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Research paper

Determination of unbound platinum after oxaliplatin administration: comparison of currently available methods and influence of various parameters

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Variations in plasma protein binding may have profound effects on both disposition and activity of drugs, especially for those which are tightly bound to proteins, such as anticancer platinum derivatives. Methods of separation of the non-protein-bound fraction and some technical parameters may influence the results. We have compared ultrafiltration and ultracentrifugation, as well as the effect of potentially interfering factors, upon the determination of the plasma unbound platinum fraction after oxaliplatin administration to cancer patients. Ultrafiltration and ultracentrifugation provided very closely correlated results, so that either technique can be used. The ultrafiltration cut-off (3000–30 000 Da) devices, the type of tube for blood sampling and the type of anticoagulant (none, lithium heparinate or EDTA) did not influence the results markedly. In contrast, results were greatly influenced by freezing: erratic results were obtained on thawed plasmas when compared with those on fresh serum or plasma. Consequences may be important in usual practice, since many pharmacokinetic studies are carried out in multicentric trials with plasma processing centralized in one reference laboratory. The methods for the determination of protein-drug binding should be standardized and guidelines elaborated where optimal conditions for each type of binding assay are given. [© 1998 Rapid Science Ltd.]

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Introduction

Drug binding to plasma proteins has a large influence on drug efficacy and clinical use. The non-protein-bound fraction of a drug is usually considered as the only active one. Thus, quantification of this fraction is very important, and reproducible and sensitive techniques are necessary for pharmacokinetic studies. Protein binding of drugs can be evaluated by several methods.[1] In particular, the method based on equilibrium dialysis has been used extensively as a direct method to measure protein binding[2] and is often considered as a reference method. However, its reproducibility is problematic and some uncertainty remains at low drug concentrations. The lack of systematic comparative studies between the methods available prevents any comparison of their efficiency.[3] The results obtained depend on the experimental conditions used and there are large variations in the literature according to the method used for the measure of protein binding of a given drug.[1]

An accurate determination of drug binding at low drug concentrations is especially important because these concentrations are relevant to drug activity.[4] Platinum-derived drugs, such as cisplatin, carboplatin and more recently oxaliplatin, which are highly efficient again to different types of tumors,[5,6] bind to proteins in important proportions and in an irreversible manner. Some studies have reported a relationship between toxicity, such as nephrotoxicity, ototoxicity or thrombopenia, and the level of unbound platinum.[7,8] In the present study, we wanted to compare the efficiency of two available methods of determination of protein binding of platinum (ultrafiltration and ultracentrifugation[4,9]) and to investigate different parameters that could influence the measurement of protein binding, such as the kind of anticoagulant, the effect of previous

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freezing and the ultrafilters cut-off. Actually, it is usual, especially in pharmacokinetic multicentric studies, to freeze plasma samples before processing. This habit may have important consequences on free drug measurement if it changes protein conformation and drug binding. For that reason, we wanted to study the influence of freezing on platinum-protein binding. Moreoever, various techniques have been used for the past 15 years under different experimental conditions for the evaluation of the unbound platinum fraction after administration of cisplatin[10] or carboplatin,[11] with quite different results. We have chosen to work on oxaliplatin, a new platinum derivative, active against some cisplatin-refractory tumors, such as colon cancer.[12,13] Its binding to plasma proteins is not well known presently.[12] We have used a new technique, inductively coupled plasma-mass spectrometry (ICPMS), for measuring platinum levels, because of its higher sensitivity as compared to classical techniques such as atomic absorption or spectrophotometry.[14-16] That very high sensitivity makes it very useful for studying very low platinum concentrations in plasma, and for comparing different techniques of sample processing and separation of free and protein-bound fractions.

Patients and methods

Patients and treatment

This study was performed with blood samples obtained from patients included in a phase II trial with pharmacokinetic and pharmacodynamic study of oxaliplatin combined with 5-fluorouracil (5-FU) and folinic acid in the treatment of advanced colorectal cancer refractory to 5-FU plus folinic acid. The patients gave written informed consent for blood sampling and analysis.

All patients had long-term venous access, established either by means of a catheter or by means of an implantable chamber device. Oxaliplatin was given at a dose of 130 mg/m^2 in 250 ml sterile 5% glucose solution, as a 2 h infusion every 3 weeks. 5-FU was administered at a dose of 1300 mg/m^2, after oxaliplatin infusion, and repeated weekly, by means of an 8 h infusion in 1 l of 0.9% saline, through a battery-operated pump. In addition, 200 mg/m^2 folinic acid was administered by i.v. bolus just before and at the fourth hour of 5-FU infusion.

