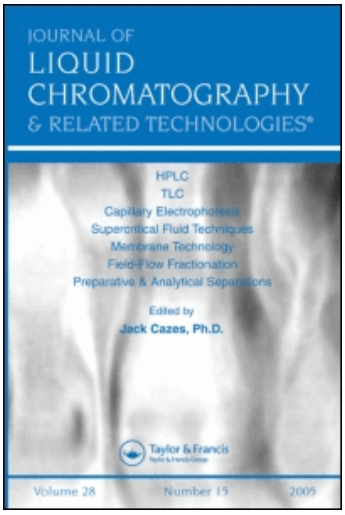

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DETERMINATION OF CIS-PLATIN IN HUMAN PLASMA BY HPLC WITH A GLASSY CARBON-BASED WALL-JET AMPEROMETRIC DETECTOR

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ABSTRACT

Previous voltammetric experiments have shown the electrooxidation of cisplatin (cis-diamminedichloroplatinum II, CDDP) and its hydrolysis products at the glassy carbon electrode. Taking into account the advantages of the oxidative detection mode(rather than reduction) and by using an easy to handle wall-jet configuration, the sensitive HPLC determination of cis-platin has been successfully performed in biological samples. Several counter-ions generally employed in ion-pair chromatography have been investigated in order to optimize the analysis of CDDP in terms of separation efficacy, retention time and compatibility with the electrochemical detection mode.The cationic detergent cetyltrimethylammonium chloride(CTACl) has been found to be the most appropriate ion-pairing

Dedicated to the memory of G.J.Patriarche.
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reagent for the determination of intact CDDP in spiked plasma.The limit of detection was 30 ng/ml and 150 ng/ml in aqueous and plasma samples, respectively. The recovery of cis-DDP in plasma was 86 ± 5 % . At a concentration of 10 µg/ml, the coefficient of variation was 6 % (within-day) .

INTRODUCTION

Cis-dichlorodiammineplatinum(II) (cis-platin, CDDP) has received considerable attention since the discovery of its anticancer activity in mice in the mid sixties (1,2). Despite some risks of nephrotoxicity, CDDP has become a leading selling anticancer drug, being most effective against testicular, ovarian, head and neck tumors (3). A great number of analytical techniques have been developed in order to study the fate of CDDP in aqueous solutions and in biological fluids and to understand its mechanism of action. Most of these techniques have been thoroughly reviewed recently (4). The difficulty in studying CDDP originates from the fact that the molecule, once dissolved, is readily transformed into several more or less potent derivatives depending on the chemical or biological environment (5-9). Moreover, because the absorbance of CDDP is low, analytical techniques using UV spectrophotometric detection exhibit poor sensitivity especially when used for biotransformation studies (4). Since protein bound-platinum is very little active, the concentration of the parent drug is of pharmacological relevance (4). Cis-platin determination is essential for the dosimetry of the drug in

each patient. It is generally accepted that the drug peak in plasma as well as its area under the curve (AUC) are closely related to toxicity. Moreover, monitoring residual plasma concentrations of cis-platin offers an objective and safe way to avoid cumulative toxicity in case of repeated administrations. Blood concentrations of cis-platin following IV administration of 100 mg/m² reach a maximum peak ranging from 5 to 10 μg/ml in plasma and then it drops with a half life of about 50 min. So far, the majority of analytical techniques have focused on the ability of high performance liquid chromatography (HPLC) in separating the intact drug from its metabolites and other interfering species. Ion-pair chromatography has been shown to be especially suitable for the determination of platinum containing drugs (4,10) . Cationic or anionic surfactants served as ion - pairing agents and have been selected for the analysis of intact CDDP (10-13) or CDDP degradation products (14,15), respectively .

Although graphite furnace atomic absorption spectrometry has been successfully interfaced off-line to HPLC for the selective and sensitive determination of CDDP, electrochemical on-line detectors have mostly been utilized in routine analysis (4). Since platinum(II) species may be oxidized or reduced electrochemically (17), several electrochemical detection modes , working electrodes and cell configurations have been investigated. Details of methods with their sensitivities have been listed in Table 1. Hanging mercury drop (HMDE), dropping mercury (DME) and

