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High-performance liquid chromatographic separation of the biotransformation products of oxaliplatin

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Abstract

A novel single reversed-phase HPLC system was developed for separating oxaliplatin and its biotransformation products formed in rat plasma. The major stable biotransformation products of oxaliplatin formed in rat plasma were identified as Pt(dach)(Cys)$_2$, Pt(dach)(Met) and free dach. The minor biotransformation products Pt(dach)Cl$_2$, Pt(dach)(GSH) and Pt(dach)(GSH)$_2$ could also be resolved from other Pt-dach complexes. Among these biotransformation products, the identification of Pt(dach)(Met) was further confirmed by LC–ESI-MS, and the identification of Pt(dach)(Cys)$_2$, Pt(dach)(GSH), Pt(dach)(GSH)$_2$ and free dach was confirmed by atomic absorption and double isotope labeling. This HPLC technique should prove useful for separating and identifying the biotransformation products of Pt-dach drugs such as oxaliplatin, ormaplatin and Pt(dach)(mal) in biological fluids. This will allow a more complete characterization of the pharmacokinetics and biotransformations of these Pt-dach drugs, which should in turn lead to a better understanding of the mechanisms leading to their toxicity and efficacy. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)] (Fig. 1) has been successfully used in chemotherapy of testicular and ovarian cancer [1]. However, cisplatin also shows severe side effects including nephrotoxicity, nausea, vomiting, ototoxicity and peripheral neurotoxicity [2]. It has been postulated that cisplatin itself is responsible for its anti-tumor activity, while some of its biotransformation products are responsible for its toxicity [3,4]. Repta and co-workers [5,6] have developed solvent generated ion-exchange high-performance liquid chromatography (HPLC) techniques to separate cisplatin from its biotransformation products. Daley-Yates and McBrien [3] subsequently utilized a similar separation methodol-

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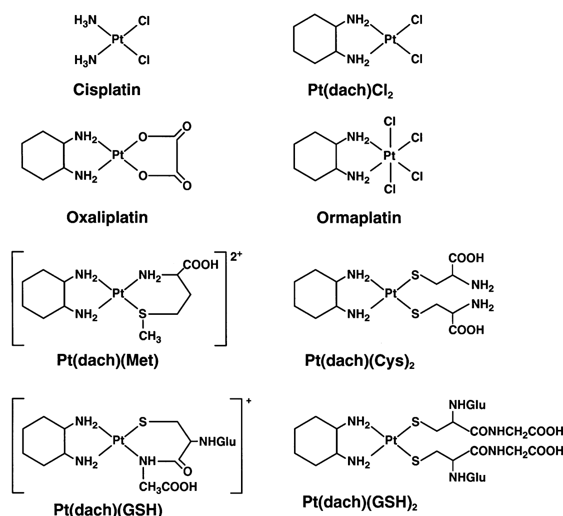

Fig. 1. Chemical structure of platinum complexes.

ogy to demonstrate that cisplatin biotransformation products had greater nephrotoxicity than the parent drug.

The understanding of the biotransformations of platinum complexes has become more advanced since the introduction of platinum compounds with a diaminocyclohexane (dach) carrier ligand that can be radioactively labeled (Fig. 1). Our laboratory has developed a two-column HPLC system (reversed-phase/cation-exchange column) capable of resolving the major biotransformation products of Pt-dach compounds [7]. This two-column HPLC separation system has been used to identify and quantitate the biotransformation products of Pt-dach complexes such as ormaplatin, Pt(dach)Cl_2 and Pt(dach)(mal) in tissue culture [8–10], rat plasma [11,12], rat tissue [13], and human plasma [14].

However, the two-column HPLC method has two major drawbacks. First, the major biotransformation products of Pt-dach drugs can only be partially resolved on the reversed-phase column. The unresolved portion needs to be further analyzed on the cation-exchange column. Therefore, resolution of Pt-dach biotransformation products on the two-column system requires extensive instrumental analysis time. Secondly, the active biotransformation products may be converted into stable biotransformation products before analysis with the cation-exchange column. Thus, it is difficult to accurately identify and quantitate the active biotransformation products. Since the active biotransformation products are likely to be responsible for the toxicity and/or anti-tumor activity of platinum drugs, we modified the elution program for the reversed-phase step of the two-column HPLC method with the goal of resolving all of the major biotransformation products on a single column.

