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DETERMINATION OF *cis*-DIAMMINEDICHLOROPLATINUM(II) IN HUMAN PLASMA USING ION-PAIR CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A thin-layer gold/mercury electrode and a hanging mercury drop electrode are compared as part of an evaluation of liquid chromatography (LC) with electrochemical detection (ED) for the determination of platinum species in human plasma ultrafiltrate. The platinum species, derived from an aged aqueous solution of the antineoplastic agent *cis*-diamminedichloroplatinum(II) (CDDP), can be separated by ion-pair chromatography. Variation of a number of parameters is described along with the limitations and advantages of each kind of electrode system. We have used our LC—ED technique to separate CDDP from its hydrolysis products and other non-platinum-containing species in human plasma ultrafiltrate with a detection limit of 62 ng/ml (ppb).

INTRODUCTION

Determination of total platinum

The recent use of platinum complexes for the treatment of several types of tumours has led to the development of a number of analytical methods for the determination of platinum in biological fluids. Most have been concerned only with the determination of total platinum and depend on sample pretreatment followed by atomic absorption spectroscopy (AAS) with electrothermal atomisation (ETA) [1—10]. Other methods for total platinum determination have included column liquid chromatography (LC) with precolumn derivatisation [11, 12] and more recently, a voltammetric technique based on differential pulse polarography (DDP) after chemical derivatisation [13]. These methods, while valid, give no information about the formal oxidation state of the metal, the nature of the ligands or the overall complex charge.

Several laboratories have developed techniques for separating proteins from plasma using centrifugal ultrafiltration membranes [14- 16]. The determination of platinum in plasma ultrafiltrate draws a distinction between protein-bound platinum and unbound (free) platinum, which may include species such as hydrolysis products and peptide- or amino acid-bound platinum.

Determination of cis-diamminedichloroplatinum(II) by LC

The most widely used platinum antitumour compound is a square planar complex *cis*-diamminedichloroplatinum(II) (CDDP), which, when formulated with sodium chloride and mannitol, is known by the trade name cisplatin. Several groups have described techniques for the specific determination of the complex in body fluids using LC to separate the parent drug from its metabolic products and other detectable substances in both plasma ultrafiltrate and urine [15, 17, 18]. Several kinds of stationary phase have been used to isolate CDDP. They include chemically bonded and solvent-generated anion-exchange columns [17], silica and C_{18} reversed-phase columns [18] and cation-exchange materials [19].

In most cases a UV absorption detector was used. One drawback with UV detection is the rather low molar absorptivity of CDDP: $\epsilon = 150$; λ max = 301 nm [18]. An improved detection limit is possible using precolumn derivatisation but this sacrifices the specificity for CDDP [12]. Many methods have utilized LC for separation and ETA-AAS for determination of platinum in eluent fractions [18, 20—22], however, the difficulties involved with interfacing LC to ETA-AAS limits its use.

Liquid chromatography with electrochemical detection (ED) for platinum antitumour compounds

The use of electrochemical detectors in LC has increased in recent years with the introduction of thin-layer solid-electrode cells [23, 24]. Most applications have involved substances which are easily oxidized at a glassy carbon or carbon paste electrode such as catecholamines [25]. Applications of reductive LC—ED have been fewer, owing to the inconvenience associated with mercury electrodes and the need to remove oxygen from the mobile phase. In the field of platinum drug analysis, thin-layer gold/mercury amalgam (Au/Hg) electrodes have been used in reductive LC—ED to detect CDDP at the 100 ng/ml (ppb) level in plasma [26, 27]. As an alternative to the thin-layer system, it is possible to interface the liquid chromatograph with a detector where the working electrode is a hanging mercury drop (HMDE). The application of such a system to the determination of platinum has been demonstrated [28, 29]. In one case [28], CDDP was separated from metabolites on a C_8 column with hexadecyltrimethyl ammonium bromide (HTAB) and detection compared for both the HMDE and dropping mercury electrode (DME). In another study, some second-generation platinum compounds (TNO-1 and TNO-6) were separated on a C_{18} column and detected by an HMDE using a DPP experiment [29].

Aqueous chemistry of CDDP

CDDP is known to undergo slow hydrolysis in aqueous solution. At low pH,

the reaction produces a number of cationic complexes which arise from nucleophilic substitution with water [30, 31]. These species may undergo base hydrolysis at high pH to form hydroxo complexes. An aged solution of CDDP is likely to contain several different platinum species in equilibrium (Fig. 1). The hydrolysis of CDDP has been well studied and it is known that the concentration of chloride ion has a marked effect on the equilibrium distribution [30, 31]. Even at moderately high chloride ion concentrations (17 m*M*), there is a stabilizing effect reducing the extent of hydrolysis [31]. For this reason the formulated drug (cisplatin) contains 90 mg sodium chloride in addition to 10 mg CDDP, which is reconstituted with 10 ml water to give 0.9% (w/v) sodium chloride or 150 m*M* Cl^- and 1000 mg/l CDDP.

