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Journal of Chromatography B, 669 (1995) 247–263

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Characterization of cisplatin–glutathione adducts by liquid chromatography–mass spectrometry Evidence for their formation in vitro but not in vivo after concomitant administration of cisplatin and glutathione to rats and cancer patients

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First received 9 November 1994; revised manuscript received 14 February 1995; accepted 20 February 1995

Abstract

After incubation of equimolar amounts of cisplatin (CDDP) and glutathione (GSH) in phosphate buffer pH 7.4 at 37°C, we detected two CDDP–GSH adducts whose structures, characterized by LC–MS, corresponded to *cis*-$[Pt(NH_3)_2Cl(SG)]$ and *cis*-$\{[Pt(NH_3)_2Cl]_2(\mu\text{-}SG)\}^+$. The latter is a new CDDP–GSH adduct, which was postulated but never structurally characterized so far. Rats and patients were given a 15-min intravenous infusion of CDDP (10 mg/kg to rats and 25 mg/m^2 to patients) preceded by a GSH intravenous administration (200 mg/kg to rats as a bolus and 1.5 g/m^2 to patients as a 15-min infusion). After the administrations, CDDP–GSH adducts were absent in rat and human plasma ultrafiltrates. The discrepancy between in vitro and in vivo findings can be explained based on pharmacokinetic considerations.

1. Introduction

Cisplatin (CDDP) is a widely used drug effective in the treatment of several solid tumors [1–3]. Clinical evidence suggested a steep dose–response curve for CDDP [1]; therefore, dose escalation and intensification are expected to improve the therapeutic outcome with CDDP, provided that the amelioration of the dose-limiting toxicity occurring during CDDP chemotherapy (neuro- and nephrotoxicity) are addressed. Among the various protective measures taken to improve the therapeutic index of CDDP, the hyperhydration and hypertonic saline (3–4%) as a vehicle for CDDP are included, to avoid or at least reduce nephrotoxicity [1]. However, peripheral neuropathy and ototoxicity are further dose-limiting adverse events of high-dose

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0378-4347/95/$09.50
SSDI 0378-4347(95)00098-4

therapy with CDDP [1,4]. The natural tripeptide thiol glutathione (GSH) was proposed as an agent of potential clinical interest for its protective activity against cisplatin-induced toxicity [5]. The therapeutic potential of GSH was confirmed by the observation of reduced CDDP renal and neurotoxicity in experimental models and clinical trials, when GSH was given in combination with CDDP [6–12]. Noteworthy, the antitumor activity of CDDP was not reduced by the combination with exogenous GSH [5–10]; on the contrary, evidence for improved CDDP efficacy was reported [6,7,9,10] and ascribed to a possible GSH effect at the tumor cell membrane level [9]. These latter findings suggest that no (or minor) direct interaction between CDDP and exogenous GSH occurs in vivo in the extracellular environment. Such an interaction, in fact, would have the effect to reduce the levels of unbound CDDP in plasma and other extracellular fluids, and consequently to reduce, at the tumor level after drug distribution, the intracellular amount of CDDP available for the interaction with DNA, thus diminishing CDDP antitumor efficacy. Our previous investigations support the lack of CDDP and exogenous GSH interaction [13,14]. In a study in cancer patients treated with 80 mg/m^2 CDDP (15 min i.v. infusion) with or without a 15-min i.v. infusion of 2.5 g GSH, given 15 min prior to CDDP, we found that CDDP pharmacokinetics, evaluated by flameless electrothermal atomic absorption spectroscopy (AAS) in terms of total and ultrafilterable platinum in plasma, is not significantly affected by GSH administration [13]. No pharmacokinetic parameter differed significantly whether CDDP was administered alone or with GSH. Also, in the same study we found that, within 2 h after CDDP administration (when CDDP concentrations were sufficiently high to be measured in the ultrafiltrates by a specific HPLC method), the curves of total ultrafilterable platinum (evaluated by AAS) and those of unchanged CDDP (evaluated by HPLC) were superimposable, without GSH and with the concomitant administration of GSH [14]. These findings indicated that the vast majority of the unbound platinum in extracellular fluids could be assigned to unchanged CDDP.

On the basis of in vitro experiments, some authors demonstrated that compounds with a strong nucleophilic moiety, like the thiol group of GSH, may react with platinum complexes [15–22]. These authors proposed the formation of adducts, whose structures were elucidated only in part. However, the question whether CDDP–GSH adducts are generated in vivo and to which extent has never been addressed.

In the present work, we undertook studies to characterize by LC–MS the chemical structure of CDDP–GSH adducts generated in in vitro experiments under different conditions, and to evaluate the in vivo relevance of the adducts formation in rats and cancer patients, after the concomitant administration of CDDP and GSH.

2. Experimental

2.1. Materials

CDDP, *cis*-dichlorodiammineplatinum (II), was purchased from Aldrich, Steinheim, Germany. [^{15}N]CDDP (isotopic purity greater than 97%) was prepared by Prof. N. Farrell, Department of Chemistry, Virginia Commonwealth University, Richmond, VA, USA. GSH was purchased from Boehringer Mannheim GmbH, Mannheim, Germany. NaH_2PO_4, Na_2HPO_4, H_3PO_4, HCOOH, and NaCl of analytical grade were purchased from Carlo Erba, Milan, Italy.

2.2. Animals

Male Sprague Dawley Crl:CD(SD)BR rats, weighing 300–350 g on the day of use, were supplied by Charles River, Calco, Italy. The animals were housed in a temperature- and humidity-controlled room. Each animal received ad libitum a standard dry diet, Altromin R-Rieper, Vandoies, Bolzano, Italy. Tap water from municipal mains was available in the cages. Rats were fasted overnight before treatment.

2.3. Instrumentation

An isocratic HPLC system Perkin Elmer, Norwalk, CT, USA, consisting of a Model 620

LC quaternary pump, an advanced LC sample processor Model ISS200, and a Model 235C diode-array ultraviolet (UV) detector focused at wavelengths of 230 and 255 nm was used. The instrumentation was controlled by a P.E. LC Analyst program, ver. 2.3, running on a 316SX DEC station. Column: Lichrosorb RP18 (25 cm × 4.6 mm I.D.), 10-μm particle size, Merck, Darmstadt, Germany. Mobile phase: 15 m*M* H_3PO_4, pH 2.2, filtered through a 0.2-μm membrane, Millipore, Bedford, MA, USA, and degassed in an ultrasonic bath. Flow-rate: 1 ml/min.

An atomic absorption spectrometer, Model SpectrAA 300, Varian, Mulgrave, Victoria, Australia, provided with graphite furnace and deuterium background correction was used for the determination of platinum in plasma, plasma ultrafiltrates, and HPLC fractions according to a validated method [23]. Before analysis, plasma samples were diluted 1:10 with 0.05% Triton solution. Ultrafiltrates and HPLC fractions were generally used without any dilution. Aliquots of 20 μl of each sample were analysed by AAS. The calibration curve was set up to 200 ng/ml and proved to be linear ($r = 0.9997$). The limit of quantitation was 10 ng/ml and the limit of detection was 5 ng/ml. The accuracy and precision, the latter expressed as coefficient of variation of the mean (C.V.), were 97.1% (C.V. 6.2%) at 25 ng/ml, 97.6% (C.V. 3.4%) at 50 ng/ml, and 98.3% (C.V. 2.7%) at 100 ng/ml.

2.4. Mass spectrometry

A triple quadrupole mass-spectrometer API III (Perkin Elmer-Sciex, Toronto, Canada) was used, operating in positive ion mode. The instrument utilizes a soft ionization technique, the atmospheric pressure ionization, and the ionization source (ionspray) was interfaced to an LC Perkin Elmer system. The column used for the liquid chromatography was the same as adopted for the HPLC–AAS analysis; the mobile phase was a 15 m*M* HCOOH aqueous solution, pH 2.8. The flow-rate was 1 ml/min and the splitting ratio for MS analysis was 1/25. A Model 22 Harvard infusion pump, operating at 2 to 5 μl/min, was used for direct sample insertion. The API III system worked in standard operating conditions. The orifice voltage applied was 50 V. For MS–MS experiments, argon was used as collision gas (300×10^{12} molecules/cm^2, collision energy 50 eV). The calibration was made with 1×10^{-4} *M* polypropyleneglycol.