Oxaliplatin was administered in association with prophylactic antiemetics, ondansetron and corticosteroids, at conventional doses. Other symptomatic treatments were allowed whenever required. Previous treatments for concomitant diseases were continued at the same dose.

Blood sampling

For each cycle, 2 × 5 ml blood samples were obtained at the following times elapsed after oxaliplatin infusion: day 1 (h0, h2 and h5), day 8, day 15 and day 22, before the next oxaliplatin infusion.

Blood samples of oxaliplatin-treated patients were collected in several types of tubes, all obtained from Becton-Dickinson (Le Pont de Claix, France) tubes without anticoagulant made of either glass, or polystyrene, glass tubes containing either lithium heparinate, or ethylene diamine tetraacetate (EDTA).

The blood samples were immediately centrifuged for 10 min at 3000 r.p.m. The plasma was split in several aliquots: one for total platinum assay, without further preparation, one for the assay of ultracentrifugeable platinum, one for the assay of ultrafiltrable platinum and one for storage at −25° C, for studying the influence of freezing on free platinum determination.

Preparation of blood samples for platinum assay

Total platinum in plasma was measured after a 20-fold dilution in a solution containing the internal standard europium at 100 µg/l (Sigma, Saint-Quentin-Fallavier, France). For the determination of ultracentrifugeable platinum, the plasma was centrifuged for 3 h at 100 000 r.p.m., with a rotor TLA 100.2 in a Beckman TL 100 ultracentrifuge. The supernatant was subsequently diluted 10-fold in the europium solution.

For the determination of ultrafiltrable platinum, we used two types of ultrafilters: Centrifree 30 000 Da (ref. 4104) and Centricon 3000 Da (ref. 4202), both from Amicon (Millipore, Saint Quentin-en-Yvelines, France). The plasma was centrifuged at 1000 *g*, at 4° C, for 1 h in Centrifree tubes and for 2 h in Centricon ones. At least 250 µl of ultrafiltrate was collected and diluted 10-fold in the europium solution.

Platinum assay

Platinum quantification was performed with ICPMS, as previously described.[17] The spectrometer used was an Elan 5000 Perkin-Elmer Sciex.

The calibration curve was performed by diluting a platinum standard solution at 1 g/l (Sigma), with a saline solution containing 8.39 g/l of NaCl and 1% (v/v) of HNO_3 (Suprapur, Merck, France). The diluted solutions were introduced into the nebulization chamber of the ICPMS, using a peristaltic pump obtained from Gilson (Villiers Lebel, France). The method was linear between 0 and 5000 μg/l of platinum. The recoveries were close to 100%. The within-day and between-day coefficients of variation were lower than 2% for total or ultracentrifugeable platinum.

Under the conditions of this study, the limit of quantification was estimated at 1 μg/l in plasma.

Statistical analysis

The comparison between different techniques was performed using the Pearson correlation test and linear regression curve coefficients.

Results

Twenty patients entered the study between June 1995 and June 1996. Ninety blood samples were collected and analyzed.

First, we compared ultrafiltration and ultracentrifugation for the unbound fraction of platinum measurement in plasma—we observed that they gave very similar results. The slope of the linear regression curve was 1.04 and the coefficient of correlation (r) was 0.98 (Figure 1).

The cut-off of the ultrafiltration devices was also studied. The results obtained with two diameters, 3000 and 30 000 Da, were compared, at very different platinum concentrations. The results were highly similar (Figure 2). The slope of the linear regression curve was 1.04 and the coefficient of correlation was 0.995. Thus, we used the devices of 30 000 Da cut-off devices for the rest of the study.

The type of the tube has been also investigated. The mean variation of platinum content between glass and plastic tubes used for blood collection was 2.2% for total platinum and 7.1% for ultrafiltrable platinum. Therefore, either type of tube could be used since it did not affect either total or ultrafiltrable platinum levels.