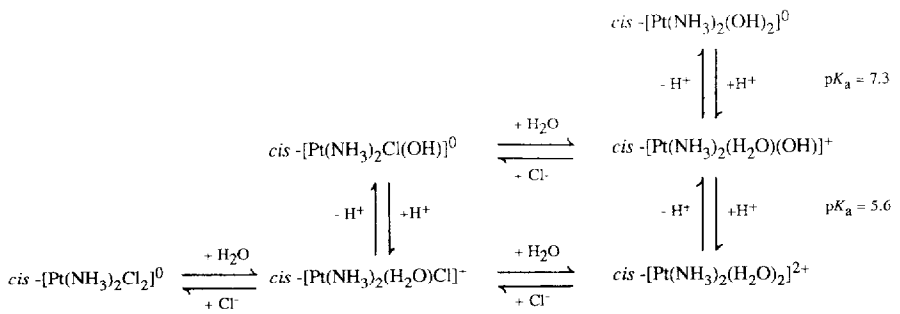

Fig. 1. Hydrolysis equilibria of *cis*-diamminedichloroplatinum(II) (CDDP) showing formation of mono- and dicationic species. At pH < 5 species reduce to three: parent CDDP, monochloroaqua and diaqua.

If an analytical method is to be useful for the study of platinum speciation, it should be able to separate the products of hydrolysis and detect low levels of CDDP (10^{-6} *M*). Ion-pair chromatography with ED appears to offer both the selectivity and sensitivity for such an assay. In addition, the use of an electrochemical detector is ideal for this application compared to ETA-AAS which, though very sensitive, does not lend itself to the rapid on-line detection of platinum required in clinical studies.

This investigation was undertaken to evaluate the suitability of on-line electrochemical detectors for the determination of platinum species separated by LC. A comparison of two kinds of electrodes (thin-layer Au/Hg electrode and HMDE) was made. Both kinds of detectors are amperometric since direct current is monitored at a fixed potential using stationary electrodes.

EXPERIMENTAL

Column liquid chromatography

A pulse damped Model 6000A solvent delivery system and Model U6K injector (Waters Assoc., Milford MA, U.S.A.) were used together with a 15 cm × 3.9 mm I.D. μBondapak C_{18} (10 μm) stainless-steel column also from Waters. The pump inlet was modified by replacing the PTFE tubing supplied with 2.25 mm I.D. stainless-steel tubing. Since PTFE is permeable to oxygen, this

procedure reduces oxygen contamination of the mobile phase. All sample injections were performed with a 25-μl blunt-tipped microsyringe (Hamilton). A Guard-Pak pre-column containing C_{18} inserts (Waters) was used to protect the column.

Electrochemical detection

Thin-layer Au/Hg electrode detector. A Model LC 4B amperometric detector (Bioanalytical Systems, West Lafayette IN, U.S.A.) was used with a Model TL-9A thin-layer transducer cell containing a gold/mercury amalgam (Au/Hg) working electrode and an Ag/AgCl reference electrode. The Au/Hg working electrode was prepared in accordance with the manufacturer's instructions [32]. Repolishing of the Au electrode was carried out initially using the polishing kit supplied. This consisted of three polishing surfaces: a 600-grit silicon mat, a 6-μm diamond polish and 0.05 μm alumina. Additional aluminium oxide polishing sheets (Moyco Industries, Philadelphia, PA, U.S.A.) were used to cover the range 63—0.3 μm.

HMDE detector. A Model 174A polarographic analyzer interfaced with a Model 303 static mercury drop electrode (SMDE), both from EG & G, Princeton Applied Research (Princeton NJ, U.S.A.), were used. The cell was modified with a Model 310 flow adaptor for LC detection. The mercury drop was dispensed in three sizes: large, medium and small corresponding to 0.0261, 0.0156 and 0.0096 cm^2 surface area [28].

Chromatograms were recorded on an Omniscribe chart recorder (Houston Instruments) with a full scale deflection (f.s.d.) of 10 V and chart speed of 2.54 cm/min (1 in./min). Polarograms were recorded on a Model 7046A X—Y plotter (Hewlett-Packard) with Y = 10 V f.s.d. X = 1.5 V f.s.d.

Atomic absorption spectroscopy with electrothermal atomisation

Total platinum determinations were carried out by ETA-AAS on plasma samples and on LC eluate fractions collected at 0.2-ml intervals using a Redirac fraction collector (LKB). An Instrumentation Laboratory Model 951 atomic absorption spectrophotometer and Model 555 controlled-temperature furnace (Allied Analytical Systems, Lexington, MA, U.S.A.) were used to determine platinum at 265.95 nm with a bandpass of 0.5 nm. The platinum hollow cathode lamp was run at 10 mA and background correction performed using a deuterium arc continuum source which was matched in energy throughput. The carbon furnace parameters, given in Table I, were optimised using pyrolytically coated tubes and a controlled-temperature feedback mode.

