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Determination of cisplatin and *cis*-diammineaqua-chloroplatinum(II) ion by liquid chromatography using post-column derivatization with diethyldithiocarbamate

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Abstract

A post-column derivatization method has been developed for the determination of cisplatin and its monohydrated form. Cisplatin was isolated on a strong anion-exchange column, while a strong cation-exchange column was used for the monohydrated complex. Diethyldithiocarbamate was used as reagent and the influence of temperature, pH and methanol content on the yield of derivative was investigated. The reaction was quantitative using a packed-bed reactor with a surrounding temperature of 115°C and a mobile phase consisting of 0.125 *M* succinic acid–sodium hydroxide buffer pH 5.2 and methanol (2:3, v/v). The resulting complex, $Pt(DDTC)_2$, was monitored photometrically at 344 nm. The precision of the determination was 11.5% (C.V.) at an injected amount of 20 ng ($n = 12$) for monoaqua and 8.0% (C.V.) at 9 ng ($n = 10$) for cisplatin. The method was used to evaluate the plasma concentration of cisplatin and its monohydrated form in a patient.

1. Introduction

cis-Diamminedichloroplatinum(II) (cisplatin) is one of the most important anticancer drugs. It is mainly active towards solid tumors and since its introduction in the late seventies the prognosis, especially for testis cancer, has improved radically. The mechanism of action is obscure, but it is generally accepted that cisplatin is hydrolysed (Fig. 1) before reacting with its target DNA. The charged monohydrated form (monoaqua) of cisplatin is regarded as the most important species in this respect [1–3]. The different platinum species have a various degree of reactivity and consequently there is a demand for selective analytical techniques for their determination. The most commonly used method for the determination of cisplatin in biological material is based on off-line atomic absorption spectroscopy performed after ultrafiltration of the sample. By this technique the total ultrafiltrable fraction, which also includes low-molecular-mass complexes of cisplatin with endogenous compounds, will be quantified. This is referred to as 'free' platinum fraction [4]. Direct UV detection at 210 nm [5,6], electrochemical detection [7], and UV detection after post-column derivatization with sodium bisulfite and potassium dichromate [8] have been used for the determination of unchanged cisplatin after initial LC separation.

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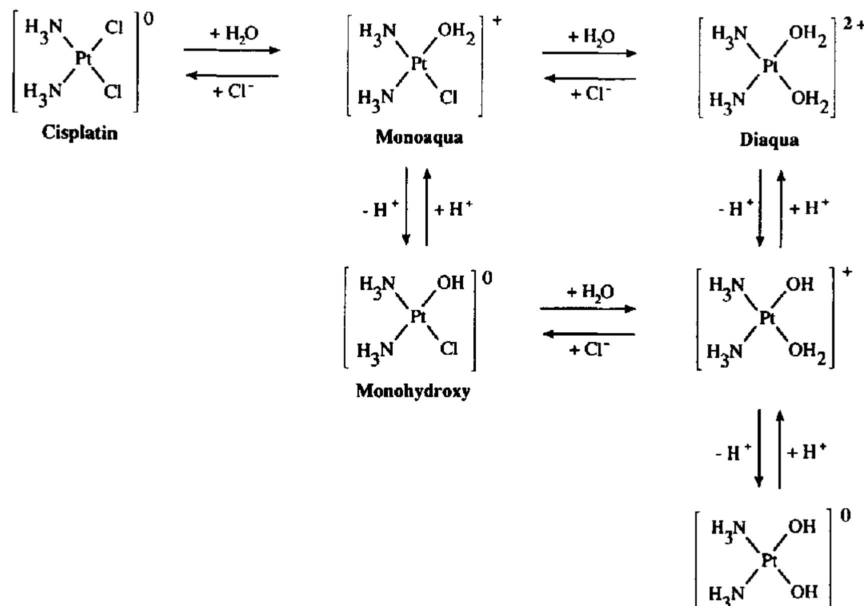

Fig. 1. Hydrolysis of cisplatin.

