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ANALYSIS OF ANTITUMOUR [1,1-BIS(AMINOMETHYL)CYCLOHEXANE]PLATINUM(II) COMPLEXES DERIVED FROM SPIROPLATIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIFFERENTIAL PULSE AMPEROMETRIC DETECTION

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SUMMARY

A reversed-phase high-performance liquid chromatographic analysis was developed for aqua[1,1-bis(aminomethyl)cyclohexane]sulphatoplatinum(II) (spiroplatin) and its hydrolysis and oligomerization products. The platinum complexes were detected by differential pulse amperometry at a hanging mercury drop electrode, at -540 mV *vs.* Ag/AgCl. The limit of detection was 0.05 μM. Aqueous solutions of spiroplatin appeared to contain the diaqua, monoaquamonosulphato, monoaquamonochloro, dichloro and hydroxo-bridged dimer complexes of [1,1-bis(aminomethyl)cyclohexane]platinum(II) in mutual equilibrium. The equilibrium shifts after dilution in infusion fluids were studied. Detection of these platinum(II) complexes in untreated human plasma ultrafiltrate and urine demonstrated the selectivity of the described analysis.

INTRODUCTION

Aqua[1,1-bis(aminomethyl)cyclohexane]sulphatoplatinum(II) (spiroplatin, TNO-6) is an analogue of *cis*-diamminedichloroplatinum(II) (cisplatin) a well known anticancer drug with renal damage as major side effect. In the search for more active and less toxic cisplatin analogues many square planar platinum(II) complexes have been synthesized and screened. Spiroplatin appeared to be less nephrotoxic and no cross-resistance with cisplatin was observed in animal studies[1–3]. Phase I and II clinical trials have been performed at the Free University Hospital[4] and several other places in Europe.

Spiroplatin (Fig. 1, I) is one of a number of [1,1-bis(aminomethyl)cyclohexane]-platinum(II) complexes synthesized by the Institute of Applied Chemistry, Utrecht,

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The Netherlands[5]. In general the N–Pt bond in square planar complexes is stable in aqueous solutions. The sulphato and aqua ligands on the other hand are susceptible to nucleophilic displacement[6]. The exchange of the sulphato ligand of spiroplatin has consequences for the dosage media employed. For example, in 0.9% NaCl the drug was rapidly converted into the corresponding dichloro complex TNO-1[7]. Therefore, it was administered in 5% glucose infusions. However, in such aqueous solutions, hydrolysis of the complex was expected (Fig. 1).

In order to investigate the influence of different media on the hydrolytic equilibria, a selective and sensitive analysis method had to be developed to discriminate between the different species. Until now, titrimetry[8], conductometry[9] and ^{195}Pt NMR spectroscopy[5,10–12] have been used to investigate hydrolysis of platinum(II) complexes in aqueous media. Separation techniques, like high voltage electrophoresis[13] and paper chromatography[14], have also been used. This paper describes an high-performance liquid chromatographic (HPLC) separation and differential pulse amperometric detection of spiroplatin and its hydrolyzed derivatives.

HPLC with flameless atomic absorption spectrophotometry (FAAS) (off-line)[15] and UV-absorption detection have been described for cisplatin[16] and for the complexes ethylenediaminemalonatoplatinum(II) (JM-40)[17] and diammine(1,1-cyclobutanedicarboxylato)platinum(II) (carboplatin)[18]. Because the molar absorptivity of antitumour platinum complexes is generally low, electrochemical detection methods were developed in various laboratories to enhance the sensitivity of detection.

Solid electrodes in oxidative as well as reductive amperometric modes[19,20] have been used for the detection of cisplatin after HPLC. Bannister *et al.*[21] obtained the lowest detection limit by using reductive amperometric detection at a hanging mercury drop electrode (HMDE). However, the practical application of their method was impeded by the necessity of maintaining the separation and detection system at a temperature of 60°C. The differential pulse amperometric detection of antitumour platinum complexes developed in our laboratory[7] combines the high sensitivity of

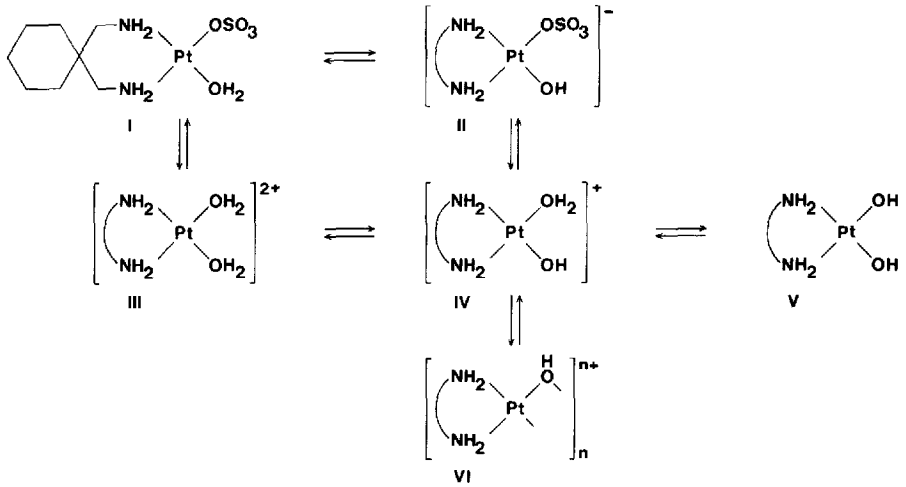


Fig. 1. Structural formulae of spiroplatin (I) and its possible derivatives in aqueous solution.

the HMDE with the inherent selectivity of this electrochemical technique. The selectivity is demonstrated by the detection of platinum complexes in biological fluids.

