Journal of Chromatography, 229 (1982) 373—386
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1190

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PLATINUM COMPLEXES ON SOLVENT GENERATED ANION EXCHANGERS

III. APPLICATION TO THE ANALYSIS OF CISPLATIN IN URINE USING AUTOMATED COLUMN SWITCHING

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(First received August 11th, 1981; revised manuscript received December 2nd, 1981)

SUMMARY

Platinum complexes are retained on solvent generated anion exchangers, prepared by coating reversed-phase (C-18) supports with a monolayer of hexadecyltrimethylammonium bromide. The retention mechanism is described in terms of ion—dipole interactions in the stationary phase, reinforced by a hydrophobic effect. The high degree of ligand selectivity exhibited by these systems arises from the use of purely aqueous mobile phases which maximize the differences in solute dipole and hydrophobic surface area. By using stationary phases of different surface characteristics and the application of automated column switching, the technique is applicable to the clinical analysis of cisplatin in urine. After chromatography, the purified cisplatin fractions are determined by atomic absorption spectrophotometry. The recovery of cisplatin from urine is 101.1% with a relative standard deviation of 3.6% and the limit of detection is 2 μg/ml.

INTRODUCTION

The discovery that platinum complexes are useful in the treatment of many solid tumors [1] has led to the increased interest in the analysis of such species in pharmaceutical formulations [2, 3] and biological fluids [1, 4—10]. At present the greatest interest surrounds the analysis of cisplatin (CDDP) which is widely used clinically [1]. It is estimated [1] that over 1100 analogues of cisplatin have been synthesized in an attempt to reduce toxicity and increase potency. Fig. 1 shows the structures of three cisplatin analogues currently undergoing clinical trials.

Although X-ray fluorescence [4] and atomic absorption spectrophotometry [6] provide the necessary sensitivity for monitoring platinum levels in

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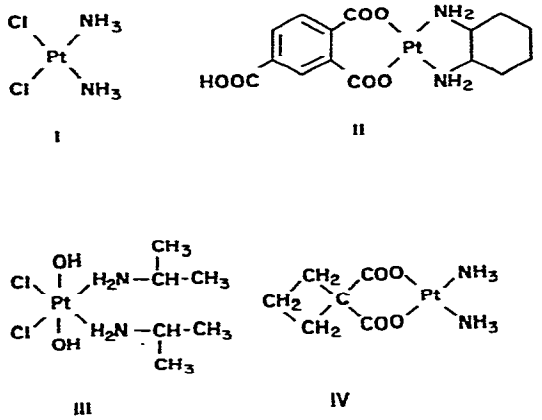


Fig. 1. Structures of some clinically investigated platinum complexes. I = *cis*-dichlorodiammine platinum(II) (cisplatin, CDDP); II = *cis*-1,2-cyclohexyldiamine-4-carboxyphthalate platinum(II); III = *cis*-dichlorodiiospropylamine-*trans*-dihydroxy platinum(IV) (CHIP); IV = *cis*-diammine-1,1-cyclobutyldicarboxylate platinum(II) (CBDCA).

clinical samples, they suffer from matrix effects when applied to urine analysis. Additionally, these techniques respond only to total levels of platinum and cannot differentiate between platinum complexes and their respective degradation and biotransformation products. The present primary need is a method for the determination of cisplatin in urine. Two high-performance liquid chromatography (HPLC) methods have been described [9, 10] for the analysis of cisplatin in urine. However, these methods require derivatization with diethyldithiocarbamate to enhance detection and respond only to total platinum. Selective HPLC methods [1, 5] have been reported for the analysis of cisplatin in plasma, involving the use of chemically bonded anion-exchange stationary phases and aqueous methanol mobile phases. The high methanol concentrations required for adequate retention of cisplatin are, however, incompatible with injections of urine. Additionally, poor peak shape, low selectivity and column instability are associated with these systems.

Very recently we have reported that solvent generated anion exchangers [11, 12] offer significant advantages over the chemically bonded stationary phases for the retention of cisplatin. These advantages include good column stability, high column efficiency and the possibility of using purely aqueous mobile phases.

The present study is concerned with the application of solvent generated anion-exchange systems to the analysis of cisplatin in urine. In addition, the effect of ligand substitution on selectivity is discussed using a functional group approach.