TABLE 1

Author	Column	Mobile Phase (M.Ph.)	Electrode	Potential V	d.l. µg/ml
I.S.Krull (19)	C18	Acetate pH 4.6 + HTABr	Au/Hg	0.00	0.1 Plasma
			GCE	+ 1.2	0.16 (A.M.)
R.P.Baldwin (13)	C18	Citrate pH 7 + HTACl + NaCl	Pt	+ 0.8	1 Serum
L.A.Sternson (18)	C 8 (T=60°C)	Citrate pH 6.5 + HTABr	HMDE DME	- 0.04 0.00	0.1 Urine
P.J.Parsons (14)	C18	Acetate pH 4.6 + HxSA	HMDE	- 0.1	0.062 Plasma
M.Smyth (15)	C18	Acetate pH 4.6 + OSA	HMDE GCE	+ 0.05 + 1.2	5 0.37 (A.M.)
W.Vandervijgh (16)	C18	Ammonium citrate pH 6.5 + HTAOH	DME	pulse 0 - 1.6	0.003 Plasma
G.J.Patriarche (this work)	C18	Acetate pH 4.3 + HpSA	GCE	+ 1.16	0.03 (A.M.) 2 Plasma
		Acetate pH 5.0 + CTACl	GCE	+ 1.16	0.03 (A.M.) 0.15 Plasma

HxSA = hexanesulfonate, HpSA = heptanesulfonate, OSA = octanesulfonate, HTAOH = hexadecyltrimethylammonium hydroxide, d.l.= detection limit, A.M. = aqueous media.

gold amalgamated mercury (Au/Hg) electrodes have been used in the reductive mode at a working potential corresponding to the proton catalytic wave (12) or to CDDP adsorption (16-19). The methods have been successfully applied to the determination of CDDP in plasma at the ppb (19) or sub-ppb (12) level. In comparison with the oxidative mode, reduction is less perturbed by interfering species, except

by dissolved oxygen. The reduction at gold/mercury surfaces, however, requires skill in electrode preparation and surface renewing (11,14,19). Moreover, sophisticated potentiostats generating pulsed waveforms and associated to computers have been required to achieve sub-ppb sensitivity (12). Oxidation at platinum (13) and glassy carbon (11,15,19) based HPLC detectors has been shown to be useful in speciation studies and for the sensitive determination of intact CDDP. We have shown earlier , that CDDP is readily oxidized at carbon electrodes and that aquo and organic ligands tend to stabilize platinum(II) complexes (20). For instance, the detection of CDDP hydrolysis products (aquo complexes) occurs at potentials more positive than CDDP oxidation potential. The high potentials applied may explain the high detection limits observed for CDDP derivatives (4,19). Glassy carbon is often used in HPLC due to its interesting electrochemical and mechanical properties. The electrode has already been used after ion-pair HPLC separation of CDDP in aqueous solutions(see Table 1), however, no application to biological fluids has been reported.

In this work, a glassy carbon working electrode confined in a wall-jet configuration has been investigated as detector for CDDP analysis in plasma and artificial fluid following ion-pair reversed phase HPLC . Special attention has been given to the sample purification procedures in order to minimize risks of interference at the detector level.

EXPERIMENTAL

Materials

Pure CDDP was obtained from Bristol Benelux N.V. and from Johnson Matthey Materials Technology . Cis-Platinol was supplied by Bristol Benelux Laboratories. All reagents were of analytical grade and supplied by Sigma or Merck. The solutions were prepared with demineralized water purified by passing through a Milli-Q (Waters/Millipore) water purification system. Cetyltrimethylammonium chloride (CTACl) and bromide (CTABr), used as ion-pairing reagents , were obtained from Aldrich Chemical Co. and Janssen Chimica ,respectively. All other ion-pairing reagents were obtained from Janssen Chimica.

Apparatus

The HPLC instrumentation was from Gilson Medical Electronics(France). It consisted of two pulse damped solvent delivery systems (model 303), an automatic injection valve with a 20 µl loop attached (Rheodyne model 7010) and an injection port model 231. The HPLC column used was a Rosil HL C18 (5µm, 250 x 4.6 mm i.d.) from Research Separation Laboratories (RSL)(Bio-Rad Belgium). The electrochemical detection system consisted in a wall-jet type configuration connected to a potentiostat Bruker model E 230. The cell was constructed and kindly obtained from Dr. L.Nagels (Rijksuniversitair Centrum, Antwerpen - B)(see Figure 1 and Ref.21). It comprised the reference,

