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High-performance liquid chromatographic procedures for the analysis of carboplatin in human plasma and urine

Robert C. Gaver and George Deeb

Dept. of Metabolism and Pharmacokinetics, Pharmaceutical Research and Development Division, Bristol-Myers Company, P. O. Box 4755, Syracuse, NY 13221-4755, USA

Summary. Specific, sensitive and reproducible high-performance liquid chromatographic procedures were developed for the quantitative analysis of carboplatin in human plasma and urine. Plasma and urine were ultrafiltered with an Amicon Centrifree™ micropartition system, and samples were injected onto a LiChrosorb™ diol column. The mobile phase was CH_3CN/H_2O (92:8, v/v) for plasma and CH_3CN/0.015% H_3PO_4 (89:11, v/v) for urine. The effluents were monitored at 229 nm. Carboplatin eluted by 10 min. The detector response was linear from 0.5 (plasma) or 5 (urine) to 500 μg/ml. The lower limit of quantification was 1.0 μg/ml plasma and 5.0 μg/ml urine. Constitutents in plasma and urine, and possible degradation products (cyclobutane mono- and dicarboxylic acids) did not interfere. Within-day precision was less than 4% for plasma and 9% and 4% for urine concentrations of 40 and 401 μg/ml, respectively. Within-day accuracy was 96% or greater for both matrices. Carboplatin was not bound to the Centrifree™ membrane and recovery was 94% for plasma and 96% for urine. The storage stability of carboplatin in water, plasma, plasma ultrafiltrate, and urine and the extent of binding to human plasma proteins were evaluated. The percentage of carboplatin reversibly bound to plasma proteins was minimal (≤ 10%) over a range of 1–50 μg/ml. In human plasma at 37 °C the drug was stable for about 2 h, but then degraded with a half-life of 32 h. Carboplatin had limited stability in water, plasma, and urine stored at −25 °C. Biological samples, therefore, should be stored frozen and analyzed within a week of collection to obtain valid results.

Introduction

Carboplatin (CBDCA, JM8, NSC-241240), *cis*-diammine (1,1-cyclobutanedicarboxylato) platinum, is a second-generation analogue of the platinum-containing antitumor agent cisplatin (Fig. 1). Preclinical studies showed carboplatin had a broad spectrum of antitumor activity similar to that of cisplatin, and was less nephrotoxic and emetogenic than cisplatin [1, 7, 8, 12, 13]. The lack of nephrotoxicity and reduced emesis was confirmed in phase I trials in humans, where myelosuppression was the dose-limiting toxicity [2–4, 10, 11]. The compound is presently in large-scale clinical trials in the United States and Europe. The pharmacokinetics of total platinum and of free, non-protein-bound, ultrafilterable platinum after IV administration of carboplatin to man have been described elsewhere [2–4, 6]. Information on the pharmacokinetics of the parent compound, carboplatin, has only recently been reported with reference to high-performance liquid chromatography (HPLC) on a silica column [6]. However, in our hands, variable results were obtained when carboplatin was applied to silica columns and eluted with CH_3CN/H_2O (90:10, v/v). We report here validated HPLC procedures for the analysis of carboplatin utilizing a diol column, and the application of these procedures to the determination of plasma protein binding and the stability of carboplatin in human plasma and urine.

Offprint requests to: R. C. Gaver

Materials and methods

Chemicals. The following chemicals were obtained from the sources indicated: HPLC grade CH_3CN and MeOH, and ACS grade 85% phosphoric acid, Fisher Scientific Co., Fairlawn, NJ; control human serum, Interstate Blood Bank, Philadelphia, Pa; 1,1 cyclobutane dicarboxylic acid, Aldrich Chemical Co., Milwaukee, Wis; cyclobutane monocarboxylic acid, Fluka AG, Chem. Fabnik, Marseilles, France; carboplatin, Bristol Laboratories, Syracuse, NY; water was generated by a Milli-Q™ system, Millipore Corp., Bedford, Mass.

