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DETERMINATION OF CISPLATIN AND RELATED PLATINUM COMPLEXES IN PLASMA ULTRAFILTRATE AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE RADIOACTIVITY DETECTION

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SUMMARY

A reversed-phase ion-pair chromatographic method with on-line radioactivity detection for the simultaneous determination of ^{195m}Pt-labelled cisplatin and related platinum complexes has been developed. With this system a good resolution of various radiolabelled platinum complexes can be achieved. The detection limit of the radioactivity detector is 10 ng of cisplatin (specific activity of 15 MBq/mg cisplatin) per millilitre of urine or plasma ultrafiltrate. The detector response is independent of both the chemical structure of the platinum complexes and the matrix composition of the samples. This method may serve as a reference system for other high-performance liquid chromatographic systems with less specific and sensitive detectors.

INTRODUCTION

Cisplatin is an important antineoplastic drug, which is widely used in the treatment of several tumours, especially of the testes and ovaries [1]. The clinical use of cisplatin, however, is limited because of its nephrotoxic potential [2]. The molecular mechanism of this nephrotoxicity is still largely unknown.

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It is also not known whether the parent compound or a metabolite is responsible for this drug-induced nephrotoxicity. The chemical structures of cisplatin and some possible metabolites are shown in Fig. 1. Studies of the metabolism and the molecular mechanism of the nephrotoxicity of cisplatin are seriously hampered by the lack of suitable analytical techniques to determine low levels of cisplatin and its metabolites in complex biological matrices.

Several high-performance liquid chromatographic (HPLC) methods have been applied to the study of the metabolism and the pharmacokinetics of cisplatin in animals and humans [3–14]. The practical applicability of these methods, however, is limited by several disadvantages. The resolving power of most chromatographic systems appears to be insufficient to achieve a good separation of complexes of cisplatin with endogenous compounds such as methionine, cysteine and glutathione. In addition, most of the on-line detection methods applied, notably UV detection with or without post-column derivatization [3,4], phosphorescence detection [9], inductively coupled plasma atomic emission spectrometric detection [11] and electrochemical detection [7], appear to lack the necessary selectivity and sensitivity for the determination of platinum complexes in biological fluids.

HPLC in combination with atomic absorption spectrometric (AAS) detection, which has been reported to be suitable for the determination of cisplatin in concentrations of 10–40 ng/ml in plasma ultrafiltrate, has the major disadvantage that detection has to be performed off-line [14]. Off-line AAS mea-

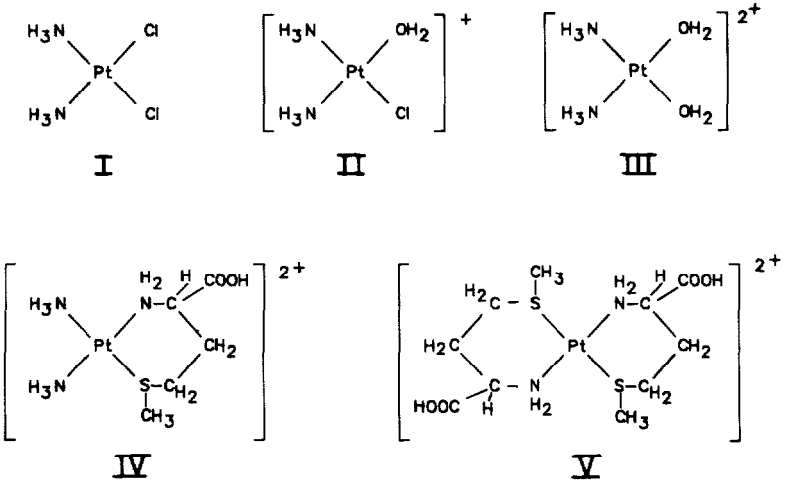

Fig. 1. Chemical structures of cisplatin and some possible metabolites. I = Cisplatin (*cis*-diamminedichloroplatinum, c-DDP); II = *cis*-diammineaquachloroplatinum(II) ion; III = *cis*-diamminediaquaplatinum(II) ion; IV = diamminemethionineplatinum(II) ion; V = dimethionineplatinum(II) ion.

surements are impractical for routine application, because they are time-consuming and laborious.

Therefore, it is clear that there is still a great need for a sensitive and selective analytical method for the determination of cisplatin and its metabolites in biological fluids. A promising approach is the application of HPLC with on-line radioactivity detection. The isotope ^{195m}Pt is especially suitable for this purpose, since it can be easily produced by irradiation of isotopically enriched ^{194}Pt in a nuclear reactor [15,16].

