

SHORT COMMUNICATION

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Comparison of ethanol plasma-protein precipitation with plasma ultrafiltration and trichloroacetic acid protein precipitation for the measurement of unbound platinum concentrations

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Abstract Sample preparation for the measurement of non-protein-bound platinum was evaluated by precipitation of plasma proteins with cold ethanol. The method was compared with the routinely used plasma ultrafiltration and with trichloroacetic acid (TCA) protein precipitation. After incubation of human plasma samples with cisplatin or carboplatin, unbound platinum concentrations were determined applying Amicon Diaflo ultrafiltration membranes and Millipore ultrafree-MC filters. For protein precipitation, 1 ml of cold (-20°C) pure ethanol was added to 0.5 ml of human plasma and the supernatant was collected after 2 h, or 0.5 ml of cold 20% TCA was added to 0.5 ml of plasma. Platinum was analyzed by atomic absorption spectrophotometry (AAS). There was no significant difference between the ethanol and ultrafiltration methods in the unbound platinum concentration. The protein content in the supernatant ($1.00 \pm 0.20\%$) was slightly higher than that in the Amicon ($0.58 \pm 0.05\%$) and Millipore ($0.55 \pm 0.04\%$) ultrafiltrates. On average, the TCA and ethanol method seemed to be equally appropriate. The ethanol precipitation method is concluded to be simple, convenient, and reproducible and has negligible costs.

Key words Cisplatin · Carboplatin · Unbound platinum

Introduction

Quantitation of the unbound, i.e., non-protein-bound, plasma concentration of platinum is important for determination of the pharmacokinetics of the platinum

drugs cisplatin and carboplatin. Only the unbound concentration of the drug is likely to be pharmacologically active. After intravenous administration, cisplatin and carboplatin become tightly bound to plasma proteins and other tissue components. Previous studies have revealed that the binding to plasma proteins is covalent and almost irreversible [3, 4]. Due to its high reactivity the binding of cisplatin to plasma proteins is very rapid, whereas the rate of binding of carboplatin is much lower [9, 10, 23, 24]. Plasma ultrafiltration for the measurement of unbound platinum applying ultrafiltration tubes is routinely used for this assay [2, 6, 7, 9, 11, 16, 17, 23]. Mostly a cutoff level for the filtration membranes of 30,000 Da is used. It has previously been suggested that precipitation of plasma proteins with cold ethanol may be a good alternative to the ultrafiltration of plasma for measurement of unbound platinum [13, 18]. In addition, trichloroacetic acid (TCA) has been used to measure unbound platinum [8, 21, 22, 25]. Herein we report on experiments in which the ethanol precipitation method was validated and compared with the results obtained with two ultrafiltration systems and the TCA precipitation method.

Subjects and methods

Cisplatin was obtained from Lederle (Wolftratshausen, Germany) and carboplatin, from Pharmachemie (Haarlem, The Netherlands). Amicon Diaflo ultrafiltration membranes (cutoff level 30,000 Da) and the MAPS-1 Starter Kit were obtained from Amicon (Beverly, Mass. USA), and Millipore filters (ultrafree-MC millipore cutoff level 30,000 Da) were obtained from Millipore (Bedford, Mass., USA). The atomic absorption spectrophotometer (AAS) was a Perkin-Elmer 3030B device (Uberlingen, Germany). Triton X-100 was obtained from Baker (Deventer, The Netherlands), and cesium chloride (CsCl) and absolute ethanol (analytical grade, Merck product 983, 99.8%) were supplied by Merck (Darmstadt, Germany). All other chemicals were obtained from Baker Deventer, The Netherlands) and were of analytical grade.

Whole-blood samples of 20 ml were obtained from four healthy volunteers and plasma was collected. In addition, plasma samples were used from 11 patients with solid tumors who were being treated

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with cisplatin in an ongoing phase II and pharmacology study for which approval was obtained from the local ethics committee.

Of the plasma obtained from the four volunteers, 10 ml was incubated with cisplatin at 5 µg/ml or with carboplatin at 50 µg/ml at 37 °C for 24 h. Immediately and at 1, 2, 4, and 24 h after addition of the platinum drug, three plasma aliquots were taken and used for ultrafiltration at 4 °C and 1,000 *g* for 30 min or for the addition of 2 vol. of cold (−20 °C) ethanol (99.8% absolute). The latter sample was mixed on a whirl mixer for 10 s and subsequently stored at −20 °C for 4 h. The sample was then centrifuged at 4 °C and 4,000 *g* for 10 min and the supernatant was carefully transferred to a clean 2-ml Eppendorf tube and stored at −20 °C until analysis. Total and unbound platinum were analyzed after dilution (total platinum with 0.2% Triton X-100 and 0.06% CsCl, and unbound platinum with deionized H₂O). The protein contents of the plasma samples prior to treatment and of the samples obtained after ultrafiltration and protein precipitation were determined by the Lowry method [14]. Also, TCA precipitation of plasma protein was compared with the ethanol method as well as with ultrafiltration. On a second occasion, 20 ml of whole blood was collected from the same volunteers and used for incubation of plasma with cisplatin and carboplatin. The periods of incubation were 1 and 24 h. An equal volume of cold 20% TCA was added to 0.5 ml of plasma, and the mixture was shaken on a whirl mixer and put on ice for 10 min as described by Siddik et al. [22]. The other assays were carried out as described above. All assays were carried out at least in triplicate.