EXPERIMENTAL

Apparatus

The basic liquid chromatograph comprised of a Model 110A Altex pump (Beckman Instruments, Berkeley, CA, U.S.A.) a fixed-wavelength UV detector (280 nm, Altex Model 153) and an Altex injector (Model 210) fitted with a

20-μl loop. Three columns, Hypersil (5 μm, 50 mm × 4.6 mm I.D., Shandon Southern, Sewickley, PA, U.S.A.), ODS Hypersil (5 μm, 200 mm × 4.6 mm, I.D.) and ODS Ultrapak (10 μm, 150 mm × 4.6 mm, I.D., Beckman) were slurry-packed using standard procedures [13, 14].

For the analysis of cisplatin in urine, a microprocessor (SLIC Systec, New Brighton, MN, U.S.A.) controlled column switching system was used in conjunction with the Hypersil and ODS Ultrapak columns, a WISP 710 autoinjector (Waters Assoc., Milford, MA, U.S.A.) and a fraction collector (Model 273, Instrumentation Specialities, Lincoln, NE, U.S.A.) (Fig. 2). The two Altex pumps were modified by Systec and interfaced with the microprocessor. The flow-cell of the Altex detector was modified by replacing all the internal tubing with PTFE microbore tubing to minimize dead volume. The two columns were linked by a high-pressure six-port valve (Rheodyne, Cotati, CA, U.S.A.) interfaced with the microprocessor, so that fractions eluting from column 1 could either run to waste or be passed to column 2. Fig. 3 summarizes the program used for the separation and collection of the cisplatin fractions which were subsequently determined by atomic absorption.

Platinum determinations were made with a Varian Techtron Model 175B atomic absorption spectrophotometer (Palo Alto, CA, U.S.A.) coupled with a CRA-90 carbon rod atomizer. The platinum line was monitored at 265.95 nm with a constant lamp current of 10 mA. A three-stage heating program of 95°C for 45 sec, 1400°C for 15 sec and 2300°C for 0.5 sec was used with a ramp rate of 600°C sec^{-1}.

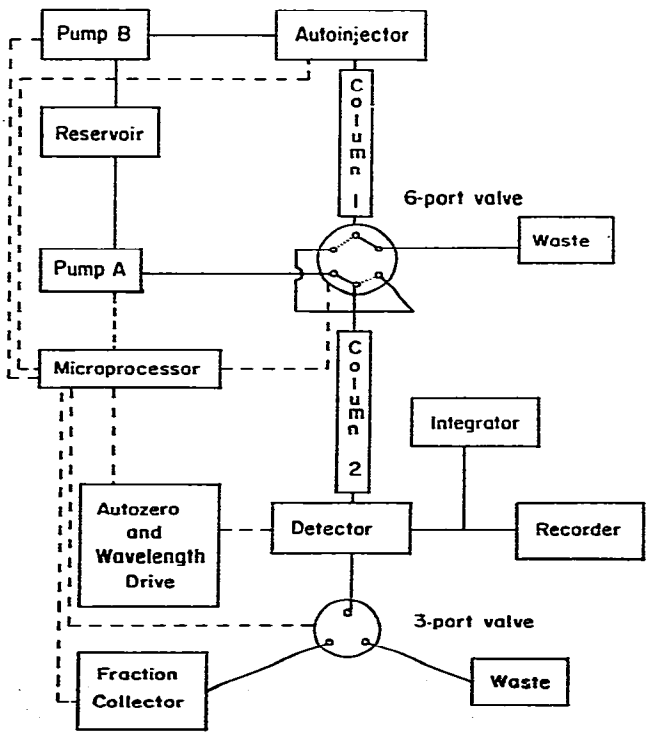


Fig. 2. Diagram of the automated chromatography system (see text for explanation).

Reagents

Reagent-grade chemicals and glass distilled water were used throughout. Crystalline samples of cisplatin (CDDP), *cis*-dichlorodiisopropylamine-*trans*-dihydroxyplatinum(IV) (CHIP), *cis*-diammine-1,1-cyclobutyldicarboxylate platinum(II) (CBDCA) and *cis*-1,2-cyclohexyldiamine-4-carboxyphthalate platinum(II) were obtained from the National Cancer Institute (Bethesda, MD, U.S.A.) and used as received.

