High-Performance Liquid Chromatography of Cisplatin

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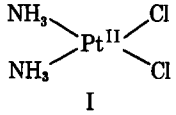
Abstract □ The retention behavior of cisplatin on a variety of stationary phases has been investigated using aqueous mobile phases modified by the addition of various electrolytes and methanol. Cisplatin is poorly retained on reverse-phase or silica columns but satisfactorily retained on chemically bonded or solvent-generated anion exchangers. The retention of the neutral complex on positively charged stationary phases is explained in terms of ion–dipole interactions and rationalized by the application of solvophobic theory. The use of solvent-generated anion exchangers for the analysis of cisplatin offers significant advantages over the chemically bonded system in terms of peak shape, column efficiency, and stability. By the use of column switching and off-line atomic absorption, solvent-generated anion exchange high-performance liquid chromatography (HPLC) is applicable to the determination of cisplatin in urine.

Keyphrases □ High-performance liquid chromatography—analysis for cisplatin in urine, solvent-generated anion exchange, column switching technique □ Cisplatin—analysis in urine, high-performance liquid chromatography using column-switching techniques

The demonstrated ability of platinum complexes to inhibit cell division in *Escherichia coli* (1) has led to extensive studies of their antitumor activity (2, 3). Although ~1300 platinum complexes have been investigated as potential anticancer agents (3), the prototype, *cis*-diamminedichloroplatinum(II) (cisplatin, structure I), remains the most widely used in the clinical setting.



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Cisplatin readily undergoes nucleophilic substitution (4, 5) with loss of chloride ion. These nucleophilic reactions have been implicated in its biological activity (3), biotransformation (3), and chemical degradation (3, 6, 7). Consequently, ligand selectivity is a necessary requirement of any analytical procedure for cisplatin. While X-ray fluorescence (8) and flameless atomic absorption spectrophotometry (9) provide the necessary sensitivities, these methods are nonselective. High-performance liquid chromatography (HPLC) offers the potential for good functional group selectivity (10); however, the poor solubility of cisplatin in nonaqueous media precludes the use of many conventional chromatographic techniques. Additionally, the UV absorptivity of cisplatin is low ($\epsilon = 150$ at 301 nm), and until a sensitive platinum detector is developed, off-line atomic absorption spectroscopy after separation is necessary for the determination of therapeutic drug concentrations in biological fluids. HPLC methods involving precolumn derivatization with diethyldithiocarbamate provide the required sensitivity (11, 12); however, they respond only to total platinum levels. Cisplatin has been retained on and eluted intact from strong anion exchangers (13); however, these systems suffer from poor peak shape and provide only moderate selectivities. Furthermore, the high concentrations of methanol required for adequate solute retention (>60%) are incompatible with injections of urine and efficient use of reaction detectors. This report describes the chromatographic behavior of cisplatin on anion-exchange columns and application to its analysis in biological fluid.

EXPERIMENTAL

Materials—Crystalline cisplatin was obtained from the National Cancer Institute[1] and used as received. All other chemicals were of reagent grade, except for the methanol which was HPLC grade[2]. Glass-distilled water was used throughout.

High-Performance Liquid Chromatography—The liquid chromatograph was comprised of a constant flow-rate pump[3], an injector[4] fitted with a 20-μl loop, and a fixed wavelength[5] (280 nm) UV detector. Six columns containing various stationary phases were used in the course of the study. Columns A[6], B[6], and C[7] contained reverse-phase material,