We report here on the development of a novel HPLC method utilizing a single reversed-phase column. We have applied this newly developed HPLC method to resolve and identify the major biotransformation products of oxaliplatin [*trans-l*-1,2-diaminocyclohexaneoxalatoplatinum(II)] formed in rat plasma in vitro. Oxaliplatin, a cisplatin analog, is one the currently most promising of the Pt-dach anti-cancer drugs. It has anti-tumor activity against colorectal carcinoma, melanoma, glioma and the tumors traditionally treated with cisplatin and carboplatin [*cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II)] [1,15–19]. It has recently been approved for the treatment of colon cancer in France and is currently being evaluated in clinical trials in this country. This newly developed HPLC technique can be utilized with ^{3}H-labeled oxaliplatin [20] for detection of labeled Pt-dach biotransformation products in pre-clinical studies and with atomic absorption for detection of unlabeled Pt-dach biotransformation products in clinical studies, as demonstrated in previous biotransformation studies of ormaplatin [8–14]. Thus this technique should facilitate the identification and quantitation of individual oxaliplatin biotransformation products in both plasma and tissues, which will allow a detailed characterization of the biotransformations and pharmacokinetics of oxaliplatin. Based on the pharmacokinetic parameters and toxicological evaluation of individual oxaliplatin biotransformation products, it should be possible to gain a much more complete understanding of the chemical reactions which lead to the characteristic toxicity and efficacy of oxaliplatin and other Pt-dach compounds.

2. Experimental

2.1. Chemicals

HPLC grade methanol, acetonitrile, 1-heptanesulfonate and KH_2PO_4 were purchased from

Fisher Scientific (Pittsburgh, PA, USA). HPLC grade water was obtained with a Barnstead Nanopure water purification system. All HPLC solutions were filtered through a 0.22-μm hydrophilic Durapore filter (GWP, Millipore, Bedford, MA, USA) before use. Amino acids and glutathione were purchased from Sigma (St. Louis, MO, USA). Sodium heparin (5000 units/ml) was purchased from the Pharmacy Store Room at UNC hospital. All the radioactive chemicals were purchased from Amersham Life Science (Arlington, IL, USA).

2.2. Drugs

^{3}H-Oxaliplatin (340 Ci/mol), ^{3}H-Pt(dach)Cl_2 (320 Ci/mol) and Pt(dach)(Met) were synthesized by Dr. Steven Wyrick (Radiosynthesis Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA) [21]. Unlabeled oxaliplatin was kindly provided by Dr. Martine Bayssas (Debiopharm, Lausanne, Switzerland). Pt(dach)(GSH) was kindly provided by Dr. Martin Graham (Sanofi Research Division, Malvern, PA, USA) and Dr. Dennis Greenslade (Sanofi Research Division, Alnwick, UK). ^{3}H-Oxaliplatin (1.0 m*M*) was prepared by dissolving the appropriate amount of drug in double distilled water. ^{3}H-Pt(dach)Cl_2 (0.4 m*M*) was prepared in 150 m*M* NaCl solution. Pt(dach)(H_2O)Cl (1.0 m*M*) and Pt(dach)$(H_2O)_2$ (1.0 m*M*) solutions were prepared by mixing Pt(dach)Cl_2 with $AgNO_3$ at 1:1 and 1:2 molar ratios in water followed by an incubation in the dark for 12 h at 37°C. HPLC analysis of the final incubation mixtures showed that the Pt(dach)(H_2O)Cl obtained from our preparation was a mixture of Pt(dach)Cl_2 (5.4%), Pt(dach)(H_2O)Cl (80.6%) and Pt(dach)$(H_2O)_2$ (14.0%). The Pt(dach)$(H_2O)_2$ obtained from our preparation was a mixture of Pt(dach)(H_2O)Cl (21.6%) and Pt(dach)$(H_2O)_2$ (78.4%).

2.3. Animals

Wistar male rats (6–8 weeks of age, ~225 g body mass) were purchased from Charles River Breeding Labs. (Raleigh, NC, USA). Prior to the experiment, they were kept on a 12-h light–dark cycle in stainless steel cages at a temperature of 22°C and allowed Purina rodent chow and water ad libitum. An acclimation period of at least one week was allowed.

2.4. Preparation of Pt-dach standard compounds

Most of the HPLC standard compounds were generated by individually incubating ^{3}H-oxaliplatin (50 μ*M*) with biologically relevant small molecules at plasma physiological concentrations. These small molecules included methionine, glutathione, cysteine, serine, threonine, lysine, aspartate, urea, citrate, citrulline, creatinine, lactate and chloride (Table 1). All incubations were conducted in a standard incubation buffer containing 23.8 m*M* $NaHCO_3$ and 5 m*M* NH_2PO_4 at pH 7.4 to mimic in vivo plasma conditions. The incubations were performed in a 5% CO_2 incubator at 37°C for 24 h. Aliquots of the incubation mixture were diluted 1:20 into cold 10 m*M* $NaClO_4$ and were subjected to reversed-phase HPLC for the characterization of retention times (t_R) of the standard compounds generated. The stoichiometry of the Pt complexes formed was inferred by varying the ratio of oxaliplatin and the biological nucleophiles in incubations. When possible, the stoichiometry was confirmed by double label experiments with ^{3}H-oxaliplatin and ^{35}S-nucleophiles. For oxaliplatin, ormaplatin, Pt(dach)Cl_2, Pt(dach)(Met), and Pt(dach)(GSH), the assignments of t_R were confirmed with chemically synthesized standards. The purity of the standard compounds has been established by elemental analysis, nuclear magnetic resonance (NMR) and mass spectrometry (MS).