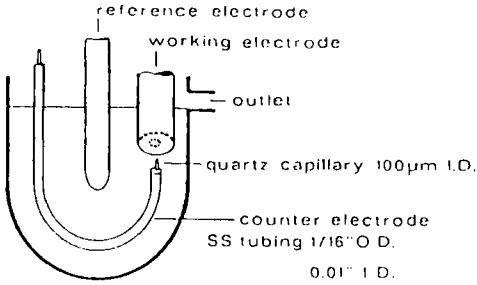

Figure 1.
Schematic structure of the wall-jet cell

counter and working electrodes. Stainless-steel chromatographic tubing served as counter electrode and as guidance for a polyimide coated fused silica capillary (100 μm i.d., 250 μm o.d.). The quartz tubing outlet extended 0.5 mm out of the stainless-steel tubing. A 0.5 mm distance was maintained between the quartz tubing nozzle and the working electrode. The other end of the quartz tubing was connected to the HPLC column. The reference electrode was a silver/silver chloride KCl saturated. The glassy carbon working electrode (GCE) was from Metrohm AG Switzerland (diameter: 3 mm).The electrochemical detector was connected to a Perkin Elmer recorder (model 165), chart speed 5 mm/min. The disposable columns (C18 cartridge , Bond-Elut Certify- 100mg mass/1 ml volume) used in the clean-up step were from Analytichem Int.

Procedure

The mobile phase selected for CDDP determination consisted of 0.01M acetate buffer adjusted at pH 5.0 ± 0.1

and contained the appropriate ion - pairing reagent (15,17,19). The anionic pairing reagents have been used within the concentration range 5mM - 13 mM ; hexane, heptane, octane or decane sulphonic acid and sodium dodecyl sulphate (SDS) have been tested. The cationic reagents CTACl and HTABr have been used at a concentration of 0.05 mM. The cationic - pairing surfactants have been adsorbed onto the hydrophobic stationary phase according to the method described by Riley et al. (10). That is, 100 ml of a 3 mM CTACl aqueous solution was passed through the Rosil C18 column at 1.0 ml/min . After pre-loading the stationary phase, a lower concentration of CTACl(0.05 mM) was used in the mobile phase to maintain the stability of the modified stationary phase (10,22). The mobile phase was maintained at 32°C in an open thermostatic bath, prior passing through the column. Daily, before starting the experiments, the glassy carbon electrode was polished manually on a soft tissue with a fine diamond paste (Diamantine - Bergeon), rinsed with Milli-Q water and sonicated during 5 min. At the end of the day, the HPLC column was washed with Milli-Q water followed by a methanol-water solution (40:60 v/v) and finally with pure methanol to remove the adsorbed surfactant. Unless otherwise stated, the potential of the working electrode was maintained at +1.16 V versus Ag/Ag^{+}. The flow rate of the mobile phase was 1.0 ml/min.

Sample preparation

Stock solutions of CDDP (1mM) were prepared in 0.1 M sodium chloride and kept one week at 4°C before renewing.

Standards were prepared daily by diluting the stock solution with 0.1 M sodium chloride in order to minimize hydrolysis of CDDP (9,13,18). Plasma as well as an artificial fluid used in isolation-perfusion of the limbs (SSPP:25% + Hartman:60% + blood:15% + 40 mEq.bicarbonate, + 20 mg Heparin, see also Reference 23) were used to validate the method.

The analysis of CDDP from plasma and artificial fluid (A.F.) involved spiking of the sample at known concentration levels.

Several sample treatments have been investigated:

- protein precipitation with trichloroacetic acid (TCA) (v/v) followed by centrifugation and (i) injection of the supernatent or (ii) clean-up on single column and injection of the supernatent
- protein precipitation with acetonitrile (v/v) followed by centrifugation and injection of the supernatent
- protein precipitation with methanol (1v/9v) followed by centrifugation , clean-up and injection
- plasma clean-up on a single column (Bond Elut) and injection
- plasma ultrafiltration, clean-up and injection.

The following procedures have been selected:

Sample treatment A: 500 µL of spiked sample was diluted with an equal volume of TCA (10 % aqueous solution) under vigorous shaking to precipitate proteins. After 10 min. centrifugation at 2200 r.p.m., the

supernatent was removed and followed by a clean-up on a C18 single column (Bond Elut) previously conditioned with methanol and acetate buffer at pH 5.0. The final solution was passed through a 0.45 μm filter and then injected into the HPLC column.

Sample treatment B: 500 μl of the sample were spiked with an equal volume of a 0.1 M NaCl solution containing CDDP. The sample was filtered through a 0.45 μm filter and ultrafiltrated over an Amicon filter (m.w. cut-off 30,000) by centrifugation for 25 min. at 5200 r.p.m.. Prior chromatographic injection , a clean-up step was made by passing the sample through 100 mg of a strong anion exchange column SAX (Bond Elut.) then through a 100 mg C18 single column. Both columns were conditioned prior to use, by passing methanol and acetate buffer pH 5.0.

