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URINE ANALYSIS OF PLATINUM SPECIES DERIVED FROM *cis*-DICHLORODIAMMINEPLATINUM(II) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOLLOWING DERIVATIZATION WITH SODIUM DIETHYLDITHIOCARBAMATE

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SUMMARY

A clinically useful method is described for the quantitative analysis of platinum species derived from *cis*-dichlorodiammineplatinum(II) in urine. The drug and its biodegradation products are derivatized directly in urine by reaction with sodium diethyldithiocarbamate (DDTC) to form a common product, a 2:1 DDTC–platinum adduct. This complex is stable and can be quantitatively extracted into 0.1 volumes of chloroform. An aliquot of the chloroform layer is then subjected to high-performance liquid chromatography on a μBondapak CN column and the eluent monitored spectrophotometrically at 254 nm. At this wavelength the DDTC–platinum adduct has a molar absorptivity of 43,000, and platinum levels of 25 ng/ml of urine can be detected with a precision of ± 2.5% and an accuracy of ± 4%.

INTRODUCTION

cis-Dichlorodiammineplatinum(II) (CDDP) has generated ever increasing interest as an anti-tumor agent since Rosenberg *et al.*[1] demonstrated its cytotoxic effects on *Escherichia coli*. CDDP is now regarded as an effective agent in the treatment of a wide variety of malignant solid tumors including testicular, ovarian, osteosarcoma, head and neck and other solid neoplasms[2,3].

Plasma levels of free circulating platinum decline rapidly after i.v. administration of the drug as it is cleared by the kidneys or alternatively becomes tightly bound to plasma protein[4,5]. Methods for measurement of urinary platinum excretion were sought by this laboratory as part of an effort to elucidate the human pharmacokinetics of CDDP after intravenous administration.

Non-flame atomic absorption spectrometry (NFAAS) is currently the most widely used technique[6,7] for determining non-radiolabelled platinum in biological samples and responds to trace (parts per 10^9) levels of platinum. The accuracy and precision of NFAAS, however, is highly dependent on the sample matrix. We have

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similarly found that wavelength dispersive X-ray fluorescence measurements[8] are subject to dramatic matrix effects. Since urine is highly variable in its physical properties and chemical content, the analysis of total platinum in untreated urine is subject to variance and irreproducibility in analytical results. The present study was directed toward the development of a clinically useful analytical method for platinum in urine with the high sensitivity of NFAAS but with a broader linear range and less interference from and dependence on the sample matrix.

Initially, the extractability of CDDP and its *in vivo* reaction products from urine into water-immiscible organic solvents was investigated as a means for circumventing the matrix effects. This approach was concluded to be unfeasible, however, due to the poor partition characteristics of the analytes between water and common organic solvents and their instability in many of these solvents. Accordingly, a reagent was sought which, when added to urine, would react quantitatively with CDDP and any other platinum species formed from CDDP *in vivo* to form a single stable platinum-containing product which would be extractable into a water-immiscible solvent. Such a product would subsequently be separated from co-extracted potential interferents by high-performance liquid chromatography (HPLC) and should have properties permitting its detection at low ng/ml levels by common detection devices which could be interfaced with the chromatograph.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000-A solvent delivery system, Model U6K injector and Model 440 absorbance detector operated at 254 nm. Detection at wavelengths other than 254 nm was done using a Varian (Palo Alto, Calif., U.S.A.) Vari-Chrom variable-wavelength liquid chromatography detector. Columns (30 cm × 1/4 in. O.D.) were all obtained from Waters and included μBondapak C_{18}, μBondapak alkyl phenyl, μBondapak NH_2 and μBondapak CN.

UV–Visible spectrophotometry was performed using a Cary (Palo Alto, Calif., U.S.A.) 219 spectrophotometer and 1-cm quartz cells. NFAAS was carried out with a Varian/Techtron 175 atomic absorption spectrometer with CRA-90 carbon-rod atomizer.