Materials, methods and sample preparation

Platinum complexes were supplied by the National Cancer Institute (Bethesda, MD, U.S.A.) and solutions made with triple-distilled and deionised water. The reagents hexanesulphonic acid, heptanesulphonic acid and octanesulphonic acid were obtained initially as concentrated solutions (Waters Assoc.) which were diluted with the mobile phase to give a concentration of 5 mM; heptanesulphonic acid, was obtained later from Kodak (Rochester, NY, U.S.A.) as the sodium salt. Sodium acetate trihydrate (HPLC grade) was supplied by J.T. Baker (Phillipsburg, NJ, U.S.A.) and triple-distilled mercury obtained from Bethlehem Apparatus (Hellertown, PA, U.S.A.).

TABLE I

INSTRUMENTAL PARAMETERS FOR THE DETERMINATION OF PLATINUM BY ETA-AAS USING THE IL 951-555 SYSTEM AFTER A 10-μl MANUAL INJECTION

Parameter		Setting
Wavelength (nm)		265.95
Band-pass (nm)		0.5
Lamp current (mA)		10.0
Furnace parameters*		
Dry_1:	Time (s)	45
	Indicated temperature (°C)	65
Dry_2:	Time (s)	45
	Indicated temperature (°C)	100
Ash_1:	Time (s)	35
	Indicated temperature (°C)	1000
Ash_2:	Time (s)	25
	Indicated temperature (°C)	1500
$Atomisation_1$:	Time (s)	0
	Indicated temperature (°C)	2700
$Atomisation_2$:	Time (s)	5
	Indicated temperature (°C)	2700
Tube clean		on
Temperature feedback		on

*Furnace parameters were established by constructing ash and atomisation curves to determine optimum temperature and observing the droplet during the drying phase.

Fresh human blood (O+), donated by P.J.P., was centrifuged to separate the plasma from red blood cells. The plasma was heparinized and incubated with 10 μg/ml CDDP, as the formulated drug cisplatin, at 37.0 ± 0.01°C in the dark. Aliquots were removed after 8 h, and again after two days and placed into Centriflo CF 50A ultrafiltration membrane cones (Amicon, Danvers, MA, U.S.A.), which were used to separate protein-bound platinum from platinum species of low molecular mass [50 000 molecular weight cut off (MWCO)]. The driving force for separation was provided by a Sorvall GLC-2B general laboratory centrifuge (DuPont Instruments) which at 2500 rpm was less than 1000 *g*.

RESULTS AND DISCUSSION

Mobile phase composition

Reversed-phase chromatography, modified with alkylsulphonic acids, appears an appropriate method to separate species of different charge such as hydrolysis products from CDDP in aged solutions of both the formulated drug (cisplatin) and the pure complex. Preliminary investigations showed that heptanesulphonic acid was most suitable for separating CDDP from other platinum-containing species. Therefore, the mobile phase composition used in subsequent studies consisted of 10 m*M* sodium acetate at pH 4.60 with 5 m*M* heptanesulphonic acid. The mobile phase was first filtered through a 0.45-μm membrane disk, degassed under vacuum and finally deoxygenated with purified nitrogen (later helium).

Mobile phase degassing and deoxygenation

The ease with which dissolved oxygen is reduced at low negative potentials presents a problem when using LC—ED in the reductive mode. The apparatus must be modified to prevent oxygen contamination of the mobile phase after it has been deoxygenated to prevent unnecessarily high background currents from interfering with the analysis. Removal of dissolved oxygen to levels compatible with a reductive electrochemical detector is achieved by passing a sparge gas through the solvent.

Initially, nitrogen was used with a four-stage scrubbing system, including an acidified solution of vanadium chloride with amalgamated zinc, as recommended by the manufacturer [33—35], followed by a distilled water wash to remove traces of the vanadium solution and then passage through an alkaline pyrogallol solution [29] followed by a solution of the mobile phase. This method substantially reduced background currents to levels typically around 100 nA, however, outgassing in the pumps and around the outlet to the electrode became a problem. Large variations in back-pressure caused by gas bubbles in the pump resulted in noisy baselines even with the pulse damper accessory installed. The appearance of gas bubbles at the outlet to the mercury drop using the Model 310 flow cell created local disturbances in the flow stream causing abrupt changes in the baseline and, in some cases, phantom peaks. These problems were overcome by changing the sparge gas to helium which is less soluble than nitrogen in aqueous solution.

Variation of mobile phase pH

A mobile phase of 10 m*M* sodium acetate at pH 4.60 was used initially. This is similar to that used by other investigators [26, 27]. The effect of varying the pH on the retention of platinum species showed no significant changes over the pH range 4.60–6.20. However, at pH 7.10, the oxygen peak broadened and samples of blank ultrafiltrate produced markedly different chromatograms. As the pH was increased, the retention of non-platinum-containing reducible species in plasma ultrafiltrate decreased and they eluted at a time which would have obscured any CDDP present. The optimum mobile phase pH was found to be 4.60.