RESULTS AND DISCUSSION

Glassy carbon has already been recommended for the oxidative detection of CDDP after ion-pair HPLC (Table 1) . CDDP detection occurs at potentials higher than + 1.10 V. vs Ag/Ag^{+}, where risks of electrolyte oxidation and surface evolution may limit the performances of the detector. This implies that in addition to the chromatographic requirements , mobile phase compositions which may be incompatible or which may destroy the electrode matrix should be avoided.

Optimization of cationic surfactant based ion-pair chromatography.

Our first attempt consisted to follow the experimental procedures and the mobile phase composition reported in the literature (11,18,19). The results were found unsatisfactory due to marked signal unstability and rapid response decline . Such problems may be related to the ease of electrooxidation of the bromide ions of the ion - pairing reagent (HTABr) with release of bromine . The latter may destroy the GCE as suspected from the progressive build up of a yellow product onto the surface. Moreover, and in accordance with the literature, we have found that bromide ions displace the chloride ions from CDDP thus accelerating its degradation (24). By selecting cetyltrimethylammonium chloride (CTACl) as ion - pairing reagent, we have been able to maintain the separation efficacy, prevent CDDP loss by degradation and minimize the electrode destruction.

The optimization of the mobile phase composition consisted to vary its pH between 3 and 6 and the CATCl concentration between 50 μM and 0.5 mM. Higher concentrations of CTACl produced severe base line changes which limited the performance of the detector.By increasing the pH , the retention of CDDP increased. Increasing the temperature of the mobile phase from 25 till 32°C improved the resolution and showed a 15 % increase of CDDP peak height (10,18). In order to shorten the separation process

30 min. for plasma and 15 min. for A.F.), a mobile phase containing 56 μM CTACl at pH 5.0 has been selected. Higher pH values may accelerate the hydrolysis of CDDP (13,18). Under such operating conditions , and by applying a potential of + 1.16 V, linear calibration graphs were obtained between 30 ng and 6 μg/ml of CDDP in 0.1 M NaCl aqueous solution. The limit of detection, based on a signal to noise ratio of 3, was found to be 30 ng/ml.

Application .

The determination of CDDP in spiked plasma and artificial fluid required deproteinization and sample clean-up steps in order to separate CDDP from oxidable interfering substances such as uric acid, ascorbic acid , amino acids, proteins,... which might be present in the sample. Several procedures have been investigated (see experimental section) but the purification of the sample by precipitating the proteins and retaining the interferents onto a C18 single column has been found to be the most appropriate (see sample treatment A) . Figure 2 shows a typical chromatogram of 0.1M NaCl (Fig. 2 a), 0.1 M NaCl + CDDP 1μg/ml (Fig.2 b), plasma (Fig.2 c) and plasma freshly spiked with CDDP (14μg/ml) (Fig. 2 d). CDDP is well resolved from a first peak at tr = 2.0 min which corresponds to the sum of chloride ions plus interfering species (including CDDP degradation products) and from broad non - identified peaks (negatively charged oxidable products) at tr higher than 20 min. The detection limit of CDDP in plasma and A.F. was 150 ng/ml.