EXPERIMENTAL

Chemicals

Ampoules of spiroplatin (10 mg in 1 ml water) were obtained from Bristol Myers, Brussels. Solid spiroplatin, $[PtCl_2R]$ (TNO-1), $[\{Pt(OH)R\}_3](NO_3)_3$ (TNO-31) and $[Pt(H_2O)_2R](NO_3)_2$ (TNO-32)* were kindly provided by Dr. H. A. Meinema of the Institute of Applied Chemistry, TNO, Utrecht, The Netherlands. All solutions were made in doubly distilled water unless otherwise indicated. 5% Glucose as sterile infusion fluid was supplied by Lansberg (Uden, The Netherlands). Mercury was of polarographic quality (Merck, Amsterdam, The Netherlands). All other chemicals were of analytical grade.

Instrumentation

The chromatographic system consisted of a Model 6000A pump (Waters, Etten-Leur, The Netherlands), a manual sample-injection valve (Valco, Chrompack, Middelburg, The Netherlands) and a stainless-steel column (11 × 0.46 cm, Valco; or 4 × 0.46 cm, Knauer, Salm & Kipp, Breukelen, The Netherlands) packed with Spherisorb S5 ODS2 (ATS chromatography, Waddinxveen, The Netherlands). The precolumn (4 × 0.46 cm) contained Permaphase ODS (DuPont, 's-Hertogenbosch, The Netherlands).

Electrochemical detection was performed with a Model 310 polarographic detector and a Model 174A polarographic analyzer (both from Princeton Applied Research, EG&G Instruments, Nieuwegein, The Netherlands). Chromatograms were recorded with a Model B-381H three-pen strip chart recorder (Rikadenki, Simac Electronics, Veldhoven, The Netherlands).

Eluent was collected with a Model 2112 RediRac fraction collector (LKB, Zoetermeer, The Netherlands). Total platinum concentrations in eluent fractions were determined with a Model 5000 atomic absorption spectrophotometer equipped with a HGA graphite furnace, an AS 40 autosampler and a PRS printer sequencer (all from Perkin-Elmer, Gouda, The Netherlands).

Methods

Analytical columns were slurry packed in dry acetone (0.16 g/ml) by upward displacement using a Knauer column packing assembly. The mobile phases consisted of methanol (10, 15, 20 and 30%, v/v) in a 0.05 *M* sodium sulphate solution acidified to pH 3 with sulphuric acid. The mobile phase and electrochemical cell were purged with deoxygenated nitrogen as described previously[7].

Standard chromatographic conditions consisted of a mobile phase of 20% methanol with a flow-rate of 1 ml/min, an injection volume of 20 μl and a 11 × 0.46 cm column. Detection occurred at an HMDE, with a medium drop size, at −540 mV *vs.* Ag/AgCl in the differential pulse mode using a pulse of −100 mV every 0.5 sec and a low pass filter of 0.3 sec. A fresh mercury drop was dispensed prior to every

* R = 1,1-bis(aminomethyl)cyclohexane.

chromatogram. All chromatograms were recorded at ambient temperature. Peak heights rather than peak areas were measured. A precolumn was used only when plasma ultrafiltrate or urine samples were analyzed.

The hydrodynamic differential pulse voltammograms were obtained by consecutive injection of a spiroplatin solution (1 mg/ml) and a TNO-1 solution (50 μg/ml) at various potentials with a pulse height of -100 mV. Peak currents (nA/nmol) were calculated from the platinum recovery from the fractionated eluent.

RESULTS AND DISCUSSION

Liquid chromatography

Spiroplatin was eluted with a low recovery (10%) from the chromatographic system described previously for TNO-1[7]. This was probably caused by adsorption or very high retention of the hydrolyzed species, possibly by nucleophilic displacement of the aqua ligand by residual free silanol groups of the Nucleosil C_{18} column.

Using the endcapped column packing material Spherisorb S5 ODS2 and 20% methanol in 0.05 *M* Na_2SO_4 as mobile phase, half of the injected spiroplatin was eluted as a single, tailing peak. Sodium sulphate was used as supporting electrolyte because of its common ion with spiroplatin. Lowering the pH of the mobile phase to 3 by use of sulphuric acid resulted in a chromatogram for spiroplatin containing several sharp peaks. These peaks comprised all of the injected material, as confirmed by atomic absorption spectrophotometric (AAS) analysis of platinum in the fractionated eluent. This change in behaviour at the acidic pH was probably caused by the (horizontal) equilibria in Fig. 1 being driven to the left, since the first pK_a of *cis*-diaquaplatinum(II) complexes is 5.6–5.8[6,8]. An additional beneficial effect of this mobile phase may be that H_3O^+ is associated with the negatively charged silanol groups[22,23].