Thin-layer solid-electrode detector

An initial study was undertaken to evaluate the thin-layer Au/Hg electrode. Aqueous solutions of both the pure complex (CDDP) and the formulated drug (cisplatin) were allowed to age at room temperature (22°C) in the dark. Samples of the fresh and aged solutions were injected onto the column and the resultant chromatograms recorded. In each case, a number of peaks were observed, some of which were later shown to be platinum-containing peaks by collecting fractions and determining the platinum content by ETA-AAS (Fig. 2). Dissolved oxygen, which is easily reduced at these low negative potentials, was identified as the peak eluting at about 3.8 min after injection (Fig. 2). The identity of the platinum-containing peaks was investigated using the HMDE detector and the results are discussed below. After several hours of use, it was apparent that some loss of sensitivity had occurred. Problems with the

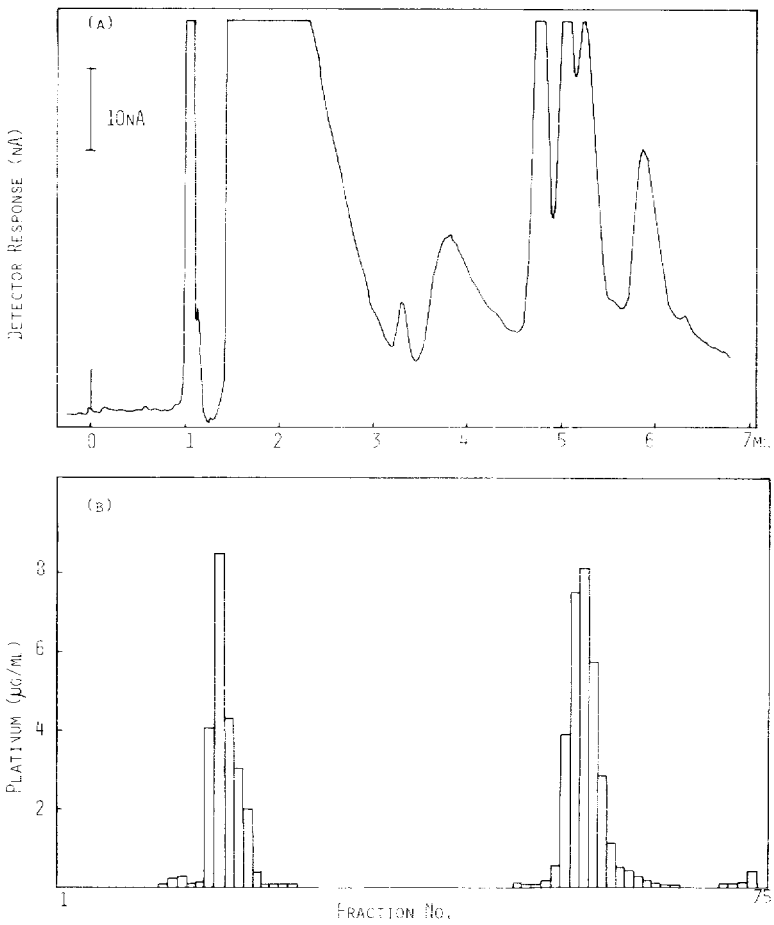

Fig. 2. Analysis of an aged solution of CDDP (10^{-4} *M*) using ion-pair chromatography to separate the platinum species with (A) electrochemical detection and (B) electrothermal atomisation atomic absorption spectrophotometry (ETA-AAS). Chromatographic conditions: column: μBondapak C_{18}; mobile phase: 10 m*M* sodium acetate containing 5 m*M* heptanesulphonic acid (pH 4.60); flow-rate: 0.5 ml/min; electrochemical detector: Bioanalytical Systems LC 4B; working electrode: Au/Hg amalgam, (potential: −100 mV); reference electrode: Ag/AgCl (RE-3); ETA-AAS detection: details given in Table I.

fouling of the Au/Hg electrode surface and day-to-day variations in sensitivity have been reported by other investigators [26, 27]. For this reason, an HMDE was investigated as an alternative to the thin-layer Au/Hg amalgam electrode.

Platinum polarography

In classical d.c. polarography the current at a DME is monitored as a function of applied potential. Much information can be obtained from the current–potential waves observed for a given electrochemical reaction. Several texts are available which describe the theory behind such experiments [36, 37]. The polarographic behavior of platinum(II) complexes, including CDDP, has been studied previously [38, 39]. Polarograms of CDDP were taken in

various modes and in different media. They agree generally with that reported by Sundholm [39]. The half-wave potential ($E_{1/2}$) for the reduction of CDDP in 0.1 *M* potassium chloride is reported to be −100 mV (vs. Ag/AgCl) at 35.5°C [38].

HMDE detector

Using the Model 310 flow cell adaptor with an HMDE, the current was monitored continuously at a fixed potential. Samples of aqueous solutions of CDDP and cisplatin, which had been aged in the dark, were injected into the same chromatographic system as used previously. The recorded chromatograms are presented in Fig. 3. The result for CDDP (Fig. 3A) shows some five peaks, of which four have been confirmed by ETA-AAS as platinum-containing; the fifth peak is dissolved oxygen in the sample.