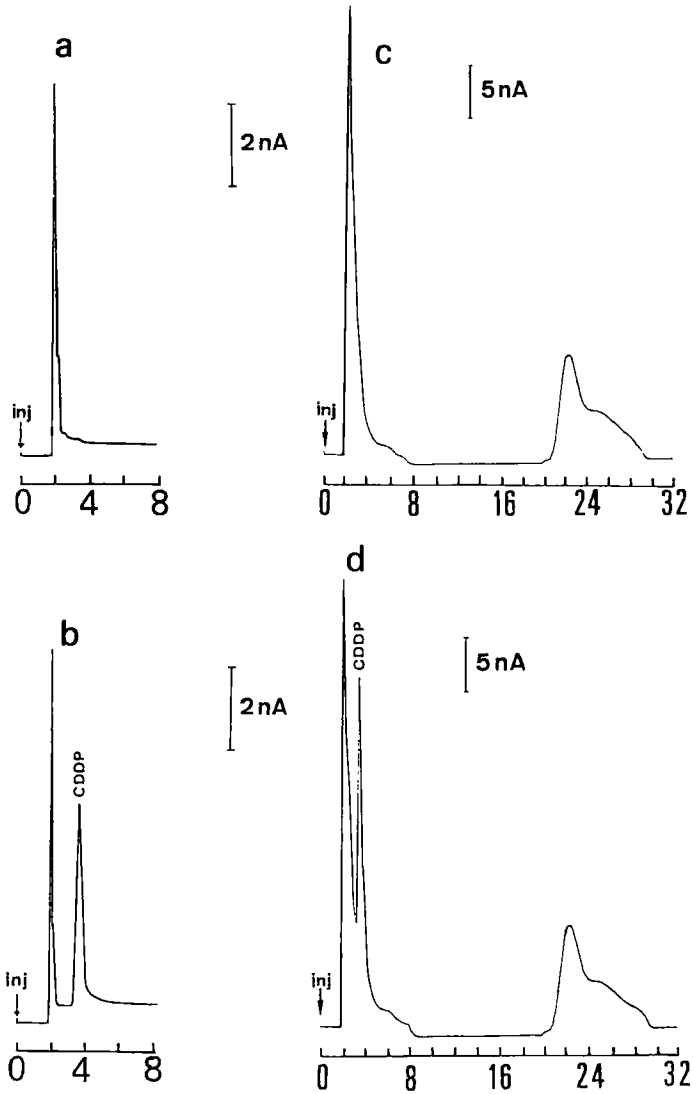

Figure 2.
Chromatograms of a: 0.1 M NaCl, b:0.1 M NaCl + CDDP (1μg/ml), c: human plasma, d: human plasma + CDDP (14 μg/ml). Mobile phase= acetate buffer pH 5.0 + 0.05 mM CTACl, 1 ml/min.

Optimization of anionic surfactant based ion-pair chromatography.

In order to determine CDDP and detect its hydrolysis products , ion-pair chromatography with anionic surfactants has been explored (14,15,25). The optimization of the mobile phase composition has been made by testing several ion - pairing reagents (see experimental section). The retention time of CDDP increased slightly by increasing the length of the hydrophobic chain of the surfactant and by raising its concentration from 2mM to 6 mM. Higher concentrations of surfactant had no influence on CDDP retention time but significantly increased the retention time of its hydrolysis products . In addition it had a positive effect on the stability of the base line .It should be pointed out , that the sensitivity towards CDDP and the interferents was significantly higher with the anionic than with the cationic surfactants. This might be due to an adsorbed layer of surfactant at the surface of the GCE which may have a negative (CTACl) or a positive (heptane sulfonic acid) effect on the detector sensitivity (26,27).

A 0.1 M sodium chloride solution showed one peak corresponding to chloride ions at tr = 2.8 followed by a non-identified peak at tr= 4.8(Fig.3 a). A fresh solution of CDDP showed, beside the chloride ion and the impurity peak , a peak which elutes at tr = 3.6 min (Fig. 3 b) while a 10 days aged solution of CDDP showed 3 additional and well-resolved peaks which may be attributed to CDDP

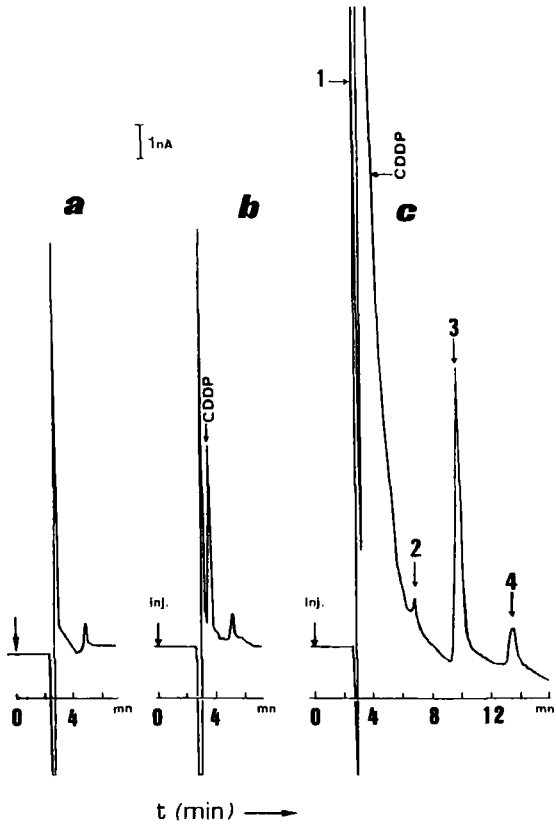

Figure 3.
Chromatograms of a: 0.1 M NaCl, b: 0.1 M NaCl + CDDP(0.48 µg/ml), c: aged solution of CDDP (30 µg/ml) in 0.1 M NaCl. Mobile phase : acetate buffer pH 4.3 + 10 mM HpSA, 1 ml/min.Peak 1 = Cl + CDDP hydrolysis product (neutral), peaks 2,3,4 : CDDP hydrolysis product (positively charged).