2.5. In vitro studies

Incubation in sodium phosphate buffer for HPLC–UV–AAS

Equimolar (3.3 m*M*) amounts of CDDP and GSH were incubated in 10 m*M* sodium phosphate buffer pH 7.4 containing 150 m*M* NaCl, at 37°C in the dark. The volume of the mixture was 3 ml. The concentrations used were much higher than those usually observed in vivo at therapeutic GSH and CDDP doses. CDDP (3.3 m*M*) was also incubated alone in sodium phosphate buffer under the same experimental conditions. Samples were taken at time zero and after 15, 30, 60, 120, 180 and 240 min incubation. An aliquot of each sample (10 μl) was analyzed by HPLC–UV. The HPLC eluates of samples taken after 15, 30, and 120 min incubation were collected in 15-s fractions. Aliquots of these fractions and of the injected samples were analyzed afterwards by AAS to determine the percent distribution of platinum in the HPLC fractions.

Incubation in buffer for LC–API-MS

Equimolar (3.3 m*M*) amounts of CDDP and GSH were incubated in 10 m*M* sodium phosphate buffer pH 7.4 containing 150 m*M* NaCl, at 37°C for 1 h in the dark. The volume of the mixture was 3 ml. Aliquots of 10 to 20 μl were injected onto the LC–MS system. The same experiment was conducted using [^{15}N]CDDP.

Incubation in rat plasma for HPLC–AAS

Blood samples were collected in heparinized tubes from untreated rats and promptly centrifuged to obtain plasma. Equimolar (3.3 m*M*) amounts of CDDP and GSH were incubated in plasma (4 ml) at 37°C in the dark. CDDP (3.3 m*M*) was also incubated alone in plasma (4 ml) under the same experimental conditions. Samples were collected at time zero and after 15 min incubation and immediately ultrafiltered through

Amicon centriflow ultrafiltration membrane CF50, cut-off 50 000 Da, by centrifuging at 1000 *g* for 10 min, at 4°C in a Minifuge T Heraeus centrifuge. Aliquots of ultrafiltrates (10 μl) were analyzed by AAS and by HPLC. The HPLC eluates were collected in 15-s fractions and analyzed afterwards by AAS.

Incubation in human plasma for HPLC–UV–AAS

Human blood samples (20 ml) were obtained into heparinized tubes from a healthy male volunteer (age 33 years, body weight 70 kg) and immediately centrifuged at room temperature to obtain plasma (10 ml). Equimolar amounts of CDDP and GSH (3.3 m*M*) were incubated in plasma (4 ml) at 37°C in the dark. CDDP (3.3 m*M*) was also incubated alone in plasma (4 ml) under the same experimental conditions. Samples were collected at time zero and after 15 and 30 min incubation. The samples were promptly ultrafiltered prior to HPLC separation as previously described for rat plasma. Aliquots of the ultrafiltrates (20 μl) were analyzed by HPLC. The HPLC eluates were collected in 15-s fractions and analyzed afterwards by AAS along with aliquots of plasma ultrafiltrates.

2.6. *In vivo studies*

Rats

Rats were anaesthetized with ethylurethane (1 g/kg i.p.) and prepared by surgical cannulation of jugular veins and carotid artery. Three rats were given 10 mg/kg (33 μmol/kg) CDDP dissolved in saline at a concentration of 1 mg/ml, by short i.v. infusion (15 min) through the jugular vein, using a peristaltic pump (Vial Medical, France). The volume of administration was 10 ml/kg. The rats were pretreated with 200 mg/kg (651 μmol/kg) GSH dissolved in saline at a concentration of 40 mg/ml, given as an i.v. bolus through the other jugular vein just before the CDDP administration. Administered GSH and CDDP doses were approximately 5 times greater than those commonly employed in humans, to make the possible adduct formation in rats more evident. At the end of the infusion and 15 min later, blood samples were taken from the carotid artery and collected into heparinized tubes. Blood samples were immediately centrifuged and the plasma was ultrafiltered as described above. These steps were performed as quickly as possible because of the time-dependent increase of platinum binding to plasma protein. Aliquots of plasma ultrafiltrates (10 μl) were analyzed by HPLC. The HPLC eluates were collected in 15-s fractions and analyzed afterwards by AAS, along with aliquots of plasma and plasma ultrafiltrates.

Patients

The plasma samples for the evaluation of the in vivo formation of CDDP–GSH adducts were obtained in the framework of a pharmacokinetic study in cancer patients (G. Pizzocaro, unpublished data, 1994). Male patients (aging from 26 to 47 years) suffering from testicular cancer, were divided on a random basis in two groups. The first group (10 patients) received a 25 mg/m^2 CDDP dose as a 15 min i.v. infusion in saline; the second group (10 patients) also received 1.5 g/m^2 GSH in saline as a 15 min i.v. infusion, just before the CDDP administration. Blood samples were immediately centrifuged and plasma samples were ultrafiltered as previously described. Total platinum concentrations in plasma samples and plasma ultrafiltrates were determined by AAS. To fulfil the purpose of the present investigation on CDDP–GSH adducts, aliquots of ultrafiltrates (20 μl) of plasma samples collected at the end of CDDP infusion and 30 min later from five patients pretreated with GSH, were analyzed by HPLC. The HPLC eluates were collected in 15-s fractions and analyzed afterwards by AAS.

3. Results

3.1. *In vitro studies*

Detection of CDDP–GSH adducts in sodium phosphate buffer

After 15, 30 and 120 min incubation at 37°C of equimolar amounts (3.3 m*M*) of CDDP and

GSH in phosphate buffer pH 7.4 (10 m*M*) containing NaCl (150 m*M*), we observed by HPLC (UV detection 230 nm) the formation of two unknown compounds, named A1 (t_R 3.4 min) and A2 (t_R 4.4 min). Their chromatographic peaks were present in the HPLC tracings along with those of unreacted CDDP (t_R 2.5 min) and GSH (t_R 5.8 min) (Fig. 1).

From a series of pilot experiments (data not shown), we found by HPLC that under the above conditions A1 and A2 peaks did not arise from CDDP hydrolysis or GSH degradation/oxidation, because they were absent when CDDP and GSH were incubated separately. Cisplatin mono and diaquoderivatives are not generated from CDDP up to 120 min incubation (differently, aquoderivatives are formed when CDDP is incubated in water or phosphate buffer pH 7.4 without NaCl), and only oxidized glutathione (GSSG, t_R 14 min) is formed from GSH after 4 h. Hence, the formation of A1 and A2 seemed to be related to the concomitant presence of CDDP and GSH in the medium and the compounds were supposed to correspond to CDDP–GSH adducts.

The HPLC tracings of platinum-containing species were reconstructed after AAS of the HPLC eluates collected as 15-s fractions. Fractions corresponding to peaks A1 and A2 contained platinum, along with fractions corresponding to unchanged CDDP (Fig. 1). After 15 min incubation of CDDP and GSH in sodium phosphate buffer pH 7.4, peaks A1 and A2 contained, respectively, 3% and 0.3% of the total eluted platinum, whereas unmodified CDDP accounted for 96.4%. After 30 min incubation, peaks A1 and A2 contained 5.6% and 0.4% of the total eluted platinum, respectively, and CDDP 92.7%. After a 120-min incubation, the percentages increased for A1 (9.9%) and A2 (4.0%), and decreased for CDDP (79.1%). These observations are consistent with the attribution of the two unknown peaks to CDDP–GSH adducts.