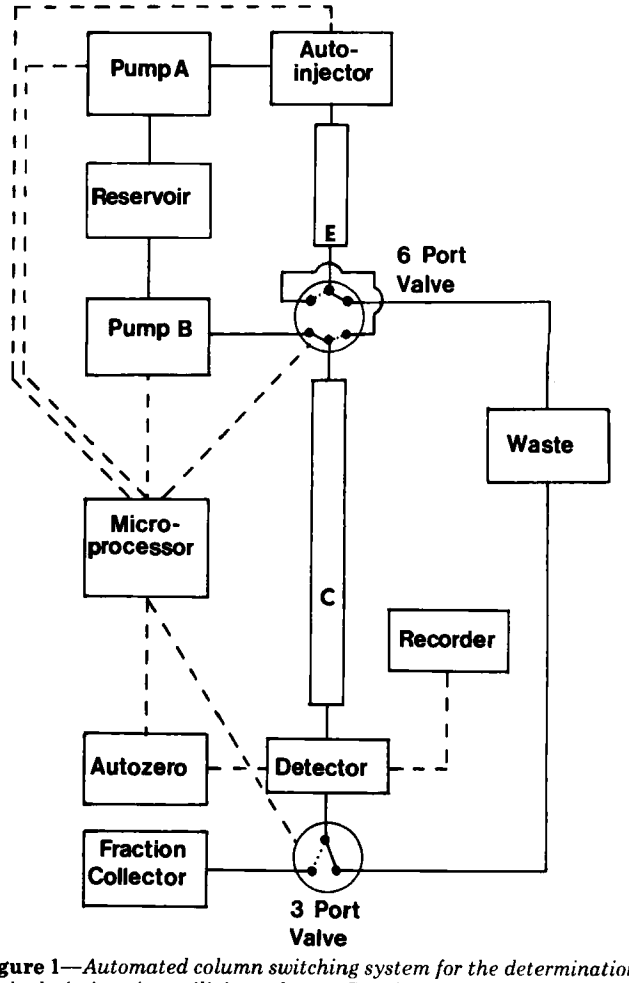

Figure 1—*Automated column switching system for the determination of cisplatin in urine utilizing columns C and E.*

[1] NCI, Bethesda, Md.
[2] Fisher Scientific Co., Fair Lawn, N.J.
[3] Altex model 110A, Beckman Instruments, Inc., Berkeley, Calif.
[4] Altex model 210, Beckman Instruments, Inc., Berkeley, Calif.
[5] Altex model 153, Beckman Instruments, Inc., Berkeley, Calif.
[6] μ-Bondapak C_{18} (10 μm, 300 mm × 3.9-mm i.d.), Waters Associates, Milford, Mass.
[7] ODS Ultrapak (10 μm, 150 mm × 4.6-mm i.d.), Beckman Instruments, Inc., Berkeley, Calif.

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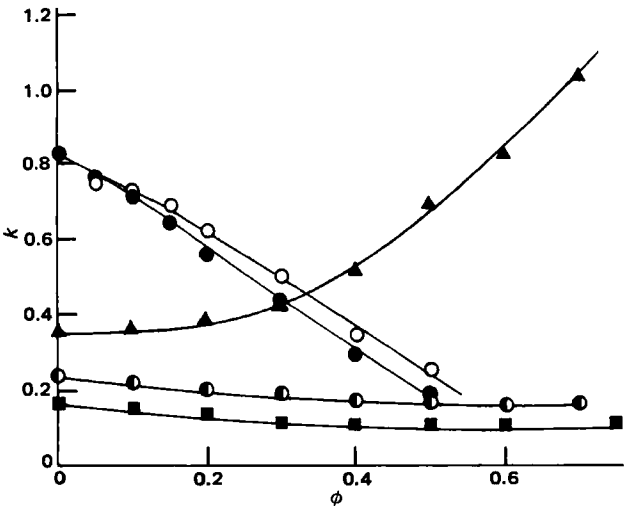

Figure 2—*The relationship between the capacity ratio of cisplatin (k) and the volume fraction (ϕ) of methanol in the mobile phase using various stationary phases at 30°. Key: (▲) column F, (■) column D, (◐) column A, (○) column A plus 0.99 μmoles/m² of 10^{-4} M hexadecyltrimethylammonium bromide, and (●) column A plus 0.99 μmoles/m² of 10^{-5} M hexadecyltrimethylammonium bromide.*

columns D[8] and E[9] contained silica gel, and column F[10] was a strong anion exchanger. Columns A, B, D, and F were obtained from commercial sources; columns C and E were slurry packed using standard procedures (14, 15).

Solute capacity ratios (k) were determined in duplicate by:

$$k = (t_r - t_0)/t_0 \qquad \text{(Eq. 1)}$$

using deuterium oxide as the unretained marker, where t_r and t_0 are the elution times of the solute and deuterium oxide, respectively.

Solvent-generated anion exchangers were prepared by the adsorption of hexadecyltrimethylammonium bromide onto the surfaces of columns A, B, C, and E. Solutions (0.5% w/v) of hexadecyltrimethylammonium bromide were pumped through the columns until a rise in baseline, attributed to equilibration of the system, was observed (16). After each working day (~10 hr) the columns were washed with water and then with successively increasing concentrations of methanol in water (up to 50%), except column F which was regenerated according to the manufacturer's specifications.