Materials

cis-Dichlorodiammineplatinum(II) was obtained from the National Cancer Institute (Bethesda, Md., U.S.A.) and was used without further purification. Sodium diethyldithiocarbamate (DDTC) was purchased from Sigma (St. Louis, Mo., U.S.A.) and was washed with chloroform, filtered under nitrogen, dried under reduced pressure and stored at −10°. Chloroform was Fisher spectral grade (Fisher Scientific. Fair Lawn, N.J., U.S.A.). Methanol, isopropanol and heptane were all Fisher HPLC Grade. Water was distilled after mixed-bed deionization and had a specific conductance of 1.0–1.3 μS. Human urine was obtained from volunteers not undergoing CDDP therapy. McIlvaine buffer (0.2 *M*; pH 7.5) was used in studies parallelling those carried out in urine.

Synthesis of diethyldithiocarbamate derivative of CDDP

CDDP (0.17 mmol; 50 mg) was dissolved in 200 ml water. Sodium diethyldithiocarbamate (DDTC) (*ca.* 23 mmol; 4 g) was added and the solution stirred for 24 h. The resulting precipitate was removed by filtration and recrystallized from methanol–chloroform (9:1). Elemental analysis (%) of the yellow crystals: calculated for $Pt(DDTC)_2$, C 24.43, H 4.10, N 5.70, S 25.09, Pt 39.68; found, C 24.88, H 4.65, N 5.35, S 25.87, Pt 39.18.

Derivatization of platinum in urine

Urine or buffer (9 ml) containing 0.25–250 μg of CDDP was placed in a 15-ml conical centrifuge tube. One milliliter of a 10% (w/v) solution of diethyldithiocarbamate prepared in 0.1 *N* sodium hydroxide solution and 2 ml of an aqueous, saturated solution of sodium nitrate were added to the tube, which was sealed thoroughly, mixed and allowed to stand at room temperature (*ca.* 23°) for 1 h.

Extraction of the platinum–DDTC adduct

The urine or buffer mixture (10 ml) was extracted with 1 ml of water-saturated chloroform for 3 min. The sample tube was then centrifuged for 5 min at 1200 *g*, and after brief vortex mixing to disrupt a congealed third phase appearing between the aqueous and organic layers, the tube was centrifuged for an additional 10 min. The aqueous layer and the stiff emulsion layer above the chloroform were removed and discarded.

Chromatography

In all runs, 30-μl aliquots of the chloroform solution containing the platinum–DDTC adduct were injected onto a μBondapak CN column (two 30-cm columns coupled in series by 0.009 in. I.D. tubing). Components were eluted isocratically from the column using heptane–isopropanol (82:18) as mobile phase at a flow-rate of 2 ml/min.

Non-flame atomic absorption spectrometry

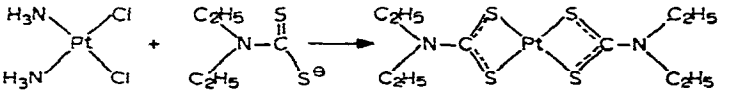
Chloroform samples (5 μl) containing the platinum–DDTC adduct were injected slowly into an atomizer held at 100°. After deposition of the sample, the atomizer was held at 100° for 45 sec, 1000° for 10 sec and 2300° for 1 sec. The temperature ramp rate from 1000° to 2300° was 600° sec^{-1}. The platinum absorbance at 265.95 nm was monitored using an element lamp current of 10 mA. Hydrogen continuum background correction was used. Nitrogen was passed through the system at 4.25 l/min.