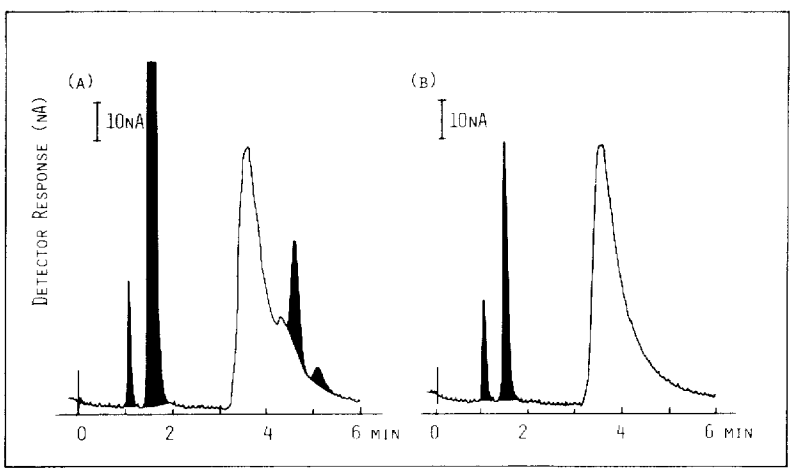

Fig. 3. Analysis of an aged solution of (A) 20 μl of 100 μ*M* CDDP (complex) and (B) 20 μl of 100 μ*M* cisplatin (formulated drug) using ion-pair chromatography to separate the platinum species and an HMDE detector. Platinum-containing peaks, as determined by ETA-AAS, are shown as shaded. Chromatographic conditions: column: μBondapak C_{18}; mobile phase: 10 m*M* acetate containing 5 m*M* heptanesulphonic acid (pH 4.6); flow-rate: 1 ml/min; detector: HMDE (EG & G, Princeton Applied Research, 174A/310), applied potential: −100 mV vs. Ag/AgCl.

A fresh solution of CDDP shows only one platinum-containing peak which elutes at 1.6 min. This peak, identified as the neutral complex CDDP, has a capacity factor (k') of 0.53. An attempt to improve retention was made by increasing the concentration of heptanesulphonic acid from 5 to 10 m*M*. Retention was improved for oxygen and the two platinum species eluting at 4.6 and 5.1 min, shown in Fig. 3A (see also Fig. 5). Capacity factors for some of these species with both 5 and 10 m*M* heptanesulphonic acid are given in Table II. The retention of CDDP was not affected by changing the concentration of ion-pair reagent; this suggests that ion-pairing does not take place with the neutral complex, but instead, the mechanism of retention probably involves hydrophobic interaction of CDDP with the reversed-phase C_{18} moieties.

An aged solution of CDDP was treated with an appropriate amount of silver nitrate to precipitate silver chloride and promote the formation of diammine-

TABLE II

CAPACITY FACTORS (k') AND RETENTION TIMES (t_R) FOR PLATINUM SPECIES SEPARATED BY ION-PAIR CHROMATOGRAPHY

Chromatographic conditions: mobile phase: 10 m*M* sodium acetate, pH 4.60; flow-rate: 1 ml/min; column: 15 cm × 3.9 mm I.D., μBondapak C_{18}; detector: EG & G, Princeton Applied Research 174A/310 polarographic cell, operated at 100 mV vs. Ag/AgCl in HMDE mode with small drop size; injector: Waters U6K with a 25-μl manual injector (precision: R.S.D. = 3.6%, n = 5).

Peak	Event	5 m*M* Heptanesulphonic acid		10 m*M* Heptanesulphonic acid	
		t_R (min)	k'	t_R (min)	k'
V_0	Solvent front/platinum (?)	1.1	-	1.1	-
V_1	CDDP	1.6	0.53	1.6	0.53
V_2	Oxygen	3.6	2.41	5.4	4.31
V_3	Platinum (?)	4.6	3.35	6.6	5.42
V_4	Diamminediaquaplatinum	5.1	3.82	7.4	6.19

diaquaplatinum(II). After 30 min, CDDP (the parent complex) could not be detected by LC—ED, but the platinum peak at 7.4 min had increased. Excess silver ions eluted between 6.4 and 7.1 min and thus obscured the platinum-containing peak at 6.6 min. It is concluded therefore, that the peak at 7.4 min is probably diaqua complex. The other platinum-containing peaks have not yet been firmly identified but are most likely some of the other hydrolysis products shown in Fig. 1. These platinum-containing peaks were unexpected, given previous reports of the polarographic inactivity of monoaqua and diaqua derivatives of CDDP [28, 38]. Further, when the chromatogram for the aged complex (Fig. 3A) is compared to the corresponding histogram of platinum content, as determined by ETA-AAS, in fractions collected from the column, it would seem that substantially more

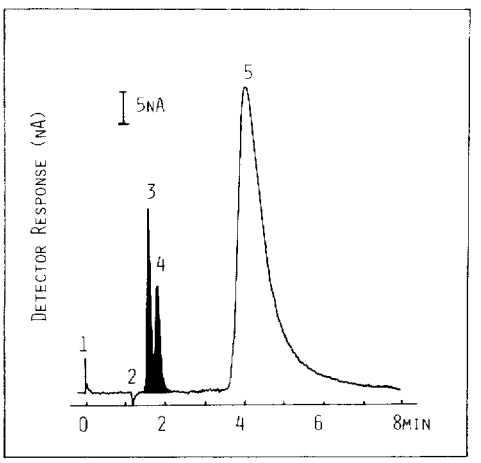

Fig. 4. Analysis of a fresh 25-μl mixture of *cis*- and *trans*-isomers of diamminedichloroplatinum(II) (5.5 μg/ml) using LC—ED at 0.0 V vs. Ag/AgCl. Peaks: 1 = injection event; 2 = solvent front; 3 = *cis*-isomer; 4 = *trans*-isomer; 5 = oxygen.