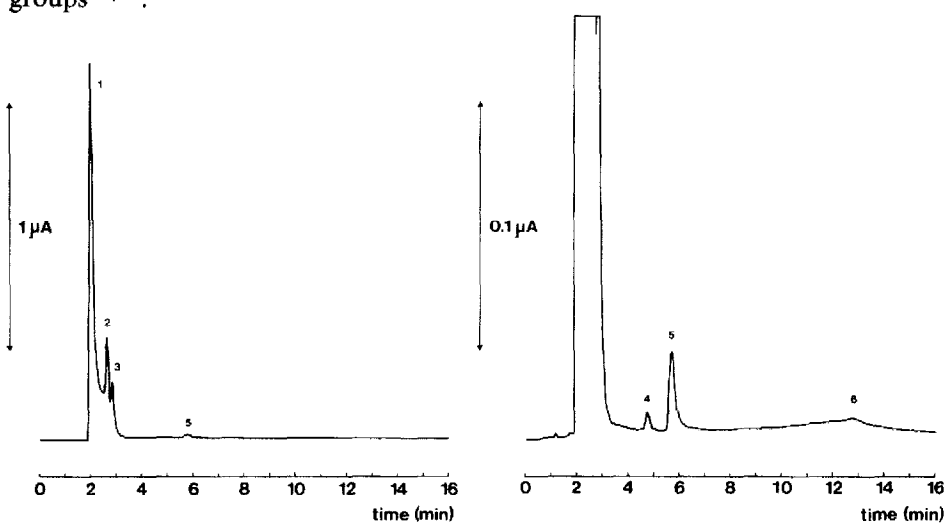


Fig. 2. Chromatogram of spiroplatin (10 mg/ml, 22 m*M*) on a Spherisorb S5 ODS2 column with 20% methanol in 0.05 *M* sodium sulphate, pH 3 as mobile phase, recorded at two sensitivities. Peaks: 1 = $[Pt(H_2O)_2R]^{2+}$; 2 = $[Pt(SO_4)(H_2O)R]$; 3 = $[PtCl(H_2O)R]^+$; 4 = $[PtCl_2R]$; 5 = $[\{Pt(OH)R\}_2]^{2+}$; 6 = unknown. R = 1,1-bis(aminomethyl)cyclohexane.

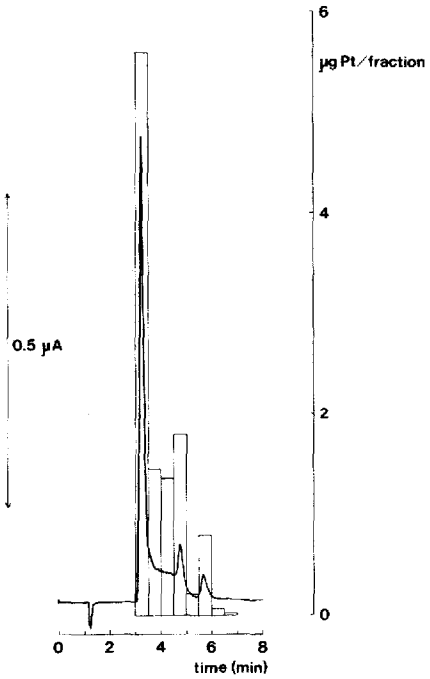


Fig. 3. Chromatogram of spiroplatin (1 mg/ml). Mobile phase: 10% methanol. Platinum was also analyzed in the fractionated eluent by off-line AAS.

An example of a chromatogram of a concentrated solution of spiroplatin is given in Fig. 2. It shows that the ampoules used for administration of spiroplatin to patients contained at least six different components, identified as the parent compound and derivatives as described below. With 20% methanol in the mobile phase, all components were eluted within 15 min. A better separation of the first three peaks was achieved with 10% methanol (Fig. 3), which allowed the collection of the individual peaks in discrete fractions. The capacity ratios of spiroplatin and its derivatives in mobile phases with various methanol contents are summarized in Table I.

TABLE I

CAPACITY RATIOS AS FUNCTION OF METHANOL CONTENT IN THE MOBILE PHASE

Methanol in 0.05 *M* Na_2SO_4, pH 3. R = 1,1-bis(aminomethyl)cyclohexane.

Complex	*Methanol (%, v/v)*			
	30	*20*	*15*	*10*
$[Pt(SO_4)(H_2O)R]$	0.8	1.5	2.25	3.7
$[Pt(H_2O)_2R]^{2+}$	0.5	0.9	1.4	2.2
$[PtCl(H_2O)R]^{+}$	0.8	1.75	2.7	4.7
$[PtCl_2R]$	1.7	3.5	5.7	9.4
$[\{Pt(OH)R\}_2]^{2+}$	2.4	4.8	10	30
$[\{Pt(OH)R\}_3]^{3+}$	15	45		

The high baseline between peaks 1 and 2 could not be eliminated by chromatographic means. It is the result of a shift of the equilibrium between the diaqua (peak 1) and the monoaquamonosulphato species (peak 2) during chromatography (Fig. 1, III and I). Sulphate ions in the mobile phase contribute to this shift and from this point of view a completely inert electrolyte would be preferable. Often used electrolytes like acetate and phosphate appeared to form new complexes when added to spiroplatin solutions. This was not observed with nitrate and perchlorate. However, mobile phases with these electrolytes did not improve the separation of peaks 1 and 2 and resulted in poorer peak shapes. Therefore sodium sulphate was maintained as supporting electrolyte.

