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Short Communication

A column-switching liquid chromatography assay for the analysis of carboplatin in plasma ultrafiltrate

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Introduction

Carboplatin (*cis*-diammine-1,1,cyclobutane dicarboxylate platinum II) is a cytotoxic drug indicated in the treatment of ovarian carcinoma and small cell lung carcinoma [1, 2]. Phase I/II clinical trials and pharmacokinetic studies on carboplatin administered in prolonged continuous infusion regimens are being conducted at this institution. Previous pharmacokinetic studies on carboplatin administered in bolus or short-term infusion regimens have utilized the non-specific determination of platinum by atomic absorption spectroscopy (AAS) [3, 4] or the specific determination of carboplatin by normal phase liquid chromatography (LC) [5, 6]. Non-specific AAS methods cannot discriminate between platinum containing species arising from degradation or metabolism of carboplatin and are of limited utility in pharmacokinetic studies. The normal phase LC assay [7] was developed for the direct determination of carboplatin in plasma ultrafiltrate with a reported limit of detection of 1 $\mu g\ ml^{-1}$.

It was envisaged that pharmacokinetic studies on low-dose continuous infusion regimens of carboplatin would require an assay method with a limit of detection of less than 100 $ng\ ml^{-1}$. Selective determination of carboplatin in the presence of degradation products or metabolites was essential for the present pharmacokinetic study which required measurement of intact carboplatin rather than total platinum. Furthermore, proposed toxicologic studies using cell culture techniques would require a reversed-phase LC method using a simple buffered aqueous mobile phase. With further development this would enable the direct biological testing of eluent containing degradation products and metabolites arising from carboplatin.

Single column isocratic reversed-phase HPLC systems with buffered aqueous mobile phase, various bonded-phase columns (C_{18}, CN, NH_2 phenyl and polymeric) and either UV or reductive electrochemical detection failed to resolve carboplatin from interfering plasma ultrafiltrate (PU) components. Attempts to develop a sample clean-up procedure were unsuccessful, with both liquid–liquid and solid-phase extraction techniques failing to eliminate interference from plasma components. Furthermore, the poor recovery of carboplatin (<50%) from the exhaustive range of clean-up systems evaluated indicated that assay sensitivity would be adversely affected. Preliminary studies indicated that the most favourable carboplatin retention and selectivity characteristics were achieved with a C_{18} bonded phase. However, increasing column length to 300 mm and combining a C_{18} column in series with either a CN, NH_2, phenyl or second C_{18} bonded phase column failed to resolve carboplatin from interfering components in PU. Pre- and post-column derivatization techniques were also likely to prove unsuccessful since preliminary experiments

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had revealed that the ammonium ligands of carboplatin were non-reactive towards reagents used to derivatize primary and secondary amino groups (e.g. OPA). In view of the difficulties experienced with sample clean-up and with derivatization, column-switching techniques were evaluated in an attempt to resolve carboplatin from endogenous PU components.

In the present study, an assay for carboplatin in PU based on an automatic column-switching method using a 'heart-cut' technique with two different C_{18} bonded-phase columns is reported. This system used a simple phosphate-buffered mobile phase and enabled carboplatin to be resolved from other plasma components with the selectivity and sensitivity required for pharmacokinetic studies on patients receiving continuous infusion dosage schedules of 20–30 mg day^{-1} for 5 days.

Experimental

Apparatus

Liquid chromatography was carried out on an isocratic system comprising a model 3000 pump, a model 3100 detector, CI4000 integrator/printer–plotter, a Promis II autosampler with a single valve switching accessory (LDC Milton Roy Ltd, Stone, Staffordshire, UK).

Chemicals

Carboplatin was supplied by Bristol Myers Ltd (Uxbridge, UK) for experimental use. All other reagents were of analytical reagent grade or LC grade as appropriate (BDH Ltd, Poole, UK).