Another method involves pre-column derivatization with sodium diethyldithiocarbamate (DDTC) to form the chromophoric complex $Pt(DDTC)_2$ [9-13]. This reaction gives the same final product for cisplatin and its hydrated forms and thus the total platinum content is determined. Furthermore, DDTC has been observed to displace glutathione (GSH) bound to platinum [14]. Thus in a pre-column derivatization method the GSH-Pt complex might be co-determined and probably also other platinum complexes with for example methionine and cysteine. It has been suggested that the use of DDTC in conjunction with pre-column derivatization will overestimate the concentration of native drug in plasma samples by up to 25% [15].

In the present study the derivatization of cisplatin and its monoaqua form with DDTC has been optimized and used in a post-column system to obtain a selective and sensitive method for the determination of the compounds.

2. Experimental

2.1. Reagents

Cis-Diamminedichloroplatinum(II) was purchased from Ventron (Karlsruhe, Germany). The *cis*-diammineaquachloroplatinum(II) ion was prepared from a hydrolysis equilibrium obtained by dissolving cisplatin in distilled water (1 m*M*) and allowing the solution to stand over night at room temperature. Under these conditions 73% of the cisplatin will be converted to the monohydrated complex [16]. Sodium diethyldithiocarbamate (DDTC) (Sigma, Darmstadt, Germany) was used without further purification. Succinic acid and methanol (Merck, St. Louis, MO, USA) were of analytical grade. $Pt(DDTC)_2$ was prepared according to Bannister *et al.* [12]. Elemental analysis revealed: found % (calculated %), C: 24.4 (24.43), H: 4.1 (4.10), N: 5.6 (5.70), S: 25.7 (25.09).

2.2. Apparatus

Liquid chromatography was carried out with two Kontron Model 420 pumps (Rotkrenz, Switzerland). A small pump head was used for the reagent phase and a medium head for the mobile phase. Samples were introduced into a Valco Model C6W injector (Houston, TX, USA) with a fixed loop volume (20 μl or 50 μl). The detectors used were an LDC SM5000 photodiode array detector and an LDC Spectromonitor D (Riviera Beach, FL, USA). The wave-

length monitored was 344 nm. Chromatographic data was collected and processed using Integration Pack (Softron ver. 3.00). The reaction yield was calculated using synthetically prepared $Pt(DDTC)_2$ as a reference compound.

2.3. Chromatographic conditions

The columns used were a strong cation- (Nucleosil SA 5 μm, 150 × 3.2 mm I.D.) and a strong anion-exchanger (Nucleosil SB 5 μm, 70 × 3.2 mm I.D. and 150 × 4.6 I.D. mm). The columns were slurry packed downwards with methanol. The mobile phase consisted of 0.125 *M* succinic acid (adjusted to pH 5.2 with sodium hydroxide)–methanol (2:3, v/v). The flow-rate was 0.50 ml/min.

2.4. Post-column reactor

Packed-bed reactors (PBR) and open tubular reactors (OTR) were used. The PBR was a 50.0 × 0.35 cm I.D. stainless steel column, dry-packed with 250-μm, 75-μm (Supelco, Bellefonte, PA, USA), or 100-μm (Werner Glas, Stockholm, Sweden), or wet-packed with 25-μm (Werner Glas) glass beads. The PBR was wrapped up with a heating band with a surrounding temperature of 115°C. The OTR consisted of 40.0 m × 0.3 mm I.D. teflon tubing knitted according to Engelhardt and Neue [17] and thermostatted at 50°C. The mixing unit was a T-piece with low dead volume (Swagelok Union Tee SS-1FO-3GC, Cleveland, OH, USA). DDTC was dissolved in methanol–water with the same methanol concentration as the mobile phase and the flow-rate was 0.17 ml/min. The concentration of the reagent was 0.02 *M*.

2.5. Plasma analysis

Blood samples were collected in heparinized tubes, immediately centrifuged at 500 *g* at room temperature for 5 min and the plasma fraction was flash frozen. Within 2 h the plasma was thawed and ultrafiltered at 4000 *g* (4°C) for 30 min using filters with a 10 000 molecular mass cut-off (Filtron). The ultrafiltrate was frozen and stored at −25°C until assayed.

The identity of monoaqua was confirmed with Californium-252 time-of-flight plasma desorption mass spectrometry after isolation on a strong cation exchanger [18].