Equipment. The following equipment was obtained from the sources indicated: Centrifree™ micropartition system, Amicon Corp., Danvers, Mass; Model 27 Cahn electronic microbalance, Cahn Instruments Inc., Cerritos, Calif; model 590 programmable solvent delivery module, model 441 absorbance detector with cadmium lamp, model 710B WISP™ sample injector, and 4 ml self-sealing WISP™ vials, Waters Associetes, Millipore Corp., Milford, Mass; model 1200 recorder, Linear Instruments Corp., Reno, Neb; Autochrom™ solvent selector, Autochrom, Inc., Milford, Mass; Merck LiChrosorb™ diol column, 250 × 4.1 mm, Alltech Associates, Inc., Deerfield, Ill; IEC model HN-SII centrifuge, Damon/IEC Div., International Equipment Co., Needham Heights, Mass; 300 μl capacity polyethylene microcentrifuge tubes, Denville Scientific, Inc., Denville, NJ; microliter syringes, Hamilton Co., Reno, Nev; B-D Vacutainer tubes (0.1 ml containing 15 mg K_3EDTA and 0.02 mg potassium sorbate), Becton-Dickin-

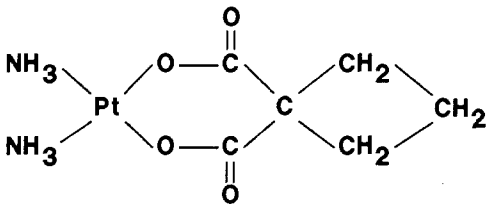

Fig. 1. Structure of carboplatin (CBDCA, JM8)

son and Co., Rutherford, NJ; and a model 3357 Laboratory Automation System, Hewlett-Packard Co., Avondale, Pa.

Preparation of ultrafiltrates. Samples of human plasma (1.0 ml) were added to the reservoir of a Centrifree™ micropartition system. The latter was capped and centrifuged for 5 min at 200 *g*. The ultrafiltrate (ca. 80 µl) was transferred to a 300-µl microcentrifuge tube and placed in a WISP™ vial. Urine samples (1.0 ml) were also routinely filtered in the Centrifree™ system to remove particles that might clog the automatic sampler/injector system.

Preparation of standards. Carboplatin was accurately weighed and diluted with water on the day of assay to give final concentrations of 10.50, 1.05 or 0.021 mg carboplatin per ml. Appropriate volumes (5–50 µl) of the stock solutions were transferred to 12 × 77 mm glass tubes. Water (0–45 µl) was then added to bring the total volume per tube to 50 µl. Blank tubes, containing only 50 µl water, were also prepared. A 1.0-ml sample of either plasma or urine was added to each tube to give final concentrations of 0.5–50 µg carboplatin per ml plasma or 5.0–500 µg carboplatin per ml urine. Duplicates of six to seven standards and a blank were prepared for each standard curve. The tubes were mixed on a vortex mixer and then filtered in the Centrifree™ micropartition units.

Liquid chromatographic conditions. Samples (6 µl) of the plasma ultrafiltrates and filtered urines were injected automatically by a WISP™ onto a LiChrosorb™ diol column conditioned with 300–400 ml water followed by 400–500 ml MeOH/CH_3CN (1 : 1, v/v). The mobile phase for plasma ultrafiltrates was CH_3CN/H_2O (92 : 8, v/v) at a flow rate of 2 ml/min. Carboplatin eluted at 7–8 min. For urine samples, the mobile phase was CH_3CN/0.015% H_3PO_4 (89 : 11, v/v) at a flow rate of 1.1 ml/min. Carboplatin eluted at 9–10 min. The column effluent was monitored at 229 nm with a range setting of 0.05 AUFS. Samples and standards were distributed in a random order for each analytical run.

Data analysis. The 1-V unattenuated output of the UV detector was interfaced via an analog-to-digital converter to a Hewlett Packard Model 3357 Laboratory Automation System. Samples were identified and the peak height data were automatically calculated and processed. The best fit line of concentration vs peak height for the standards was determined by the method of least squares. The slope, intercept (with 95% confidence limits), and correlation coefficient were tabulated. Sample concentrations were determined by inverse prediction.