Assay procedures

The LC system was allowed to equilibrate for at least 4 h and the column switching time was determined by connecting the Spherisorb ODS-1 column directly to the detector and injecting a carboplatin standard solution (0.5 μg ml^{-1}). The pre-column eluate was switched to the analytical column at least 5 s prior to detection of the carboplatin peak and was switched back to waste at least 5 s after baseline recognition. A pneumatic column-switching value was employed to automate this system. The 'heart-cutting' configuration used for column switching is shown in Fig. 1. The chromatographic conditions were: stainless steel pre-column (a) packed with Spheriorb ODS-1 (5 μm, 250 × 4.6 mm); stainless steel analytical column (b) packed with Apex ODS (3 μm, 250 × 4 mm); mobile phase phosphate buffer (pH 6.1, 0.07 M) at flow rates of 0.57 ml min^{-1} (pump A) and 1.2 ml min^{-1} (pump B). Carboplatin was detected at a wavelength of 210 nm (0.001 AUFS). At this flow rate carboplatin eluted from the pre-column between 10.5 and 11.5 min. The autosampler was therefore programmed to switch the valve and direct the flow from the pre-column to the analytical column during this time interval.

Sample preparation

Blood samples from patients were collected in Vacutainer tubes containing heparin and were immediately centrifuged (1000*g* for 15 min). The plasma layer was aspirated and transferred to Centrisart (Sartorius) ultrafiltration tubes (m. wt. cut off 10,000) which were centrifuged immediately (2000*g* for 30 min). PU was aspirated from the tubes and either analysed immediately for carboplatin or stored at −25°C.

Preliminary experiments on the stability of carboplatin in PU had demonstrated there was no loss of carboplatin from PU spiked with carboplatin (0.2 μg ml^{-1}) stored frozen (−25°C) for up to 7 days. This was in agree-

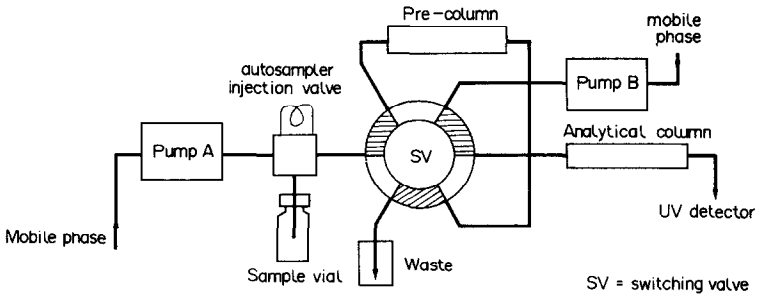

Figure 1
Column-switching configuration for LC analysis of carboplatin in PU.

ment with a previous study [7] which reported that there was no loss of carboplatin (5 μg ml^{-1}) in PU stored frozen (−25°C) for up to 6 days.

Standard solutions of carboplatin in PU were prepared by volumetric dilution of a stock solution of carboplatin (0.1 mg ml^{-1}). Duplicate injections of PU samples from patients were 'bracketed' by a freshly prepared external standard solution of PU spiked with carboplatin (0.5 μg ml^{-1}).

Data analysis

The linearity of the carboplatin peak area with concentration of carboplatin PU was determined by constructing an eight-point calibration plot over the concentration range 0.1–1.0 μg ml^{-1} and evaluated using unweighted linear regression. The limit of detection (LOD) was calculated from the calibration plot using the equation:

$$S_y/x = \frac{(y_i' - y_i)^2}{n - 2}, \quad (1)$$

where LOD is the point on the x axis extrapolated from that point plus 2 S_y/x on the y axis.

The relative error (RE) of carboplatin at concentrations of 0.2, 0.5 and 0.7 μg ml^{-1} was calculated from the calibration plot from

$$\text{RE} = \frac{C_s}{C_c} \times 100\%, \quad (2)$$

where C_s is the concentration of a standard solution of carboplatin in PU and C_c is the concentration of carboplatin calculated for standard solution in PU from substitution of carboplatin peak area into the regression of equation of the calibration plot. The precision of the system was determined by assay of six replicate injections of a solution of carboplatin in PU (0.5 μg ml^{-1}).

The recovery of carboplatin from spiked plasma at concentrations of 0.2 and 1.0 μg ml^{-1} was determined by subjecting seven replicate samples to the assay procedure. Samples were injected in duplicate and 'bracketed' by injections of an external standard solution (PU spiked with carboplatin at 0.2 or 1.0 μg ml^{-1}). The mean percentage recovery and the precision of the method were then calculated. The inter-day and the intra-day precision of the assay were calculated for PU spiked with carboplatin (0.5 μg ml^{-1}, $n = 5$).