Chromatography was performed either at room temperature (~22°) or at 30° in which case the column was thermostated by the use of a water jacket[11] and a recycling water heater[12].

For the analysis of cisplatin in urine, an automated column-switching system was used (Fig. 1). Columns C and E were connected in series *via* a six-port switching valve[13], which was positioned so that the eluent from column E either could be passed to waste or transferred to column C. A three-port switching valve[14] was positioned after the detector so that the eluent from column C either could be passed to waste or transferred to the fraction collector[15]. All chromatographic events were controlled externally by the use of a microprocessor[16]. A mobile phase of 10^{-2} M citrate buffer (pH 7.0) containing 10^{-4} M hexadecyltrimethylammonium bromide was delivered from a common reservoir by two pumps[3] at a rate of 1.0 ml/min through both columns.

The urine samples were injected automatically[17] onto column E and eluted to waste for 1.6 min, after which time the valve was switched and the fractions containing cisplatin were loaded onto column C. After loading column C, the valve was returned to its original configuration and the remaining endogenous material eluting from column E was vented to waste at a high flow rate (3.0 ml/min). At the same time, cisplatin was separated on column C from the endogenous material with which it coeluted from column E. After passing through the detector, the eluent fraction containing cisplatin was collected and subsequently analyzed for platinum content by atomic absorption. The cisplatin concentration was determined by comparison with calibration curves prepared by treating standard solutions of cisplatin (2–200 μg/ml) in 0.9% NaCl (normal saline) in an identical manner.

Platinum Determination—Platinum determinations were made on an atomic absorption spectrophotometer[18] fitted with a carbon rod atomizer. A constant lamp current of 10 mA was used and the platinum line monitored at 265.95 nm. A three-stage heating program was used: 95° for 45 sec (dry), 1400° for 15 sec (ash), and 2300° for 0.5 sec (atomize) with a ramp rate of 600°/sec. Depending on concentration 2–20-μl samples were injected directly onto the carbon rod.

Clinical Urinalysis—Urine samples (10 ml) were taken from three patients treated with cisplatin (50 mg/m²) for ovarian cancer. Urine specimens were divided (providing duplicate samples), frozen rapidly, and stored over solid CO_2 prior to analysis. The samples were thawed, sonicated for 2 min, passed through 3-μm filters[19], and analyzed immediately. The cisplatin concentration was determined by the aforementioned HPLC procedure, and the total platinum concentration was determined directly by atomic absorption after appropriate dilution with normal saline.

Table I—Relationship Between τ Values of Electrolytes Added to the Mobile Phase and the Capacity Ratios of Cisplatin [a]

Electrolyte	τ [b]	k [c]
Citrate buffer (pH 7)	3.12	3.97
Sodium sulfate	2.73	3.22
Sodium nitrate	1.32	0.86
Sodium bromide	1.06	0.74

[a] Mobile phase: 10^{-4} M hexadecyltrimethylammonium bromide, 0.1 M electrolyte. Stationary phase: 1.31 μmoles/m² hexadecyltrimethylammonium bromide supported by column B. Temperature: 30°. [b] Constants describing the effect of electrolyte on the surface tension of water (Eq. 3, Ref. 21). [c] $k = (t_r - t_0)/t_0$ (Eq. 1).

RESULTS AND DISCUSSION

The Chromatography of Cisplatin—Due to its very low solubility in organic solvents, the chromatography of cisplatin is limited to systems employing aqueous mobile phases modified by polar solvents such as methanol. The retention of cisplatin on various stationary phases was investigated using aqueous methanol mobile phases (Fig. 2). Cisplatin was poorly retained (k = 0.25, Eq. 1) on both silica (column D) and hydrophobic (column A) reverse-phase columns, and its retention decreased slightly with an increase in methanol concentration. In contrast, cisplatin was significantly retained on stationary phases possessing cationic functionalities in the form of either chemically bonded (column F) or physically adsorbed (column A modified by a monolayer of hexadecyltrimethylammonium bromide) quaternary ammonium groups. By the observation of breakthrough times (16) it was found that a monolayer of 0.99 μmole/m² hexadecyltrimethylammonium bromide was adsorbed onto the hydrophobic stationary phase (column A). With a purely aqueous mobile phase, the stability of the modified stationary phase was maintained by a low concentration (10^{-4} or 10^{-5} M) of surfactant in the mobile phase. The addition of methanol to the mobile phase resulted in displacement of some of the surfactant from the stationary phase. With 10^{-5} M hexadecyltrimethylammonium bromide in the mobile phase, partial displacement of the adsorbed surfactant was observed when the methanol volume fraction ϕ exceeded 0.10, and successive increases in methanol concentration produced further displacement. With 10^{-4} M surfactant in the mobile phase no displacement of adsorbed surfactant was observed at $\phi \leq 0.15$. These observations indicate that the surface of the modified reverse-phase column consisted of a mixture of adsorbed surfactant and methanol, the proportions of which were determined by the composition of the mobile phase.

