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Determination of cisplatin and some possible metabolites by ion-pairing chromatography with inductively coupled plasma mass spectrometric detection

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ABSTRACT

A sensitive method is described for measuring cisplatin and some possible metabolites. The method combines reversed-phase ion-pairing liquid chromatography (LC) with inductively coupled plasma mass spectrometry (ICP-MS) for platinum-specific detection. Separation conditions for cisplatin hydrolysis products and the reaction products of cisplatin with methionine, cysteine, and glutathione have been investigated with sodium dodecylsulfate or heptanesulfonate as the ion-pairing agent. The detection limit for cisplatin was found to be 0.1 ng, corresponding to a concentration detection limit of 1 ng/ml when using an injection volume of 100 μl. This study has demonstrated the usefulness of LC–ICP-MS for cisplatin metabolism studies.

INTRODUCTION

The discovery that platinum-containing complexes show antitumor activity has promoted great interest in the analysis of such complexes [1]. Numerous Pt complexes have been synthesized in an attempt to increase the efficacy as well as reduce toxicity. Currently, there is a great deal of interest in analysis of cisplatin [*cis*-diammine-dichloroplatinum(II)] and its metabolites, since this complex is widely used in cancer chemotherapy. Cisplatin increases cure rates dramatically for several human cancers [2]. However, the dose of cisplatin is limited because of its toxic side-effects such as nephrotoxicity and severe nausea. The toxicity of the drug may be linked to specific metabolites [3]. Several studies have been undertaken to understand the cause of nephrotoxicity and to correct the problems [4–6].

High-performance liquid chromatography (HPLC) is currently the most widely used analytical technique for pharmacokinetic and metabolic studies of cisplatin. However, detection of Pt at low concentrations is a major obstacle and its improvement is a challenge. With off-line detection, such as flameless atomic absorption spectroscopy (AAS), the detection limit (for the best case) was 0.8 ng [7]. The off-line flameless AAS methods are cumbersome and time-consuming, and the resolution of the separation deteriorates because of the necessary fraction collection. On-line detection techniques have overcome some of these problems. These techniques include inductively coupled plasma atomic emission spectrometry (ICP-AES) [8], UV detection after post-column derivatization [9], reductive amperometric detection [10], and others [11,12]. ICP-AES provides a metal-specific detector but its detection

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limit for cisplatin biological samples is far from that desired. Reductive amperometric detection [10] (with a hanging mercury drop electrode) has good detection limit (70 pg), however, the method suffers severe baseline noise for real samples and only detects cisplatin (*i.e.*, it does not detect cisplatin metabolites). Thus no convenient method using HPLC with traditional detection schemes has shown adequate detection limits to determine cisplatin and its various metabolites.

Inductively coupled plasma mass spectrometry (ICP-MS) is now an established technique for trace-metal analysis. It has been used for measuring total Pt in blood or urine from patients on chemotherapy [13–16]. Recent work in our laboratory has demonstrated that the combination of HPLC with ICP-MS is an excellent technique for the determination of gold drug metabolites in biological samples [17,18]. Using ICP-MS as the metal-specific detector, excellent sensitivity and high selectivity are obtained without matrix component interferences. It appeared that LC–ICP-MS would have the capability of speciating Pt complexes at the low ng/ml levels necessary to study the metabolism of cisplatin in real samples as well. In this paper, the determination of cisplatin and several of its possible metabolites using the LC–ICP-MS system is described.

EXPERIMENTAL

Materials

Cisplatin was purchased from Johnson Matthey Electronics (Ward Hill, MA, USA) and its solutions were prepared in 0.9% sodium chloride. L-Methionine, L-cysteine and glutathione were purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were purchased from various commercial sources and used as received. Water used in the preparation of mobile phase and other solutions was purified with a Barnstead Nanopure system (Milford, MA, USA) equipped with a 0.2-μm filter.