Unknown concentrations of carboplatin in PU samples were calculated by using an external standard in which the concentration of carboplatin was known. The mean peak area of the two standard injections 'bracketing' a particular sample was used to calculate the concentration of carboplatin in the sample.

Results and Discussion

Validation

The relationship between peak area (A) and carboplatin concentration was linear over the concentration range 0.1–1.0 μg ml^{-1} satisfying equation (3):

$$C_s = 190A - 107, \; r = 0.999, \; n = 8. \quad (3)$$

Table 1 shows the theoretical concentration of carboplatin in PU spiked with carboplatin at 0.2, 0.5 and 0.7 μg ml^{-1}, the corresponding observed concentrations determined from the calibration plot and the REs which were 5.7, 1.7 and 1.2%, respectively. The RSD at a carboplatin concentration of 0.5 μl ml^{-1} was calculated to be 0.52%. The limit of detection of carboplatin in plasma ultrafiltrate was 14 ng ml^{-1}.

The recovery of carboplatin from spiked plasma at a concentration of 0.2 μg ml^{-1} was 97.2%, RSD = 2.5% ($n = 7$); and 1.0 μg ml^{-1} was 102.2%, RSD = 1.6% ($n = 7$). The intra-day and the inter-day precision for PU spiked with carboplatin (0.5 μg ml^{-1}) were RSD = 2.24 and 3.33% ($n = 5$), respectively.

Table 1
Accuracy and precision of the method

C_s* (μg ml^{-1})	C_c† (μg ml^{-1})	RSD (%)	RE‡ (%)
0.210	0.198	–	5.7
0.520	0.536		
	0.527		
	0.534	0.52	1.7
	0.523		
	0.525		
	0.530		
0.728	0.719	–	1.2

*C_s = Concentration of a standard solution of carboplatin in PU.

†C_c = Concentration of carboplatin calculated for standard solution in PU from substitution of carboplatin peak area into regression equation of the calibration plot.

‡RE = relative error.

Assay development

Initial development of the LC system focused on the resolution of carboplatin from plasma components afforded by various column packing materials. Chromatograms of carboplatin (5.0 μg ml^{-1}) in PU were obtained with a mobile phase of 0.02 M phosphate buffer (pH 6.1) at 1 ml min^{-1} and a column packed with either Spherisorb ODS-1 (5 μm) or Apex ODS (3 μm). These chromatograms are shown in Fig. 2(a) and (b), respectively. Both the retention of carboplatin and the degree of interference from PU differed for these two columns. For example, the Spherisorb ODS-1 column provided greater resolution of carboplatin from interfering components from PU, whereas the Apex ODS column exhibited a reduced peak area for the interference peak complex eluting after carboplatin. The Spherisorb ODS-1 column was therefore selected as the precolumn for column-switching and the Apex ODS column was the analytical column. With a flow rate of 1 ml min^{-1}, eluant fractions containing the carboplatin peak were collected from the precolumn between 5.8 and 6.2 min and injected onto the analytical column. Only minor interference at the tail of the carboplatin peak was observed. A column-switching valve was employed to automate this system (Fig. 1) and resolution was further improved by reducing the flow rate of the mobile phase through the precolumn from 1 to 0.57 ml min^{-1}. To prevent excessive pressure changes on the analytical column when flow of mobile phase was diverted from the precolumn to the analytical column, the flow rate to the analytical column was set at 1.2 ml min^{-1}.

Typical chromatograms of carboplatin aqueous standard (0.5 μg ml^{-1}), blank PU and PU containing carboplatin (0.5 μg ml^{-1}) are shown in Fig. 3(a)–(c), respectively. A typical chromatogram of a PU sample from a patient receiving carboplatin (30 mg day^{-1} for 5 days)