Although cisplatin was retained on both chemically bonded and solvent-generated anion exchangers, Fig. 2 indicates that the retention mechanism and the influence of methanol was different in the two systems. Initially, with a purely aqueous mobile phase, cisplatin was retained better on the solvent-generated anion exchanger than on the chemically bonded anion exchanger. Increasing the methanol concentration de-

[8] Partisil 5, (5 μm, 250 mm × 4.6-mm i.d.), Whatman, Inc., Clifton, N.J.
[9] Hypersil (5 μm, 50 mm × 4.6-mm i.d.), Shardon Southern, Sewickley, Pa.
[10] Partisil 10 SAX (10 μm, 250 mm × 4.6-mm i.d.), Whatman, Inc., Clifton, N.J.
[11] Alltech Associates, Deerfield, Ill.
[12] Haake, Saddlebrook, N.J.
[13] Rheodyne 7001, Cotati, Calif.
[14] Systech, Inc., New Brighton, Minn.
[15] Model 273, Instrument Specialties, Lincoln, Nebr.
[16] SLIC 1400, Systec, Inc., New Brighton, Minn.
[17] WISP 710, Waters Associates, Milford, Mass.
[18] Varian Techtron 175B, Varian Assoc., Palo Alto, Calif.
[19] Millipore Corp., Bedford, Mass.