High-performance liquid chromatography

The HPLC system consisted of Waters Assoc. HPLC pumps and a controller (Milford, MA, USA), and a Valco C6W injector with a 20-μl loop. Separations were performed on a Burdick & Jackson OD5 C_{18} column, 150 mm × 4.6 mm I.D., 5 μm particle size (McGaw Park, IL, USA). A 50 mm × 4.6 mm I.D. guard column was packed using Spherisorb ODS-2 5-μm particles (Norwalk, CT, USA). The negatively charged ion-pairing agents sodium dodecylsulfate (SDS) or 1-heptanesulfonate were used in the mobile phase. The pH of the mobile phase was adjusted to 2.6 in order to retain the thiol-containing Pt complexes [8]. Prior to use, the mobile phase was filtered through a 0.45-μm Nylon-66 membrane filter. The flow-rate was 1 ml/min, and the column temperature was maintained at 30°C with a water jacket and a thermostated water bath.

Inductively coupled plasma mass spectrometry

Eluent from the HPLC column was analyzed on-line with a Sciex Elan 250 ICP-MS system (Toronto, Canada). Coupling of the column to the ICP-MS was accomplished with PTFE capillary tubing (96 cm × 0.1 mm I.D.). This tubing has a volume of approximately 0.08 μl/cm and will have a minimal effect on extra-column peak broadening. The Pt m/z 195 was monitored, *i.e.*, peaks appearing in chromatograms are Pt-containing species. Typical ICP-MS operating conditions were used: RF power, 1.25 kW; nebulizer argon flow-rate, 0.9 l/min; auxiliary argon flow-rate, 1.2 l/min; coolant flow-rate, 12.5 l/min; spray chamber, 5°C.

Preparation of platinum complexes with thiol compounds

Complexes of cisplatin with methionine, cysteine, and glutathione were prepared using procedures similar to those described by Baldew *et al.* [19]. Typically, cisplatin was allowed to react with the above thiols separately in saline solution. Reactions were carried out at 37°C for about 4 h. The ratio of thiol to cisplatin was 20:1 and the concentration of cisplatin was 0.1 mM. Reaction mixtures were diluted to the desired concentrations with the mobile phase and used immediately. The mono- and diaquo forms of cisplatin were generated by incubating cisplatin in aqueous solution.

RESULTS AND DISCUSSION

In an attempt to identify metabolite peaks for future investigation, these studies deal with hydrolysis of cisplatin as well as reactions of cisplatin with several biologically relevant thiols such as methionine, cysteine, and glutathione. The objective is to elucidate the metabolism of cisplatin, through monitoring of reaction products using ICP-MS as a selective detector. Reversed-phase ion-pairing chromatography was chosen as the separation scheme in order to retain ionic and neutral cisplatin derivatives. The method was devised assuming the cisplatin metabolites of interest were neutral complexes or hydrophilic and positively charged complexes. For example, mono- and diaquo cisplatin products are 1+ charged and 2+ charged species, respectively.

Cisplatin hydrolysis products

Aquo metabolites of cisplatin may form in cells as the labile chloro groups are successively displaced by water molecules. As has been previously suggested [20], they may contribute to nephrotoxicity. In this study, cisplatin was incubated in water for about 3 h. Since our purpose was to show that a quantitative (baseline) separation could be effected and that qualitative identification of the products could be established, rather than to measure the equilibrium constant or the kinetics for the hydrolysis reaction, we made no attempt to quantify the proportions of the drug and its hydrolysis products.

SDS was used as the ion-pairing agent and 3% *n*-propanol was added to the mobile phase to improve the separation efficiency [21,22]. Fig. 1 shows the cisplatin hydrolysis products separated with a SDS concentration of 0.2 m*M*. Peak C is cisplatin, which represents the unchanged material in the sample. The shoulder on the front of the cisplatin peak may be transplatin which exists as an impurity in the reagent. Two peaks, H_1 and H_2, are the mono- and diaquo derivatives of cisplatin and their capacity factors were 5.67 and 8.57, respectively (eluting at 14 and 20.1 min, respectively). Under the conditions used, the separation was more than adequate but the retention