The period of storage of the ethanol-treated sample at −20 °C prior to collection of the supernatant was varied to study the influence of storage on the remaining protein content and unbound platinum recovery in the supernatant. Four 5-ml plasma samples were treated with cisplatin as outlined above and stored at −20 °C. Aliquots were taken at 0, 1, 2, 4, and 16 (overnight) h after storage and treated as described above.

Unbound platinum concentrations were also determined in the plasma of 11 patients who were being treated with a 70-mg/m² dose of cisplatin given on a weekly schedule by 3-h infusion. A 4-ml volume of whole blood was taken at 0.5 and 3 h after the end of the cisplatin infusion and was immediately processed according to the outlined ultrafiltration and ethanol precipitation methods. Students two-sided *t*-test was used to evaluate differences between the treatment methods.

Results

The concentration of unbound platinum detected in the Amicon ultrafiltrate immediately after the addition of cisplatin to plasma was 2.71 ± 0.08 µg/ml, that measured in the Millipore ultrafiltrate was 2.83 ± 0.31 µg/ml, and that determined in the supernatant after ethanol treatment was 2.79 ± 0.08 µg/ml [*n* = 3, not significant (NS)]. After carboplatin addition the concentrations were 26.51 ± 1.24 (Amicon), 27.64 ± 1.02 (Millipore), and 27.53 ± 1.4 (ethanol; NS). The variation coefficient obtained with the ethanol method was lower or of the same order as that obtained with the ultrafiltration methods (Fig. 1). The concentrations of the total platinum detected in the plasma samples were 3.07 ± 0.12 µg/ml after incubation with cisplatin and 25.14 ± 0.75 µg/ml after incubation with carboplatin. The recovery of unbound platinum in the ultrafiltrates and supernatant after ethanol precipitation is given in Table 1. The protein content of the samples after ultrafiltration or ethanol precipitation is also given in Table 1. The amount of protein measured

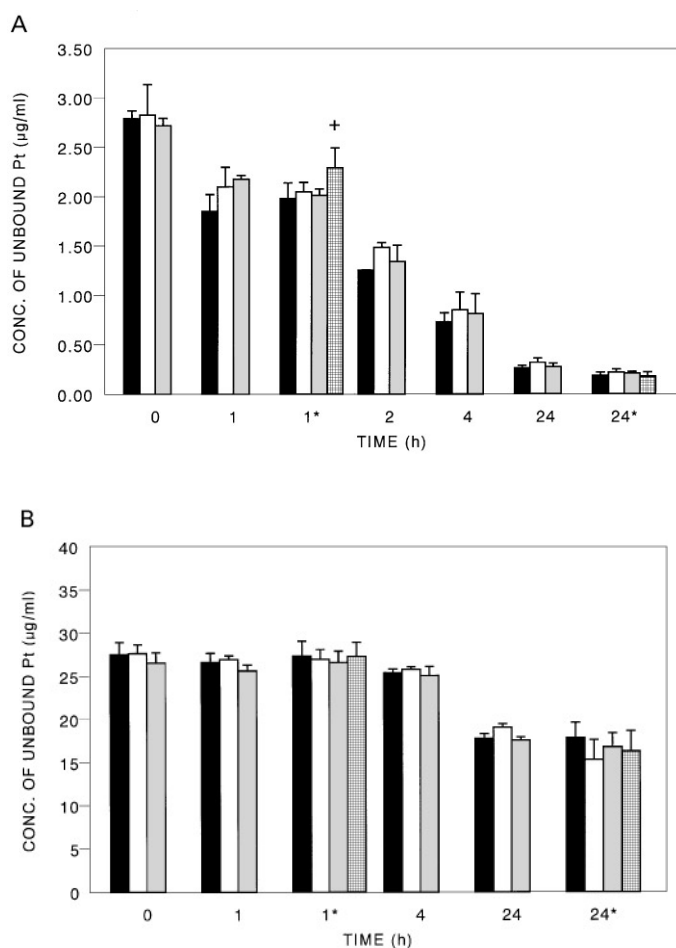


Fig. 1 A Unbound platinum concentrations determined in plasma supernatants (black bars, cold ethanol protein precipitation) and in plasma ultrafiltrates using Millipore (white bars) and Amicon (dotted bars) ultrafiltrates. Plasma samples were incubated with cisplatin at 5 µg/ml *in vitro* and analyzed at different time points after the start of the incubation. On a second occasion the *in vitro* incubation experiments were repeated using plasma from the same volunteers, which was incubated for 1 (*1**) and 24 h (*24**) with cisplatin. The TCA method (hatched bars) was compared with the other three methods. B Unbound platinum concentrations determined in plasma supernatants (black bars, cold ethanol protein precipitation) and in plasma ultrafiltrates using Millipore (white bars) and Amicon (dotted bars) ultrafiltrates. Plasma samples were incubated with carboplatin at 50 µg/ml *in vitro* and analyzed at different time points after the start of the incubation. On a second occasion the *in vitro* incubation experiments were repeated using plasma from the same volunteers, which was incubated for 1 (*1**) and 24 h (*24**) with carboplatin. The TCA method (hatched bars) was compared with the other three methods. ⁺*P* < 0.05 as compared with the other three methods

in the supernatant after ethanol protein precipitation was slightly, albeit significantly, higher than that found in the ultrafiltrates.