RESULTS AND DISCUSSION

CDDP is a square planar molecule that undergoes bimolecular substitution reactions (with preservation of stereochemistry) at platinum, similar to reactions observed at the electrophilic carbon atoms of other alkylating agents[9]. Accordingly, a nucleophilic reagent was sought which would react with CDDP and its *in vivo* reaction products directly in urine to form a single, stable product that would meet the analysis criteria delineated above. We have previously demonstrated[10] that sodium diethyldithiocarbamate (DDTC), 2, reacts readily and quantitatively with the epoxide, dianhydrogalactitol, directly in blood plasma to form a stable product, which is

significantly more hydrophobic than the parent and with pronounced UV absorbance at 254 nm.

To determine the applicability of this reagent to CDDP analysis, CDDP was mixed with DDTC in aqueous buffer (pH 7.5) solution. A yellow precipitate formed rapidly and was isolated by filtration. The recrystallized product was shown by elemental analysis to represent the addition of 2 moles of DDTC to 1 mole of CDDP and is presumed to be structure 3. Similar chelates have been shown to form between DDTC and a variety of metal ions, including platinum(II)[11], and have proven useful in chromatographic separation of metals[12]. The adduct(s) strongly absorbs UV light with wavelength maxima at 254 nm (a_m = 43,000 M^{-1} cm^{-1}) and 347 nm (a_m = 22,000 M^{-1} cm^{-1}).



1 2 3

The platinum–DDTC chelate is a neutral species, that is nearly insoluble in water, acid (pH 1) and base (pH 13) but freely soluble in chloroform. Thus it was presumed that the chelate, 3, would partition favorably into chloroform from an aqueous phase. Using a 10:1 ratio of McIlvaine buffer (pH 7.5) to chloroform, the adduct was quantitatively retained in the chloroform phase after vigorous agitation of the mixture for 1 h. Partitioning efficiency was pH-independent, *i.e.*, quantitative retention of 3 in chloroform was observed when the pH of the aqueous phase was varied between 1 and 13. The affinity of the adduct for chloroform and its incompatibility with water allows for the ten-fold concentration of the analyte (3) which is achieved by extraction.

The reaction was also found to proceed directly in urine containing 25–15,000 ng of CDDP per ml, in which the product, 3, was isolated by extraction into chloroform. Extraction of 3 from urine by chloroform was shown to be quantitative, when the ratio of urine to chloroform was 10:1. Larger aqueous:organic phase ratios could not be used because of emulsification problems occurring at the interface after extraction, which hampered sampling of the organic phase.

The nature of the derivatization was investigated further in urine in order to optimize reaction conditions. The formation of 3 from 1 is apparently an A $\xrightarrow{k_1}$ B $\xrightarrow{k_2}$ C process, where B represents the product(s) formed from the addition of 1 mole of DDTC to 1 and C is compound 3 which will ultimately be monitored spectrophotometrically. The reaction was carried out in urine containing 0.025–15 μg of CDDP per ml, by the addition of a 10% solution of DDTC (prepared in 0.1 *M* sodium hydroxide solution) to give a final concentration of 1% DDTC in the urine; and the formation of 3 was monitored as a function of time by HPLC. At 25°, in urine, CDDP was quantitatively converted to 3 in 10 min. When the reaction was repeated in McIlvaine buffer (0.2 *M*; pH 7.5), the formation of 3 with time was slower than that observed in urine and followed apparent first-order kinetics with a half-life of < 10 min. The apparent simple first-order behavior for formation of 3 indicates that one of the rate constants is much greater than the other, since no lag phase is observed. The yield of 3 obtained in 1 h at room temperature decreased with decreasing pH (Table 1.

TABLE I

EFFECT OF pH OF REACTION MEDIA ON THE OVERALL RECOVERY AND ANALYSIS OF CDDP FROM BUFFER

The recovery of platinum was measured spectrophotometrically at 254 nm, as $Pt(DDTC)_2$, using the procedure described in the text. McIlvaine buffers made from combinations of 0.2 *M* Na_2HPO_4 and 0.1 *M* citric acid solutions were used. Ionic strength uncontrolled. The buffer solutions were spiked with three different concentrations (5.1, 20.3, 50.6 μg/ml) of CDDP. Each solution was prepared in duplicate.

pH of buffer	*Recovery of platinum from buffer (%)*	r^{2*}
5.29	86	0.991
6.46	92.4	0.998
7.05	94.8	0.999
8.08	95.8	0.995

* Regression coefficient for platinum response.