The type of anticoagulant had little influence on the evaluation of total platinum concentration (Figure 3) or on the percentage of ultrafiltrable platinum. Results obtained either without anticoagulant, or with lithium heparinate, or with EDTA, were not significantly different (Figure 4). The concentrations obtained with

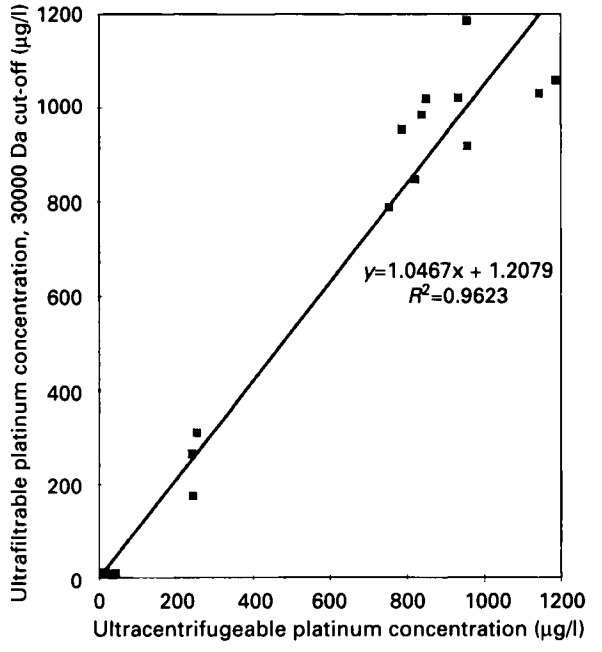

Figure 1. Comparison of unbound fractions measured after separation either by ultrafiltration, 30 000 Da cut-off or ultracentrifugation. The results obtained by both methods were closely correlated (r=0.98). However, for very low values, ultracentrifugation appeared more sensitive.

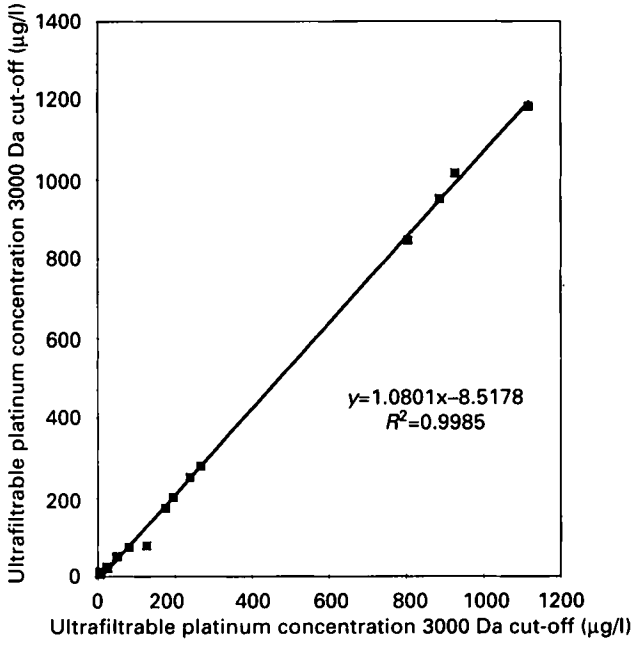

Figure 2. Comparison of unbound fractions measured after separation either by ultrafiltration, 30 000 Da cut-off, or ultrafiltration, 3000 Da cut-off. The results were identical, whatever the time of sampling. Therefore, the proteins involved in platinum binding were of high molecular weight >30 000 Da.

dry tubes and lithium heparinate tubes were highly correlated: the slope of the linear regression curve was 0.93 and the coefficient of correlation was 0.98. The same comparison was done between dry tubes and EDTA-containing tubes: the linear regression curve coefficient was 1.01 and the coefficient of correlation was 0.99.

In contrast, whereas sample freezing before processing did not affect total platinum concentration, it did have a marked effect on the determination of unbound platinum. Whatever the type of anticoagulant used, the concentrations of ultrafiltrable or ultracentrifugeable platinum were no longer valid after freezing (Figure 5).