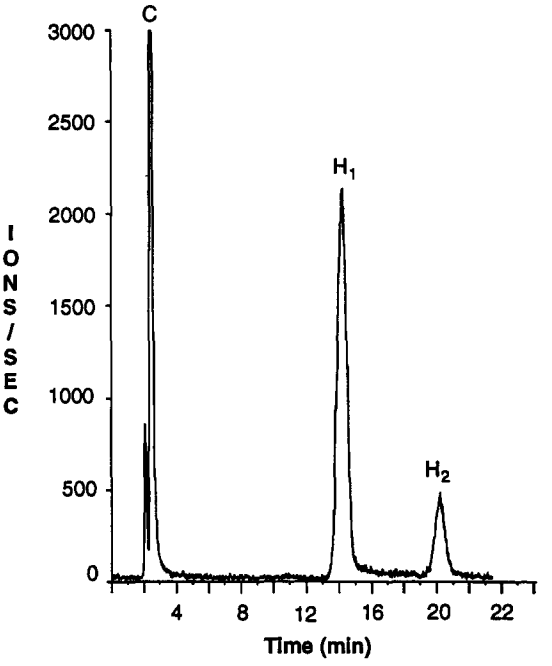

Fig. 1. Chromatogram of cisplatin hydrolysis products. Peak C is cisplatin, peaks H_1 and H_2 represent the mono and diaquo derivatives of cisplatin. Mobile phase: 0.2 m*M* SDS, 3% *n*-propanol, 0.1% trifluoroacetic acid, pH 2.6.

was too long for the aquated species. In such a system, retention may be decreased by increasing the organic modifier concentration in the mobile phase. To ensure the stability of the argon plasma of the ICP-MS, the organic content was purposely kept low. Alternatively, reduced retention can be achieved by reducing the concentration of SDS.

The use of a lower SDS concentration (0.1 m*M*) had no significant effect on the retention of cisplatin, but retention of the hydrolysis products was significantly decreased (capacity factors were 3.05 and 4.43, respectively). The separation was acceptable, however, the retention times for other Pt complexes of interest were too long. Another less hydrophobic ion-pairing agent, 1-heptanesulfonate, was investigated.

Heptanesulfonate produced a faster separation of the cisplatin hydrolysis products. As shown in Fig. 2, the mono- and diaquo complexes eluted more rapidly but are still baseline-resolved. Cisplatin retention appeared to remain unchanged.

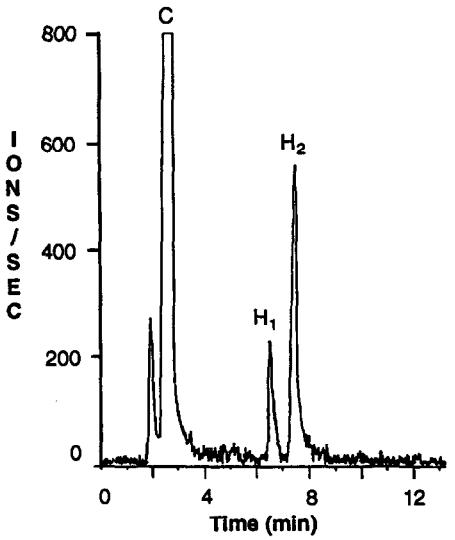

Fig. 2. Chromatogram of cisplatin hydrolysis products. Peak C is cisplatin, peaks H_1 and H_2 are cisplatin hydrolysis products. Mobile phase: 5 m*M* heptanesulfonate, 10% methanol, 0.1% trifluoroacetic acid, pH 2.6.

However, there is some variation in the ratio of mono- and diaquo complexes. Obviously this ratio is affected by several factors including ionic strength and the solvent components of the mobile phase used for dilution of the prepared cisplatin hydrolysis products to the desired concentration for column injection (*i.e.*, 100 ng/ml as Pt). To confirm that this is the case the comparison study was conducted by using water and the mobile phase for the dilution. Fig. 3 shows the comparison of chromatographic results of different solvents used. Significant changes in the ratio of three species are obvious. The ratio of peak H_1 to H_2 for water as diluting solvent is much higher than that using mobile phase as the diluting solvent. Also the degree of cisplatin hydrolysis is higher for water as the solvent. A kinetic study of the mobile phase dilution effect indicated that the shifts in the equilibrium of hydrolysis was slow (Fig. 4). Therefore this type of mobile phase effect can be avoided by diluting the sample immediately before injection.