platinum elutes after oxygen than suggested by a simple ratio of the platinum peak heights or areas. It is known that the polarographic activity of square planar platinum II complexes is highly dependent on the nature of the ligands which are coordinated to the metal ion [38- 40]. At the moment, it has not been possible to quantitate the other platinum species derived from the parent compound, but this problem is still under investigation.

A chromatogram of an aged solution of cisplatin (formulated drug), in Fig. 3B, shows only two platinum-containing peaks, one of which is the neutral complex CDDP. The other species, which elutes with the solvent front, has not been firmly identified but is also found in the chromatogram of the aged complex CDDP shown in Fig. 3A. The corresponding geometrical isomer, *trans*-diamminedichloroplatinum(II) (NSC 131558), which does not exhibit antineoplastic activity, elutes with a retention time of 1.8 min and is well

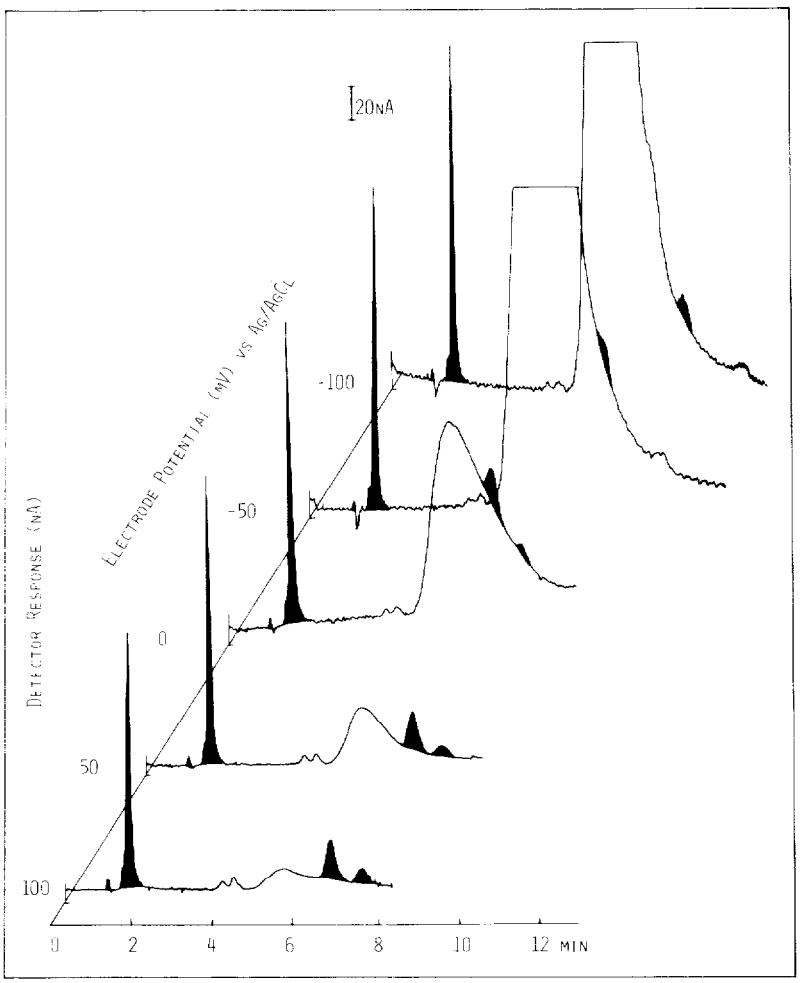

Fig. 5. Variation of cell current as a function of applied potential (E). The resultant effect on the chromatogram of 20 μl aged CDDP (5.5 μg/ml) for the potential range +100 mV to −100 mV vs. Ag/AgCl. The heptanesulphonic acid was increased from 5 to 10 mM to increase the retention of the paltinum species eluting after oxygen.

resolved from the *cis*-isomer when a fresh mixture of the two is chromatographed (Fig. 4).

Variation of applied potential (E)

A Model 310 flow cell with an HMDE was used to detect platinum species and the applied potential was varied from +100 to - 400 mV (vs. Ag/AgCl) in steps of 50 mV. The resultant chromatogram, recorded at each potential, changes in various ways. As E becomes more negative, the baseline noise increases and the oxygen peak becomes much larger whilst the platinum peaks increase only slightly (Figs. 5 and 6). The effect of changing E on the peak caused by dissolved oxygen has been reported by others [29].