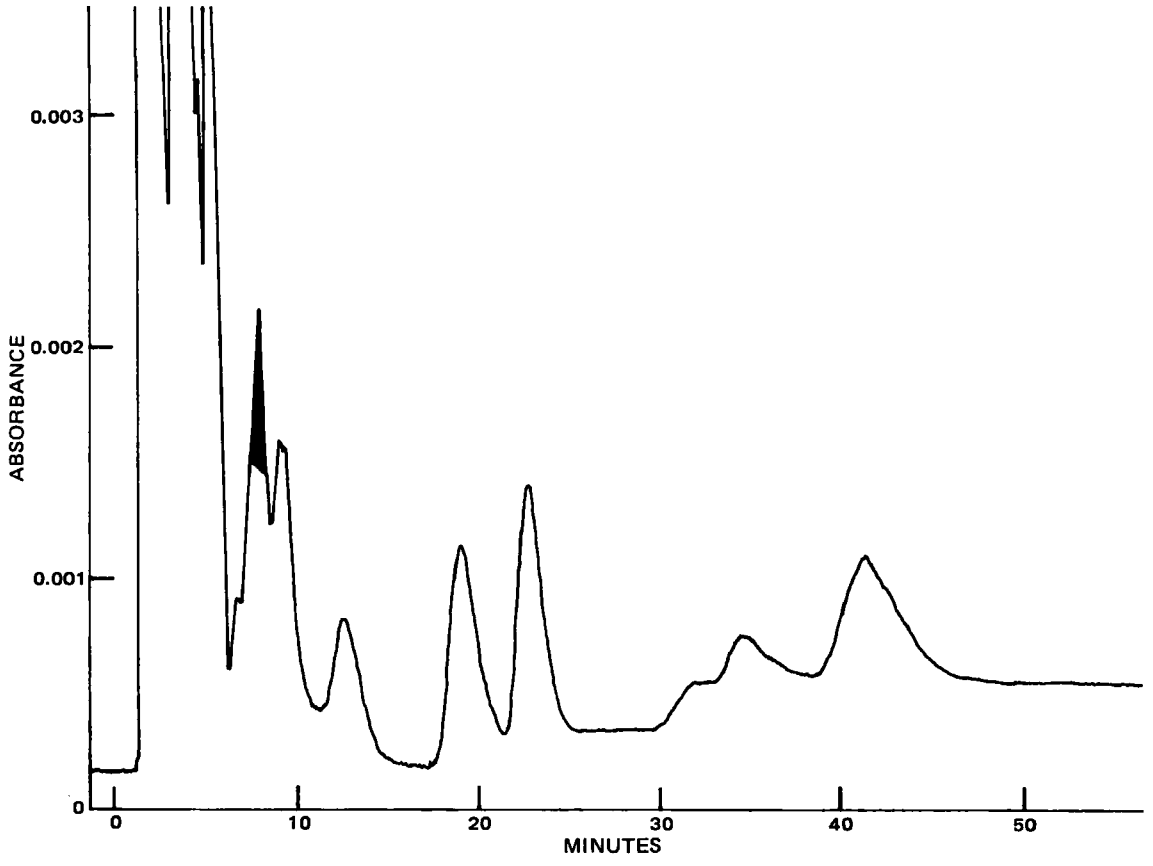

Figure 3—*Chromatogram of human urine spiked with 200 μg/ml of cisplatin on a reverse-phase column coated with 1.96 μmoles/m² of hexadecyltrimethylammonium bromide. Other chromatographic conditions were: mobile phase, 10^{-2} M citrate buffer (pH 7.0) + 10^{-4} M hexadecyltrimethylammonium bromide; flow rate, 1.0 ml/min; and ambient temperature. Arrow indicates cisplatin peak (darkened).*

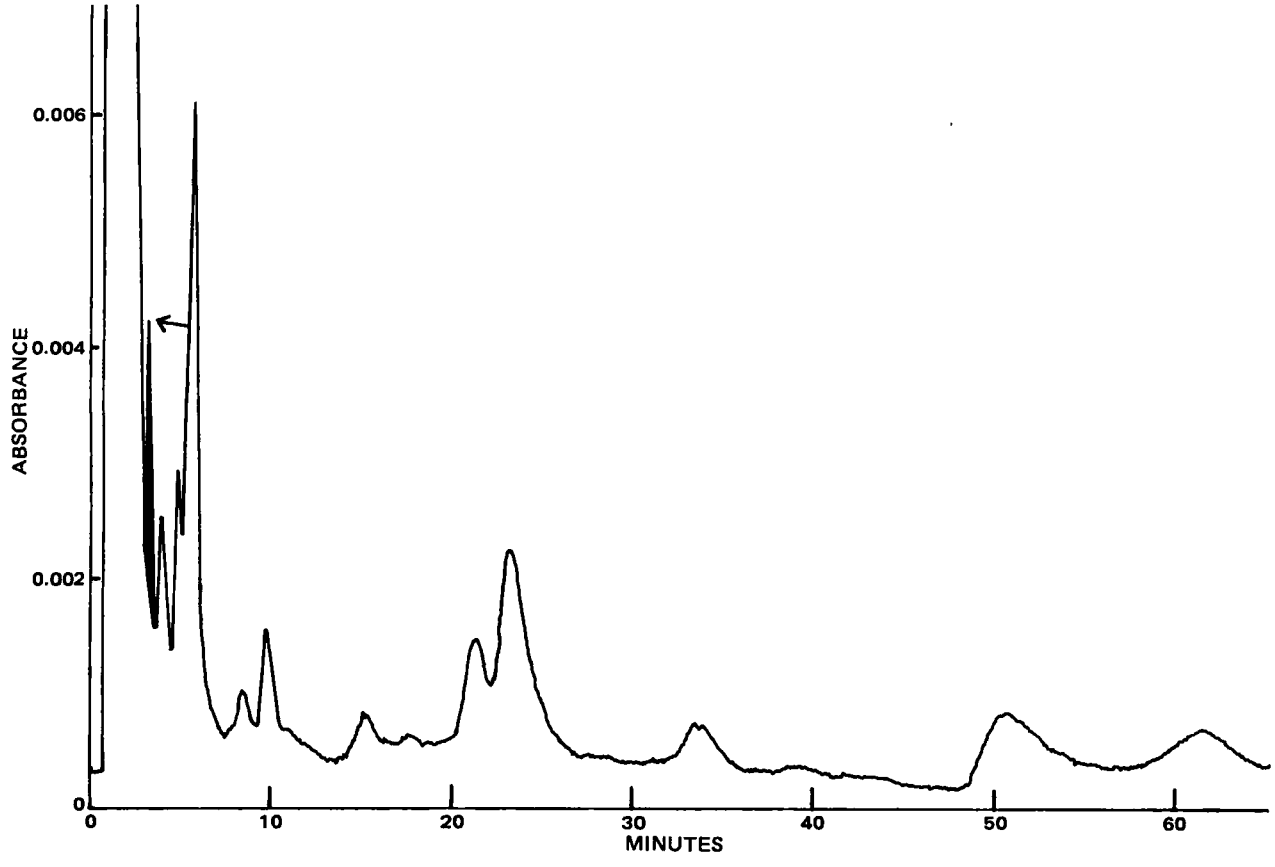

Figure 4—*Chromatogram of human urine spiked with 200 μg/ml of cisplatin on silica gel coated with 0.63 μmoles/m² of hexadecyltrimethylammonium bromide. Arrow indicates cisplatin peak (darkened). Other chromatographic conditions as Fig. 3.*