3. Results and discussion

The reaction between DDTC and cisplatin has been extensively used for pre-chromatographic derivatizations [9–13]. However, the reaction conditions have not been systematically investigated, for example, the reaction times used varies between 10 and 60 min. In the present study the formation of $Pt(DDTC)_2$ in batch experiments in methanol–water (93:7, v/v) was initially followed with LC. The reaction was relatively slow with a maximum yield of 90% after one hour at 70°C (5 m*M* DDTC). To make this useful in a post-column derivatization it was necessary to increase the reaction rate. Optimization studies were performed using the whole post-column system, since the reaction rates can be significantly different in a closed pressurized reactor [19]. The influence of temperature, pH and methanol content in the mobile phase on the reaction yield was investigated.

3.1. Influence of methanol content

The reaction yield increased with increasing methanol content in the mobile phase (Fig. 2). This might be due to an improved mixing efficiency of the reagent and the LC phases due to a change in viscosity. At *ca.* 40% methanol in water the viscosity is maximal and then decreases with increasing methanol content [20]. It is also possible that the reaction medium *per se* affects the reaction rate. At 80% methanol the peak of the monoaqua compound was deformed and a correct measurement of the peak area was not possible.

3.2. Influence of pH

The optimum pH of the mobile phase for the reaction yield is between 4.5 and 6 for the monoaqua compound (Fig. 3). It is not possible

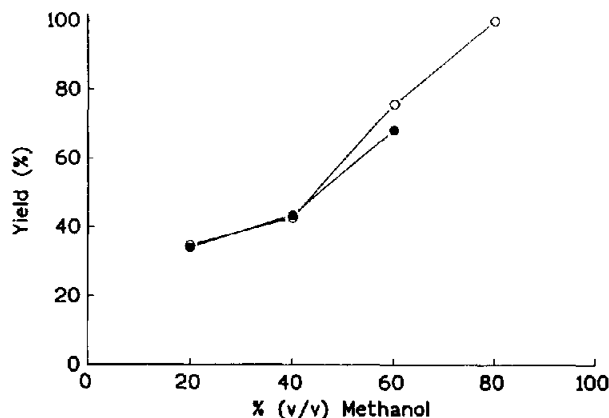

Fig. 2. Influence of methanol content on yield of $Pt(DDTC)_2$ after derivatization of monoaqua (●) and cisplatin (○). Temperature: 100°C, pH: 5.0.

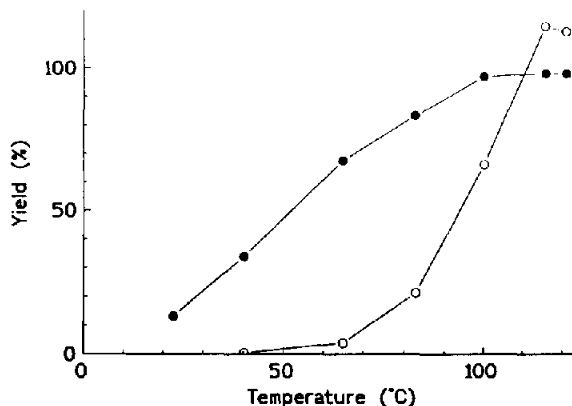

Fig. 4. Influence of surrounding temperature on the yield after derivatization of monoaqua (●) and cisplatin (○). Methanol content: 60% (v/v), pH: 5.2.

to work at lower pH since the reagent is quickly decomposed in acidic medium ($t_{1/2} = 30$ s at pH 4) [21] and it is highly reactive only in its deprotonized form (pK_a 5.5 in 60% ethanol) [21]. The decreased yield at pH > 6 is probably due to the fact that the monohydroxy form is considerably less reactive than the corresponding monoaqua form (pK_a 6.56) [18].

The reaction yield for the derivatization of cisplatin was also influenced by the pH (Fig. 3). At low pH the effect of the instability of the reagent was pronounced, while at pH > 5 no substantial effect of pH was observed.

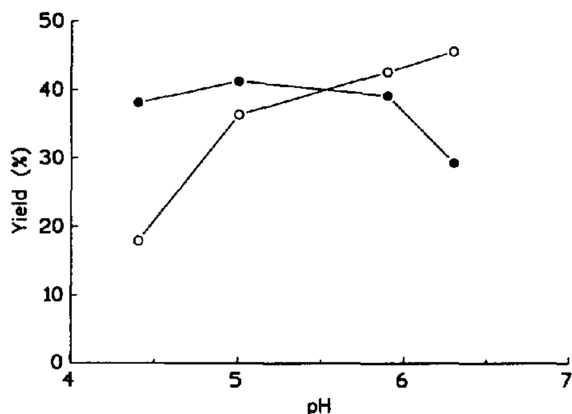

Fig. 3. Influence of pH on yield of $Pt(DDTC)_2$ after derivatization of monoaqua (●) and cisplatin (○). Temperature: 95°C, methanol content: 80% (v/v).