The identities of peaks H_1 and H_2 were elucidated by varying the chloride concentration of the hydrolysis product solution. Without addition of Cl^-, the peak H_1 dominated, compared to the later-eluting peak H_2. As the chloride concentration increased, the H_1 peak was markedly reduced and the H_2 peak increased. Since the equilibrium of the hydrolysis reaction is shifted to the monoaquo complex by increasing Cl^-, the later-eluting peak H_2 appears to be the monoaquo complex and H_1 the diaquo complex. The identification of H_1 and H_2 was further confirmed by comparison to the retention time of the diaquo complex $[Pt(NH_3)_2(H_2O)_2]^+$, which was generated by addition of $AgNO_3$ to an aqueous solution of cisplatin to remove Cl. The result was consis-

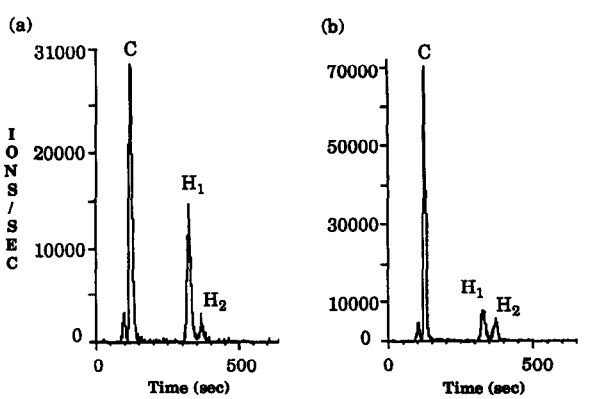

Fig. 3. Chromatogram of cisplatin hydrolysis product sample diluted with (a) water and (b) mobile phase. Peak C is cisplatin, peaks H_1 and H_2 are cisplatin hydrolysis products. Mobile phase: 5 m*M* heptanesulfonate, 10% methanol, 0.1% formic acid, pH 2.6. The guard column was 2 cm in length.

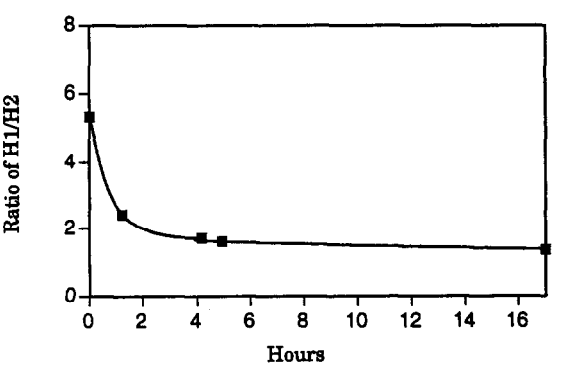

Fig. 4. Changes in ratio of diaquo to monoaquo cisplatin *versus* time of cisplatin hydrolysis species in mobile phase. Mobile phase: 5 m*M* heptanesulfonate, 10% methanol, 0.1% formic acid, pH 2.6.

tent with the conclusion drawn from the Cl^- concentration study. Obviously, the retention times for the two hydrolysis products are not simply a function of their charges, and the elution order cannot be inferred from charges in ion-pairing chromatography. The mechanism of retention in this system is complex including both hydrophobic interaction and charge interaction.

Cisplatin and thiol mixtures

Cisplatin is highly reactive in the body, and its biotransformation products may have activity and toxicity behavior different from that of the parent drug. At present, the biotransformation products of cisplatin have not been well characterized. However, methionine–cisplatin complexes have been found in the urine of patients receiving cisplatin therapy [23]. Also, there is some evidence indicating that metabolites in the kidney cytosol and urine of rats might be adducts of cisplatin with cysteine and glutathione [24].