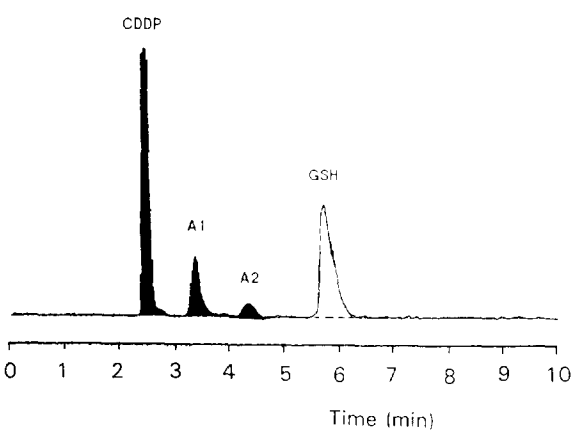


Fig. 1. Representative HPLC–UV tracing obtained after 30 min incubation of equimolar amounts (3.3 m*M*) of CDDP and GSH in 10 m*M* pH 7.4 sodium phosphate buffer–150 m*M* NaCl (3 ml). Darkened peaks correspond to platinum-containing species. A1 and A2 correspond to CDDP–GSH adducts.

Platinum recovery from the chromatographic column was complete, suggesting that no platinum-containing species are retained by the column and no other platinum-containing species are present in the incubation medium.

Mass spectra of CDDP and GSH

The molecular structure of the two unknown peaks was elucidated by using a mass spectrometer provided with ionspray source operating at atmospheric pressure. Before analyzing A1 and A2 structures, we studied CDDP and GSH fragmentation patterns by direct injection of the respective solutions (infusion of 5 μl/min). CDDP was dissolved in water at a concentration of 1 mg/ml. The mass spectrum of CDDP is shown in Fig. 2. The protonated molecule $[M+H]^+$ appeared as a cluster of ions related to the isotopic distribution of Pt and Cl (Table 1). The most abundant peak of this cluster was found at m/z 301. Similarly, the most abundant peaks of ion clusters associated to the adducts of CDDP with NH_4^+ and with Na^+ were observed, respectively, at m/z 318 and m/z 323. The CDDP–NH_4^+ cluster shows the typical isotopic distribution of $PtCl_2$ derivatives, with relative intensities of 70% for M, 75% for M + 1, 100% for M + 2, 47% for M + 3 and 58% for M + 4 (theoretical abundances: 71% for M, 73% for M + 1, 100% for M + 2, 47% for M + 3, and 58% for M + 4). The MS–MS spectrum obtained by fragmentation of the most abundant peak m/z 318 (M +

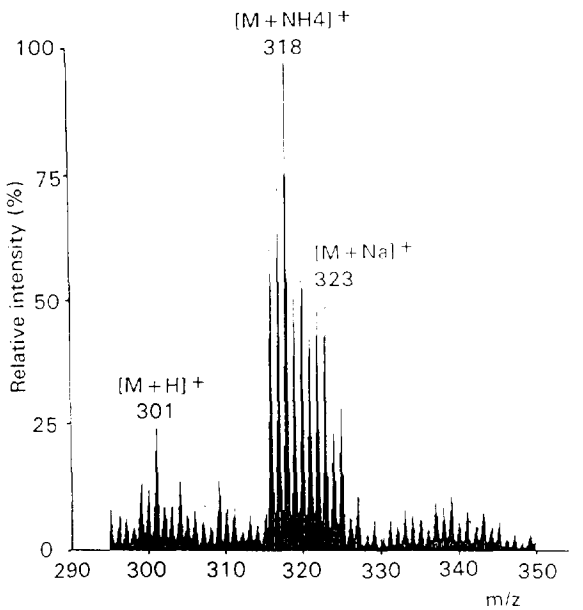


Fig. 2. Mass spectrum of CDDP dissolved in water (1 mg/ml). The infusion rate was 5 μl/min.

2), shows ions that may be easily associated to CDDP–NH_4^+ loss of Cl and NH_3 (Fig. 3).

Glutathione was dissolved in water at a concentration of 1 mg/ml. The mass spectrum of GSH shows the protonated molecule ion $[M + H]^+$ at *m/z* 308, with two isotopic peaks, M + 1 and M + 2, at *m/z* 309 and 310, and a GSH–Na^+ adduct at *m/z* 330 (Fig. 4). The MS–MS spectrum of the precursor ion (*m/z* 308) shows the presence of characteristic peaks, mainly originating from peptide bond breakage with formation of a glycine residue (*m/z* 76) and the complementary fragment cysteinyl-glutamic acid (*m/z* 233), a glutamic acid residue (*m/z* 130) and the complementary fragment cysteinyl-glycine (*m/z* 179), and a cysteine residue (*m/z* 102) (Fig. 5).

Table 1
Theoretical isotopic distribution of platinum and chlorine

Pt		Cl	
Mass	Abundance (%)	Mass	Abundance (%)
193.96	32.9	34.97	75.8
194.96	33.8	36.97	24.2
195.97	25.3		
197.97	7.2		

Characterization of A1 and A2

After 1 h incubation at 37°C of equimolar amounts (3.3 m*M*) of CDDP and GSH in phosphate buffer pH 7.4 (10 m*M*,) containing NaCl (150 m*M*), LC–MS tracings showed the presence of the same four peaks already depicted in Fig. 1. According to the expected retention times and to the mass spectra, two peaks proved to correspond, respectively, to unchanged CDDP and GSH. One of the two unknown peaks, A1, gives a protonated molecule ion at *m/z* 570, $[M + H]^+$, and its isotopic distribution (Fig. 6) is characteristic for a [PtClS] species, theoretical values: M (77%), M + 1 (90%), M + 2 (100%), M + 3 (43%), M + 4 (45%) and M + 5 (8%); found values: M (87%), M + 1 (93%), M + 2 (100%), M + 3 (46%), M + 4 (45%) and M + 5 (4.4%). The ion at *m/z* 570 is consistent with a monosubstitution of Cl with GSH in the CDDP molecule. When submitted to MS–MS analysis, the ion at *m/z* 570 generates a complex spectrum (Fig. 7) with a variety of peaks typical of either the CDDP or GSH moiety. The most abundant peak was the ion at *m/z* 308, which may be attributed to GSH; the complementary ion is at *m/z* 263, $[PtCl(NH_3)_2]^+$ (Fig. 7). The majority of fragments with *m/z* values ranging from 50 to 250 (*m/z* 76, 130, 162, 179, 233, 245) were typical of GSH fragmentation (see also Fig. 5). Ions of higher masses originated from the precursor ion for loss of NH_3 (*m/z* 553), of Cl (*m/z* 535), of NH_3 and Cl (*m/z* 517 and 500), and of a glutamic acid residue (*m/z* 442). According to these results, the structure of A1 is *cis*-diamminechloro(glutathionato-S)platinum, *cis*-$[Pt(NH_3)_2Cl(SG)]$, a CDDP–GSH (1:1) adduct (Fig. 7). The structure of A1 was confirmed by LC–MS analysis after incubation of $[^{15}N]$CDDP and GSH in sodium phosphate buffer, under the same experimental conditions used for unlabelled CDDP. The isotopic cluster of A1, as the $[M + NH_4]^+$ adduct shifted 2 mass units in comparison to that of the unlabelled adduct (Fig. 8), confirmed the presence of two NH_3 groups deriving from CDDP. The MS–MS

