This conclusion is well in agreement with a previous report showing a half-life of cisplatin in plasma of 52 h at −20 °C and stability for at least 21 days at −70 °C [1].

The patterns of decline in free Pt were very similar for storage of whole blood and that of plasma (Fig. 2), but the levels of free Pt were consistently slightly more than 2 times higher in plasma than in whole blood. A possible explanation for the lower Pt concentration measured in whole blood could be dilution by fluid from ruptured erythrocytes. Comparison of the 17 pairs of measurements of free Pt in whole blood and plasma, respectively, showed an excellent linear correlation ($r = 0.96$) between the concentrations in the two compartments. Thus, it is possible that free Pt in whole blood may be as valuable as a pharmacokinetic marker as free Pt in plasma. However, no definite conclusion can be drawn from the present study since this comparison was restricted to whole blood

and plasma samples that had been stored under various conditions after 4 h of incubation with cisplatin. It is not certain that the correlation would be equally good in blood containing a greater proportion of unbound drug. This issue needs to be studied further.

On the basis of our study and the literature data, we conclude that a blood sample drawn for analysis of free plasma Pt from a patient treated with cisplatin may be stored for at least 1 h on ice or in the refrigerator before plasma centrifugation without compromising the final result. If deproteinization is not performed immediately, the sample can be frozen at −70 °C for several months until processing, whereas −20 °C is not sufficiently cold. Precipitation of plasma with ethanol is a simple and cost-effective alternative to ultrafiltration for deproteinization.

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