Assay validation. Possible interferences from endogenous constituents of human plasma ultrafiltrates and urine were evaluated. Urine and plasma samples obtained from at least ten different donors were analyzed by the appropriate procedure. The response (peak height) of material eluting at the retention time of carboplatin was recorded. The retention times of potential degradation products of carboplatin, cyclobutane mono- and dicarboxylic acids, were determined.

The lower limit of detection was determined for plasma and urine. Duplicate or triplicate samples (1.0 ml) of plasma and urine from at least ten different donors were added to 12 × 77 mm tubes. Water (50 µl) was added to one tube (blank), and water (50 µl) containing 0.25 µg (plasma), 0.50 µg (plasma), 1.05 µg (urine), or 5.25 µg (urine) carboplatin was added to the other tubes (samples). The blanks and samples were analyzed by the appropriate procedure and the peak height response (µV.s) for each blank and sample were recorded. The results were evaluated by a paired-comparison *t*-test.

The range of reliable response was established by preparing at least five duplicate standards in plasma covering the concentration range of 0.5 to 500 µg carboplatin/ml. Similar standards ($n=6$) were prepared in urine covering the range of 1.0–500 µg/ml. Each sample, as well as a blank containing no added carboplatin, was analyzed and the results were evaluated by least-squares analysis.

Within-day accuracy and precision of the assay were determined by preparing and analyzing ten replicate samples of two concentrations of carboplatin in plasma (6.3, 37.8 µg/ml) and in urine (39.9, 400.5 µg/ml). Carboplatin for the standard curve was weighed and diluted on each assay day. The mean observed concentration, the percentage relative standard deviation, and the percentage of the theoretical concentration were calculated.

Recovery was determined by preparing appropriate standards ($n=7$) in water and in either plasma (0.5–50 µg/ml) or urine (5–500 µg/ml). The standards in water were analyzed directly, while the standards in plasma and urine were ultrafiltered prior to analysis. Standards in water (5–500 µg/ml) were also analyzed after being ultrafiltered, to determine whether the Centrifree™ membrane adsorbed any of the drug. The concentrations of carboplatin in the ultrafiltered plasma, urine and water samples were predicted from the standard curve generated from the unfiltered water standards. The slopes, intercepts, and correlation coefficients of the standard curves prepared in unfiltered and filtered water, plasma, and urine were calculated and compared.

Storage stability at −25 °C was evaluated by repeated analysis of the urine and plasma samples prepared for accuracy and precision determinations. In addition, a standard of carboplatin in water (1.05 µg/ml) was prepared and immediately added to plasma to generate a standard curve, and the samples were analyzed. Samples of the solution were then stored at −25 °C and were removed at weekly intervals for preparation of additional standard curves in plasma. Identical standards were prepared from carboplatin weighed and diluted on the day of assay. The slopes and 95% confidence limits of the standard curves generated from the stored and freshly prepared solutions were calculated and compared.

Samples of carboplatin in plasma ultrafiltrate (5 and 40 µg/ml) were prepared, stored at −25 °C in microcen-

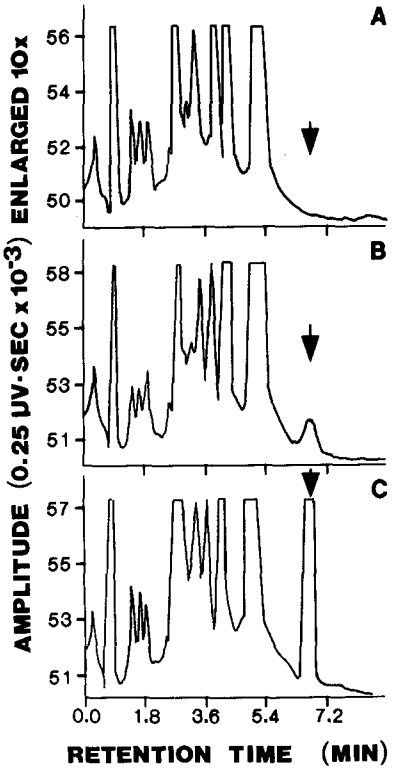

Fig. 2A–C. Computer-amplified chromatograms of carboplatin standards in plasma ultrafiltrates: **A** 0 μg/ml; **B** 5 μg/ml; **C** 50 μg/ml

trifuge tubes, and analyzed at various times from day 0 to day 20.