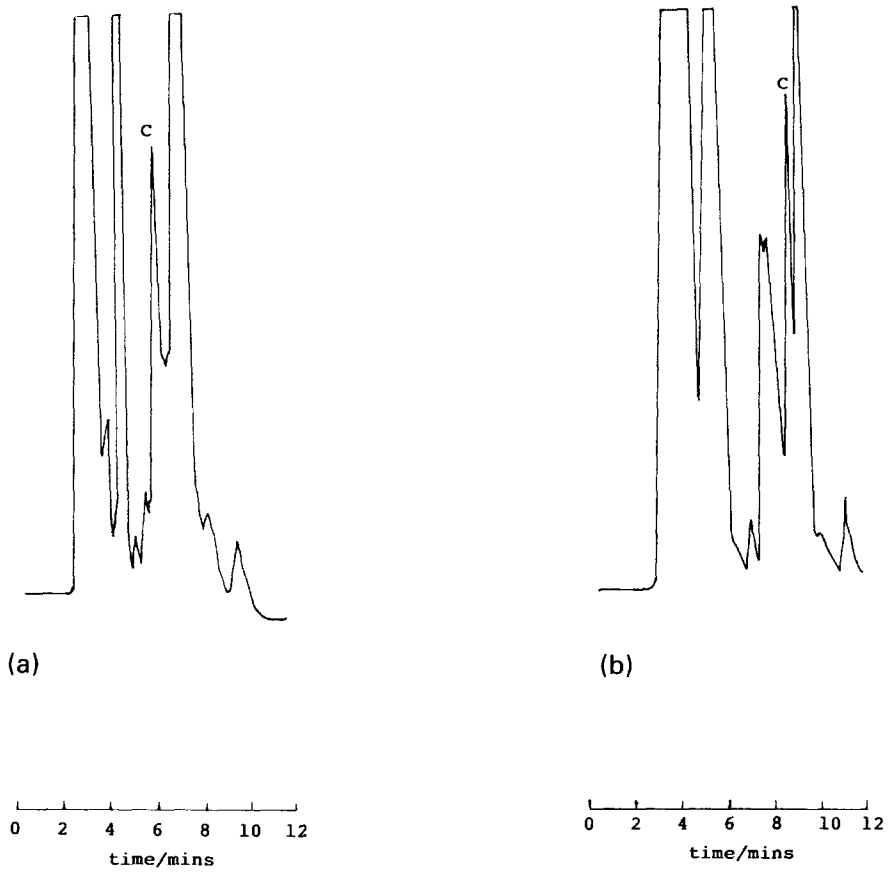

Figure 2
(a) LC chromatogram of PU containing carboplatin (5.0 μg ml^{-1}) using a Spherisorb ODS-1 column (250 × 4.6 mm, 5 μm). (b) LC chromatogram of PU containing carboplatin (5.0 μg ml^{-1}) using an Apex ODS column (250 × 4.6 mm, 3 μm).