Immediate collection of the supernatant after the addition of cold ethanol to plasma and vortexing resulted in a higher amount of remaining protein (2%) and in higher platinum recovery (5–10%). After 2 h of

Table 1 Comparison of three plasma pretreatment methods for measurement of unbound platinum (CDDP cisplatin, CBDCA carboplatin)^a

Method	Unbound Pt recovery (%) (CDDP)	Unbound Pt recovery (%) (CBDCA)	Remaining protein (%)
Millipore filter	92 ± 10	110 ± 1	0.58 ± 0.05
Ethanol precipitation	91 ± 6	109 ± 4	1.00 ± 0.20*
Amicon filter	88 ± 3	105 ± 2	0.55 ± 0.04

* $P < 0.05$ as compared with Millipore and Amicon

^aData represent mean values ±SD ($n > 3$)

storage at -20°C the amount of protein decreased to 1%, which did not decrease further upon longer storage. A total of four Millipore filters were needed to obtain the same volume of ultrafiltrate acquired with one Amicon unit. With one Millipore filter a 10- μl volume of ultrafiltrate could be obtained. Analysis of the Amicon and Millipore ultrafiltrates resulted in much higher background signals on the AAS as compared with the supernatant after ethanol protein precipitation.

The concentrations of unbound platinum measured after incubation with cisplatin for 1 h were slightly (12%), albeit significantly, higher after TCA protein precipitation (Fig. 1A). At 24 h after incubation there was no significant difference between any of the four methods. After incubation with carboplatin, no significant difference in unbound platinum concentrations was observed (Fig. 1B). The concentrations of unbound platinum detected in the patients' samples at 0.5 h after the end of the cisplatin infusion were 568 ± 182 (ethanol), 561 ± 231 (Millipore), and 511 ± 198 ng/ml (Amicon; $n = 11$, NS). At 3 h post-infusion the concentrations were 69 ± 17 (ethanol), 74 ± 19 (Millipore), and 68 ± 17 ng/ml (Amicon; $n = 11$, NS).

Discussion

Measurement of the unbound platinum concentration after treatment with cisplatin and carboplatin is essential for assessment of the pharmacokinetics of these drugs [1, 15, 19, 23]. Previous studies have revealed significant relationships between the area under the platinum plasma concentration-time curve (AUC) and the likelihood of tumor response and toxicity [1, 5, 12, 15, 19, 20]. The results of the present study illustrate that treatment of plasma with cold ethanol to precipitate proteins is adequate for preparation of the sample for the measurement of non-protein-bound platinum. A comparison of this technique with the routinely used ultrafiltration methods revealed that the recovery of

unbound platinum observed for ethanol treated samples was not significantly different from that recorded for the Amicon and Millipore ultrafiltrates. The percentage of protein remaining in the supernatant after ethanol protein precipitation was slightly higher than that remaining in the ultrafiltrates. The magnitude of the difference seems to be unimportant, because the recovery was not significantly different from that recorded for the ultrafiltration method. The concentration of unbound platinum detected in the samples of patients who were being treated with cisplatin also showed no difference between the three methods. The recovery of platinum after the addition of cisplatin in vitro is slightly lower than 100% (Table 1). The most plausible explanation for this is that during sample workup the binding process of cisplatin to plasma proteins continues. The recovery of carboplatin was slightly higher than 100% for all methods.

The TCA method resulted in a slightly higher unbound platinum concentration after incubation of plasma for 1 h with cisplatin. This was similar to the result obtained when plasma supernatant was immediately collected by the ethanol precipitation method. This could be explained by partly reversible binding of cisplatin or incomplete precipitation of proteins. Another possibility would be that during the longer sample workup involved in the other three methods, cisplatin plasma-protein binding continues, thereby leading to an underestimation of the unbound concentration. No significant difference was obtained with carboplatin, although the percentage bound was much lower than that recorded for cisplatin. The result is in contrast with previously published data [22]. However, in that study another species was evaluated and the binding kinetics of carboplatin were found to be much faster than they were in the present study. On average, the TCA method and the ethanol method seem to be equally appropriate.

The optimal period of storage for the ethanol-treated sample at -20°C appears to be 2 h. After that time, no further decrease in the amount of protein remaining could be observed. The yield of ultrafiltrate noted for the Millipore filters was only one-quarter of the amount obtained with the Amicon filters. The background signal on the AAS was lowest for the ethanol method, indicating that the sample was relatively clean as compared with the ultrafiltrates. This may be beneficial in cases of very low plasma concentrations. Cold ethanol precipitation of plasma proteins is a simple, practical, reproducible, and extremely inexpensive method for preparation of samples for the measurement of unbound platinum.

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