Platinum can form complexes that may be cationic, anionic or neutral. Differently charged complexes can be separated on chemically bonded or solvent-generated ion-exchange columns. De Waal et al. [11] have successfully used reversed-phase ion-pair chromatography for the separation of cisplatin and methionine–platinum complexes in aqueous solutions. The primary aim of the present study was to develop a sensitive analytical method, based on their system [11], with a good resolving power for ^{195m}Pt-labelled platinum complexes and on-line radioactivity detection, and further to evaluate the applicability of this method to studies of the pharmacokinetics and metabolism of cisplatin.

EXPERIMENTAL

Chemicals

^{195m}Pt-labelled cisplatin, with a specific activity of 15 MBq/mg cisplatin, was synthesized as follows: platinum metal, isotopically enriched to 80% ^{194}Pt (Intersales Holland, Hengelo, The Netherlands) was irradiated in a thermal neutron flux of $2.0 \cdot 10^{14}$ cm^{-2} s^{-1} for 168 h. The resulting ^{195m}Pt was converted into [^{195m}Pt]cisplatin according to the synthetic and test procedures described by Hoeschele et al. [15]. The radionuclide ^{195m}Pt decays with a half-life of 96.5 h to stable ^{195}Pt [17]. It emits various gamma lines, which are substantially converted. In addition to conversion electrons, Auger electrons and X-rays also are emitted, in coincidence with the gamma rays or conversion electrons with most of the radioactivity signal between 30 and 120 keV. This allows measurement of ^{195m}Pt with a high efficiency via liquid scintillation counting. The half-life of the labelled complex was measured in a standard apparatus for liquid scintillation counting (Packard Instruments, U.S.A.), yielding a value of 95.6 ± 1.6 h. Thus, the ^{195m}Pt-labelled cisplatin may be considered of sufficient radiochemical purity.

Chemicals for preparing the mobile phase of the chromatographic system were of HPLC grade. Other chemicals were of analytical grade.

Chromatography

The liquid chromatograph consisted of an LKB 2150 HPLC pump and a Rheograde 7125 high-pressure injection valve. Gradient elution was performed with an LKB 2152 LC controller and an LKB 11300 Ultrograd mixer driver

(LKB-Pharmacia, Woerden, The Netherlands). Separations were performed on a 100 mm×3.0 mm I.D. column, packed with 5-μm Spherisorb ODS (Chrompack, Middelburg, The Netherlands). Several mobile phases and gradient programmes were evaluated. The flow-rate was 0.25 ml/min and the injection volume 20 μl.

The detector consisted of a Berthold LB 506 C HPLC monitor and a Bernhard 1. Stadler E.100.1 scintillator pump (Betron Scientific, Rotterdam, The Netherlands). The LB 506 C HPLC monitor contained a radioactivity flow-through monitor LB 506 C with a computer (Motorola 6809 cpu) for data reduction and a personal computer (Tandon TM 7516) for data handling. Measurements of radioactivity were made with the admixture method. A liquid scintillator, Lumagel (Lumac, Landgraaf, The Netherlands), was aspirated with the scintillator pump, and mixed continuously with the HPLC eluate, and the resulting mixture was passed through a flow-through cell, arranged between the cathodes of two photomultipliers operating in coincidence to reduce the background. Cells of different volumes (1.0, 0.5, 0.2 ml) were tested. Data processing, including peak-area measurements, was performed with the LB 506C HPLC software.

In vitro experiments

^{195m}Pt-labelled cisplatin (1 m*M*) was incubated with several thiols in a cisplatin-to-thiol molar ratio of 1:2 at 37°C for 2 h. The thiols used were: glutathione (GSH), N-acetylcysteine (NAC), methionine (METH) and cysteine (CYS). HPLC analysis of each reaction mixture was performed immediately after the respective incubation.

Animal experiments

Male Wistar rats (200 g) were obtained from the Central Institute for the Breeding of Laboratory Animals, Harlan Sprague Dawley (CPB/HSD), Zeist, The Netherlands, and fed water and standard laboratory chow ad libitum. The rats were treated intravenously with solutions of ^{195m}Pt-labelled cisplatin (5 mg/kg) in physiological saline. At selected intervals blood and urine were collected. Blood was obtained through heart-puncture under diethyl ether anaesthesia and immediately centrifuged for 5 min at 1000 *g* to obtain plasma. These plasma samples were immediately ultrafiltered over YMT filters in an Amicon MPS-1 micropartition system (Amicon, Oosterhout, The Netherlands) for 20 min at 2000 *g*. Ultrafiltrates were immediately injected on the HPLC column. Urine samples were obtained under diethyl ether anaesthesia, filtered through 0.45-μm Millipore filters (Nihon Millipore Kogyo Yonezawa, Japan) and stored in liquid nitrogen. Urine samples were analysed within 2 h and were shown to be stable for at least 24 h.