Reactions of CDDP with human plasma

Formulated CDDP (10 μg/ml) was incubated with fresh heparinized human

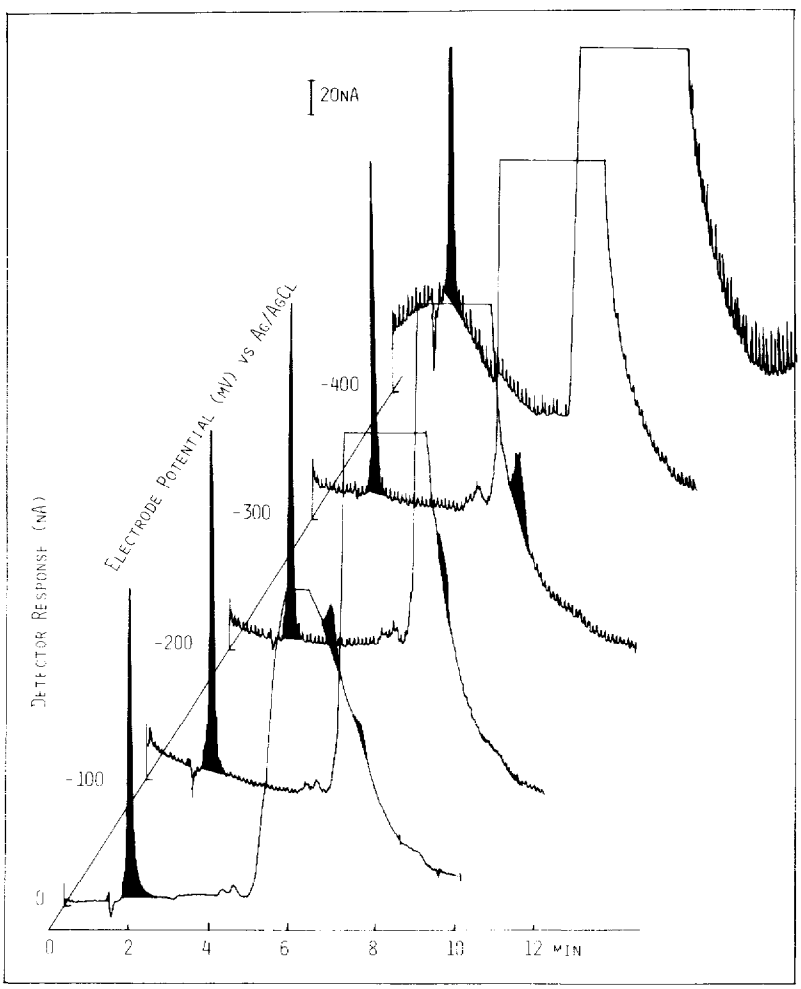

Fig. 6. Variation of cell current as a function of applied potential (E). The resultant effect on the chromatogram of 25 μl aged CDDP (5.5 μg/ml) for the potential range 0.0 mV to −400 mV vs. Ag/AgCl.

plasma at 37.0 ± 0.01°C in the dark. Aliquots were removed after 8 h and again after two days. Ultrafiltration, with CF 50A membrane cones (Amicon), was employed to separate the protein-bound platinum from the unbound platinum (MWCO: 50 000). The ultrafiltrate was injected directly without pretreatment into the system which also contained a C_{18} guard column. The recorded chromatograms are presented in Figs. 7 and 8. The presence of several oxidizable substances in human plasma caused several large negative peaks when E was 0.00 V (vs. Ag/AgCl). Moving E to more negative potentials reduced these oxidation peaks but both the baseline noise and the peak from dissolved oxygen in the sample increased. Dissolved oxygen in the sample was not removed for a number of reasons: its peak did not overlap with that of CDDP, the principal platinum species of interest; moreover, it was required that the preparation time be as short as possible for later kinetic studies.