The complete recovery of injected platinum in the effluent and the linearity of the signal–concentration curve indicate that the stainless-steel surfaces of the column and capillary tubing do not react to a measurable extent with the solute under the conditions used.

Electrochemical detection

All [1,1-bis(aminomethyl)cyclohexane]platinum(II) complexes investigated were monitored by differential pulse amperometric (DPA) detection at an HMDE. The sensitivity of the detection for the different compounds in a solution of spiroplatin was determined by AAS analysis of separately collected peaks. Fig. 3 shows a chromatogram of a 1 mg/ml spiroplatin solution of which fractions were collected at 0.5-min intervals. Total platinum measured in the fractions from 3 to 7 min and from 30 to 33 min, comprising peaks 1–3 and 5 respectively, contained 97% of the injected amount; 47% was retained in the fraction of 3–3.5 min (peak 1). From the peak areas and the platinum concentrations in the corresponding fractions it could be calculated that the sensitivity of the electrochemical detector was 1.5 times higher for peak 1 than for peaks 2 and 3.

Under standard operating conditions, the linearity of the assay was determined for the first peak. A range of solutions was chromatographed immediately after dilution of a 1 mg/ml spiroplatin stock solution. A linear dynamic range of 0.002–2.22 mM total spiroplatin was achieved when plotted on a double logarithmic scale (r = 0.999). The slow shift of the equilibrium between peaks 1 and 2 during the analysis did not influence the linearity of the response to peak 1. For peak 1 the sensitivity was 55 nA/μg injected spiroplatin.

The detection limit was determined using a 50-μl injection loop and large mercury drop size. The baseline current was 2 nA. With a peak-to-peak noise of 50 pA, a practical detection limit of 0.05 μM spiroplatin (20 ng/ml) was achieved for peak 1. This value is lower than those obtained for cisplatin with UV absorption and FAAS detection[15] and with amperometric detection at solid electrodes[19,20]. Still lower quantities could be detected[21] only by reductive amperometric detection at an HMDE at 60°C.

Repeated injection (n = 9) of 0.22 mM spiroplatin revealed within-day coefficients of variation (C.V.) of 3.4 and 2.5% for peaks 1 and 5, respectively. The between-day C.V. (n = 5) were 12.5 and 7.5%, respectively. For a solution of TNO-1 (0.11 mM in 1.5 M sodium chloride) the within-day C.V. was 1.2% (n = 5) and the between-day C.V. was 8% (n = 3).

The DPA mode was used because of its higher selectivity compared to direct

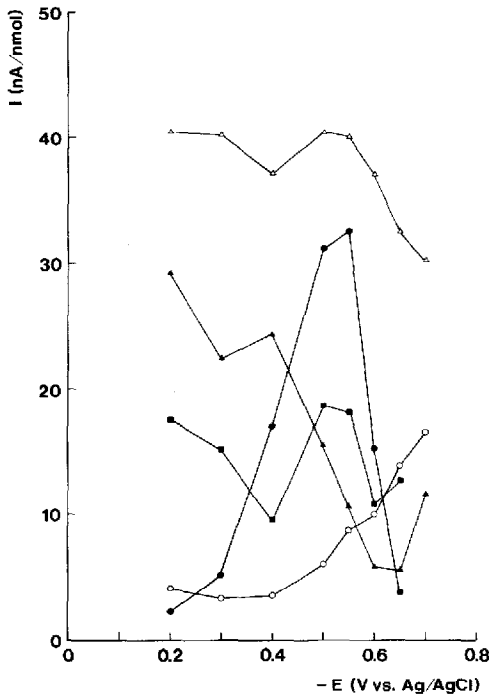


Fig. 4. Hydrodynamic differential pulse voltammograms of the peaks of Fig. 2: ●—●, peak 1; ○—○, 2; ▲—▲, 3; △—△, 4; ■—■, 5.

current amperometric detection. This selectivity is especially advantageous in the case of biological samples. Furthermore, at the applied potential, the oxygen peak (k' = 2.0) was 100 times lower (3 *vs.* 300 nA) than with sampled direct current detection at −650 mV *vs.* Ag/AgCl, while the current of peak 1 was only two-fold lower in the DPA mode compared to the direct current method. The limits of detection were comparable for the two methods. The hydrodynamic differential pulse voltammograms in Fig. 4 show that maximum sensitivity for the species of peaks 1, 4 and 5 of Fig. 2 is achieved at a potential of −540 mV *vs.* Ag/AgCl and with a pulse amplitude of 100 mV. Besides, the selected potential is a satisfactory compromise between the ascending wave of peak 2 and the declining wave of peak 3.

Maximum peak currents were obtained with a pulse height of 100 mV (maximum instrument setting). The irreversibility of the platinum(II) reduction[24] permits the assumption that at higher pulse amplitudes the sensitivity of detection will be increased. On the other hand, the selectivity of the DPA detection mode will decrease with increasing pulse amplitude. Therefore, for a given sample and a given HPLC resolution a pulse amplitude has to be chosen which provides the best compromise between sensitivity and selectivity.