RESULTS AND DISCUSSION

Chromatography

The primary aim of the present study was to develop a sensitive HPLC method, with on-line radioactivity detection, for the separation of ^{195m}Pt-labelled platinum complexes and further to evaluate the applicability of this method to studies of the pharmacokinetics and metabolism of cisplatin. In order to achieve optimal resolution of cisplatin–GSH and cisplatin–NAC complexes, the reversed-phase ion-pair HPLC system recently developed by de Waal et al. [11] for the separation of cisplatin–METH complexes was modified: the concentration of 2-propanol (the organic modifier) in the second eluent of the gradient was increased and the linear gradient of sodium phosphate–2-propanol was expanded. The best results were ultimately obtained with the following two mobile phases: (I) 5 m*M* sodium dodecyl sulphate (SDS), 10 m*M* sodium phosphate buffer (pH 2.6); (II) 5 m*M* SDS, 25% 2-propanol, 60 m*M* sodium phosphate buffer (pH 2.6). The following gradient programme was used: a linear gradient from 100% I to 100% II in 45 min; 10 min at 100% II; a linear gradient from 100% II to 100% I in 5 min; 10 min at 100% I. The chromatographic conditions were evaluated with in vitro incubation mixtures of cisplatin and several biologically relevant thiols, notably GSH, CYS, METH and NAC. Fig. 2 clearly illustrates the resolving power of this modified chromatographic system. Within 1 h, various reaction products of cisplatin could be separated from cisplatin and from each other in one run.

To study the possibility of reactions between the platinum complexes and constituents of the eluents, the reaction mixtures were diluted (1:1) with water or eluent II. After standing for 1 h at room temperature, radiochromatograms were recorded again. There were no significant differences between the radiochromatograms of the water-diluted and those of the mobile phase-diluted reaction mixtures. Apparently, the analyses were not significantly disturbed by reactions between platinum complexes and constituents of the mobile phases during the chromatographic run. This observation is in accordance with previous results of de Waal et al. [11], who also used a phosphate buffer–2-propanol based HPLC system.

The identities of the various platinum complexes, shown in Fig. 2, remain to be established. Two cisplatin–METH complexes already have been identified by others [18,19], viz. the diamminemethionineplatinum(II) ion and the dimethionineplatinum(II) ion (structures IV and V, Fig. 1). In these complexes the chlorine atoms and ammonia molecules of cisplatin have been substituted by the sulphur and nitrogen atoms of methionine. GSH, CYS and NAC may form similar complexes with cisplatin. For example, NAC possesses nucleophilic groups, capable of substituting one or more chlorine atoms in cisplatin. Incubation of cisplatin with NAC in an aqueous solution resulted in a