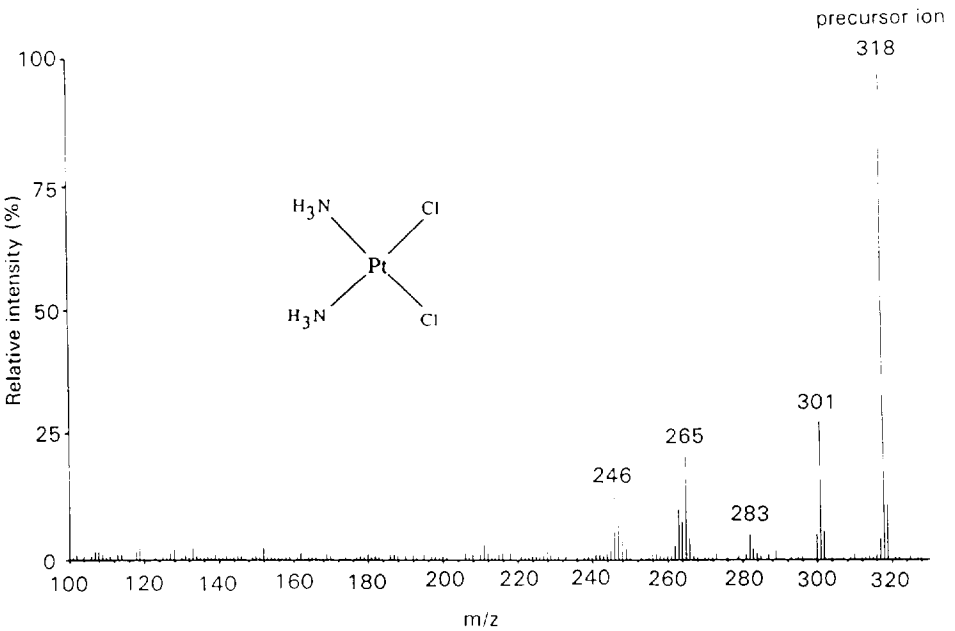


Fig. 3. MS–MS spectrum of the precursor ion $[M + NH_4]^+$ at m/z 318 of CDDP.

spectrum of the precursor ion $[M + NH_4]^+$ m/z 590 and 588, respectively, of labelled and unlabelled A1 were compared. The fragmentation pattern of the ion at m/z 590 shows ions at m/z 553 + 1, 500, 308, 263 + 2, which are consistent with the proposed structure for A1 (Fig. 9).

The other unknown peak, A2, provides a molecular ion at m/z 832, $[M]^+$, and a different

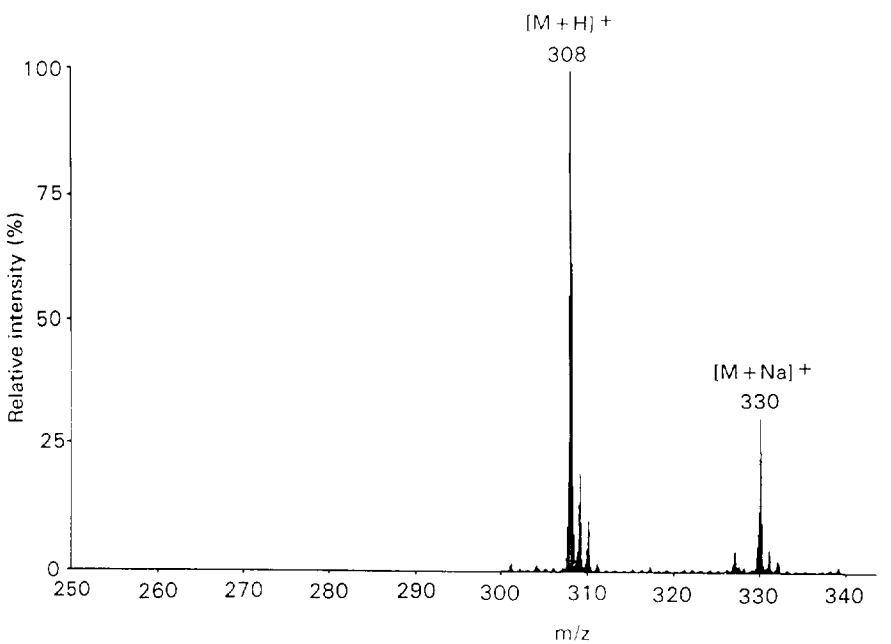


Fig. 4. Mass spectrum of GSH dissolved in water (1 mg/ml). The infusion rate was 5 μl/min.

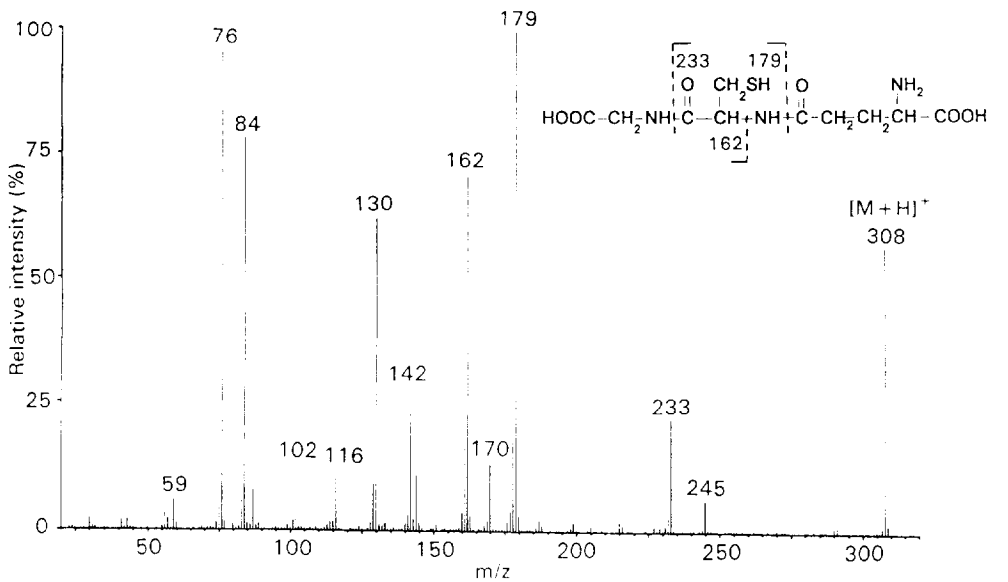


Fig. 5. MS–MS spectrum of the precursor ion $[M + H]^+$ at m/z 308 of GSH.

isotopic distribution as compared to that of A1 (Fig. 10). The pattern of the relative intensities of the individual isotopic peaks corresponds to that of a theoretical $[C_{10}H_{28}O_6N_7Cl_2SPt_2]$: M (29.5%; found 33.5%), M + 1 (61.5%; found 67.4%), M + 2 (100%; found 99.6%), M + 3 (96%; found 93.2%), M + 4 (95%; found 95.8%), M + 5 (63%; found 68.1%), M + 6 (47%; found 56.1%), M + 7 (21%; found 23.4%), M + 8 (14%; found 15.1%). This observation leads to the conclusion that the ion at m/z 832 may originate from the reaction of a GSH molecule with two CDDP molecules, and the proposed structure is μ-S-glutathionyl-bis-(*cis*-diamminechloroplatinum), *cis*-{$[Pt(NH_3)_2Cl]_2(\mu$-SG)}. The MS–MS spectrum for A2 was obtained by fragmentation of ion m/z at 835 (M + 3), one of the most abundant peak of the cluster (Fig. 11). Sequential loss of NH_3 groups from the precursor ion generates the ions at m/z 818, 801, and 784. Ions at m/z 764 and 748 come from m/z 801 and 784 via a Cl loss. The most abundant peak at m/z 709 may correspond to $[M\text{-}3NH_3\text{-Glycine}]^+$, whereas ions at m/z 726 and 681 may, respectively, be $[M\text{-}2NH_3\text{-Glycine}]^+$ and $[M\text{-}3NH_3\text{-}CONHCH_2COOH]^+$.

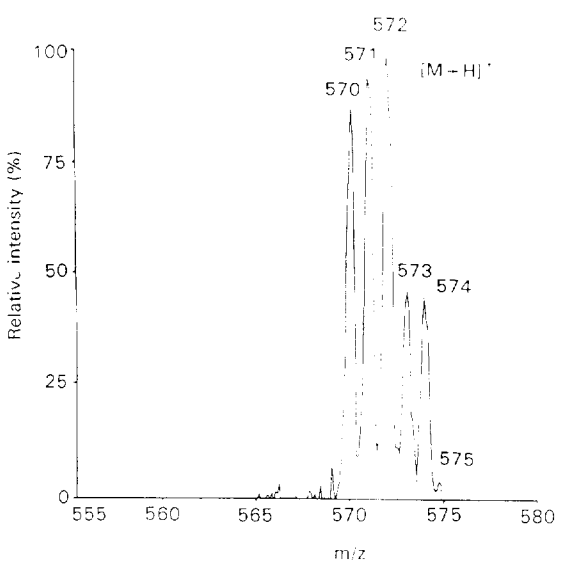


Fig. 6. Mass spectrum of the adduct A1 over a limited mass range. Isotopic cluster of $[M + H]^+$.