When the polarographic detector is used in the DPA mode, the time constant of the polarograph also has to be considered. With a clock time of 0.5 sec, which means that every 0.5 sec a pulse is generated and the differential current is measured, the width of a chromatographic peak has to be at least 12.5 sec[25]. This requirement was just attained for the first peak of the spiroplatin chromatogram under standard

operating conditions. DPA detection with a clock time of 1 sec, recommended for detection at a droppping electrode (static mercury drop electrode, SMDE), was not feasible. This minor drawback of the DPA detection mode can be eliminated by shortening the circuit time constant of the polarograph[26,27]. With a clock time of 0.5 sec, detection at a SMDE was possible, but at an HMDE a lower noise level was achieved (50 pA *vs.* 1 nA), probably because of an undisturbed flow pattern around the electrode.

Identification of the chromatographic peaks

The identities of peaks 1–5 in the chromatogram of Fig. 2 were elucidated. Using the described chromatographic method, the influence of salt, acid or base on the hydrolytic equilibria (Fig. 1) was followed. Fig. 5 shows the effect of 23 m*M* sodium sulphate on the constitution of a spiroplatin solution (0.23 m*M*) in water. The height of the second peak increased and that of the first peak decreased in the presence of sodium sulphate. Peaks 3 and 5 remained constant and peak 4 was undetectable at this concentration. The equilibrium shift was completed within 1 h, reducing the hydrolysis of the sulphato complex I. This strongly suggests that peak 1 corresponds to the diaqua complex III and peak 2 to the monoaquamonosulphato complex I, *i.e.*, the original spiroplatin. ^{195}Pt NMR spectra of concentrated solutions of spiroplatin with and without sodium sulphate enable the same conclusions to be drawn[5,28]. Another indication as to the identities of peaks 1 and 2 was the appearance of peak 1 and the absence of peak 2 in chromatograms of a solution of $[Pt(H_2O)_2R](NO_3)_2$ (TNO-32) which is present as the diaqua species III in aqueous solutions.

The assumption, based on its retention time, that peak 5 represents the dimer species VI (n = 2, Fig. 1) was confirmed by chromatograms of a solution of the chemically pure trimer TNO-31 (VI, n = 3).A fresh solution of this compound in water yielded one peak with k' = 45. The trimer decomposed in acidic solution (pH = 3), yielding components with retention times corresponding to peaks 1 and 5 of Fig. 2, *i.e.*, monomer (III) and dimer (VI). Furthermore, the height of peak 5 decreased in acidified spiroplatin solutions and increased in dilute spiroplatin solutions of intermediate pH, in which the prevalence of IV is favoured[8]. These results are in

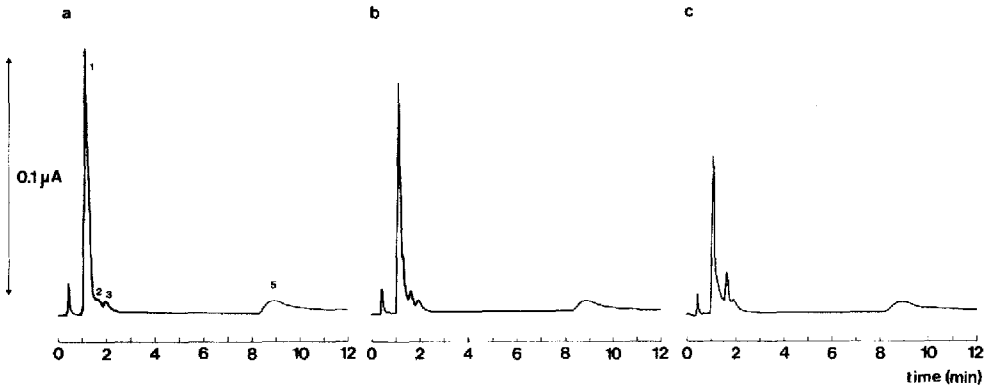


Fig. 5. Chromatograms of spiroplatin (0.23 m*M*) in water (a) and in 23 m*M* Na_2SO_4, 1 min (b) and 1 h (c) after preparation. Column: 4 cm. Mercury drop size: small.