3.3. *Influence of temperature*

Fig. 4 shows the reaction yield as a function of temperature. At 110°C the yield was quantitative for both cisplatin and monoaqua. Except from the increase in reaction rate the increase in temperature will also decrease the viscosity and thereby contribute to a better mixing.

3.4. *Reactor design*

Initially a knitted open tubular reactor (OTR) was used. The problem encountered was a 'pulsing' baseline, which originated from the difference in absorbance between the reagent and the LC mobile phases at the wavelength monitored combined with an incomplete mixing of the two phases [22,23]. Different pumps and mixing devices were investigated, but the baseline noise could not be improved to a satisfactory level. This 'pulsing' was, however, drastically reduced when a PBR was used, but the band broadening was increased compared to the OTR. Table 1 shows that the contribution to band broadening from the post-column reaction system is approximately 40% for both systems, but the residence time in the OTR is notably longer than in the PBR.

An increase in temperature influenced the band broadening and baseline noise in the PBR.

Table 1
Contribution to band broadening

		Column + residual	Post-column reactor	Total
OTR	$\sigma^2(\mu l^2)$	10 430	5880	16 310
	$\sigma^2(\%)$	64	36	
PBR	$\sigma^2(\mu l^2)$	7120	4710	11 830
	$\sigma^2(\%)$	60	40	

Column: cation exchanger. Sample: monoaqua compound. Flow-rate: 0.5 ml/min. Room temperature. OTR 40 m × 0.30 mm I.D., residence time in reactor (t_v): 350 s, $k' = 5.2$.
PBR 50 cm × 3.5 mm I.D., $t_v = 270$ s, $k' = 2.2$.

Changing the temperature from 23 to 80°C resulted in a 20% decrease in band broadening. This has been noted before [24] and is proposed to be due to decreased adsorption to the glass beads at elevated temperatures. It was observed that the baseline noise decreased with increasing temperature, probably as an effect of better mixing.

3.5. *Influence of the particle size*

The studies of the contribution to band broadening were initially carried out with the monoaqua complex and $Pt(DDTC)_2$, since both forms are present in the PBR. They differ widely in their polarity, which might affect the dispersion. It was, however, found that the difference between the monoaqua compound and $Pt(DDTC)_2$ in the band broadening studies was not significant. A comparison of the influence of the different particle sizes of silanized and non-silanized glass beads indicated that the least band broadening was obtained with the silanized 75-μm particles (Table 2). Theoretically, the smallest particle size would be expected to give least band broadening [25]. However, more dispersion was observed with the 25-μm particles compared to the 75-μm beads, which might be due to the difficulty of packing small particles in the relatively long post-column reactor. An advantage of

Table 2
Contribution to band broadening

		Column + residuals	Post-column reactor	Total
25 μm silanized	$\sigma^2(\mu l^2)$	5750	14 320	20 070
	$\sigma^2(\%)$	29	71	
75 μm non-silanized	$\sigma^2(\mu l^2)$	5500	6240	11 740
	$\sigma^2(\%)$	47	53	
75 μm silanized	$\sigma^2(\mu l^2)$	7120	4710	11 830
	$\sigma^2(\%)$	60	40	
100 μm silanized	$\sigma^2(\mu l^2)$	5750	11 290	17 040
	$\sigma^2(\%)$	34	66	
250 μm silanized	$\sigma^2(\mu l^2)$	7120	17 950	25 070
	$\sigma^2(\%)$	28	72	

Experimental conditions: see Table 1.

using the larger beads is the possibility to dry-pack the column. The risk of clogging of the reactor is also minimized, which may be a problem in on-line derivatization techniques [26]. The largest band broadening was observed with beads of the largest particle size. Silanized beads were better than non-silanized beads (Table 2), possibly because the silanized beads are easier to pack or because the non-silanized beads interact with the analyte.