Stability in plasma and plasma ultrafiltrate at 37 °C. Carboplatin was added to fresh human plasma obtained from normal volunteers to give a final concentration of 25 μg/ml. The plasma (25 ml) was incubated at 37 °C, and samples (1.0 ml) were removed for analysis at the following times: 0, 0.5, 1, 2, 4, 6, 24, 48, and 72 h. Carboplatin was also added to fresh human plasma ultrafiltrate (PUF) to give final concentrations of 5 and 40 μg/ml. The PUF (25 ml) was incubated at 37 °C and samples (80 μl) were removed for analysis at the following times: 0, 0.5, 1, 2, 4, 6 and 24 h.

Results

Plasma analysis

Carboplatin was separated from endogenous constituents of human plasma ultrafiltrate (PUF) on a LiChrosorb diol column with a mobile phase of CH_3CN/H_2O (92:8, v/v) (Fig. 2). Most of the material absorbing at 229 nm eluted before the carboplatin peak, but a minor peak was seen at 15–16 min. Interference from this material was avoided by injecting samples at 13-min intervals. The cyclobutane mono- and dicarboxylic acids, possible degradation products of carboplatin, had retention times of less than 2 min in this system. No significant peaks were found at the retention time of carboplatin in control plasma from ten volunteers. This system, therefore, completely separated carboplatin from endogenous PUF constituents and from two potential degradation products.

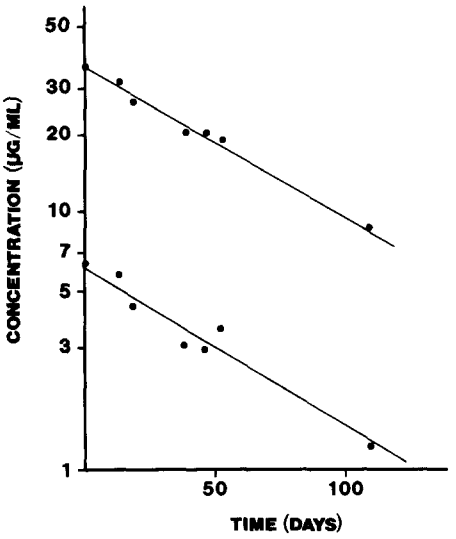

Fig. 3. Concentrations of carboplatin (μg/ml) in human plasma as a function of storage time at −25 °C

Standard curves ($n=6$), covering a range of 1–50 μg carboplatin per ml plasma, had correlation coefficients of greater than 0.98 and a mean (±SD) slope of 162±9. Peak height was linear with concentration up to at least 500 μg/ml. At 0.5 μg/ml the peak height was shown to be significantly different (*t*-test) from the responses given by control plasma ($n=10$). The lower limit of quantification for plasma was 1.0 μg/ml, since in some assays the responses given by 0.5 μg/ml were not significantly different from those obtained with control plasma. The within-day precision ($n=10$) was 3.5% and 3.0% and within-day accuracy, 100% and 97% at concentrations of 6.3 and 37.8 μg/ml plasma, respectively. Recovery was 94% according to the ratio of the slopes of standard curves prepared in water and in plasma.

Stability of frozen samples

Carboplatin had limited stability in fresh human plasma stored at −25 °C (Fig. 3). At concentrations of about 6 and 38 μg/ml plasma, the half-life values for carboplatin were 49 ($r=0.965$) and 54 ($r=0.993$) days, respectively. After 18 days of storage only about 70% of each of the original concentrations was present as carboplatin. These results show that plasma samples from human patients must be analyzed within a few days of collection to obtain results representative of the concentrations in patients.

Carboplatin also degraded when stored at −25 °C in PUF. At concentrations of about 5 and 40 μg/ml PUF, 100% of the added carboplatin was present after 6 days of storage. However, the carboplatin then degraded, with estimated half-lives of 17 ($r=0.91$) and 36 ($r=0.93$) days at concentrations of 5 and 40 μg/ml, respectively. Samples of carboplatin in PUF, therefore, should be stored at −25 °C and analyzed within a week of preparation.