Cisplatin was incubated *in vitro* with solutions of single thiol compounds and the products were separated by LC. A mobile phase containing 10 m*M* heptanesulfonate, 10% methanol, and 0.1% formic acid, pH 2.6 adjusted with sodium hydroxide, was used for the separation. Fig. 5 shows the chromatogram of a mixture of cisplatin and methionine. Peak assignments were based on chromatographic retention times. By comparing with the cisplatin standard chromatogram, peak C was identified as unchanged cisplatin, and peaks H_1 and H_2 as the diaquo and monoaquo species respectively. The other two peaks, M_1 and M_2, eluted at 17 and 35 min, are assumed to be monomethionine and dimethionine substitution complexes of cisplatin. Norman *et al.* [25] have reported that up to eight products of 1:1 and 1:2 cisplatin–L-methionine have been identified using NMR spectroscopy. Clearly, the cisplatin derivatives and their relative amounts vary dramatically with the concentration of methionine [25]. Relatively high ratios of methionine to Pt will be expected in physiological fluids, thus no effort was made to investigate the reactions at low thiol/Pt ratios.

Chromatograms of mixtures of Pt complexes

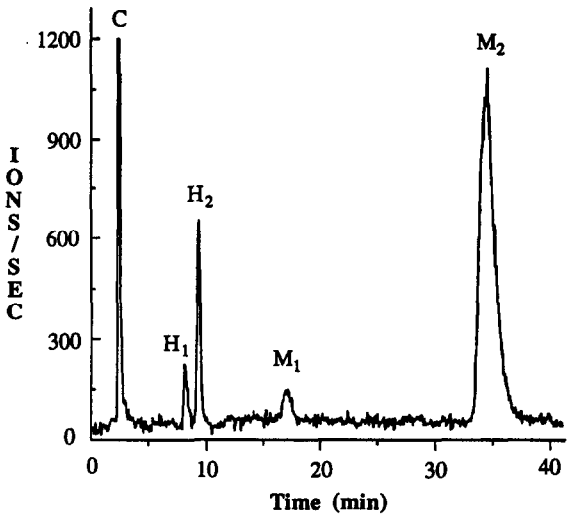

Fig. 5. Chromatogram of platinum complexes obtained by incubation of cisplatin and methionine. Peak C is cisplatin, peaks H_1 and H_2 are cisplatin hydrolysis products. Peak M_1 and M_2 are monomethionine and dimethionine substitution complexes. Mobile phase: 5 m*M* heptanesulphonate, 10% methanol, 0.1% formic acid, pH 2.6. The guard column was 5 cm in length.

obtained by incubation of cisplatin with glutathione and cysteine are shown in Figs. 6 and 7, respectively. For the cisplatin and glutathione mixture, most of the cisplatin and its hydrolysis products are still present. Only a small amount of cisplatin complexed with glutathione and formed glutathione-based complexes, G_1 and G_2. In contrast to that with glutathione, incubation with cysteine showed that most cisplatin was converted to a late-eluting peak (cysteine–cisplatin substitution complex) as shown in Fig. 7. Only a very small amount of the initial cisplatin (peaks C) remained. The hydrolysis products (peak H_1 and H_2) were still present. Since we wished to show that quantitative (baseline) separation and element-specific detection of the Pt-containing products of this reaction are possible we did not further probe either the structures or the formulas corresponding to the product peaks shown in Figs. 6 and 7. Methionine is known to complex with Pt through its sulfur and nitrogen atoms [23]. Cysteine may bind to Pt in a similar fashion since it also possesses amine groups.

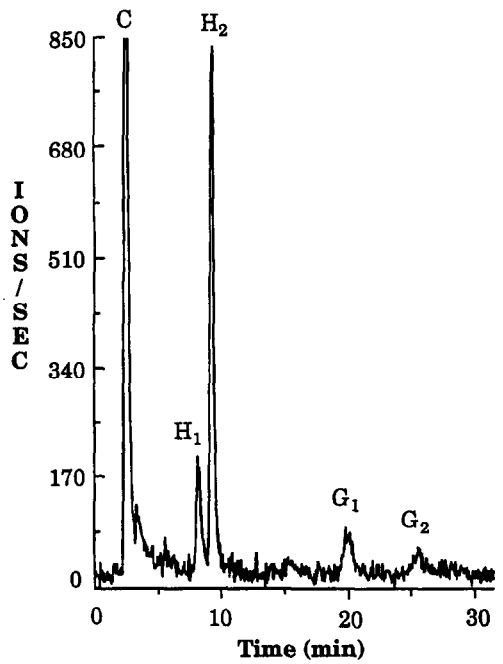

Fig. 6. Chromatogram of cisplatin and glutathione mixture. Peak C is cisplatin, peaks H_1 and H_2 are cisplatin hydrolysis products. Peak G_1 and G_2 are glutathione–cisplatin complexes. Mobile phase: 5 m*M* heptanesulfonate, 10% methanol, 0.1% formic acid, pH 2.6.