Liquid chromatography

Solvent generated anion exchangers were prepared by pumping 0.5% (w/v) hexadecyltrimethylammonium bromide (HTAB) through the columns until a rise in baseline attributed to equilibration of the system, was observed. Mobile phases were filtered through 0.45-μm Millipore filters (Millipore, Bedford, MA, U.S.A.) and degassed in vacuo. Solute capacity ratios, k, were determined at least in duplicate according to eqn. 1 using ^2H_2O for the determination of t_0.

$$k = (t_r - t_0)\, t_0^{-1} \qquad (1)$$

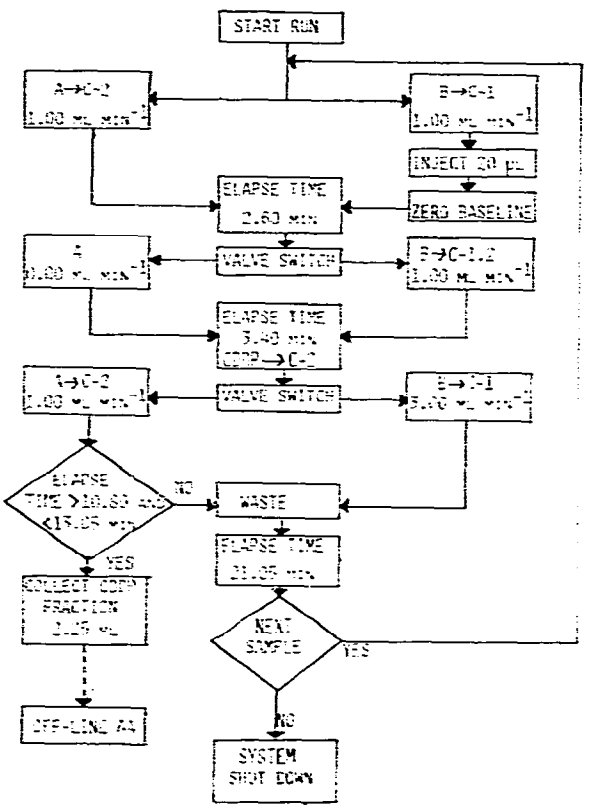


Fig. 3. Flow diagram of column switching program for the HPLC separation of cisplatin in urine. A, B, C-1 and C-2 refer to pumps A and B, column 1 (Hypersil) and column 2 (ODS Ultrapak), respectively. Arrows indicate the flow through the columns. The elapse times are from the point of injection. For further details see text and Fig. 2.

Divalent platinum complexes

Aqueous solutions containing mixtures of $[Pt(NH_3)_2Cl_2]$, $[Pt(CH_3)_2ClY]$ and $[Pt(NH_3)_2Y_2]$ (where Y = H_2O, Br, I, N_3) were prepared by incubating cisplatin in water and aqueous solutions of NaBr, NaI and NaN_3 (at 0.1 mol dm^{-3}) at 30°C for 2 h [15].

Urine analysis

Clinical urine samples (5 ml) were frozen rapidly and stored over solid carbon dioxide prior to analysis. The samples were thawed, sonicated for 2 min, filtered through 3-μm Millipore filters and chromatographed immediately. The purified cisplatin fractions, collected from the chromatograph were subsequently determined by atomic absorption spectrophotometry. Each sample was determined in duplicate and compared with calibration curves prepared by treating standard solutions of cisplatin in 0.1 mol dm^{-3} sodium chloride in an identical manner.

RESULTS AND DISCUSSION

Ligand selectivity

Previously the application of solvent generated anion exchanger HPLC has been restricted to cisplatin and no precedent exists for predicting the chromatographic behavior of cisplatin analogues and their biotransformation products. Accordingly, we have investigated the effect of ligand substitution on retention using divalent platinum complexes as model solutes and applying solvophobic theory [16] and a functional group approach [17—23] to the data. For this study a solvent generated anion exchanger consisting of 2.09 μmol m^{-2} HTAB adsorbed onto ODS Hypersil was used in conjunction

TABLE I

CHROMATOGRAPHIC RETENTION DATA FOR SOME DIVALENT PLATINUM COMPLEXES

Stationary phase: ODS Hypersil + 2.09 μmol m^{-2} HTAB; mobile phase: 10^{-4} mol dm^{-3} HTAB.