3.6. Chromatographic system

The retention of monoaqua on the SCX column was regulated with the pH of the mobile phase (Fig. 5) and the methanol content (Fig. 6). Cisplatin is not retained very well on the cation exchanger (Fig. 6). It eluted with the solvent front except at high (>80%) methanol concentration where a slight retardation was obtained. An anion exchanger was used for the analysis of cisplatin. Its retention increased with increasing methanol content (Fig. 6). The mechanism of retention is proposed to be an ion–dipole interaction [27]. Increasing the methanol content decreases the dielectric constant. The attraction between the ion exchanger and the dipole cisplatin will increase and therefore also the retention increases. Changes in pH had only a minor effect on the retention of cisplatin on the anion exchanger (Fig. 5).

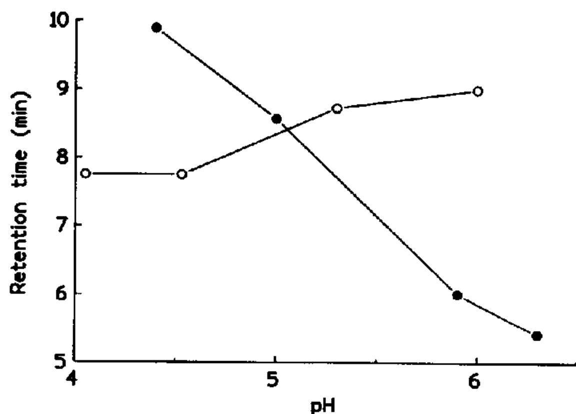

Fig. 5. Influence of pH on the retention time for monoaqua compound on a cation exchanger (●) and cisplatin on an anion exchanger (○).

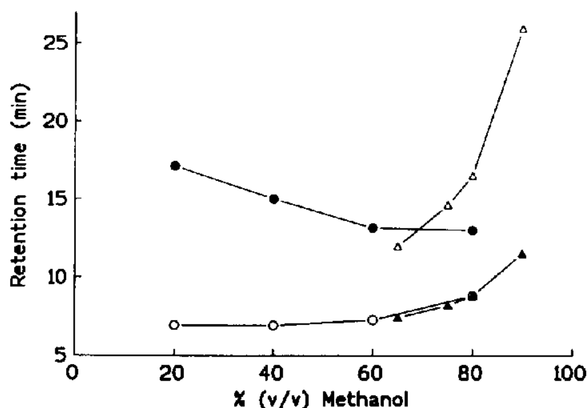

Fig. 6. Influence of methanol concentration on retention time of monoaqua on a cation exchanger (SCX) (●), cisplatin on SCX (○), monoaqua on anion exchanger (SAX) (▲), and cisplatin on SAX (△).

Since neither of the columns could separate both cisplatin and monoaqua, a coupled column system was used (Fig. 7). This system has only proven to be useful for the analysis of aqueous solutions, *e.g.* pharmaceutical preparations of cisplatin. Analysis of biological samples is com-

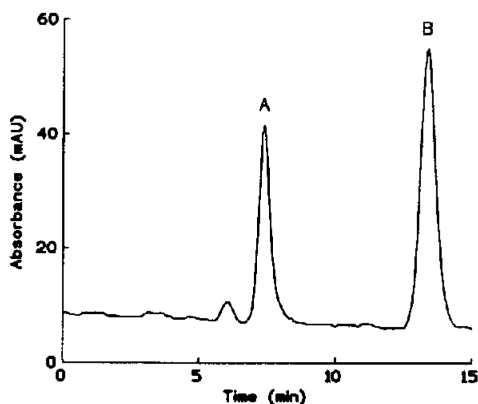

Fig. 7. Chromatogram obtained from coupled-column system. Columns were SAX (70 × 3.2 mm I.D.) and SCX (150 × 3.2 mm) with a mobile phase consisting of 0.125 *M* succinic acid/sodium hydroxide buffer at pH 5.0 and methanol (2:3, v/v). Reactor temperature: 100°C. A 2-μl volume of the hydrolysis mixture (2 m*M*) was injected. A: cisplatin, B: monoaqua compound.

plicated by interference from endogenous compounds.