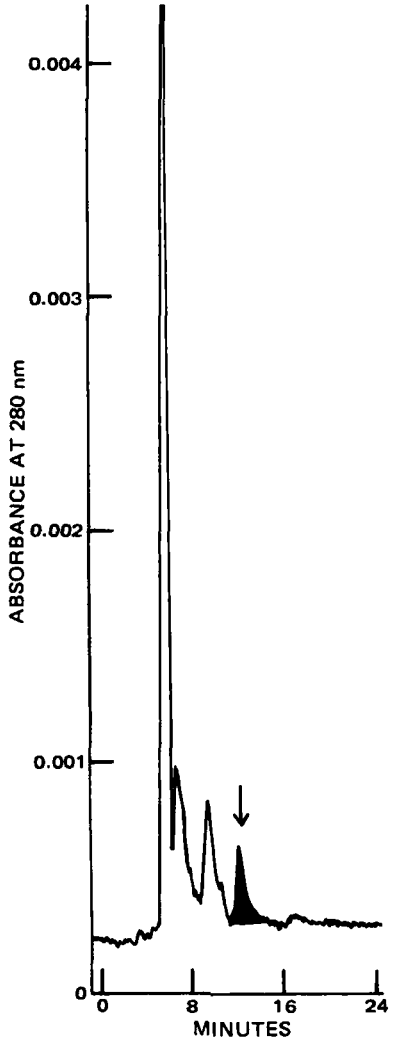

Figure 5—*Chromatogram of clinical urine sample containing 96 μg/ml of cisplatin using a column-switching configuration as in Fig. 1 and mobile phase conditions as described in Fig. 3. Arrow indicates cisplatin peak (darkened).*

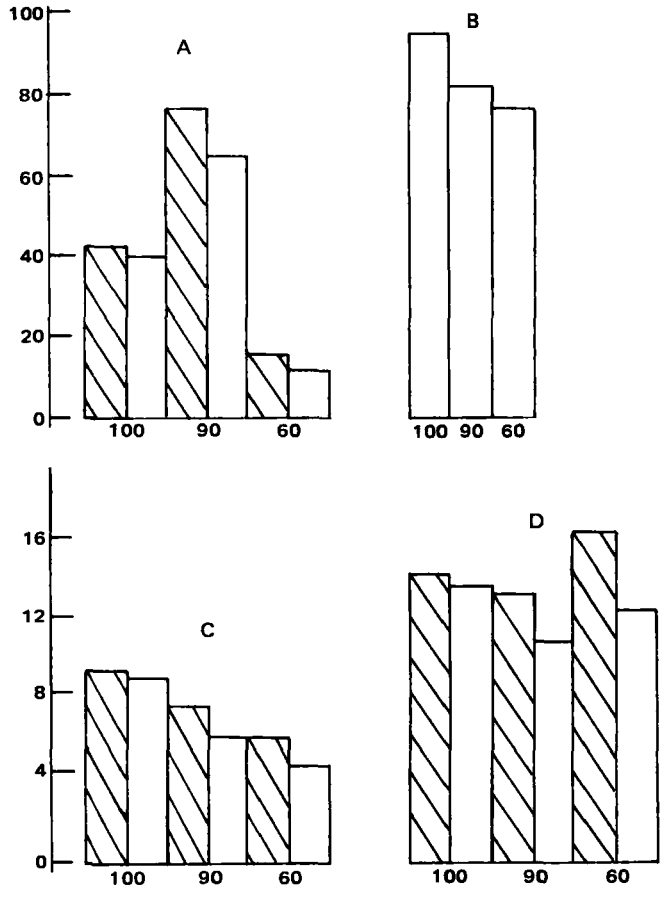

Figure 6—*Urine analysis of three patients treated with cisplatin for ovarian cancer, showing the amounts and concentrations of unchanged drug and total platinum excreted 30 min after infusion (50 mg/m² iv at 1 mg/min). Key: (A) urinary concentration of total platinum ▨ and cisplatin □ μg/ml; (B) ratio of cisplatin–total platinum for three patients, %; (C) absolute amount of total platinum ▨ and cisplatin □ present in the urine samples, μg; and (D) fraction of the administered dose excreted as total platinum ▨ and cisplatin □, %. The doses of drug administered to each patient are given under each histogram in milligrams of cisplatin; amounts and concentrations excreted are given in terms of free metal (Pt).*