Solute	k^*	τ^{**}
cis-$[Pt(NH_3)_2Cl_2]^0$	1.05	—
trans-$[Pt(NH_3)_2Cl_2]^0$	0.30	—0.54
cis-$[Pt(NH_3)_2ClH_2O]^+$	0.10	—1.02
cis-$[Pt(NH_3)_2Br\,Cl]^0$	2.15	0.34
cis-$[Pt(NH_3)_2Br_2]^0$	4.63	0.64
cis-$[Pt(NH_3)_2Cl\,I]^0$	8.00	0.88
cis-$[Pt(NH_3)_2I_2]^0$	49.0	1.67
cis-$[Pt(NH_3)_2Cl\,N_3]^0$	3.00	0.46
cis-$[Pt(NH_3)_2(N_3)_2]^0$	4.55	0.63

*$k = (t_r - t_o)t_o^{-1}$ (eqn. 1).

**$\tau = \log(k_j \cdot k_i^{-1})$ (eqn. 2), where k_i = $Pt(NH_3)_2Cl_2$.

with a mobile phase of 10^{-4} mol dm^{-3} HTAB in water (Table I). The functional group contribution term, τ, is related to the chromatographic selectivity factor, α, by

$$\tau = \log \alpha = \log (k_j \cdot k_i^{-1}) \quad (2)$$

where the subscripts j and i refer to substituted and unsubstituted molecules, respectively. In the present study, cisplatin is taken as the reference compound (i.e., $j = [Pt(NH_3)_2ClY]$ or $[Pt(NH_3)_2Y_2]$ and $i = [Pt(NH_3)_2Cl_2]$).

The relationship for τ according to solvophobic theory is given by [16, 18, 19, 21, 22]:

$$\tau = [(a_j - a_i) + \gamma(\Delta A_j - \Delta A_i)]\ [2.3\,RT]^{-1} \quad (3)$$

where $(a_j - a_i)$ represents the differences in overall effects of dipole, charge and polarizability of the solutes, $(\Delta A_j - \Delta A_i)$ is the difference in hydrophobic surface area (HSA) of the solutes and γ is the mobile phase surface tension. The high selectivity of solvent generated anion exchanger systems for divalent platinum species (Table I) presumably arises from the use of a purely aqueous eluent which maximizes these differences in addition to providing a high surface tension (γ_{H_2O} = 72.0 mNm^{-1}). The retention order of the halogenated complexes indicates that hydrophobic differences play the major role in governing selectivity in these systems, the replacement of two chloride ions on cisplatin by two bromide ions produced a τ value which was approximately twice that obtained for the replacement of one chloride ion. Similarly, the τ value for the diiodo species was twice that obtained for the monoiodo species. In contrast, non-equivalency of the two azide groups was observed, indicative of different intramolecular interactions within one or both of the azide complexes, compared with cisplatin. The poor retention of the aquo species ($[Pt(NH_3)_2ClH_2O]^+$) arises primarily from electrostatic repulsion of the solute from the stationary phase by the adsorbed cationic surfactant. The weak retention of the *trans*-isomer of cisplatin arises from its lack of a molecular dipole and a reduced HSA.

In addition to rationalizing retention behavior, functional group analysis in HPLC has been used in drug design models [21, 23—30] and for the identification of drug metabolites and degradation products [23]. Consequently, this approach should prove useful in future studies of noble metal anti-tumor agents. Fig. 4 shows the preliminary results of the application of solvent generated anion exchanger HPLC to the analysis of three cisplatin analogues and demonstrates further the flexibility of this approach. With a purely aqueous mobile phase, III and IV were unretained, since presumably, they present a lowered HSA compared with cisplatin. In contrast the anionic complex, II, was strongly retained as a result of electrostatic interactions. The addition of 10^{-2} mol dm^{-3} citrate buffer (pH 7.0) enhanced the retention of III and IV due to an increase in the mobile phase surface tension [12, 13, 31] while decreasing the retention of II as predicted by conventional ion-exchange theory. The 4-carboxyphthalado complex, II, was found to be very unstable in aqueous solution and two major and two minor peaks were observed in a sample which was injected immediately after dissolution. The peak corresponding to