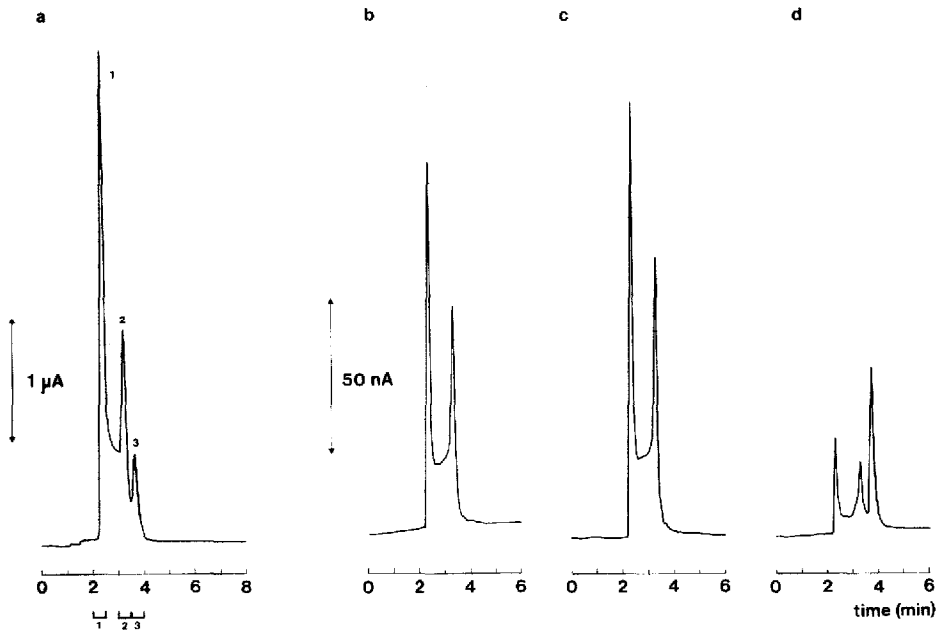
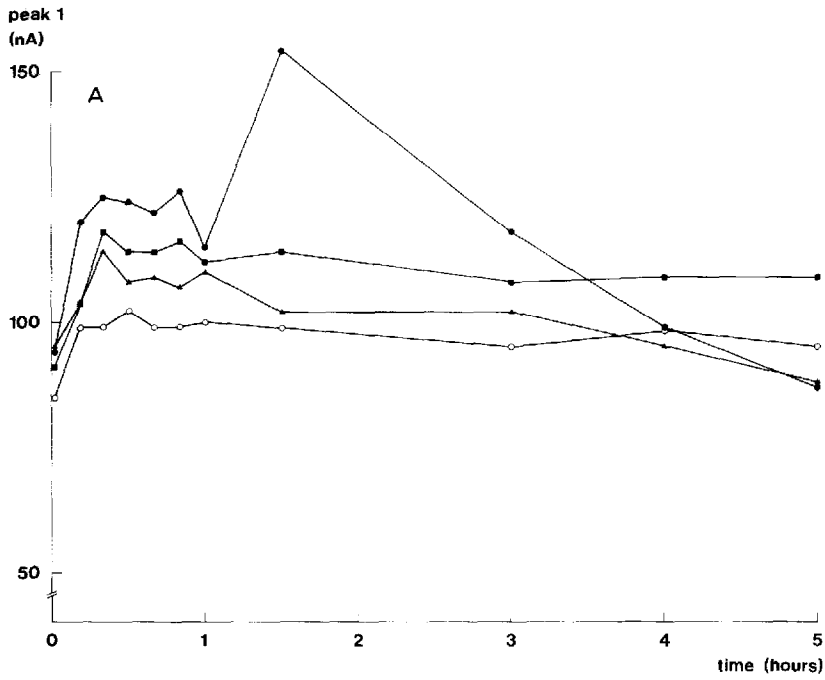


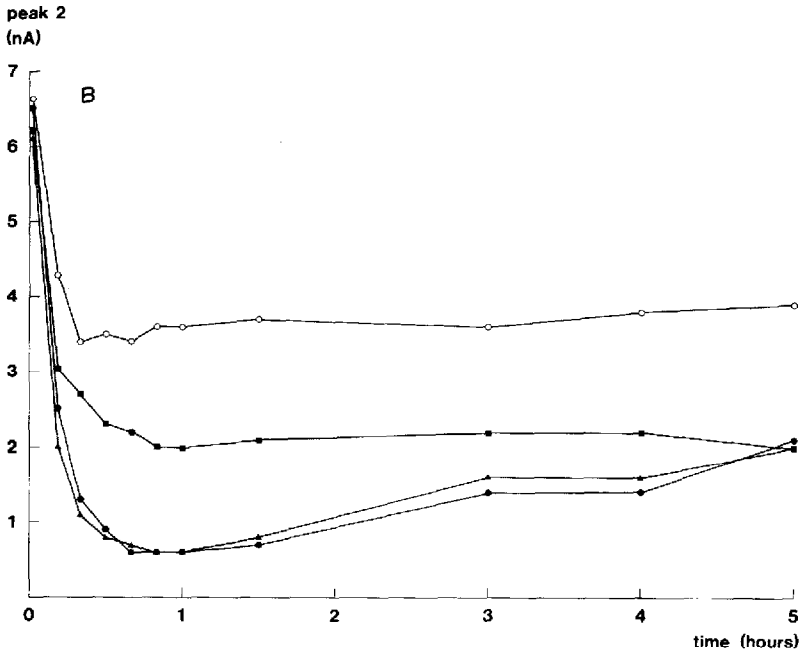
Fig. 6. Chromatogram of spiroplatin (20 mg/ml) (a) and rechromatograms of fractions containing peaks 1, 2 and 3 respectively (b, c and d) 1.5 h after collection. Mobile phase: 15% methanol.

complete agreement with those of the ^{195}Pt NMR experiments on cisplatin and some congeners performed by other investigators[5,10,12].