creased retention on the former while increasing retention on the latter. Below $\phi = 0.10$, cisplatin retention on the modified reverse-phase column was independent of surfactant concentration in the mobile phase, which indicates that the contribution to retention of solute–surfactant interactions in the mobile phase was negligible. At higher concentrations of methanol, cisplatin retention was dependent on the concentration of hexadecyltrimethylammonium bromide in the mobile phase due to displacement of the adsorbed surfactant. However, this desorption does not explain the initial decrease in retention with increasing methanol concentration on the modified reverse-phase column.

The retention of cisplatin on either of the cationic stationary phases is explained most readily in terms of ion–dipole interactions between the neutral platinum complex and the positively charged stationary phase. The differences between the two systems may be explained in terms of the different environments in which these interactions occur and the influence of methanol on those environments. The cationic groups on column A are situated in an apolar, hydrophobic environment, whereas the quaternary ammonium groups on the chemically bonded column (F) are in a polar environment in close proximity to a silica backbone. In both systems methanol is adsorbed at the stationary phase–mobile phase interface (17, 18), which decreases the polarity of the cationic groups of column F while increasing the polarity of the corresponding groups on column A.

The effects of methanol may be further rationalized by the application of solvophobic theory. According to solvophobic theory, retention in HPLC systems employing aqueous mobile phases may be described by:

$$\ln k = a + b + \frac{\gamma \Delta A}{RT} \qquad \text{(Eq. 2)}$$

which is a summation of all the possible solute–solvent–stationary phase interactions contributing to retention (19, 20). The first term, a, is related to the properties of the mobile and stationary phases and is independent of the nature of the solute. In the present system, b may be taken as a measure of the ion–dipole interactions which contribute to retention. The third term is a measure of the hydrophobic interactions where γ is the mobile phase surface tension and ΔA is the decrease in hydrophobic surface area on binding of the solute to the stationary phase. Since the chemically bonded anion exchanger has little hydrophobic character, the contribution of the third term is probably negligible in this system, and the increased retention observed with increasing methanol concentration arises from an increase in the contribution of b.

In the solvent-generated system, increasing the methanol concentration may influence both b and the hydrophobic term ($\gamma \Delta A/RT$). The increase in polarity of the environment in which the interactions occur, due to the presence of methanol, can be expected to result in a decrease in the contribution of b and decreased retention. Also, the addition of methanol lowers the mobile phase surface tension (19), further reducing retention. As indicated previously, a third effect is introduced at higher methanol concentrations at which hexyldecyltrimethylammonium bromide is displaced from the column, reducing the contributions of both a and b.

Solvophobic theory may be applied also to the explanation of the effects of electrolyte on the retention of cisplatin on solvent-generated anion exchangers (Table I). In this study column B was used as support for the adsorbed surfactant. This column produced a higher uptake of surfactant (1.31 μmoles/m²) which resulted in a correspondingly increased retention of cisplatin. The addition of the monovalent electrolytes

sodium bromide and sodium nitrate (both 0.1 M) to the mobile phase decreased the retention of cisplatin. Conversely, the addition of the polyvalent electrolytes, sodium citrate and sodium sulfate, produced a significant enhancement of retention. These effects may be explained in terms of the effect of the added salts on the mobile phase surface tension as described by the relationship:

$$\gamma = \gamma_0 + \tau m \qquad \text{(Eq. 3)}$$

where m is the molality of the electrolyte, γ_0 is the surface tension of the pure solvent and τ is a constant for the particular electrolyte. Combining Eqs. 2 and 3 gives a prediction of the linear relationship between ln k and τ at a fixed salt concentration:

$$\ln k = a + b + \frac{(\gamma_0 + \tau m)\, A}{RT} \qquad \text{(Eq. 4)}$$

The relationship between ln k and τ (21) at an electrolyte concentration of 0.1 M for the cisplatin solvent-generated anion exchange system was found to be:

$$\ln k = 0.85\,\tau - 1.23 \quad r = 0.998,\ n = 4 \qquad \text{(Eq. 5)}$$

Increasing the ionic concentration of the mobile phase may also reduce the thermodynamic activity of the cationic stationary phase binding sites due to ion–ion interactions which in turn leads to a decreased contribution of a and b (Eq. 4) and decreased retention of cisplatin. The excellent linearity of Eq. 5 indicates that this latter contribution is constant for the different salts examined at a concentration of 0.1 M and is more than compensated for by the increase in mobile phase surface tension produced by the polyvalent electrolytes having large values of τ.