Accordingly, reaction conditions were established wherein 1 ml of reagent solution [containing 10% DDTC (w/v) in 0.1 *N* NaOH] was added to 9 ml of urine and the reaction allowed to stand at room temperature for 1 h. Sodium hydroxide was present in the reagent to elevate the pH above 7, where maximum and reproducible yield of 3 was achieved. Precipitation of inorganic salts occurs with some urine samples upon the addition of base, however, this sediment does not affect the yield of 3 obtained. After the 1-h reaction period, the adduct, 3, was quantitatively extracted from the urine into 0.2 volumes of chloroform.

Compound 3 showed no detectable deterioration in chloroform over 2 months and no breakdown in urine (containing 1% DDTC) was observed after more than 72 h.

Chromatography

The high molar absorptivity of the adduct lends itself to spectrophotometric detection. To unsure selective monitoring of 3 in the chloroform extract, the mixture was fractionated by HPLC. The objectives of the separation step were: (1) to resolve the peak resulting from 3 from peaks arising from urine constituents and products formed from decomposition of DDTC which are co-extracted into chloroform; (2) to allow monitoring of the column effluent at 254 nm rather than 347 nm to achieve greater sensitivity, since the molar absorptivity of 3 at 254 nm is twice that observed at 347 nm and the light intensity generated by a mercury vapor lamp at 254 nm is significantly greater than that achievable at 347 nm (with either a mercury vapor or deuterium lamp); and (3) to achieve symmetrical, well-shaped peaks with capacity factors (k') in the range 3–6.

Attempts to achieve these goals were unsuccessful using reversed-phase (C_{18} or alkyl phenyl) μBondapak columns. However, using a μBondapak NH_2 or CN column these aims could be realized. An evaluation of various stationary phases is summarized in Table II. Unfortunately, the μBondapak NH_2 column is not an inert phase and after repeated injection of chloroform extracts (*ca.* 200) irreproducible peak heights, loss in peak symmetry (tailing) and increased retention volumes (V_r) for 3 were observed. Column performance could be partially regenerated by successive washes with acetic acid (0.1 *N*; 100 ml), water (200 ml), isopropanol (100 ml) and

TABLE II

SUMMARY OF COLUMN PERFORMANCE FOR THE ANALYSIS OF PLATINUM IN URINE AS $Pt(DDTC)_2$

The analysis was carried out as described in Experimental. Analytes were detected spectrophotometrically at 254 nm, unless otherwise indicated. μBondapak columns (30 cm × 3.9 mm I.D.) were used. The mobile phase was optimized by solvent-flow programming (Waters Model 660 solvent programmer), adjusting the composition of the indicated binary mixtures from 0 to 100%.

Stationary phase	*Mobile phase (composition)*	k'	N[c]	R_s[d]	*Comments on performance*
C_{18}	methanol–water (70:30)	3.1[a]	1070	[e]	[f,g]
Alkyl phenyl	methanol–water (70:30)	4.8[a]	1700	[e]	[f,g]
	methanol–phosphate buffer (0.005 *M*; pH 3) (70:30)	4.3[a]	1100	0.5	[f,h]
NH_2	heptane–isopropanol (87:13)	4.1[b]	490	0.7	[f,i,j]
CN	heptane–isopropanol (82:18)	3.1[b]	810	0.9	[f,j]
CN (two columns)	heptane–isopropanol (82:18)	3.0[b]	1600	1.3	[k]

[a] Capacity factor for $Pt(DDTC)_2$ calculated as $(t_r - t_0)/t_0$ where t_r is the retention time of $Pt(DDTC)_2$ and t_0 is the time for elution of a nonretained component (determined from a injection of 30 μl of chloroform).