The structure of this second CDDP–GSH adduct was confirmed by LC–MS analysis after incubation in sodium phosphate buffer of [^{15}N]CDDP and GSH, under the same experimental conditions used for unlabelled CDDP. The isotopic cluster of labelled A2

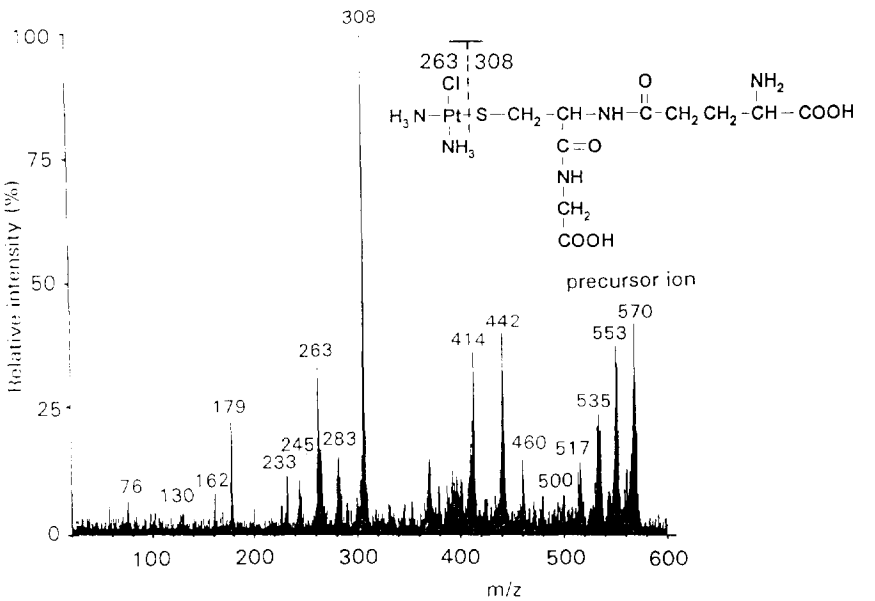


Fig. 7. MS–MS spectrum of the precursor ion $[M+H]^+$ at m/z 570 of adduct A1.

shifted 4 mass units in comparison to that of the unlabelled adduct (Fig. 12), confirming the presence of four NH_3 groups deriving from two CDDP molecules. MS–MS spectra of the $[M+2]^+$ precursor ions at m/z 834 and 838 of labelled and unlabelled, respectively, A2 were compared. The fragmentation pattern of labelled A2 shows ions at m/z 800 + 2, 783 + 1, 747 + 1, 725 + 2, 708 + 1 and 308, which are consistent with the proposed structure for A2 (Fig. 13).

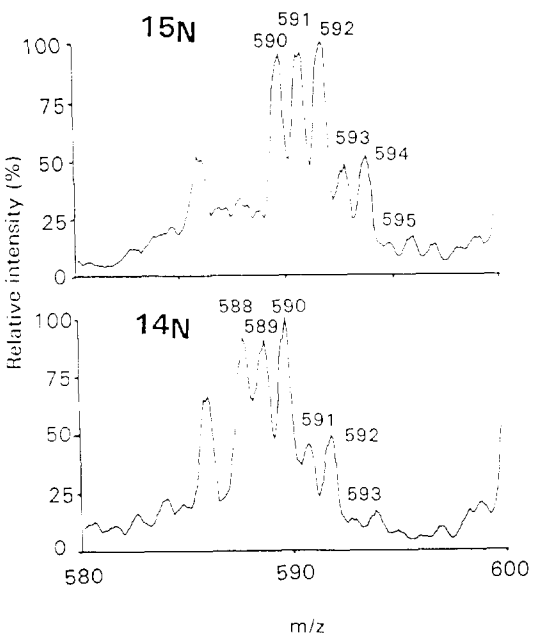


Fig. 8. Comparison of mass spectra over the limited mass range 580–600 of the adduct A1 obtained after incubation of GSH with [^{14}N]CDDP or [^{15}N]CDDP.

Detection of CDDP–GSH adducts in rat and human plasma

After 15 min incubation of equimolar amounts (3.3 mM) of CDDP and GSH in rat and human plasma, HPLC–UV tracings of plasma ultrafiltrates showed the presence of unchanged CDDP, GSH, and two unknown peaks whose retention times corresponded to those of A1 and A2, observed after incubation in sodium phosphate buffer. HPLC–AAS analysis proved that the fractions corresponding to peaks of CDDP and the A1 and A2 adducts contained platinum. After 15 min incubation of CDDP alone (3.3 mM) in rat and human plasma, A1 and A2 were absent. All of the platinum that was contained in the ultrafiltrates collected after 15 and 30 min incubation was found in the fractions corresponding to the CDDP peak. These findings

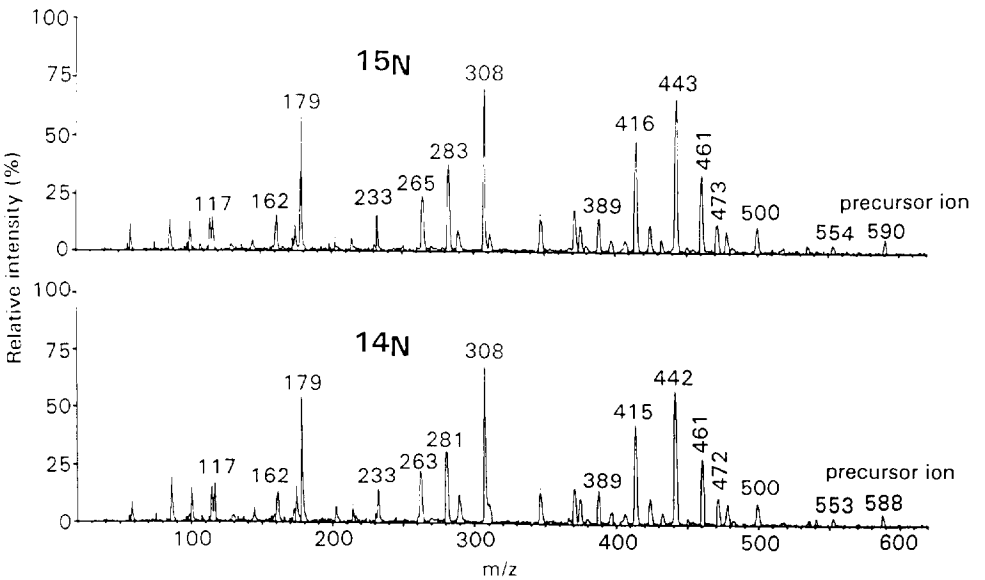


Fig. 9. Comparison of the MS–MS spectra of the two precursor ions of the adduct A1, 590 and 588, for ^{15}N and ^{14}N, respectively.

confirmed that CDDP–GSH adducts may be formed in vitro also in rat and human plasma. A1, A2, and unchanged CDDP accounted, respectively, for 7.1%, 2.1% and 86.3% of total platinum in rat plasma ultrafiltrates, and 5.2%, 1.7%, and 90.9% of total platinum in human