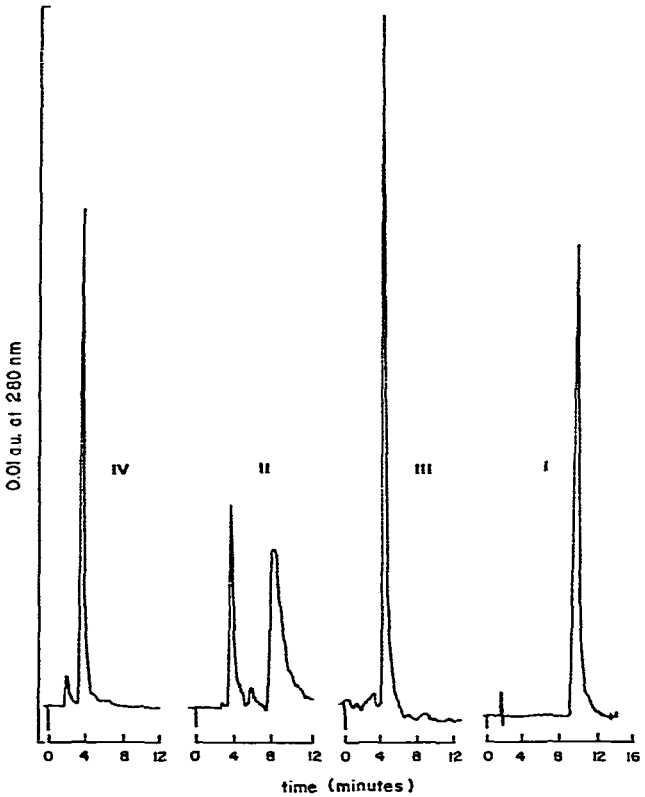


Fig. 4. Chromatograms of some clinically investigated platinum complexes. Stationary phase: ODS Hypersil + 2.09 μmol m^{-2} HTAB. Mobile phase: 10^{-2} mol dm^{-3} citrate buffer (pH 7.0)—10^{-4} mol dm^{-3} HTAB. Peak identification as Fig. 1.

II itself was broad and asymmetrical indicating significant degradation during chromatographic migration. Further studies at different wavelengths of detection have revealed several other degradation products with long retention times in solutions of this complex, II.

Nature of the stationary phase

A number of reversed stationary phases were investigated as supports for the adsorbed cationic surfactant (HTAB). Previous studies [11, 12] were performed on μBondapak C_{18}; however, the instability of this material with prolonged use and the considerable batch-to-batch variations between columns led to the subsequent use of ODS Hypersil and ODS Ultrapak. These spherical alkylsilicas having no residual silanols proved to be much more stable: for example, over 1000 injections of cisplatin in solutions of high ionic strength (0.5 *M*) were made on the ODS Hypersil column without any observable column deterioration. Additionally, ODS Ultrapak and ODS Hypersil gave similar uptakes of HTAB (ca. 2 μmol m^{-2}) which were higher than those observed for μBondapak C_{18} (ca. 1.3 μmol m^{-2}). This increased uptake of HTAB was associated with a proportional increase in the retention of cisplatin.

For the analysis of cisplatin in urine, a silica column (Hypersil) was used

for the primary clean up of the samples. Significant amounts of HTAB (0.63 μmol m^{-2}) were adsorbed onto the silica surface resulting in cisplatin retention. In the absence of adsorbed HTAB, cisplatin is not retained on silica gel [11] which is deactivated due to hydrogen bonding with water molecules. Thus, it appears that the silica column in this study is behaving primarily as a reversed-phase material with a reduced retention capacity [32].