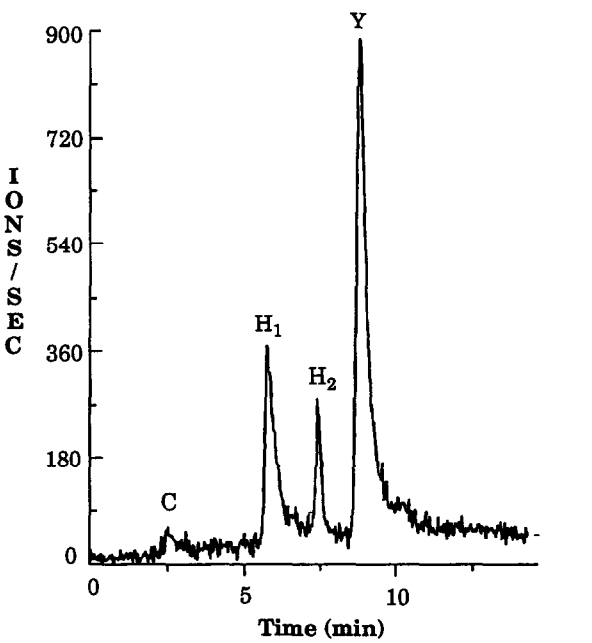

Fig. 7. Chromatogram of cisplatin and cysteine mixture. Peak C is cisplatin, peaks H_1 and H_2 are cisplatin hydrolysis products. Peak Y is cysteine–cisplatin substitution complex. Mobile phase: 5 m*M* heptanesulfonate, 10% methanol, 10 m*M* trichloroacetic acid, pH 2.6.

Quantitation of cisplatin

A calibration curve for cisplatin was obtained using standard cisplatin solution with excess chloride ions present. A chloride concentration will suppress the hydrolysis of cisplatin [26], and the solution was prepared immediately prior to injection. Quantitation of cisplatin was based on peak-height calculations. The correlation coefficient of the regression line was 0.997 between 2 ng and 20 μg. The reproducibility of peak height was good as indicated by the small relative standard deviation (R.S.D.) of 8% (n = 3). The limit of detection defined as that amount of solute producing a signal three times the standard deviation of the noise was 0.1 ng Pt, which for a 100-μl injection corresponds to a concentration detection limit of 1 ng/ml.

Since the ICP-MS detector response is nearly independent of the molecular form of the analyte, the detection limits for other Pt species should be similar, apart from increased dilution for more strongly retained species. The typical total Pt concentration found in plasma ultrafiltrate and urine obtained from patients undergoing chemotherapy is in the range 0.25–50 μg/ml [27]. Consequently, any Pt-containing metabolite with a concentration above 2.5 ng/ml (>1% of total Pt) can be detected using this method.

As expected, a blank urine sample gave no signal and the chromatogram of a urine sample spiked with cisplatin was identical to that of the cisplatin standard. This indicates there is no interference from the urine matrix due to use of Pt-specific detection. Our previous studies of gold-containing drug metabolism [28] have shown that blood plasma samples add no interferences to the separation provided the sample is filtered through a 10 000 molecular mass cut-off membrane to remove proteins.

CONCLUSION

The present work indicates that on-line ICP-MS detection for HPLC is simple, reliable, and compatible with biological samples. Compared to conventional detection techniques, ICP-MS detection of Pt species is much more efficient with regard to both selectivity and sensitivity. The typical concentration of Pt in clinical samples is far above the detection limit of this method and thus low-concentration metabolite species can be easily detected. With heptanesulfonate as an ion-pairing agent, all Pt complexes of interest were efficiently resolved. It is apparent that this method can be successfully applied to the quantitative determination of cisplatin and its metabolites in biological samples such as urine or blood ultrafiltrate.