[b] Capacity factor for $Pt(DDTC)_2$ calculated as $(t_r - t_0)/t_0$ where t_0 was determined with an injection of 75 μl of heptane.

[c] Plate count calculated from an injection of $Pt(DDTC)_2$ (16 μg Pt per ml) in chloroform as $16\,(t_r/t_w)^2$ where t_w is the width of the $Pt(DDTC)_2$ peak at the baseline and t_r is its retention time.

[d] Resolution of the $Pt(DDTC)_2$ peak from its closest neighboring peak, calculated as $2(t_2 - t_1)/(w_1 + w_2)$ where t_1 and t_2 are the retention times of bands 1 and 2 and w_1 and w_2 are their baselines widths defined by the tangents to the inflection points of a given curve.

[e] Grossly inadequate ($R_s \leqslant 0.5$).

[f] Inadequate resolution of analyte from co-extracted materials.

[g] Very complex chromatogram.

[h] Chromatogram simplified; analyte can be detected spectrophotometrically at 347 nm. Detection limit: 1 μg CDDP per ml.

[i] Rapid column degeneration.

[j] Platinum can be quantitated spectrophotometrically at 254 nm as $Pt(DDTC)_2$ to levels approaching 350 ng of CDDP per ml of urine.

[k] Good performance; platinum can be quantitated as $Pt(DDTC)_2$ to levels of 25 ng CDDP per ml of urine.

then reintroduction of mobile phase (200 ml), which is consistent with azomethine formation. However, this procedure is time-consuming and does not completely restore chromatographic characteristics. These column performance problems could be circumvented by using a μBondapak CN column. To achieve minimum analysis time with adequate resolution of components, a mobile phase for isocratic separation on a CN column was selected by starting with pure isopropanol and adding sufficient heptane to increase k' to 3–6 (Fig. 1) while maintaining the resolution of 3 from contaminating peaks. Complete resolution could only be achieved with columns 60 cm long. A mobile phase of isopropanol–heptane (18:82) was selected in which 3 elutes with $V_r = 24$ ml, $k' = 3$ and the number of theoretical plates, N, is 1600 (based on 3).