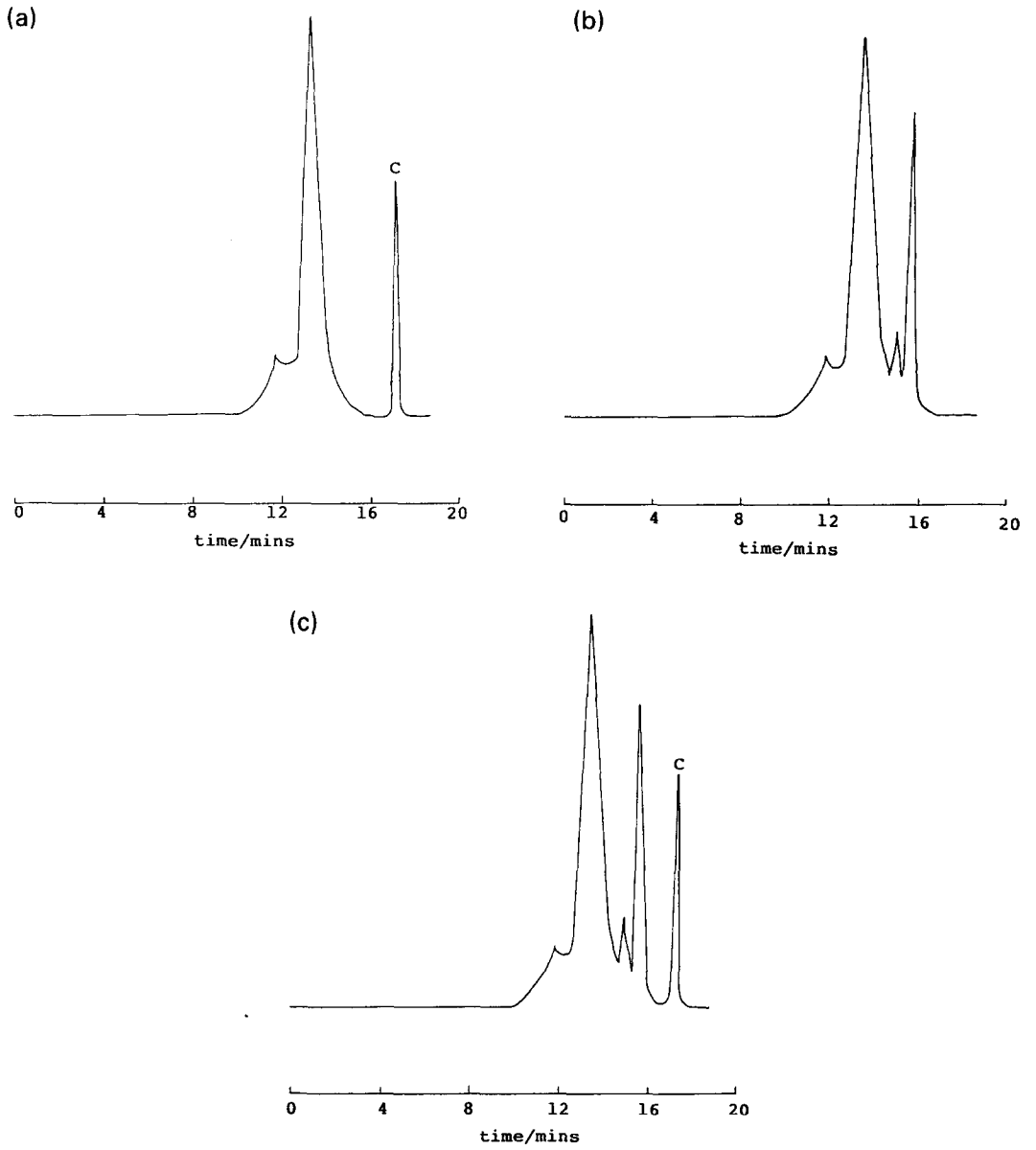

Figure 3
(a) LC chromatogram of an aqueous standard of carboplatin (0.5 μg ml^{-1}). (b) LC chromatogram of PU. (c) LC chromatogram of PU containing carboplatin (0.5 μg ml^{-1}).

by continuous infusion is shown in Fig. 4. The sample from which PU was prepared was taken 48 h after the start of the carboplatin infusion. Figure 5 shows a typical PU carboplatin concentration–time profile (computer fitted curve) for a patient receiving carboplatin by continuous infusion (30 mg day^{-1} for 5 days).

Clinical analysis

The column-switching assay has facilitated phase I pharmacokinetic studies on carboplatin administered in continuous infusion regimens where steady-state concentrations of carboplatin in PU were <0.5 μg ml^{-1}. This study has subsequently enabled the development of pharmacokinetic/toxicity relationships and comparison of pharmacokinetic data with those obtained for bolus regimens. Although the assay is time consuming (20 min per sample) a limited sampling strategy is under development to reduce the number of blood samples taken during the course of a 5-day infusion. Similar limited sampling strategies have been validated in previous pharmacokinetic studies [8, 9].

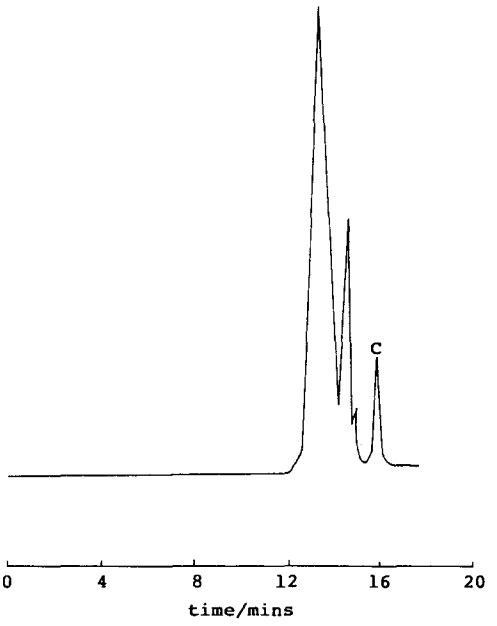

Figure 4
Typical LC chromatogram of a PU sample from a patient receiving carboplatin by continuous infusion (30 mg day^{-1} for 5 days).