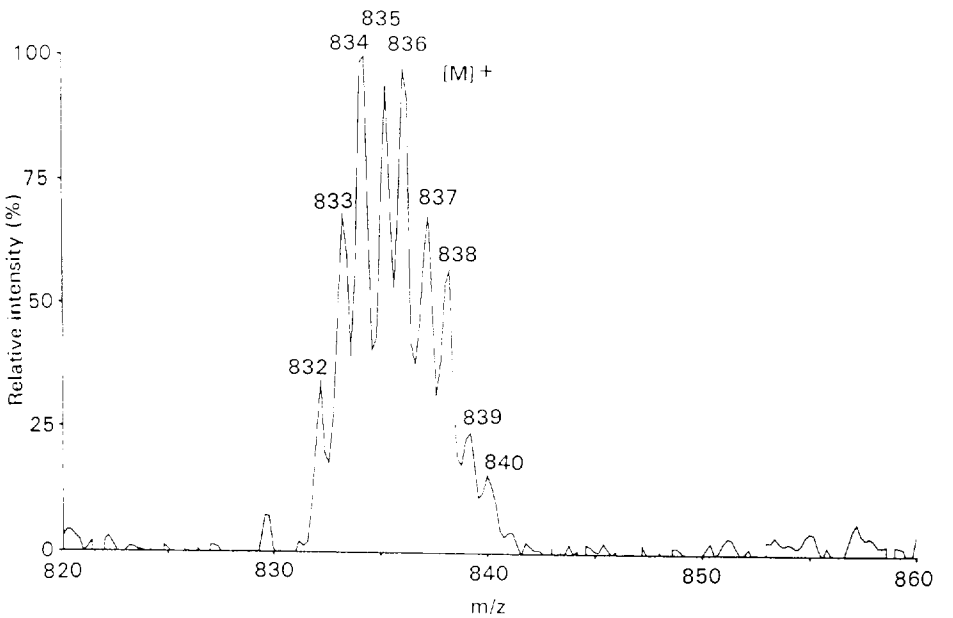


Fig. 10. Mass spectrum of the adduct A2 over a limited mass range. Isotopic cluster of $[M]^+$.

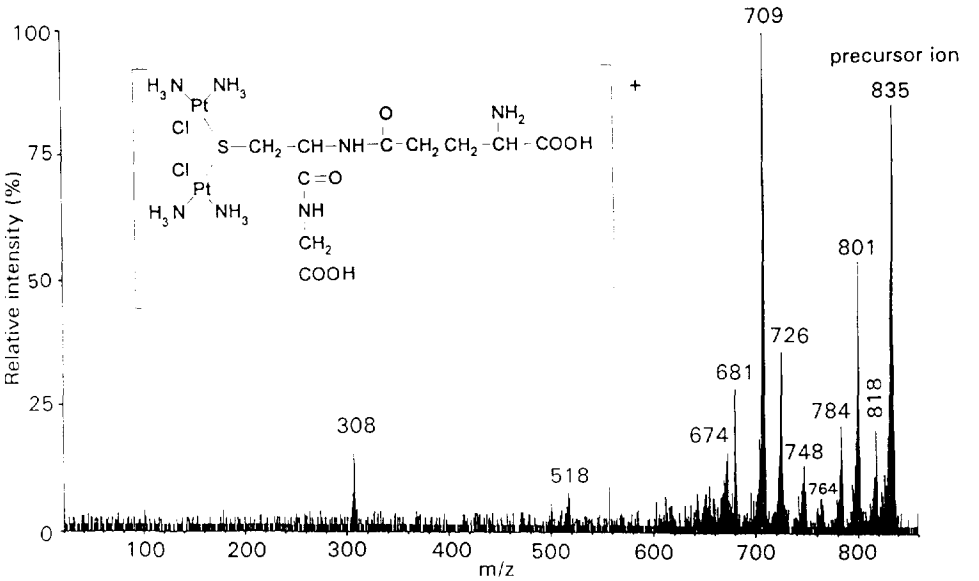


Fig. 11. MS–MS spectrum of the precursor ion 835 of the adduct A2.

plasma ultrafiltrates. In human plasma ultrafiltrates, the percentages of A1 and A2 increased after 30 min incubation to 7.5% and 4.1%, respectively, whereas the percentage of CDDP decreased to 84.1%.

Recovery of injected platinum from the chro-

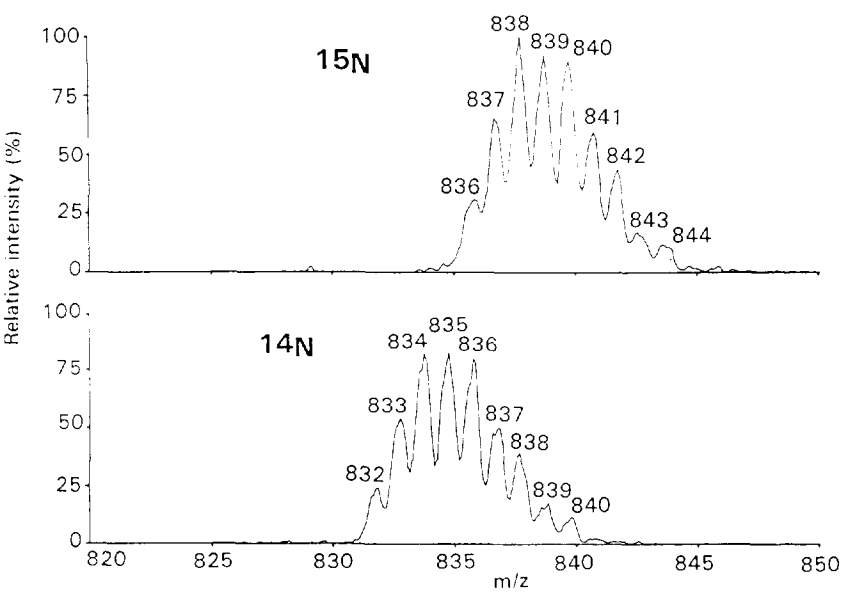


Fig. 12. Comparison of mass spectra of the adduct A2 obtained after incubation of GSH with [^{14}N]CDDP or [^{15}N]CDDP over a limited mass range.

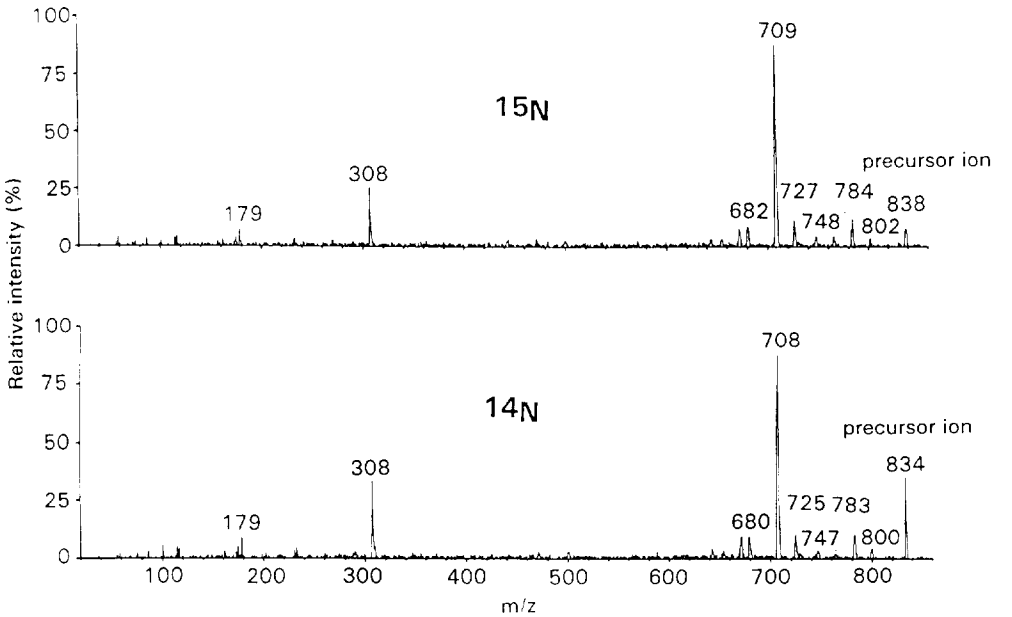


Fig. 13. Comparison of the MS–MS spectra of the precursor ions of the adduct A2, 834 (^{14}N) and 838 (^{15}N), after incubation of GSH with [^{14}N]CDDP or [^{15}N]CDDP.

matographic column was complete, both for rat and human plasma ultrafiltrates, suggesting that no platinum-containing species are retained by the chromatographic column and no other platinum-containing species are present in the plasma ultrafiltrates.