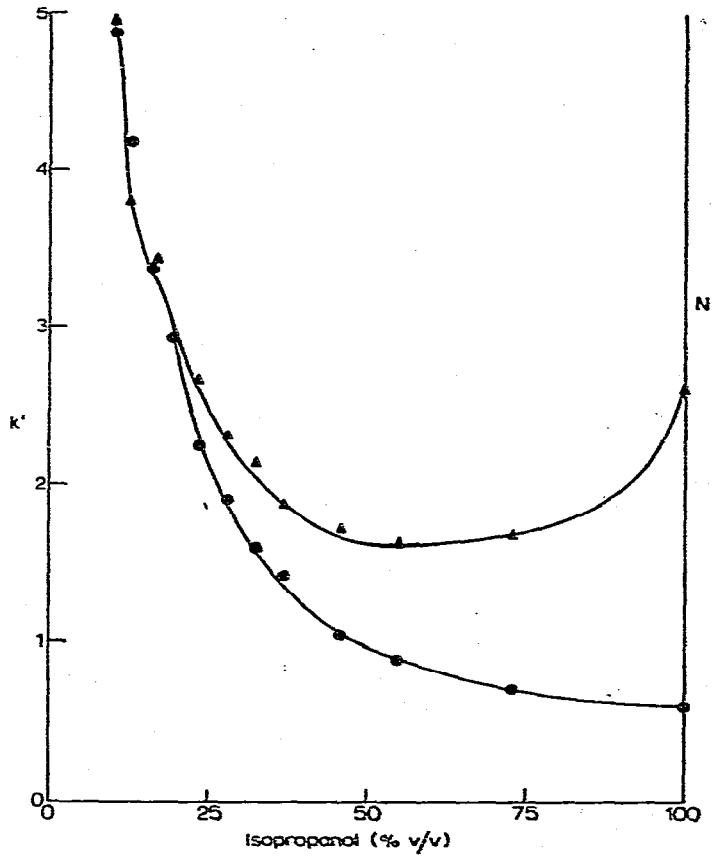


Fig. 1. The effect of variation in isopropanol–heptane mobile phase composition on the capacity factor (k', ○) and column efficiency (N, △) for separation of the CDDP–DDTC adduct (3) (16 μg Pt/ml) on a μBondapak CN column (60 cm × 3.9 mm I.D.). Flow-rate: 2 ml/min.

A minimum is observed in the curve describing the change in plate count (N) with mobile phase composition. At high isopropanol concentrations, the large plate count apparently results from minimum retention of 3 by the stationary phase and therefore minimum band broadening. The increasing plate count also observed at low isopropanol concentrations may result from the decreased viscosity (η) of the mobile phase ($\eta_{\text{heptane}} = 0.386$ cP; $\eta_{\text{iso-propanol}} = 1.811$ cP at 25°) produced by the addition of the heptane.

Fig. 2 shows the analysis of 25 ng of CDDP per ml of urine as its DDTC adduct. Injection volumes greater that 50 μl of chloroform changed the retention characteristics slightly, accelerating the elution of 3.

Standard curves and sensitivity

A standard curve was prepared by analyzing twelve urine samples to which CDDP had been added at different concentrations ranging from 25 to 500 ng/ml. Over this concentration range, chromatogram peak height was linearly related to CDDP concentration, and is described by the line $y = 0.65x + 2.15$ (with a zero-