Similar studies with solutions of carboplatin in water (1 mg/ml) showed the compound to be stable for at least 1 week when stored at −25 °C. However, by 2 weeks and at 3 weeks, the slopes of standard curves prepared from this solution were significantly different from the slope of a standard curve prepared from a freshly prepared standard. Stock solutions of carboplatin in water, therefore, should

not be stored for more than 7 days at −25 °C. Solutions for the preparation of standards are best prepared on the day of assay.

Stability at 37 °C

Carboplatin at a concentration of 25 μg/ml appeared to be stable for about 2 h in fresh human plasma at 37 °C. At 0, 0.5, 1, and 2 h, 92%, 89%, 94% and 95% of the nominal concentration, respectively, was recovered in the PUF. At 6, 24, 48, and 72 h, the recovery decreased to 86%, 53%, 34% and 20%, respectively. The estimated in vitro half-life in human plasma was 32 h. This result is in good agreement with the 29 h in vitro plasma half-life reported by Harland et al. [6] at a concentration of 37 μg/ml. The initial 2 h stability of carboplatin in human plasma, however, was not reported by these authors. The recovery of greater than 90% of the carboplatin in the PUF at the zero time point indicated that only 10% or less of the carboplatin was rapidly and reversibly bound to human plasma proteins.

Carboplatin appeared to degrade faster in PUF than in plasma at 37 °C. After a 1-h incubation at 37 °C only 85% of the original PUF concentrations of 5 and 40 μg/ml remained. By 2 h, only 63% and 77% of the low and high concentrations, respectively, remained. After 2 h, the percentage of each initial concentration remained relatively constant up to 24 h.

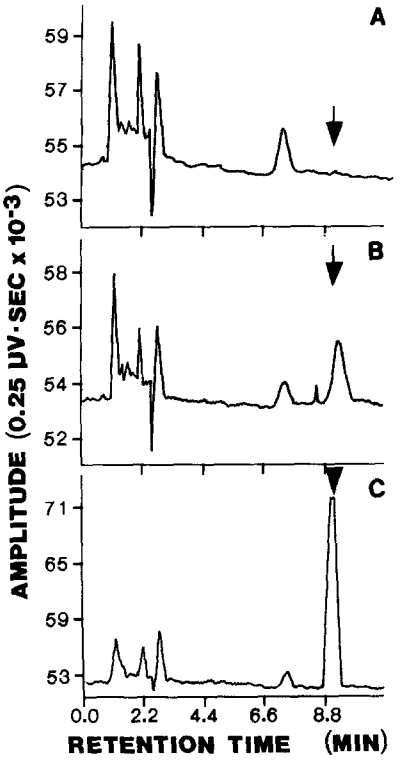

Fig. 4 (A–C). Chromatograms of carboplatin standards in urine: **A** 0 μg/ml; **B** 5 μg/ml; **C** 50 μg/ml

Stability during analysis

At 22 °C, these same concentrations of carboplatin in PUF were stable for at least 2 days, but then degraded with estimated half-lives of about 13 days (5 μg/ml) and 28 days (40 μg/ml). These results show that carboplatin was sufficiently stable in human PUF for overnight HPLC analyses in an automatic sampler/injector. Additional studies demonstrated that PUF could be analyzed and then stored at 3–7 °C for up to 48 h without degradation. A set of samples and standards were analyzed during an overnight run of 16 h, stored in a cold room at 3–7 °C for 48 h, and then reanalyzed by another overnight run. The mean (±SD) recovery on day 4 was 100%±6% ($n=18$) of the initial values, and the slopes and intercepts for the two standard curves were not significantly different. Therefore, if a malfunction occurred during an overnight analysis, the standards and samples could be stored for at least 2 days in the cold while repairs are made.