hydrolysis products (Fig. 3 c) (28). By varying the pH between 2.5 and 6 it has been found that a decrease in pH increases the retention time of interfering species, CDDP retention being not affected. To reduce the equilibration time between analysis, a mobile phase at pH 4.3 has been

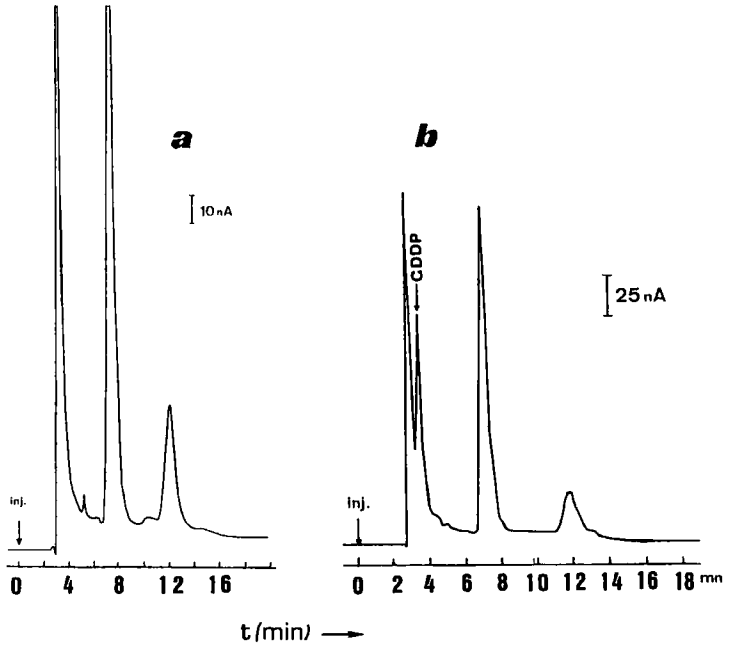

Figure 4.
Chromatograms of a: human plasma, b: human plasma + CDDP (18 µg/ml).
Mobile phase: acetate buffer pH 4.3 + 10 mM HpSA, 1 ml/min.

selected. Under these conditions , and in the presence of 10 mM heptane sulfonic acid (HpSA), the linear calibration range was comprised between 30 ng/ml and 13 µg/ml of CDDP in 0.1 M NaCl solution, the detection limit was 30 ng/ml.

Application.

One major drawback in anionic ion-pair chromatography is that the negatively charged interferents such as urate ,ascorbate, chloride ions... elute at tr = 2.8 min, i.e., close to CDDP elution (tr = 3.6 min) (Fig.4 a,b). Sample clean - up consisted to reduce proteins and oxidable

molecules to concentration levels compatible with the sensitivity required. Following sample preparation B (interfering anions retained on single column, CDDP and positively charged hydrolysis products eluted) the detection limit of CDDP was approximately 150 ng/ml and 2 μg/ml in A.F and plasma, respectively. The low sensitivity observed in plasma may be due to the presence of interfering anions not entirely eliminated by the clean up step.

This comparative study has shown that ion-pair HPLC with a cationic surfactant is the most appropriate mode for the sensitive and selective determination of intact CDDP in plasma . Under these conditions and by applying the sample treatment described above, the recovery of CDDP was 90 + 5% for the A.F and 86 ± 5% for plasma. The reproducibility of CDDP analysis in 0.1 M sodium chloride has been determined by performing successive injections (N=6) at different concentrations of CDDP. The coefficient of variation ranged between 2 and 4%. Due to the progressive response decline (surface fouling), the GCE was polished daily (15). The coefficient of variation of four measurements of CDDP in plasma (10 μg/ml) was 6 % .Since the slope of the electrode response varied considerably from day to day (the linear range and reproducibility remaining unchanged), calibration plots and sample analysis were made within the same day .

The study of the stability of CDDP in the filtrate of pretreated plasma and A.F. has shown that CDDP was stable at least for a period of 2 hrs (coefficient of variation (N= 6) ranging between 4 to 6 %).

Works are in progress to determine the pharmacokinetic profile of intact CDDP in plasma of patients which are under intensive care.

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