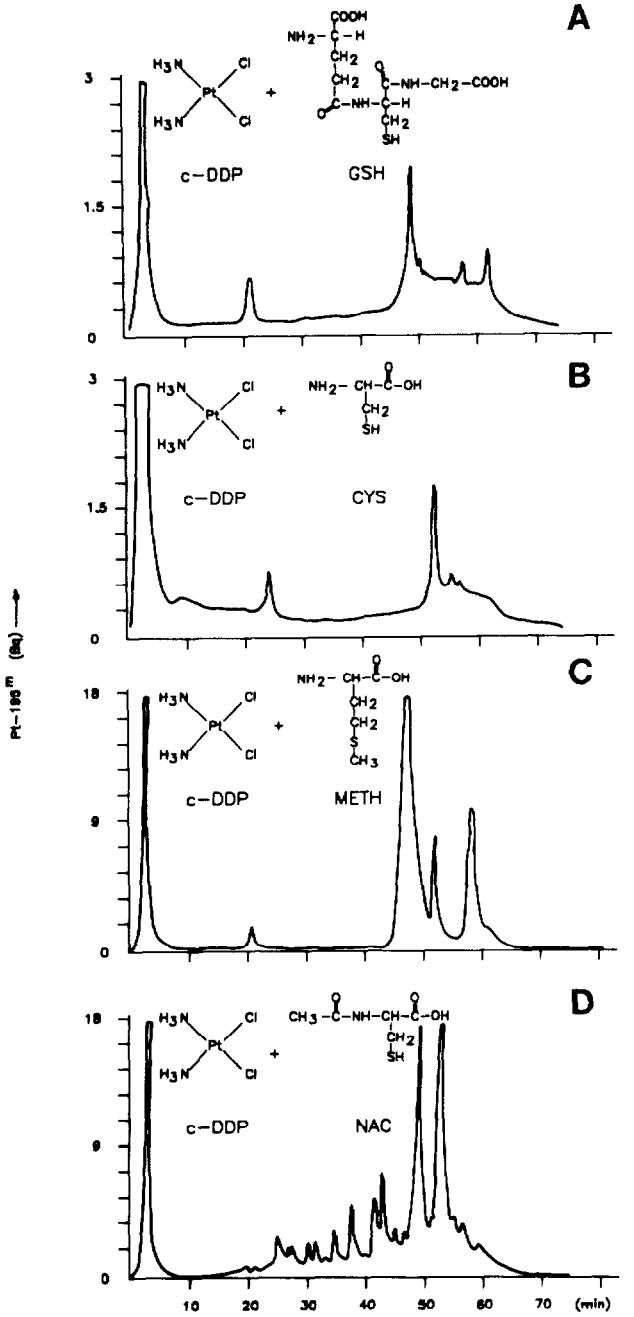

Fig. 2. Radiochromatograms of several mixtures of platinum complexes, obtained after incubation of ^{195m}Pt-labelled cisplatin (c-DDP) at 37°C for 2 h with (A) glutathione (GSH), (B) cysteine (CYS), (C) methionine (METH) and (D) N-acetylcysteine (NAC). Incubations were performed in a molar ratio of 1.2 (c-DDP to thiol). Chromatographic conditions are described in the text.

mixture of three major and several minor platinum complexes, which could be separated from each other with our HPLC system (Fig. 2).

The recovery of cisplatin from urine, spiked with $5 \cdot 10^{-7}$ *M* ^{195m}Pt-labelled cisplatin was determined as $95 \pm 5\%$ ($n=3$). For plasma ultrafiltrate samples, spiked with ^{195m}Pt-labelled cisplatin, the recovery was also found to be $95 \pm 5\%$ ($n=3$). Recoveries of cisplatin–thiol incubation mixtures and of urine samples and plasma ultrafiltrate samples of rats treated with cisplatin were all at least 95%. These results demonstrate that this chromatographic system is suitable for the resolution of various platinum complexes in several matrices.

Detection

The sensitivity of the radiodetector is dependent on the residence time in the measuring cell, the efficiency of the detector and the specific activity of the radioactive compound. Basically, two types of measuring cells can be distinguished: cells with solid scintillator beads and flow-through cells designed for liquid scintillation counting. Cells with solid beads are not suitable for the determination of ^{195m}Pt-labelled compounds, because they are easily contaminated, owing to adsorption of platinum on the beads. Therefore, and because liquid scintillation measurements of ^{195m}Pt are more sensitive, the measurements were made with liquid scintillation counting in a flow-through cell. With this method, the possibility of contamination is virtually excluded.

Optimal conditions for sensitivity and resolution were determined by repeated injections of cisplatin–NAC and cisplatin–GSH incubation mixtures, using various cell volumes and various discriminator settings. The best results were obtained with a Z-1000-4 flow-through cell with a volume of 1 ml. The optimal conditions are summarized in Table I.

The necessity for correction for differences in quenching in eluents I and II was studied by measuring the radioactivity of ^{195m}Pt-labelled cisplatin in both

TABLE I

OPERATING CONDITIONS FOR THE LB 506C ON-LINE RADIOACTIVITY HPLC MONITOR

Dwell time	13 s
High energy window	
Lower level	225
Upper level	700
Measuring cell	Z-1000-4
Cell volume	1.00 ml
Flow-rate	1.55 ml/min
Efficiency	Constant
Scintillator	Lumagel
Scintillator flow-rate	1.3 ml/min

eluents with a liquid scintillation counter. No differences were observed and therefore no corrections were necessary.

Under optimal conditions, the efficiency, the detection limit, the linearity of detector response and the reproducibility of the measurements were determined.