3.2. *In vivo studies*

Rats

Rats were given CDDP (10 mg/kg) as a 15-min i.v. infusion with GSH pretreatment (200 mg/kg, i.v. bolus). At the end of the CDDP infusion, total platinum plasma concentrations averaged 84.7 μM (S.D. 17.9 μM). Platinum in plasma was almost completely unbound, the mean unbound fraction being 97.6% (1.4%). Fifteen minutes after the infusion, total platinum plasma concentrations decreased to 54.7 μM (13.7 μM), and the unbound fraction to 81.7% (2.3%) due to the time-dependent increase of platinum binding to plasma protein (albumin). already reported in the literature [13].

At both observation times, HPLC–UV tracings of plasma ultrafiltrates from all treated animals did not show peaks corresponding to the A1 and A2 adducts. The AAS analysis of HPLC eluates showed that those fractions corresponding to the unchanged CDDP peak retention time accounted for the whole platinum contained in plasma ultrafiltrates, whereas no platinum was detected in those fractions corresponding to the retention times of A1 and A2 peaks. These findings confirmed that no other platinum species other than unchanged CDDP is present in the ultrafiltrates, or at least that, if other species were present at concentrations that could not be detected by the analytical methodology used (AAS detection limit 25 nM), then they individually would account for far less than 0.5% of total ultrafilterable circulating platinum levels.

Patients

The investigation on the presence of CDDP–GSH adducts in human plasma ultrafiltrates was conducted in five patients of the group receiving CDDP (25 mg/m^2 as a 15-min i.v. infusion) and a GSH pretreatment (1.5 g/m^2 as a 15-min i.v. infusion). At the end of the CDDP infusion,

total platinum plasma concentrations averaged 10.9 μM (S.D. 3.4 μM) and the unbound fraction was 87.1% (9.0%). Thirty minutes after the end of the infusion, total platinum plasma concentrations decreased to 5.0 μM (0.6 μM) and the unbound fraction to 57.4% (14.7%). At the end of the GSH infusion, GSH plasma concentrations averaged 114.7 μM (20.5 μM); 15 min after the end of the GSH infusion (the time corresponding to the end of the CDDP infusion), GSH concentration was 37.8 μM (32.2 μM); 45 min after the end of the GSH infusion (the time corresponding to 30 min post-CDDP infusion), GSH concentration was 14.4 μM (9.7 μM)

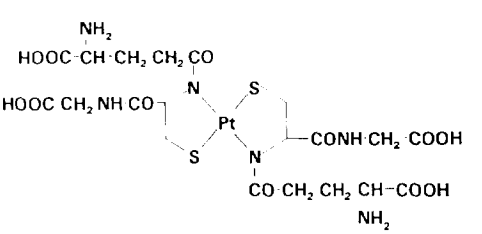
In HPLC eluates of plasma ultrafiltrates obtained at the end of CDDP infusion and 30 min later, platinum was found only in the fractions corresponding to the unchanged CDDP peak retention time. Absence of platinum in other HPLC fractions indicated that CDDP–GSH adducts A1 and A2 were not present in the systemic circulation or, if present at concentrations that could not be detected by the analytical methodology used, they individually would account for less than 3% of total ultrafilterable circulating platinum levels (as before, this calculation is based on the AAS detection limit).

4. Discussion

GSH, the major intracellular tripeptide thiol, is involved in cell protection from oxidant injury and alkylating agents [24]. Administration of exogenous GSH has been proposed to reduce CDDP adverse events in patients undergoing chemotherapy, thus increasing the therapeutic index of the anticancer drug [8,9]. Animal studies [5,12] and clinical trials [6,7,10,11] demonstrated that pretreatment with exogenous GSH reduced the CDDP-induced neuro- and nephrotoxicity, without reducing the antitumor activity. No (or minor) direct interaction between CDDP and exogenous GSH seems therefore to occur in vivo in the extracellular environment. Such an interaction, in fact, would have the effect of reducing levels of unbound CDDP in plasma and other extracellular fluids and, after drug distribution, to reduce at the tumor level the intracellular amount of CDDP available for the interaction with DNA. However, because platinum compounds are known to have affinity for nucleophilic sulfur ligands such as GSH [15–22], we performed experiments to investigate more deeply (i) the rate and extent of CDDP–GSH adducts formation in vitro, through studies performed in phosphate buffer and in rat and human plasma, (ii) the chemical structure of CDDP–GSH adducts, and (iii) their in vivo relevance in extracellular fluids after administration of CDDP to rats and patients pretreated with high-dose GSH.

Several in vitro investigations on the reaction between CDDP and GSH have been reported [17–22]. The results of those studies lead to controversial conclusions, possibly due to the different experimental conditions used by the various authors and the use of different and in some cases scarcely specific (e.g. elemental analysis, IR) analytical techniques. In the present work, we succeeded to unequivocally characterize labile compounds such as CDDP, GSH and their adducts in liquid phase, using an LC–MS system with soft ionization.

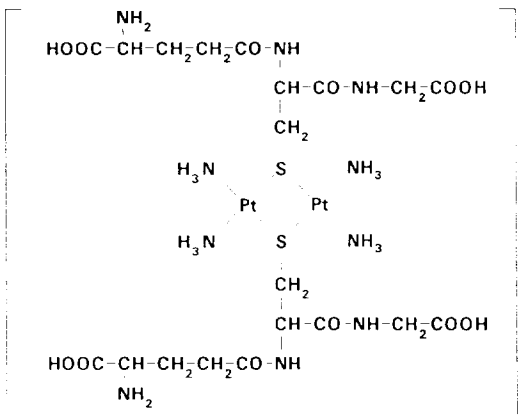
By incubating for 4 to 5 days at 37°C CDDP (3.33 mM) and GSH (6.67 mM) in saline (100 ml), Odenheimer and Wolf suggested, on the basis of elemental analysis and infrared spectra, the formation of a CDDP–GSH chelate complex, bis-(glutathionato)-platinum [17].



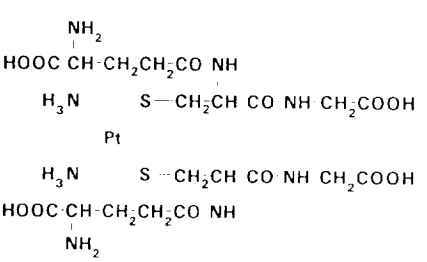
The formation of this complex was demonstrated later on by mass spectrometry and NMR after incubation of a mixture of CDDP (1.67 mM) and GSH (3.33 mM) in PBS (15 ml) at 37°C for 48 h [18]. This complex, which has a Pt–GSH molar ratio of 1:2, was found also in L1210 leukemia

cells exposed to CDDP. The complex was considered a major CDDP metabolite in L1210 cells and was suggested to be involved in the inhibition of protein synthesis and CDDP elimination from cells [18].

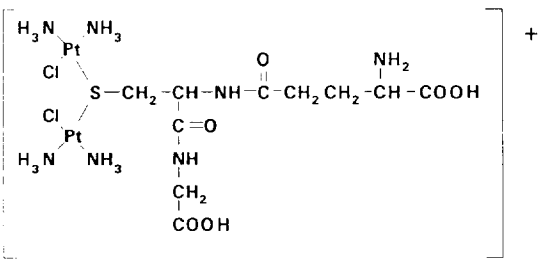
Appleton et al. [19] showed by NMR that the reaction of GSH with *cis*-diamminediaquaplatinum (II) gives a sulfur-bridged dimeric complex, *cis*-$[\{Pt(NH_3)_2(\mu\text{-SG})\}_2]^{2+}$:



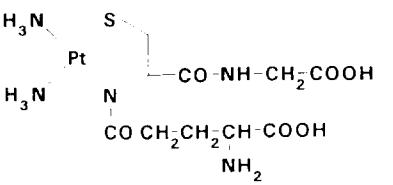
After incubation at 23°C for 7 h of GSH (150 m*M*) and CDDP (50 m*M*) dissolved in a sodium phosphate (500 m*M*) + NaCl (500 m*M*) pH 7.2 solution (2 ml), Berners-Price and Kuchel [20] followed the formation kinetics of different Pt–GSH species. One adduct was identified by NMR and corresponded to the 1:1 adduct between CDDP and GSH, *cis*-$[PtCl(SG)(NH_3)]$. Other possible species mentioned by the authors, whose structures were proposed but not characterized, were *cis*-$[Pt(NH_3)_2(SG)_2]$:



cis-$\{[(NH_3)_2Pt(\mu\text{-SG})]_2\}^{2+}$, equivalent to that indicated by Appleton et al. [19], *cis*-$\{[Pt(NH_3)_2Cl]_2(\mu\text{-SG})\}^{2+}$:



and *cis*-$[Pt(NH_3)_2(S\text{-NG})]$:



Other authors reported that the final product of the reaction between CDDP (3 m*M*) and GSH (6 m*M*) in saline [21], in pH 7.2 buffered solutions [20] or in human red blood cells [22] is consistent with high-molecular-mass polymers with a 1:2 Pt–GSH stoichiometry in which coordination is almost exclusively via the S atom. The final compounds were obtained after several days of incubation.