2.5. High-performance liquid chromatography

Reversed-phase HPLC was carried out on a 250× 4.5 mm Whatman Partisil ODS-3 column with a 50×4.5 mm guard column packed with the same material. Solvent A was 5 m*M* 1-heptanesulfonate, pH 3.4, and solvent B was 90% methanol in water. The flow-rate was 1 ml/min. The HPLC separation scheme described here was based on the previously described two-column HPLC separation of Pt-dach complexes. The rationale for the development of that system has been described previously [7]. In brief, 5 m*M* 1-heptanesulfonate was included in solvent A to provide solvent-generated cation-exchange sites in addition to the hydrophobic sites on the ODS-3

Table 1
Characterization of standard biotransformation products of oxaliplatin

Pt-dach complexes	Retention time (min)	Nucleophiles	Plasma concentration[a] (m*M*)	Reactivity[b] (%)
Pt-dach standards[c]				
Pt(dach)Cl_2	13	–	–	–
Oxaliplatin	18	–	–	–
Pt(dach)(H_2O)Cl	35	–	–	–
Pt(dach)(Met)	48	–	–	–
Pt(dach)(GSH)	53	–	–	–
Free dach	58 (major)	–	–	–
	68 (minor)	–	–	–
Pt(dach)$(H_2O)_2$	68	–	–	–
Generated Pt-dach complexes[d]				
Pt(dach)Cl_2	13	Chloride	108	67.8
Pt(dach)(GSH)	53	Glutathione	0.05	69.0
Pt(dach)$(GSH)_2$	28, 50			
Pt(dach)(Cys)	62	Cysteine	0.05	56.2
Pt(dach)$(Cys)_2$	32			
Pt(dach)(Met)	48	Methionine	0.05	59.5
Pt(dach)(serine)	39	Serine	0.19	10.4
Pt(dach)(threonine)	39	Threonine	0.40	26.7
Pt(dach)(lysine)	65	Lysine	0.40	6.1
Pt(dach)(aspartate)	65	Aspartate	0.05	3.3
Pt(dach)(urea)	65	Urea	3.2	8.1
Pt(dach)(citrate)	36	Citrate	0.14	12.3
Pt(dach)(citrulline)	36	Citrulline	0.06	2.9
Pt(dach)(creatinine)	65	Creatinine	0.05	12.0
Pt(dach)(lactate)	36	Lactate	0.02	13.0
Pt(dach)$(H_2O)_2$	68	Water	55 000	13

[a] The values were obtained from by Altman [24].
[b] Reactivity of nucleophile was defined the percentage of oxaliplatin consumed after a 24-h incubation. The incubation was performed in standard incubation buffer containing 24 m*M* $NaHCO_3$, 5 m*M* NaH_2PO_4, pH 7.4.
[c] Oxaliplatin, Pt(dach)Cl_2, Pt(dach)(Met), Pt(dach)(GSH) and free dach were authentic standards; Pt(dach)(H_2O)Cl and Pt(dach)$(H_2O)_2$ were prepared as described in Section 2.4.
[d] The generated Pt-dach complexes were prepared by individually incubating ^{3}H-oxaliplatin with nucleophiles at plasma concentrations in standard incubation buffer as described in Section 2.4.

column, as originally described by Riley et al. [6] and Daley-Yates and McBrien [3]. As might be expected, the 1-heptanesulfonate was found to be essential for elution of the positively charged Pt-dach biotransformation products such as Pt(dach)(Met) [7]. The aquated biotransformation products were better separated when the pH of solvent A was 3.4 and when buffer B was methanol rather than acetonitrite [7]. However, the two-column separation scheme still gave inadequate separation of the major Pt-dach biotransformation products, and a second chemically-bonded cation-exchange column was required for complete separation of Pt-dach biotransformation products.