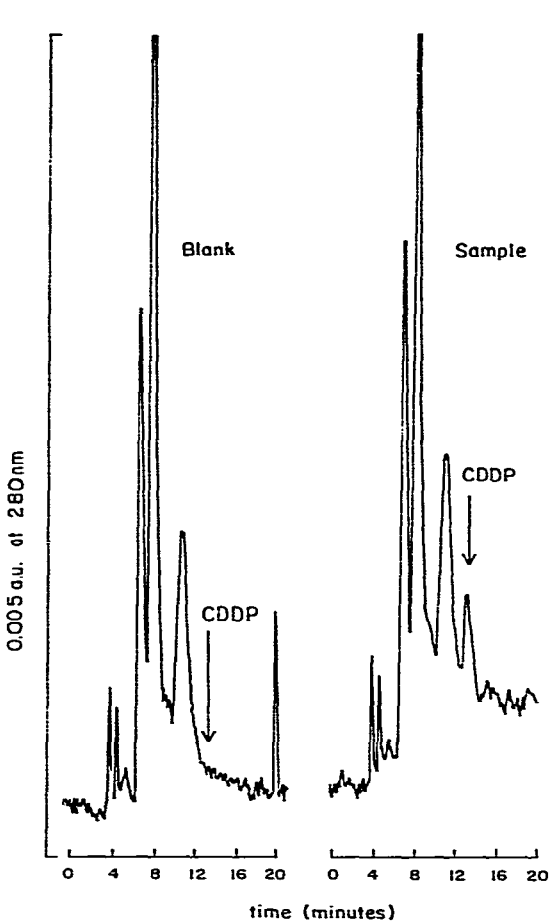
Analysis of cisplatin in urine

Column switching. A priori, purely aqueous mobile phases are preferred for the analysis of cisplatin since they produce the potential for maximum selectivity combined with optimum solute retention. However, water is the weakest solvent in reversed-phase systems. As a result cisplatin co-eluted with several urine components from the solvent generated anion exchanger (ODS Ultrapak + HTAB) using a mobile phase of 10^{-4} mol dm^{-3} HTAB in a citrate buffer (10^{-2} mol dm^{-3}, pH 7.0). The problem was exacerbated by the high retention of several urine components which were presumably anionic in character. The use of a silica pre-column (Hypersil + HTAB) only partially solved the problem. However, it was observed that cisplatin was retained on the silica pre-column and co-eluted with different urine components, i.e., the two stationary phases had different selectivities. Therefore, by use of these two columns and the application of column switching it was possible to resolve cisplatin from the urine components (Fig. 5).

The switching was designed so that only the fraction containing cisplatin was transferred from column 1 (Hypersil + HTAB) to column 2 (ODS Ultrapak + HTAB). After transferring cisplatin to column 2, the remaining material was vented from column 1 to waste at a higher flow-rate (3.00 ml min^{-1}). The required time for this clean-up step was measured by monitoring the eluent from column 1 by UV spectroscopy at 280 nm and determined the overall analysis time, which was 20 min. Some of the urine components were irreversibly adsorbed onto column 1, which had to be repacked after about 50 injections of urine. The same ODS Ultrapak column was used throughout this study without overall changes in performance. This stability reflects the superior quality of the packing material and the fact that very little of the urine was actually transferred to column 2. The different selectivities of the two columns were such that cisplatin eluted through the detector after all the material with which it co-eluted from column 1.

The relationship between cisplatin peak height and concentration was linear; however, its low absorptivity (ϵ ca. 100, 280 nm) limited this method of measurement to samples containing more than about 100 μg ml^{-1}. Therefore, measurement of cisplatin by off-line atomic absorption was preferred. By means of the three-port switching valve positioned after the detector (and also under microprocessor control), the cisplatin was collected as a single fraction (2.25 ml) and measured subsequently by atomic absorption. Fig. 6 shows that there was little lag time and sample dilution between the detector and the fraction collector. Thus, the window of the fraction collector could be set accurately by observation of the UV detector output.

The relationship between the platinum absorbance (A) and the concentration of cisplatin injected onto the chromatograph was linear over the range 0—250 μg ml^{-1} (eqn. 4)



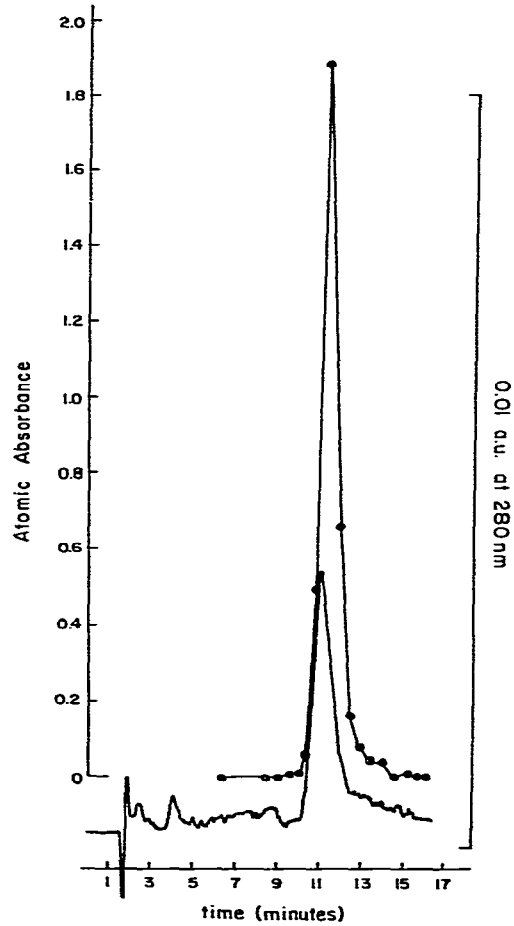


Fig. 5. Chromatogram of urine spiked with 100 μg ml^{-1} cisplatin (see text for conditions).