CDDP is well resolved both from the oxidizable and reducible non-platinum

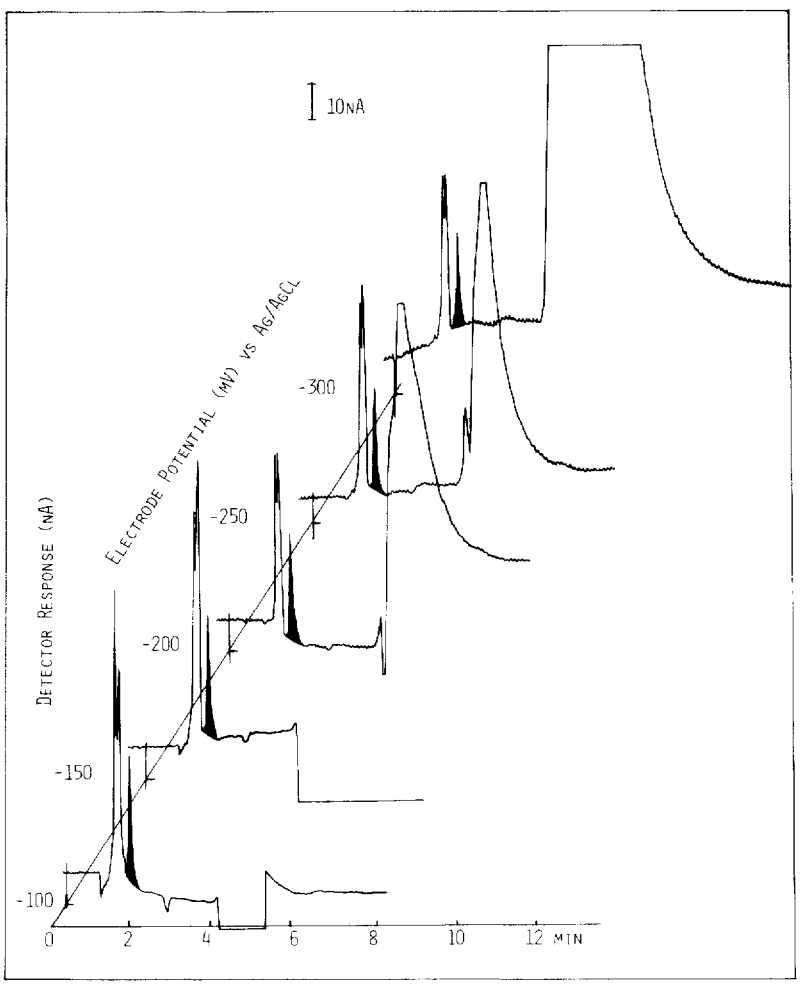

Fig. 7. Variation of cell current as a function of applied potential (E). The resultant effect on the chromatogram of 25 μl plasma ultrafiltrate 8 h after in vitro incubation with 10 μg/ml cisplatin for the potential range −100 mV to − 300 mV vs. Ag/AgCl.

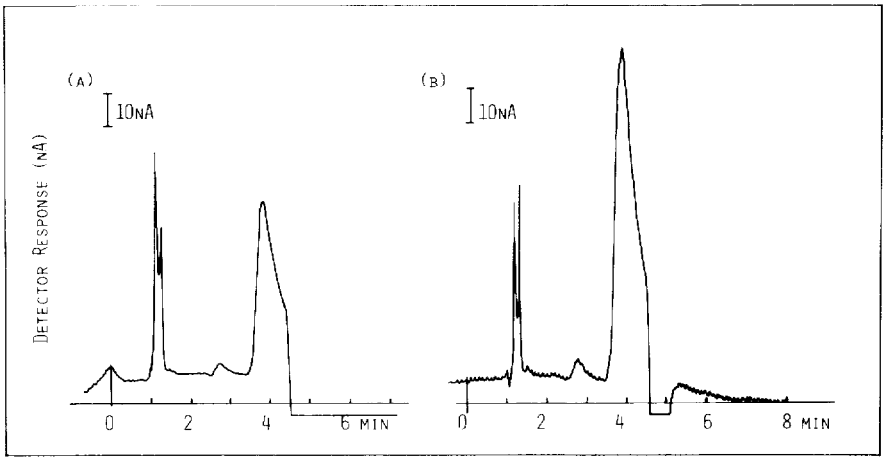

Fig. 8. Analysis of 25 μl plasma ultrafiltrate two days after in vitro incubation with 10 μg/ml cisplatin using an HMDE at (A) −100 mV and (B) −250 mV vs. Ag/AgCl. Note: fractions were collected from a similar sample of plasma ultrafiltrate and the platinum content determined by ETA-AAS. A trace amount of platinum (10 ppb) was detected in the fraction which eluted at 1.6 min, i.e., that corresponding to parent CDDP.

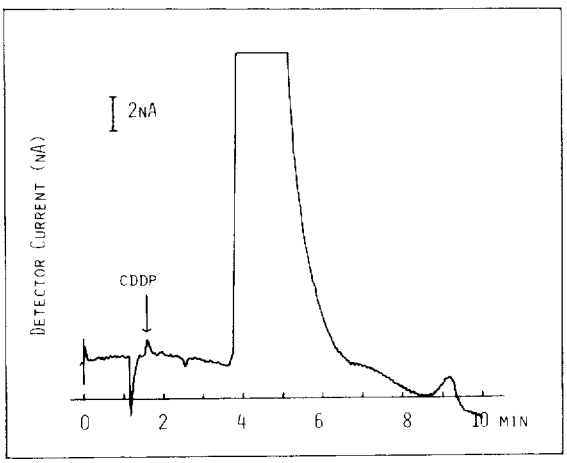

Fig. 9. Analysis of a fresh solution of 100 ng/ml CDDP using a large-drop HMDE at −100 mV vs. Ag/AgCl.

artifacts in human plasma. The working detection limit, based on a signal which is twice the noise, is 62 ng/ml CDDP with a 25-μl injection and using a large-drop HMDE detector (Fig. 9). Calibration curves were linear over several orders of magnitude for both small- and large-drop modes, moreover, a significant increase in sensitivity is achieved using the HMDE in the large mode.

Analysis of the plasma after two days incubation showed that CDDP had fallen below the detection limit of the LC—ED technique (Fig. 8). When fractions were collected and subjected to ETA-AAS, a trace of platinum (10 ppb) was found in the fraction where CDDP elutes (1.6 min). Since no other fraction contained detectable platinum, we must conclude that any other platinum species formed in plasma, which may be present in the ultrafiltrate, are either below the detection limit of the present technique or more likely, retained on the column.

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