The present LC method using UV detection gives a LOD of 14 ng ml^{-1}. This has enabled steady state plasma concentrations and post-infusion pharmacokinetic studies to be carried out. However, a further increase in sensitivity would be valuable for more detailed post-infusion studies. Techniques based on the same LC system described but using reductive electrochemical detection are currently under evaluation. It is anticipated that this will increase the sensitivity of the assay by 10–100 fold.

Other applications

In separate studies [10] on the degradation of carboplatin in aqueous solution in the presence and absence of chloride ions (0.4 M), where the LC assay system utilized a Spherisorb ODS-1 column identical to the precolumn in the present study, it was shown that degradation products of carboplatin eluted close to the solvent front. It was possible to resolve two of these degradation products on a stainless steel column (250 × 4.6 mm) packed with Ultracarb 5 ODS using the phosphate buffer mobile phase described previously (Fig. 6). It has been proposed [11] that the same degradation species would be produced by the *in vivo* metabolism of carboplatin since hydrolytic and nucleophilic substitution mechanisms would be involved in both cases. Further studies will

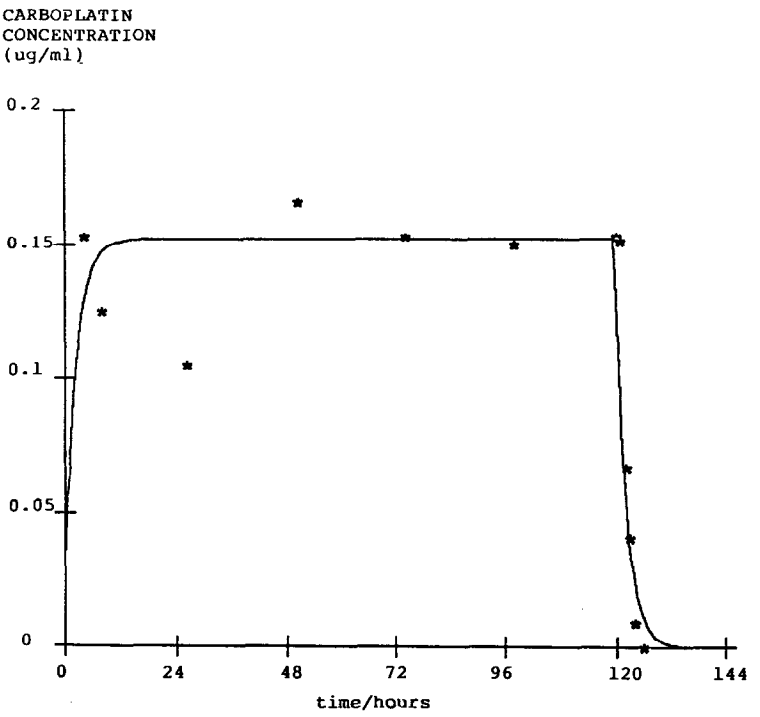

Figure 5
Typical PU carboplatin concentration–time profile for a patient receiving carboplatin by continuous infusion (30 mg day^{-1} for 5 days).

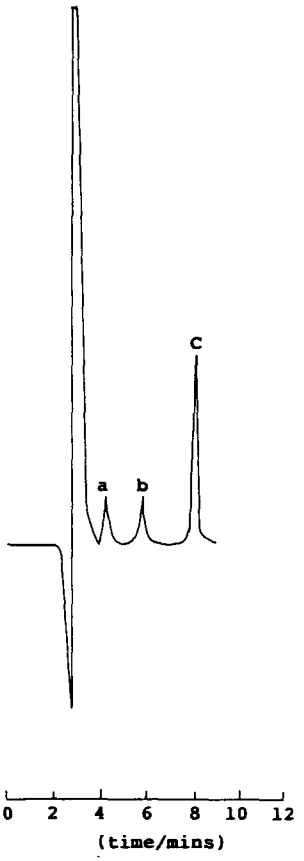

Figure 6
LC chromatogram showing the degradation of carboplatin (1.0 μg ml^{-1}) in an aqueous solution containing chloride ions (0.4 M) after 12 h incubation at 60°C.

examine the incorporation of the Ultracarb ODS column into the column-switching system to isolate carboplatin metabolites for biological testing in cell culture systems. The phosphate buffered aqueous mobile phase will permit the direct introduction of eluent fractions into cell culture systems without sample pre-treatment.

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