Fig. 6. Chromatograms of cisplatin obtained by on-line UV and off-line atomic absorption (•). The atomic absorption data were obtained by collecting 500-μl eluent fractions. CDDP concentration: 500 μg ml^{-1}.

$$A = (6.49 \cdot 10^{-4})\,[\mathrm{CDDP}] - 0.0011 \quad (r = 0.999,\ n = 6) \qquad (4)$$

At concentrations above 250 μg ml^{-1}, CDDP overloading of column 1 resulted in reduced efficiency of solute transfer from column 1 to column 2 and negative deviation from linearity. The limit of detection of cisplatin in urine injected onto the chromatograph was 2 μg ml^{-1} which could be measured by injecting 20 μl of the collected fraction onto the carbon rod atomizer of the atomic absorption spectrophotometer.

Cisplatin stability in urine

Cisplatin was found to be unstable when incubated in the urine of healthy volunteers at room temperature (ca. 22°C) over a 6—8-h period (Fig. 7). Considerable subject variation was observed (Fig. 7) and the amount of cisplatin lost varied between about 40% and 80%. The rate of loss of cisplatin on the

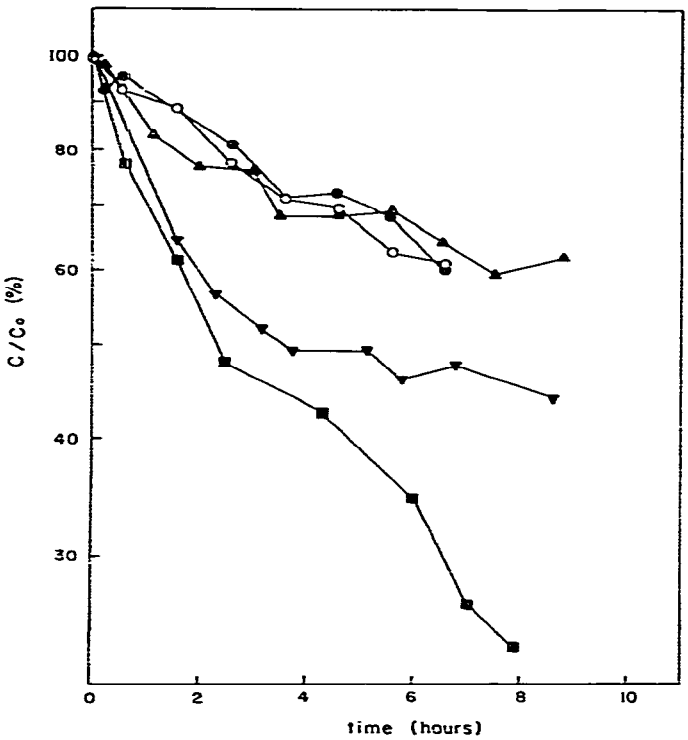


Fig. 7. The effect of subject variation on the loss of cisplatin from urine. Initial CDDP concentration: 200 μg ml^{-1}. The different symbols represent the data from samples of urine taken from four healthy volunteers. Open symbols (○), off-line atomic absorption measurements. Closed symbols (■, ▲) UV peak height measurements.

TABLE II

STABILITY OF CISPLATIN IN URINE AT —11°C AND —60°C

Temperature			
—11°C*		—60°C**	
Time (h)	CDDP concentration*** (μg ml^{-1})	Time (h)	CDDP concentration*** (μg ml^{-1})
0	51.6	0	46.2
11	41.4	1	45.8
23	36.2	2	45.4
30	32.6	3	48.4
48	32.1	4	48.4
		5	47.7
		8	46.2
		28	48.8
		48	43.8

*Refrigeration.
**Stored in solid carbon dioxide.
***Determined by HPLC and off-line atomic absorption.

other hand was independent of solute concentration. The data obtained from one subject was measured both by peak height and off-line atomic absorption (Fig. 7). There was no difference in the observed rate of loss of cisplatin by the two techniques but there was more scatter by peak height measurement, confirming off-line atomic absorption to be the method of choice for quantitation.