Standard curves were linear in the concentration range studied, 0.14–78 μM for cisplatin and 1.3–130 μM for the monohydrated compound. Least squares linear regression analysis yielded slopes and intercepts of 4.13 ± 0.03 (S.E.M.) and -0.05 ± 0.92 (S.E.M.) for cisplatin ($r^2 = 0.9997$, $n = 8$) and 4.03 ± 0.02 (S.E.M.) and 1.61 ± 1.20 (S.E.M.) for monohydrated compound ($r^2 = 0.9999$, $n = 6$). There is no significant difference in the standard curves, which is to be expected when the yields are quantitative. Therefore it should be possible to use the same standard curve for both compounds. The precision of the determination was 11.5% (C.V.) at the 20 ng level ($n = 12$) for monoaqua and 8.0% (C.V.) at the 9 ng level ($n = 10$) for cisplatin.

The method was applied for evaluating the plasma concentrations in a patient receiving cisplatin 100 mg/m^2 (Fig. 8). The chromatograms of cisplatin and monoaqua are shown in Figs. 9 and 10, respectively. No interfering peaks were detected in blank plasma. From this study it can be concluded that it should be possible to perform pharmacokinetic studies in man not only of intact cisplatin but also of its monoaqua complex. The method has also been used to follow the concentration of cisplatin in the perilymphatic compartment in guinea pig [28].

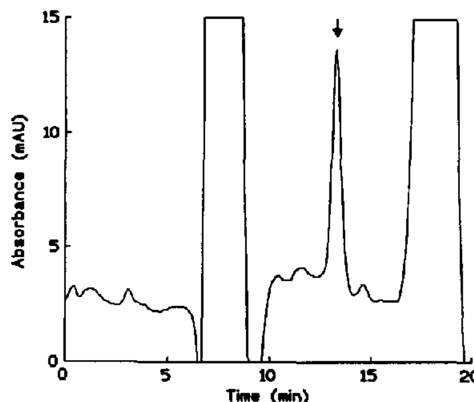

Fig. 9. Chromatogram of cisplatin in a plasma sample taken 30 min after the start of the infusion. Concentration of cisplatin 1.9 μg/ml. Chromatographic system: Column SAX 15 × 0.46 cm I.D., mobile phase 0.125 M succinic acid/sodium hydroxide buffer at pH 5.2 and methanol (2:3, v/v) Post-column system: PBR 50 × 0.35 cm I.D.; 75-μm silanized glass beads; reactor temperature 115°C.

4. Acknowledgments

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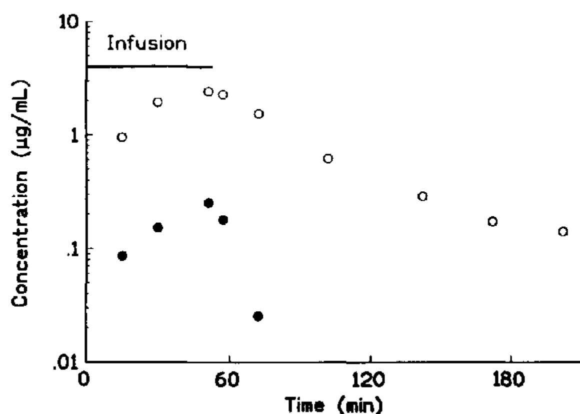

Fig. 8. Plasma concentration curves from a patient receiving 100 mg/m^2 as a 52-min infusion in physiological sodium chloride. Cisplatin (○) and monoaqua compound (●).

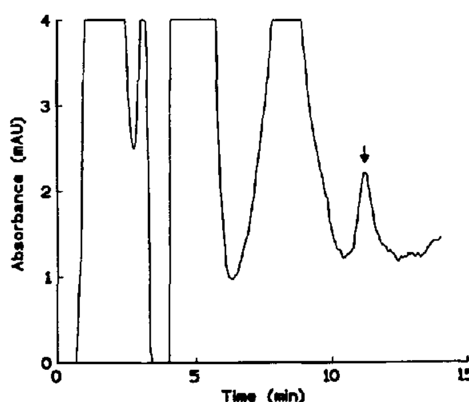

Fig. 10. Chromatogram of monoaqua compound in a plasma sample taken 30 min after the start of the infusion. Concentration of monoaqua 0.15 μg/ml. Column: SCX 15 × 0.46 cm I.D., additional conditions see Fig. 9.

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