The efficiency of the Z-1000-4 flow-through cell was established as $61.3 \pm 0.3\%$ ($n=3$) by comparing the measurements of ca. 24 000 Bq ^{195m}Pt-labelled cisplatin, with those measured on a germanium–lithium detector, calibrated with a solid-disc point source (QCD.1) from Amersham. The efficiency of the Berthold HPLC detector was found to be somewhat lower than that of a liquid scintillation counter (efficiency 95%), owing to the smaller volume, the geometry and the composition of the tube of the flow-through cell.

The absolute detection limit, defined as a signal-to-noise ratio of 3, was found to be as low as 3 Bq, i.e. 0.2 ng of cisplatin (specific activity 15 MBq/mg). This allowed the determination of cisplatin concentrations of 10 ng/ml in urine and plasma ultrafiltrate. In principle, the detection limit may be improved by using cisplatin with a higher specific activity or by off-line counting in a liquid scintillation counter.

Calibration curves of standard solutions of known amounts of cisplatin in physiological saline were found to be linear in the range 10^{-7}–10^{-4} M, corresponding to 8–30 000 Bq ($r=0.997$, $n=15$). The lower detection limit was de-

TABLE II

COMPARISON OF THE DETECTION LIMITS OF SEVERAL ON-LINE HPLC DETECTORS FOR THE DETERMINATION OF CISPLATIN

HPLC detection	Sample	Detection limit[a]		Reference
		ng/ml	ng	
UV 208	UF[b]	150	15	3
Quenched room temperature phosphorescence in liquids	Urine	90	1.8	9
Post-column reaction with sodium bisulphite (UV)	UF	40	0.8	4
Inductively coupled plasma atomic emission spectrometry	UF	35	3.5	11
Electrochemical detection with solid electrodes	UF	10[c]	0.2	7
^{195m}Pt radioactivity (on-line)	Urine, UF	10[d]	0.2	This study

[a]Concentration or absolute quantity of cisplatin injected (signal-to-noise ratio = 3).
[b]UF = plasma ultrafiltrate.
[c]Dependent on the chemical structure of the platinum complex: detection limit for carboplatin is 5000 ng/ml.
[d]For a specific activity of 15 MBq/mg cisplatin. This detection limit is independent of both the chemical structure of the platinum complexes and the matrix composition of the sample.

termined by the background and the counting time, the upper by the dead time. Standard solutions of $5 \cdot 10^{-7}$ *M* cisplatin in 0.9% sodium chloride could be determined with a precision of $\pm 2.2\%$ (S.E.M.; $n=5$).

A survey of detection limits of several on-line HPLC detectors, previously used by others for the determination of cisplatin and related platinum complexes, is given in Table II. It is important to note that the detector response of the radioactivity detector described in this paper is independent of the chemical structure of the platinum complexes, in contrast to the response obtained with other detectors, such as electrochemical detectors [7,8]. Obviously, this is an important advantage when one is interested in the simultaneous quantitative determination of different platinum complexes. The response of the radiodetector is also independent of the matrix. This is again in contrast to the response obtained with other detectors, such as the quenched phosphorescence detector [10] and off-line atomic absorption detection [14].

Animal studies

In order to evaluate the applicability of the developed HPLC method, plasma ultrafiltrate samples and urine samples obtained from male Wistar rats 10 min or 2 h after treatment with 5 mg/kg cisplatin were analysed. In the plasma ultrafiltrate samples two radiolabelled platinum complexes were detected (Fig. 3A and B): the first (retention time 2.1 min) was identified as unchanged cisplatin on basis of co-elution with authentic reference compound. The chemical structure of the second complex (retention time 19.2 min) still remains to be established.

In urine samples, several radiolabelled platinum complexes were detected, with unchanged cisplatin being predominant (Fig. 3C); the chemical structures of the others are unknown. Platinum–METH complexes, previously identified in human urine [19,20], might also be present in the urine of rats treated with cisplatin (Fig. 3C). It is not known, however, whether these platinum complexes are formed in the urinary tract by chemical reactions between cisplatin and pro-urine or whether they are formed as metabolites in other parts of the body.