Both peaks 3 and 4 appeared to be chloride complexes, originating from the precursor used for the synthesis of spiroplatin[5]. After the addition of an equimolar concentration of sodium chloride to a solution of 0.22 m*M* spiroplatin both peaks 3 and 4 increased, the former becoming the highest. In high chloride concentrations (0.11 m*M* spiroplatin in 0.15 *M* sodium chloride) ultimately all species were converted into one complex having the same capacity ratio as peak 4. Its retention behaviour is unaffected by pH or the supporting electrolyte (Na_2SO_4, $NaClO_4$ or $NaNO_3$) in the mobile phase, indicative of a neutral, *i.e.*, dichloro, complex. After dilution of a concentrated spiroplatin solution, peak 4 disappeared and peak 3 increased (Fig. 7C), probably because of hydrolysis of the dichloro species to the monoaquamonochloro complex represented by peak 3.

In order to confirm the identity of the first three peaks, they were collected separately and rechromatographed at various times after injection. Fig. 6 shows chromatograms of the original spiroplatin solution (20 mg/ml) and of the fractions, containing the individual peaks, rechromatographed *ca.* 1.5 h after collection. By that time the new equilibria were established. Chromatograms obtained at various times after isolation showed that, in fraction 1, peak 1 (diaqua species) decreased and peak 2 (monoaquamonosulphato species) increased. This was expected, because of the sodium sulphate present in the mobile phase. In fraction 2 the opposite occurred, resulting in the same equilibrium as in fraction 1. In fraction 3, peaks 1 and 2 increased and peak 3 decreased. Thus, reactions occurred between the species of peaks 1–3, but peak 3 was not formed from fractions 1 and 2 in this medium, which cor-


peak 1
(nA)
150
A
100
50
0
1
2
3
4
5
time (hours)


peak 2
(nA)
7
B
6
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2
1
0
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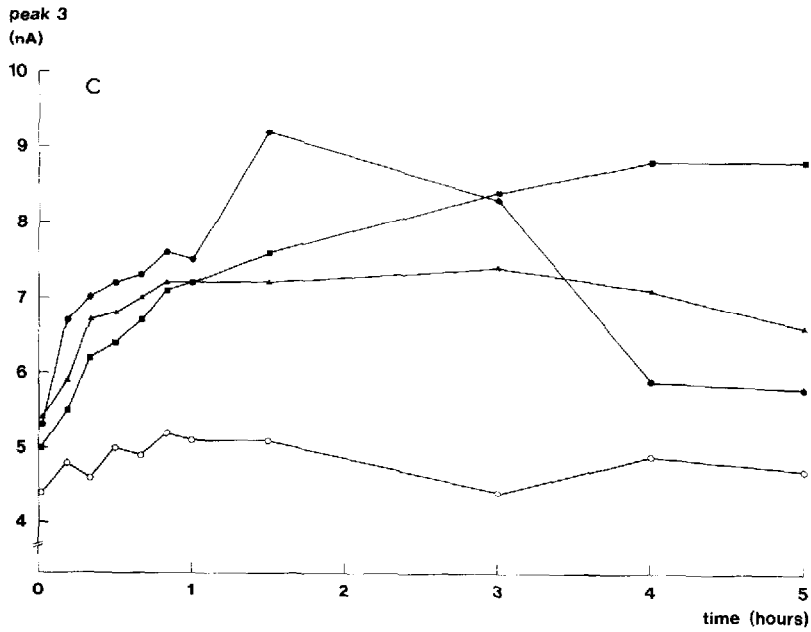
time (hours)

Fig. 7.



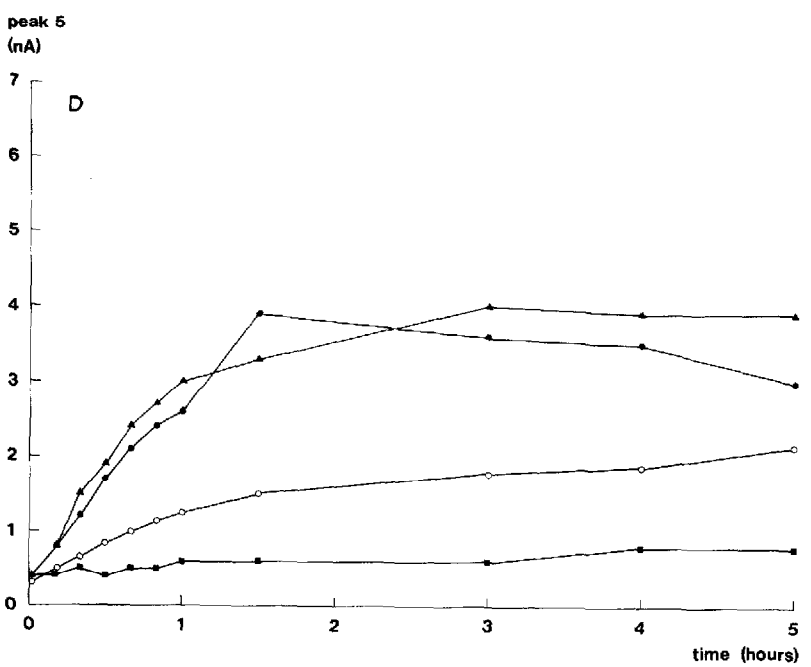


Fig. 7. Peak heights *vs.* time after dilution of spiroplatin from an ampoule in 5% glucose: 0.1 mg/ml, pH 4.0 (●), 4.6 (▲), 2.9 (■); 1 mg/ml, pH 3.5 (○, nA × 0.1).

responds well with peak 3 being the monoaquamonochloro species. Elemental analysis of spiroplatin revealed 0.6% Cl (0.073 atom Cl per molecule spiroplatin)[5], which is in agreement with the recovery of 6.7% Pt from peak 3 in Fig. 3.