The results of our work showed that, after incubation of equimolar amounts of CDDP and GSH in pH 7.4 sodium phosphate buffer–NaCl, two CDDP–GSH adducts are formed, named A1 and A2. After a 15 to 120 min incubation, A1 represented 3% to 9.9% of total platinum in the medium and A2 0.3% to 4% of total platinum. Except for CDDP, no other platinum-containing species seemed to be present in the incubation medium. The molecular structure of the two unknown peaks was assigned by using a mass spectrometer provided with ionspray source operating at atmospheric pressure, a new soft ionization technique, which makes the characterization of polar or thermal labile compounds possible. This technique proved to be particularly useful for the analysis of platinum-containing compounds.

LC–MS spectra of A1 and A2, MS–MS spectra of the $[M + H]^+$ or the M^+ ions, respectively at *m/z* 570 (A1) and 832 (A2), were consistent with the following structures (see also Figs. 7 and

11): A1, *cis*-[$Pt(NH_3)_2Cl(SG)$]; A2, *cis*-{$[Pt(NH_3)_2Cl]_2(\mu$-SG)}$^+$. The first structure corresponds to that studied by NMR by Berners-Price and Kuchel [20]. The second structure was predicted, but not characterized, by the same authors.

Before starting in vivo experiments, we verified through in vitro studies whether the adduct formation might also occur in biological fluids spiked with CDDP and GSH. In rat and human plasma, after incubation of equimolar amounts of CDDP and GSH at 37°C for up to 30 min, A1 and A2 were still formed. No other platinum-containing species were present in plasma ultrafiltrates. This observation indicated that plasma components do not affect the adduct formation.

In vivo studies were performed in rats and in humans after GSH and CDDP administration to establish whether the presence of GSH–CDDP adducts may also occur in actual treatment conditions. At the end of CDDP infusion and 15 (rat) or 30 min later (man), the unchanged CDDP peak accounted for the whole platinum contained in plasma ultrafiltrates. No other platinum-containing species were present in plasma ultrafiltrates. A1 and A2 adducts were not detectable in rats and humans receiving CDDP with a GSH pretreatment and their presence (if any) was therefore lower than 0.5% (rat) or 3% (man) of the total ultrafilterable circulating platinum levels.

The in vivo assessment of CDDP–GSH adduct formation was made in the plasma compartment in a limited time interval, the end of CDDP infusion and 15 or 30 min later, for two reasons. Firstly, GSH undergoes rapid disposition processes and has a short half-life, approximately 2 to 7 min in rats (A. Bernareggi, unpublished data, 1994) and 7 min in patients [26]; therefore, its concentration in the systemic circulation is expected to remain greater than basal values only for a limited time interval (less than 1 h) after GSH administration. By taking 7 min as the GSH half-life for rat and man, at 15 and 30 min after the end of the CDDP short i.v. infusion (15 min), we should expect a GSH plasma concentration respectively 20 and 90 times lower than the initial concentration (i.e., the concentration at the end of GSH i.v. infusion or i.v. bolus). Secondly, free CDDP concentration in the plasma compartment rapidly decreases due to a prompt drug disposition and to the irreversible CDDP binding to plasma protein, which increases progressively as a function of time: 2 to 4 h after CDDP administration this drug is almost completely bound. As a consequence of these facts, a significant interaction between GSH and CDDP, if any, is likely to be limited to a narrow time interval after CDDP administration.

We can explain the discrepancy between the observation of the in vitro formation and the in vivo absence of CDDP–GSH adducts through the following considerations. After CDDP and GSH administration, maximum concentrations of both drugs achieved in rat and human plasma were much lower than those used for the in vitro experiments. In patients treated with 25 mg/m^2 CDDP and 1.5 g/m^2 GSH, CDDP and GSH plasma concentrations observed at the end of the CDDP infusion were, respectively, 10.9 μM and 37.8 μM, i.e., about 300 times (CDDP) and 90 times (GSH) lower than those used in in vitro experiments (3.3 mM). In rats given 10 mg/kg CDDP, mean maximum CDDP plasma concentration (84.7 μM) was about 40 times lower than that used in in vitro experiments. We did not measure GSH plasma concentrations in rats after a 200 mg/kg i.v. bolus. However, on the basis of our own previous studies in rats given 250 mg/kg i.v. (A. Bernareggi, unpublished data, 1994), we may predict that the GSH concentration at the end of CDDP infusion would be about 20 times lower than that used in the in vitro trials reported in this paper. Also, as mentioned before, the occurrence of prompt disposition processes which both GSH and CDDP undergo after their administration, and of the time-dependent irreversible binding of CDDP to plasma proteins, results in a very rapid and progressive decrease of GSH and unbound CDDP plasma concentrations, a fact that further limits the possibility of a significant in vivo reaction between CDDP and GSH in the extracellular fluids. The formation of CDDP–GSH adducts, observed in vitro

in the present study after incubation of CDDP and GSH at fixed and high concentrations, does not seem to occur in plasma of patients and rats after CDDP and GSH concomitant administration. These findings confirm our previous observations in patients treated with CDDP alone (80 mg/m^2) or in combination with GSH (2.5 g), in which platinum in plasma was found to be associated only to the parent drug with no evidence of significant CDDP conjugation to GSH [13]. The plasma ultrafiltrate concentration–time curve of total platinum, evaluated by AAS, overlapped that of unchanged CDDP evaluated by HPLC with electrochemical detection [14]. Furthermore, the plasma pharmacokinetic profile of total and unbound platinum observed in patients after administration of CDDP with GSH pretreatment did not differ from that depicted after administration of CDDP alone [13].

Our results indicate that the co-administration of GSH does not reduce the unbound CDDP plasma concentration, available for tissue distribution and activity. No direct interaction seems to occur between CDDP and exogenous GSH in the extracellular environment. Also, previous studies indicated that the pharmacokinetics of total and unbound CDDP is not affected by the concomitant administration of GSH [13]. Therefore, after CDDP + GSH administration, normal tissue and tumor cells would be exposed to the same CDDP concentration obtained after administration of CDDP alone. The protective effect against CDDP injury exerted by high-dose exogenous GSH in patients, with no decrease of CDDP antitumor activity [6,7,10,11], might be explained by considering that exogenous GSH is not able to cross the cell membrane and it is taken up to an appreciable extent only by cells with substantial expression of γ-glutamyl-transpeptidase on the membrane surface (i.e. the kidney cells) [24]. In this type of cells, we expect to see an increased GSH content after GSH administration and, therefore, a protective effect against CDDP injury due to mechanisms such as a GSH-dependent reversion of CDDP-related mitochondrial protein sulphydryl alteration and decreased mitochondrial calcium uptake [27], or a GSH-dependent CDDP metabolism with formation of intracellular complexes [18,22]. No (or a modest) GSH content increase is expected in common tumor histotypes, including ovarian cancer (e.g. A2780 and IGROV-1 cells), which express relatively low levels of γ-glutamyl-transpeptidase [9]. It has been reported that only a substantial increase of intracellular GSH content can produce a decreased tumor cell susceptibility to CDDP [9]. Therefore, the cytotoxic activity of CDDP against tumor cells would not be reduced by the concomitant administration of GSH.

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