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REFERENCES

1 U. R. Tjaden and E. A. De Bruijn, *J. Chromatogr.*, 531 (1990) 235.
2 C. R. Hamilton, J. M. Bliss and A. Horwich, *Eur. J. Cancer Clin. Oncol.*, 25 (1989) 185.
3 C. L. Litterst, *Toxicol. Appl. Pharmacol.*, 61 (1981) 99.
4 F. Zunno, G. Pratesi, A. Micheloni, E. Cavalletti, F. Sala and O. Tofanetti, *Chem. Biol. Interact.*, 70 (1989) 89.
5 M. M. Jones and M. A. Basinger, *Anticancer Res.*, 9 (1989) 1937.
6 J. E. Melvik and E. O. Pettersen, *Inorg. Chim. Acta*, 137 (1987) 115.
7 P. T. Daley-Yates and D. C. H. McBrien, *Biochem. Pharmacol.*, 33 (1984) 3063.
8 W. A. J. de Waal, F. J. M. J. Maessen and J. C. Kraak, *J. Chromatogr.*, 407 (1987) 253.
9 M. Kinoshita, N. Yoshimura and H. Ogata, *J. Chromatogr.*, 529 (1990) 462.
10 S. J. Bannister, L. A. Sternson and A. J. Repta, *J. Chromatogr.*, 273 (1983) 301.
11 R. Kizu, S. Higashi and M. Miyazaki, *Chem. Pharm. Bull.*, 33 (1985) 4614.
12 C. Gooijer, A. C. Veltkamp, R. A. Baumann, N. H. Velthorst and R. W. Frei, *J. Chromatogr.*, 312 (1984) 337.
13 P. Tothill, L. M. Matheson, J. F. Smyth and K. McKay, *J. Anal. At. Spectrom.*, 5 (1990) 619.
14 B. Casetta, M. Roncadin, G. Montanari and M. Furlanut, *At. Spectrosc.*, 12 (1991) 81.
15 P. Tothill, K. McKay, L. M. Matheson, M. Robbins and J. F. Smyth, in G. Holland and A. N. Eaton (Editors), *Applied Plasma Source Mass Spectrometry, Sel. Pap. Int. Conf., 2nd Meeting 1990*, Royal Society of Chemistry, Cambridge, 1991, pp. 89–95.
16 P. Allain, S. Berre, Y. Mauras and A. Le Bouil, *Biol. Mass Spectrom.*, 21 (1992) 141.
17 R. C. Elder, W. B. Jones and K. Tepperman, *ACS Symp. Ser.*, 479 (1992) 309.
18 Z. Zhao, W. B. Jones, K. Tepperman, J. G. Dorsey and R. C. Elder, *J. Pharm Biomed. Anal.*, 10 (1992) 279.
19 G. S. Baldew, K. J. Volkers, J. J. M. De Goeij and N. P. E. Vermeulen, *J. Chromatogr.*, 491 (1989) 163.
20 P. T. Daley-Yates and D. C. H. McBrien, *Biochem. Pharmacol.*, 34 (1985) 2363.
21 J. G. Dorsey, M. T. DeEchegaray and J. S. Landy, *Anal. Chem.*, 55 (1983) 924.
22 J. P. Foley and W. E. May, *Anal. Chem.*, 59 (1987) 110.
23 C. M. Riley, L. A. Sternson, A. J. Repta and S. A. Slyter, *Anal. Biochem.*, 130 (1983) 203.
24 P. Mistry, C. Lee and D. C. H. McBrien, *Cancer Chemother. Pharmacol.*, 24 (1989) 73.
25 R. E. Norman, J. D. Ranford, and P. J. Sadler, *Inorg. Chem.*, 31 (1992) 877.
26 Y. Cheung, J. C. Cradock, B. R. Vishnuvajjala and K. P. Flora, *Am. J. Hosp. Pharm.*, 44 (1987) 124.
27 O. H. Drummer, A. Proudfoot, L. Howes and W. J. Louis, *Clin. Chim. Acta*, 136 (1984) 65.
28 R. C. Elder, Z. Zhao, Y. Zhang, J. G. Dorsey, E. V. Hess and K. Tepperman, *J. Rheumatol.*, 20 (1993) 268.