The origin of the rise in baseline designated as peak 6 in Fig. 2 is not yet known. It is only observed in chromatograms of solutions of concentration $\geqslant 3$ mg/ml.

The pH of the spiroplatin solutions did not affect the retention times of the aquated species. Protonation and deprotonation of the aqua ligands are fast processes[8,10,11]. Therefore complexes I and II as well as III–V cannot be separated chromatographically. The slow rate of ligand exchange of platinum complexes allowed the chromatographic separation of mixtures of complexes with different ligands (H_2O, Cl^-, SO_4^{2-}) and oligomers.

Composition of spiroplatin in infusion fluids

The ligand exchange of spiroplatin in 0.9% NaCl solution was described previously[7]. Using the present analysis method we studied the stability of spiroplatin in 5% glucose.

The clinical situation was imitated by diluting spiroplatin from an ampoule (10 mg/ml) in 5% glucose to 0.1 mg/ml. This concentration was used when spiroplatin was administered to patients as 4-h i.v. infusions. The pH of the resulting solution was 4.0. Chromatograms were recorded at regular intervals for 5 h after preparation. The same experiment was carried out with solutions acidified with sulphuric acid to pH 2.9, alkalinized to pH 4.6 with NaOH or containing a higher concentration of spiroplatin (1 mg/ml, pH 3.5). The concentrated solution was used during phase I trial in the case of 10-min i.v. infusions.

The change in peak heights with time is shown in Fig. 7. The main conclusion is that more diaqua and dimer species are administered during the 4-h infusion (0.1 mg/ml) than during the 10-min infusion (1 mg/ml). The opposite is true for the sulphato complex (peak 2). In acidified solution the formation of the dimer is limited. The relatively high content of the sulphato complex (peak 2) in the acidified 0.1 mg/ml solution is a result of the added H_2SO_4. The increase in peak 3 (monoaquamonochloro complex) is caused by hydrolysis of the dichloro complex (peak 4, Fig. 2). This effect is more pronounced after dilution to 0.1 mg/ml than after dilution to 1 mg/ml, as expected. Similar results were obtained after dilution of the spiroplatin solution from the ampoule in water only, which means that the shift of the equilibria is caused by concentration and pH effects and not by the presence of glucose.

Due to hydrolysis, spiroplatin (I) in aqueous solutions is only present to a small extent. In a 1 mg/ml solution only 15% of the complex is present in this form (peak 2, Fig. 3). However, when the hydrolytic equilibria are reached the resulting solution of various complexes is stable for at least 1 year. The total amount of aquated and oligomerized products administered to patients depends on the time after dilution and the concentration of the complexes in the infusion fluid. This is important for the evaluation of (pre)clinical studies, because the presence of more or less hydrolyzed products may influence the toxicity and antitumour activity of the drug[10,12].

The observed differences in chemical composition of the infusion solutions initiated a study of the possible relationship between the composition of the spiroplatin solution and nephrotoxicity[29].

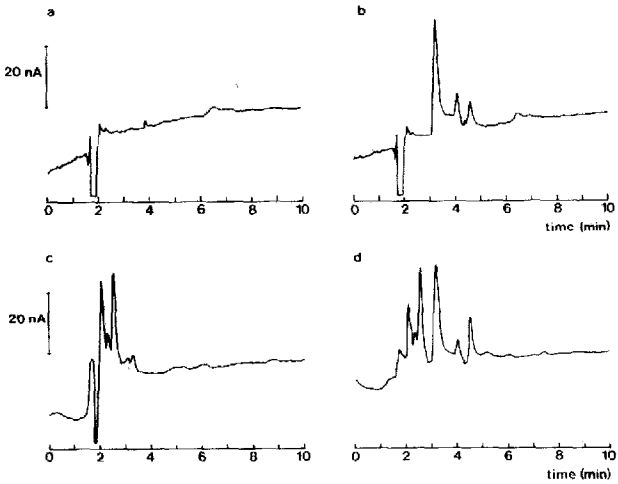


Fig. 8. Chromatograms of blank and spiked (0.22 m*M* spiroplatin) plasma ultrafiltrate (a, b) and urine (c, d). Column: 15 cm. Precolumn: Permaphase ODS, 4 cm.

Detection in body fluids

The ability of the described method to analyse spiroplatin in biological samples is shown in Fig. 8. Human plasma ultrafiltrate and urine samples were spiked with spiroplatin from a 10 mg/ml stock solution and immediately analyzed without prepurification. The selectivity of the applied electrochemical detection mode is clearly demonstrated by the chromatogram of blank plasma ultrafiltrate (Fig. 8a). Urine constituents were detected but I, II and the monoaquamonochloro complex were separated from them (Fig. 8c and d). Actual samples from patients were not analyzed because the use of spiroplatin was not continued in the clinic when this analysis became available. Anyhow, our procedure has the selectivity and sensitivity to quantify antitumour platinum complexes and their derivatives in body fluids.

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