The loss of cisplatin in urine could not be totally arrested by storage at —11°C such that about 40% was lost after 48 h from a sample originally containing 50 $\mu g\ ml^{-1}$ (Table II). Cisplatin could only be successfully kept without significant degradation by storage over solid carbon dioxide (ca. —60°C). Statistical evaluation of the data in Table II revealed that the recovery of cisplatin from urine stored over solid carbon dioxide was 101.1% and the relative standard deviation of the assay was 3.6%.

Clinical samples

The developed methodology was applied to the determination of cisplatin in urine taken from three patients suffering from ovarian cancer. Cisplatin was administered (50 mg m^{-2}) by slow intravenous (i.v.) infusion at a rate of ca. 1 mg min^{-1} after prior dosage with cyclophosphamide (ca. 600 mg m^{-2} i.v.) and hydration with 0.45% sodium chloride and 5% dextrose (i.v.). Urine was voided before administration of cisplatin and the samples taken 30 min after completion of infusion. This procedure was designed to minimized the residence time of cisplatin in the bladder.

The urine concentration of unchanged cisplatin varied from 16.3 to 96.3 $\mu g\ ml^{-1}$ CDDP (equivalent to 10.6—62.6 $\mu g\ ml^{-1}$ Pt); however, the amounts and percentages of the total doses excreted were remarkably similar for each of the three patients (Table III). The total amount of platinum was determined by dilution of the sample (1:10 to 1:50 in water) to minimize matrix effects and direct atomic absorption spectrophotometry. It was found that between 75% and 95% of the drug was excreted unchanged in the urine (Table III).

CONCLUSIONS

The retention of platinum complexes on solvent generated anion exchangers is influenced by solute charge and ligand substitution. For neutral complexes, retention and selectivity are controlled by solute dipole and hydrophobic effects, whereas electrostatic effects dominate the retention of charged complexes such that cations are poorly and anions are strongly retained. The extrathermodynamic approach taken towards rationalizing the retention behavior of platinum complexes in reversed-phase systems employing secondary equilibria should prove useful in future studies, particularly in those aimed at identifying degradation and biotransformation products.

The inherent high selectivity of solvent generated anion exchanger systems towards platinum species may be attenuated by exposing the solutes to stationary phases of different surface properties during chromatographic migration. Furthermore, the use of this HPLC system together with a specific platinum detection system (off-line atomic absorption) produces a technique

TABLE III

URINE ANALYSIS OF THREE PATIENTS TREATED WITH CISPLATIN FOR OVARIAN CANCER

Patient	Total dose*,** (mg)	Urine analysis***						
		Concentration ($\mu g\ ml^{-1}$)		Amount (mg)		Percentage of dose		CDDP/Pt ratio (%)
		As CDDP**,§	Total Pt§§	As CDDP**,§	Total Pt§§	As CDDP	Total Pt	
1	39 (60)	10.6 (16.3)	13.4	4.7 (7.3)	6.2	12.2	16.1	75.8
2	65 (100)	39.1 (60.2)	41.0	8.8 (13.5)	9.2	13.5	14.2	95.1
3	58.5 (90)	62.6 (96.3)	75.6	6.2 (9.6)	7.6	10.6	13.0	81.5

*50 mg m^{-2} by i.v. infusion at ca. 1 mg min^{-1}.
**Values refer to amounts or concentrations of Pt. Values in parentheses refer to amounts or concentrations of CDDP.
***Urine collected ca. 30 min post infusion.
§ Determined by HPLC and off-line atomic absorption.
§§ Determined by direct atomic absorption.

capable of the unambiguous determination of cisplatin in urine. This technique requires minimal sample preparation and is easily automated.

The clinical results indicate that large amounts of cisplatin are excreted unchanged within the first 2 h of administration and that previous results [1] reporting low levels of cisplatin in urine, attributed to extensive biotransformation, may be due to degradation during storage either in the bladder or after sample collection. Finally, the presence of relatively high concentrations of cisplatin in urine may be related to the clinical findings of its utility in the management of bladder cancers [33].

ACKNOWLEDGEMENTS

This work was partially supported by grants CH-149 from the American Cancer Society, and CA-09242 and CA 24834 from the National Institutes of Health. The technical assistance of Ms. Stephanie A. Slyter is gratefully acknowledged.

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