Daley-Yates and McBrien [14] have reported the observation of several platinum complexes in protein-free plasma and urine samples of rats treated with cisplatin. However, the extensive biotransformation of cisplatin reported by these authors is in contrast to the results obtained by Safirstein et al. [21] and Riley et al. [22], who found that cisplatin was excreted predominantly unchanged in urine. De Waal et al. [11] have shown that 'on-column' formation of new platinum complexes in the HPLC method used by Daley-Yates and McBrien [14] cannot be excluded, however, owing to the presence of acetonitrile in their mobile phase. Acetonitrile, in contrast to other organic modifiers, such as 2-propanol and methanol, reacts with platinum complexes to form

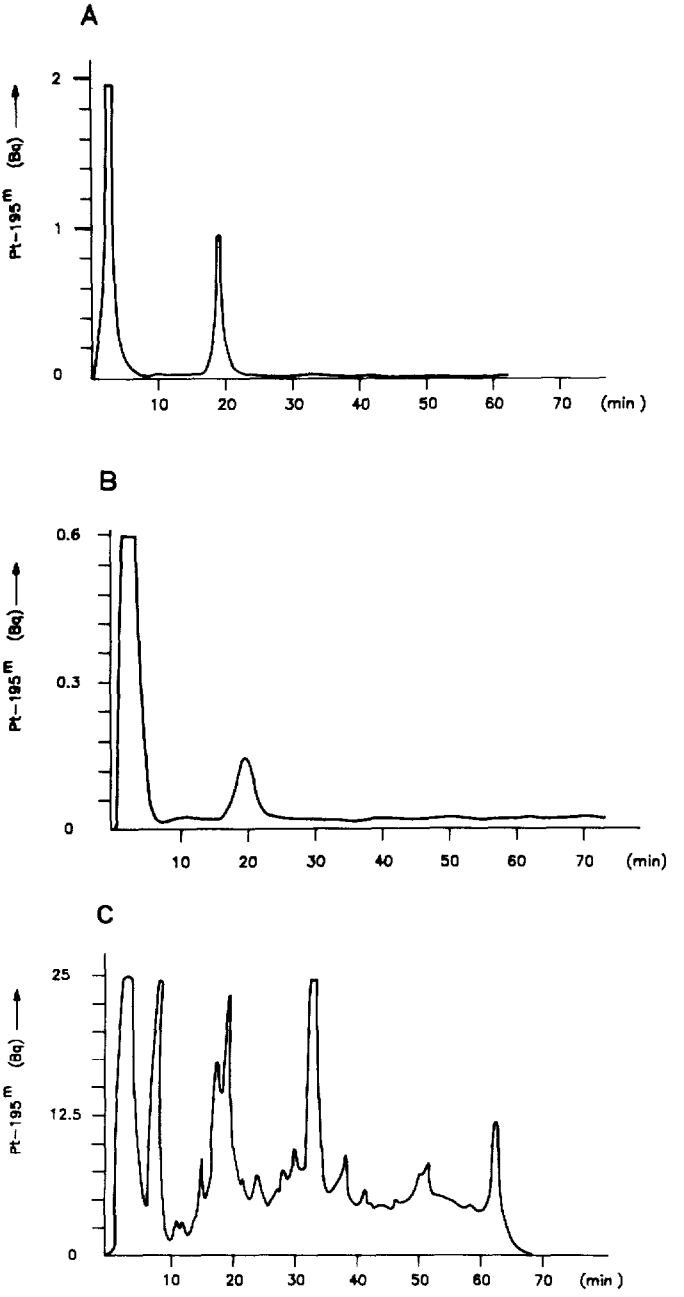

Fig. 3. Radiochromatograms of plasma ultrafiltrate (A, B) and urine samples (C) from Wistar rats obtained 10 min (A) and 2 h (B, C) after intravenous treatment with cisplatin (5 mg/kg). Chromatographic conditions are described in the text. In both chromatograms of the plasma samples (A, B), two platinum complexes were observed. The first complex (retention time 2.1 min) was identified as cisplatin. In urine samples (C), several platinum complexes were observed, with unchanged cisplatin being the predominant compound.

several products [11]. The radio-HPLC method described in this paper might be a valuable research tool to elucidate these contradictory results.

CONCLUSIONS

The resolving power of the radio-HPLC system described in this paper enables the determination of various platinum complexes in one run. HPLC with on-line radioactivity detection is superior to HPLC with other detectors for platinum complexes, in terms of selectivity and sensitivity. The signal response of the radiodetector, measuring ^{195m}Pt radioactivity, was found to be independent of both the chemical structure of the platinum complexes and the matrix composition of the samples. Therefore, this method is an excellent reference method for other, less specific and sensitive methods for the detection of platinum complexes. Animal experiments demonstrated that our radio-HPLC method might be a powerful research tool in studies of the pharmacokinetics and metabolism of cisplatin and might open new perspectives in elucidating the molecular mechanism of cisplatin nephrotoxicity.

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