Urinalysis—The use of columns A and B was associated with short column lives and considerable batch-to-batch variation. Consequently, column C was used as the support for the cationic surfactant in the analysis of cisplatin in urine. This material produced a higher coverage of adsorbed surfactant (1.96 μmoles/m^2) and a correspondingly greater retention of cisplatin. As a result, it was possible to use a lower concentration of citrate (10^{-2} M) in the mobile phase and still maintain adequate retention of cisplatin (k = 4.68).

Despite optimal retention of cisplatin on column C coated with hexadecyltrimethylammonium bromide, the drug still coeluted with several endogenous urine components (Fig. 3). Additionally, a number of highly retained peaks were observed, resulting in long analysis times. These interfering compounds were not satisfactorily removed by the use of a silica precolumn (column E). However, it was observed that cisplatin was significantly retained (k = 2.83) on this precolumn and that on silica it coeluted with different urine components (Fig. 4). Since it has been shown that cisplatin was virtually unretained on silica (Fig. 2) it was concluded that the silica had been modified by the presence of hexadecyltrimethylammonium bromide and that it was behaving as a solvent-generated anion exchanger with a different selectivity from that produced by adsorbing the surfactant onto a reverse-phase column. This hypothesis was confirmed by uptake studies which revealed significant amounts of surfactant (0.63 μmole/m^2) adsorbed onto the silica surface.

By the application of column-switching techniques (Fig. 1) the different selectivities of the two solvent-generated anion exchangers bonded to silica and hydrophobic supports were utilized and complete resolution of cisplatin from urine was achieved (Fig. 5). The system was fully automated, under microprocessor control, and designed so that only the cisplatin-containing fraction which eluted from column E was transferred to column C. After column transfer, cisplatin was separated from the components with which it coeluted from the first column. The remaining urine components were eluted from the first column at a higher flow rate (3.0 ml/min) and vented to waste.

Due to the poor sensitivity provided by UV spectroscopy, off-line flameless atomic absorption was preferred for the analysis of urine containing cisplatin at <100 μg/ml. After chromatography, the fraction containing cisplatin was collected and determined by atomic absorption spectrophotometry. The platinum absorbance of the collected fraction A_{Pt} was linear with respect to the amount of cisplatin injected (calibration curve prepared as described in *Experimental*), as defined by:

$$A_{Pt} = 0.65\ [\text{cisplatin}] + 0.001 \quad r = 0.999,\ n = 6 \qquad \text{(Eq. 7)}$$

and offered a detection limit of 2 μg/ml. Although the use of off-line atomic absorption introduces an extra step in the analysis, it provides a specific platinum-detection system which potentiates the selectivity of the HPLC.

It was found that urine samples containing cisplatin could not be satisfactorily stored in a freezer (−11°) since >30% was lost from one sample over a period of 48 hr. No degradation of cisplatin was observed in urine stored over solid CO_2 (~ −60°) for 48 hr. The recovery of drug (50 μg/ml) from urine samples stored in this manner was 101% with a relative *SD* of 3.6% (n = 9).

The developed methodology was applied to the analysis of the urine taken from three patients treated with cisplatin for ovarian cancer (Fig. 6). The amount of unchanged cisplatin excreted was determined by HPLC, and total platinum concentration was determined directly by atomic absorption spectroscopy. There was wide variation in the concentration of cisplatin found; the amounts and percentages of the total dose excreted unchanged were similar. The ratios of cisplatin–total platinum excreted varied between 75 and 95%, indicating that the previous results (3) reporting low levels of cisplatin in urine may be due to degradation during storage, either in the bladder or after sample collection, rather than extensive biotransformation. Furthermore, the presence of high concentrations of unchanged cisplatin in urine may be related to its clinical utility in the treatment of bladder cancers (3, 22).

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