Urine analysis

The mobile phase developed for the analysis of carboplatin in human plasma was not suitable for the analysis of the compound in human urine, because of interferences from endogenous substances. Carboplatin in urine was analyzed on a LiChrosorb™ diol column with a mobile phase of CH_3CN/0.015% H_3PO_4 (89:11, v/v). The major peaks in human urine eluted prior to the carboplatin peak (Fig. 4). A late-eluting substance was present at 21 min, but sample injection at 25-min intervals prevented interference from this peak. No significant peaks at the retention time of carboplatin were found in control urines from ten subjects.

Standard curves ($n=6$), covering a range of 5–500 or 10–500 μg carboplatin per ml urine had correlation coefficients of greater than 0.99, intercepts that were not significantly different from 0, and a mean (±SD) slope of 364±41. Peak height was linear with concentration up to at least 500 μg/ml. The lower limit of quantification was 5 μg/ml, although in some analyses 1 μg/ml samples gave responses that were significantly different from the responses obtained with control urine. The within-day precision ($n=10$) was 9.4% and 3.7%, and within-day accuracy was 96% and 97% at concentrations of 39.9 and 400.5 μg/ml urine, respectively. Recovery was 99% based on the ratio of the slopes of standard curves prepared in water and in urine.

Stability in urine

Carboplatin had limited stability in human urine stored at −25 °C. After 11 days, 89% and 94% of the original concentrations of 40 and 400 μg/ml, respectively, were present as carboplatin. By 28 days, the percentage of original carboplatin dropped to 77% and 87%, respectively. The estimated half-lives were 84 and 159 days, respectively, for concentrations of 40 and 400 μg/ml. These results show that urine samples should be stored at −25 °C and analyzed within a few days of collection to obtain results representative of the amounts excreted by patients.

Analysis of urine samples and standards ($n=20$) on day 1 followed by storage at 3–7 °C for 48 h and reanalysis on day 4 gave a mean (±SD) recovery of 100%±12%. However, in contrast to the similar experiment with plasma, the slopes of the day-1 and day-4 standard curves were significantly different. According to the peak heights, approximately 30% of the carboplatin degraded between analyses. Nevertheless, if samples and standards are stored together, valid results can be obtained after 2 days at 3–7 °C.

Binding to Centrifree™ membranes

Carboplatin was not adsorbed to the Centrifree™ membranes over a concentration range of 5–500 μg per ml water. The mean (±SD) percentage recovery of standards ($n=14$) prepared in water and filtered in the Centrifree™ units was 101%±4% according to a standard curve prepared from the same water standards that were not filtered. The corresponding mean (±SD) percent recovery of standards ($n=12$) in human urine after filtration was 96%±8% over the concentration range of 10–500 μg/ml. The standard curves prepared in urine and water had slopes that were not significantly different and correlation coefficients of greater than 0.99. The intercepts were not significantly different from 0. At 5 μg per ml urine, 52% of the added carboplatin was recovered after filtration in the Centrifree™ units. This indicated possible degradation of carboplatin. Therefore, a urine concentration of less than 10 μg/ml should be interpreted with caution.

Discussion

The procedure described here for the analysis of carboplatin in human PUF was developed when we were unable to obtain reproducible retention times and separation factors with silica columns and CH_3CN/H_2O mobile phases. This variability may have been due to inactivation of the silica by the water in the mobile phase and was not seen with the diol column. The mobile phase of CH_3CN/H_2O (92:8, v/v) and the detection wavelength (229 nm) used with the diol column were similar to the mobile phase (90:10, v/v) and wavelength (225 nm) reported by Harland et al. [6] for a silica column. The linear range reported for the latter was 3.7–37.1 μg carboplatin/ml plasma, while the linear range with the diol column was 1.0–500 μg/ml. However, the plasma concentrations of carboplatin in patients given the maximum tolerated dose of about 400 mg/m^2 apparently will not exceed 50 μg/ml [6], and the results reported here were obtained with standard curves and sample concentrations in the range of 1–50 μg/ml.

Human urine samples could not be analyzed for carboplatin with the CH_3CN/H_2O mobile phase, because of interfering endogenous constituents. The urine concentration could be determined, however, with a mobile phase of CH_3CN/0.015% H_3PO_4. The range of the standard curve (5–500 μg carboplatin/ml urine) was similar to the reported linear range of 3.7 to 557 μg/ml on a silica column [6].