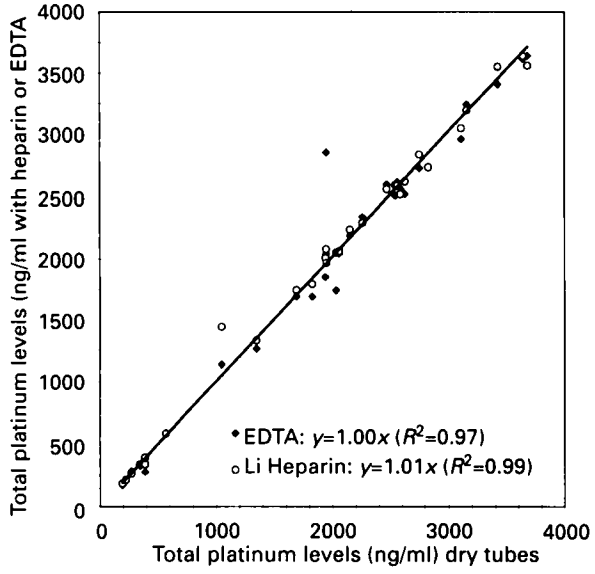

Figure 3. Influence of anticoagulant on total plasma platinum determination. The results were identical whatever the anticoagulant.

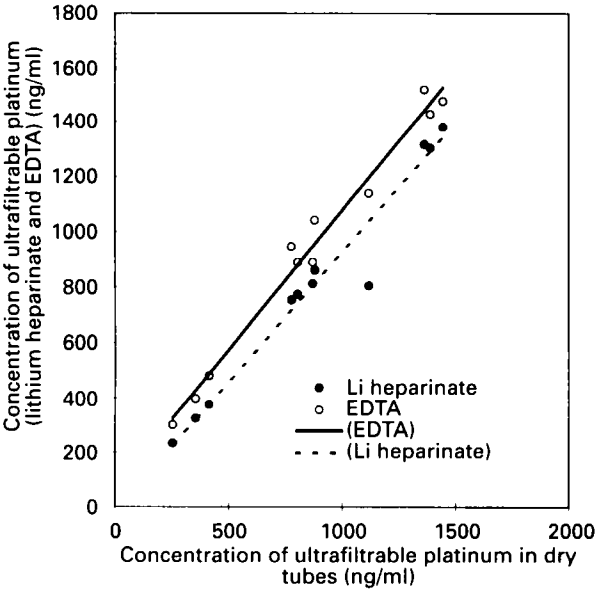

Figure 4. Influence of anticoagulant on plasma unbound platinum determination. Ultrafiltration was performed immediately after collection. The results were highly correlated whatever the anticoagulant (lithium heparinate, EDTA or dry tube). The correlation between EDTA and dry tubes (r=0.99) was a little better than that between lithium heparinate and dry tubes (0.98).

Discussion

Plasma protein binding may have profound effects on both disposition and action of drugs. Several techniques are available for the investigation of drug binding and the estimation of the relative importance of the unbound fraction may be influenced by the method used.[1] Besides, other parameters may lead to discrepancies in the results obtained. The effects of changes in the extent of protein binding are especially significant for drugs which are irreversibly bound to proteins, since even a relatively small change in the

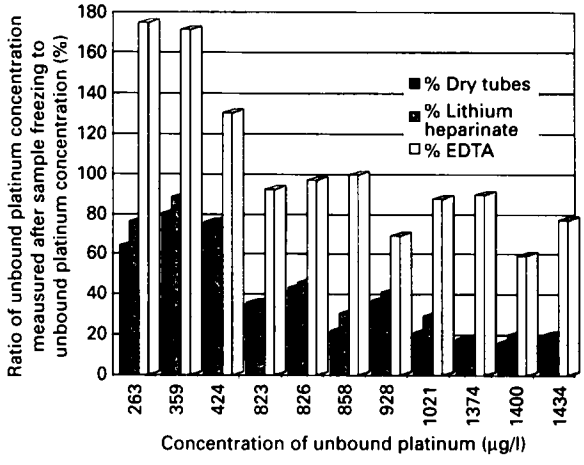

Figure 5. influence of plasma sample freezing on unbound plasma platinum determination. A high discrepancy was observed in unbound fraction levels measured after freezing. We observed that EDTA combined with ultrafiltration for separating bound and unbound drug gave results which were less erratic but anyway not accurate when compared to those obtained before freezing. We can hypothesize that platinum bound to such low molecular weight proteins was not retained by the pores of the 30 000 Da ultrafilter membranes. In addition, EDTA, as a chelator agent, can bind metals such as platinum, and consequently move the equilibrium between unbound and bound platinum toward an apparent increase of the unbound fraction. Therefore, EDTA could restore a higher proportion of the unbound fraction by lowering artificially the platinum amount bound to small proteins and peptides.

degree of binding may have a dramatic effect on the unbound fraction.[4] This is the case for anticancer platinum derivatives, such as cisplatin, carboplatin and oxaliplatin.[5,10,11] Therefore, an accurate determination of drug binding at low drug concentrations is very important. We investigated here some factors potentially able to interfere with the determination of the non protein-bound fraction of a new platinum derivative, oxaliplatin.