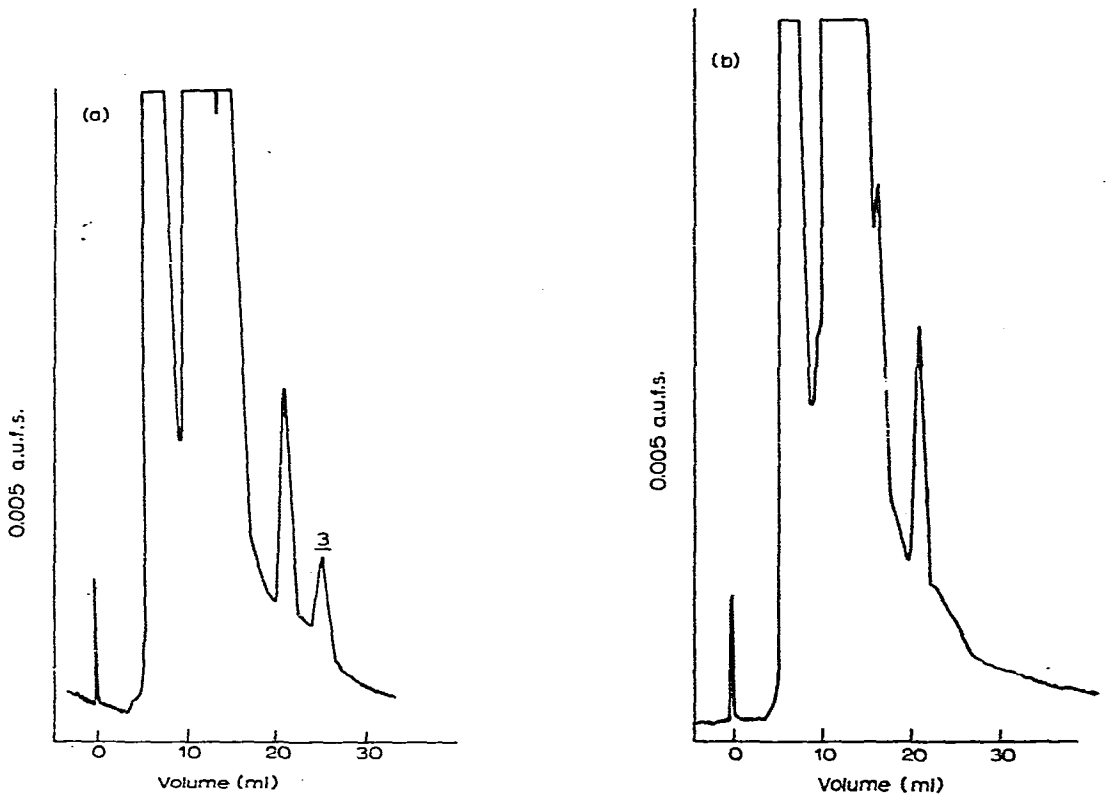


Fig. 2. Chromatograms of the CDDP–DDTC adduct (3) obtained by carrying out the methodology described in the text on a 9-ml urine sample containing 25 ng of CDDP (a) and of a drug-free urine sample subjected to the assay (b).

order correlation coefficient of 0.99). Total recovery of CDDP from urine was $> 97\%$ over this range, and measurements could be made with an accuracy of $\pm 4\%$ and precision of $\pm 2.5\%$. The minimum amount of platinum detected by the method was approximately 25 ng/ml of urine. The identical standard curve was obtained for analysis of platinum immediately after CDDP addition to urine and for samples allowed to remain untreated in urine for 36 h prior to the addition of DDTC. Thus, although CDDP is unstable in urine, it must be converted to species which react with DDTC in a manner similar to CDDP to form 3. Immediate processing of samples is therefore not necessary.

There is still some uncertainty with regard to the spectrum of platinum complexes that react with DDTC to form 3. We have found that CDDP, CDDP incubated with urine for 35 h and the biodegradation products of CDDP which have been isolated from plasma[13] (but not yet identified) respond identically with the reagent to form 3. Furthermore, Fackler *et al.*[11] have shown that 3 "reacts only slowly (hours) with diphenylmethylphosphine", a reagent known to react very rapidly with other divalent platinum complexes, supporting the contention that the complex 3 is stable and DDTC is able to displace most ligands bonded to platinum.

Atomic absorption detection

Alternatively, an aliquot of the chloroform extract containing 3 can be introduced directly into the graphite furnace of an atomic absorption spectrophotometer and the platinum levels measured. By this detection method, platinum levels approaching 10 ng/ml of urine can be followed quantitatively. Unfortunately, the imprecision and instrumental variability associated with this technique made it unreliable and thus less advantageous than the HPLC method.

CONCLUSION

A clinically-useful method has been developed for measuring non-radiolabelled platinum in urine following administration of CDDP to patients, while circumventing the matrix effects associated with other analytical methods (X-ray fluorescence and atomic absorption spectrometry). By taking advantage of the electrophilicity of the divalent platinum atom and the lability of its ligands, it is possible to convert CDDP as well as a number of its biodegradation products to a common compound with hydrophobic character, permitting its isolation from biological fluid by extraction which coincidently provided a ten-fold concentration of the sample. Further separation of the platinum adduct was afforded by HPLC. The platinum–DDTC complex strongly absorbs UV light ($\lambda_{max.}$ 254 nm; a_m 43,000) and could be detected in the column effluent to levels approaching 25 ng of platinum per ml of urine with a precision of $\pm$ 2.5% and accuracy of $\pm$ 4%. The chromatographic step was essential in providing sufficient specificity to permit spectrophotometric monitoring of the adduct at either 254 or 347 nm since other absorbing species are also extracted from urine by chloroform. Attempts to measure platinum in urine by other techniques fell far short of these statistics, apparently due to sample matrix effects and instrumental variability. The method is rapid, inexpensive, requires minimum sample manipulation and is suitable for automation to accommodate large numbers of samples.

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