These HPLC procedures were developed so that the pharmacokinetics of parent compound could be determined in cancer patients. Since the results were to be part of a registrational package, it was necessary to validate the methods by determining the specificity, precision, accuracy, lower limit of quantification, linear range, and storage stability for both plasma and urine. The assays appear to have good specificity according to the separation of carboplatin from the potential degradation products, cyclobutane mono- and dicarboxylic acids, and to the loss of the compound on incubation or storage in plasma, PUF, and urine. Final verification of specificity, however, will have to be demonstrated with clinical samples to assure that metabolites or degradation products do not cochromatograph with carboplatin in these systems. This can be accomplished by analyzing the material eluting at the retention time of carboplatin by an independent method (e.g., mass spectrometry), to demonstrate that it contains only carboplatin.

The results presented here illustrate why a determination of the storage stability of a compound should be part of the validation procedure for an analytical method. Harland et al. initially reported the instability of carboplatin in human plasma and stated that their PUF samples were analyzed within 6 h of plasma collection. They also reported carboplatin to be unstable in human urine at 37 °C, with half-lives ranging from 20 to about 460 h at a concentration of 371 μg/ml. The results presented here show that urine samples of carboplatin are unstable even when stored at −25 °C. Aqueous solutions of carboplatin were reported to be stable for at least 69 h at 37 °C [6]; but we found that significant decomposition occurred between 1 and 2 weeks of storage at −25 °C. Carboplatin has limited stability in water, plasma, PUF, and urine. Therefore, samples of these matrices should be stored at −25 °C and analyzed within a week of collection for the results to be representative of the concentrations in patients.

In the procedure for plasma, samples were ultrafiltered through a membrane that excluded proteins with a molecular weight of greater than 30 000 daltons, and the protein-free PUF was analyzed for carboplatin. Therefore, the percentage of drug recovered in the PUF is the percentage of the plasma carboplatin present as free, non-protein-bound drug. Conversely, since carboplatin was shown not to be bound to the Centrifree™ membrane, the percentage of plasma carboplatin not recovered in the PUF is the percentage of the drug bound to plasma proteins. The mean recovery of plasma carboplatin in the PUF was 94% relative to unfiltered aqueous standards. In addition, after incubation in fresh human plasma for 0–2 h at 37 °C, 89%–95% of the added carboplatin was recovered in the PUF. Therefore, only about 10% or less of the plasma carboplatin was bound to human plasma proteins.

Since carboplatin is only minimally bound to plasma proteins, the decrease in plasma carboplatin concentrations with increasing incubation time at 37 °C ($T_{1/2}=32$ h) must be due to the formation of degradation products or metabolites of carboplatin. These products apparently become bound to protein, according to the report by Harland et al. [6] that the mean (±SD) in vitro plasma half-lives for free, non-protein-bound, platinum (30±1 h) and for carboplatin (29±1 h) were essentially the same. In other words, essentially all the free, unbound platinum is accounted for by carboplatin. The initial products may be the same highly reactive mono- or diaquated species of diammine platinum as are formed from cisplatin. These products would rapidly react with sulfhydryl groups of plasma proteins and become essentially irreversibly bound. This binding is in contrast to the reversible equilibrium characteristic of normal drug protein binding.

A failure to distinguish between these two types of protein binding and between the binding of parent compound and of total platinum has resulted in varied literature reports on the binding of carboplatin to human plasma proteins: Priego et al. [9] reported 60%–80% bound in 1 h; Wooley et al. [14] reported 0–60% bound in 2 h; and Harland et al. [5] reported 0–29% bound in 4 h, with 85%–95% bound by 24 h. The results presented here demonstrate that the parent compound is only minimally bound (≤10%) to plasma proteins and that the increased binding of plati-

num with time is due to degradation products of carboplatin.

Conclusions

Methods were developed and validated for the HPLC analysis of carboplatin in human plasma and urine. The results demonstrate the importance of storage stability studies as part of the validation procedures for analytical methods, and show that carboplatin is only minimally bound to human plasma proteins.

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