We compared two rapid and easy methods for the separation of the unbound platinum fraction, ultracentrifugation and ultrafiltration. Ultrafiltration is usually considered as a reference method and has been compared to equilibrium dialysis.[1,3] It is simple to use in routine practice. However, there are interferences due to Donnan and membrane effects, and it can be influenced by pH, temperature variations[4] and pressure gradient. Besides, the protein-free phase is forced through a semi-permeable membrane using centrifugal force and this could interfere with protein binding.[3] On the other hand, ultracentrifugation has been reported as being technically simple and having a high degree of reproducibility. This technique has been compared with others and was found comparable to the reference technique, equilibrium dialysis, in terms of reliability and superior in terms of reproducibility, especially for measuring low unbound drug concentrations.[2,9] Moreover, ultracentrifugation, as a physical method, does not involve a membrane. The protein-free phase is separated by spinning the sample at high speed.[3] It does not take into account the binding of drugs to low molecular weight proteins or peptides, as has been shown with low density lipoproteins.[2] Thus, this technique appears particularly adapted to the measurement of unbound platinum. Our methodological study compared ultracentrifugation and ultrafiltration. A close correlation was found between the two techniques (Pearson correlation test: r=0.98) for a large range of values. Therefore, one technique can be used for unbound platinum determination. However, it is important to note that for very low values, at late sampling times after oxaliplatin administration, ultracentrifugation gave consistently higher results than ultrafiltration. This observation has already been made when comparing ultracentrifugation and equilibrium dialysis.[2,9] In conclusion, ultracentrifugation seems to be more accurate than either one of the other methods of separation for measurement of very low values.

The size of the membranes used in ultrafiltration may play a role in the determination of the unbound drug fraction. It has been previously shown for cisplatin that at early times of sampling, within the few hours after infusion, the binding of platinum to proteins was not specific, almost all protein species being involved, especially the low molecular weight thiols.[18-21] At later sampling times, the binding became more specific and platinum was found to bind solely to proteins of molecular weight higher than 25 000 Da, such as albumin, γ-globulins and transferrin.[10,18-20] For carboplatin, unbound platinum levels were high and the ratio unbound/total platinum was around 30%.[22,23] Tosetti *et al.*[20] compared protein binding of cisplatin and carboplatin. They observed with cisplatin a significant platinum binding to plasma components with molecular weight over 25 000 Da and a rapid decrease of the unbound platinum fraction during the first 2 h after infusion. In contrast, no evidence of platinum binding to plasma components with molecular weight over 25 000 Da was noted with carboplatin and the unbound platinum fraction did not change significantly after treatment. In the present study, no difference was found between results obtained by ultrafiltration performed with 3000 and 30 000 kDa cut-off membranes after oxaliplatin administration, whatever the sampling time. Therefore, platinum appeared to quickly and specifically bind to high molecular weight proteins, even within few hours after infusion. We would propose to combine both 3000 and 30 000 Da cut-off membranes for measuring the protein-bound fraction when studying platinum derivatives, since their binding to plasma proteins may differ from one compound to the other.

The tube, glass or plastic, had no influence, neither had the type of anticoagulant or even its presence. The results were identical, provided that the separation of free and bound platinum was done immediately.

Freezing introduced a high discrepancy in the results, whatever the type of anticoagulant used. The important increase of the bound fraction of platinum after freezing is probably due to a conformational change of proteins when plasma is frozen and then thawed. That change could modify the binding sites of proteins. The consequences are important in practice. Freezing plasma samples first can generate dramatic errors and the results we show might probably be extrapolated to some other drugs. Yet, pharmacokinetic studies are now frequently performed in multicentric trials. For avoiding processing samples with different techniques in different laboratories, samples are often collected and then processed in a reference laboratory. Methodology must be rigorous and the unbound form of the drug should be separated immediately after blood withdraw, before freezing. In that situation, ultrafiltration should be preferred to ultracentrifugation because it can be done more easily, but this technique may be less sensitive in the case of very low concentrations.

Conclusion

The dependence of the results upon the technique used emphasizes that the methods for determining drug binding should be standardized. Guidelines should be elaborated in which optimal conditions for each type of binding assay are given.

Acknowledgments

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