Studies utilizing this two-column HPLC separation scheme had identified Pt(dach)Cl_2, Pt(dach)(Met), Pt(dach)(Cys), Pt(dach)(GSH) and free dach as the major plasma and intracellular biotransformation products of the Pt-dach compounds ormaplatin [11,12] and oxaliplatin (data not shown). Therefore, we systematically altered the elution profile of the first column to maximize the separation of these biotransformation products. Oxaliplatin and Pt(dach)Cl_2 were well resolved by isocratic elution with buffer A. Initially, a very shallow linear gradient from solvent A to solvent B over 2 h was used to maximize the separation of Pt(dach)(Cys), Pt(dach)(Met), Pt(dach)(GSH) and free dach. The

elution profile was then modified by using steeper gradients for those portions of the elution profile in which no significant amounts of the plasma biotransformations products of oxaliplatin were observed to elute. The modified elution profile gave comparable resolution of the major plasma biotransformation products, but required a shorter run time (70 min). The optimal elution program, expressed as the relationship of the elution time with the proportion of solvent A, was as follows: t=0 min, 100% solvent A (start of elution); t=0–10 min, linear isocratic elution with 100% solvent A; t=10–20 min, linear gradient elution from 100% solvent A to 80% solvent A; t=20–25 min, linear gradient elution from 80% solvent A to 75% solvent A; t=25–38 min, linear gradient elution from 75% solvent A to 70% solvent A; t=38–40 min, linear gradient elution from 70% solvent A to 56% solvent A; t=40–48 min, isocratic elution with 56% solvent A; t=48–62 min, linear gradient elution from 56% solvent A to 50% solvent A; t=62–64 min, linear gradient elution from 50% solvent A to 0% solvent A; t=64–70 min, isocratic elution with 0% solvent A; t=70 min, 0% solvent A (end of elution).

A 20-min isocratic elution with buffer A was required to re-equilibrate the column between runs.

Aliquots of samples containing ~10^5 cpm were injected onto the reversed-phase HPLC column. Fractions of 1 ml were collected throughout the whole elution. Aliquots (0.2–0.5 ml) of each fraction were mixed with 5 ml of Scinti Verse and counted in the LKB 1215 liquid scintillation counter to quantitate Pt-dach complexes in each fraction. The chromatograms were obtained by plotting the radioactive counts of aliquots (cpm) of all fractions versus time.

2.6. Identification of biotransformation products of oxaliplatin

A Wistar male rat was anesthetized with ether in a desiccator. The thorax of anesthetized rat was cut longitudinally along the midline through the sternum using surgical scissors. The blood was withdrawn from the heart and transferred to a pre-cooled heparinized tube followed by immediate centrifugation at 2000 g for 10 min at 4°C to separate the plasma and red blood cells (RBCs). Typically 0.25 ml of the ^{3}H-oxaliplatin stock solution (400 μg/ml or 1 mM) was gently mixed into 4.75 ml of rat plasma. This mixture was incubated in a 5% CO_2 incubator at 37°C for up to 24 h. At various times, aliquots of 50 μl were removed from the incubation mixture, and mixed with 950 μl of cold 10 mM $NaClO_4$ to quench the reaction. The diluted plasma samples were filtered through a YMT30 membrane (M_r cut off=30 000 000, Amicon, Danvers, MA, USA) with a centrifugation at 1700 g for 15 min. The obtained plasma ultrafiltrate (PUF) was immediately frozen at −80°C until HPLC analysis.

2.7. LC–ESI-MS analysis of mono-methionine Pt-dach complex in PUF

The LC–ESI-MS system was composed of a Pharmacia liquid chromatographic system with dual pumps 2248 (Pharmacia LKB Biotechnology, Uppsala, Sweden) coupled to a Finnigan 4000 quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA, USA) retrofitted with a pneumatic electrospray source (Analytica of Branford, Branford, CT, USA). Chromatographic separation was accomplished on a capillary C_{18} column (150×0.8 mm I.D.; Hypersil, 3 μm particle size; LC Packings) with an isocratic elution of water–methanol (95:5) supplemented with 0.5 mM ammonium acetate. The flow-rate through the capillary column was controlled at 20 μl/min by a microflow processor (LC Packings, San Francisco, CA, USA). Both the standard mono-methionine Pt-dach solution and the putative mono-methionine Pt-dach sample obtained from the HPLC analysis of PUF were injected (5 μl) via a Valco injector (Valco Instruments, Houston, TX, USA). Following the chromatographic separation, the eluent was directed to the electrospray needle through a fused-silica capillary column (30 cm×50 μm I.D.×375 μm O.D.). A voltage of 2.85 kV was applied to the electrospray needle and 70 p.s.i. (1 p.s.i.=6894.76 Pa) nebulizer gas (N_2) was employed to stabilize the spray. The voltage difference between the exit of the glass capillary and the first skimmer in the differential pumping region was optimized at 100 V for the signal of the [M+H]+ ion. Full-scan mass spectra were obtained by scanning from m/z 100 to m/z 650 every 2 s. Data were acquired and processed by a Technivent Vector 2 data system (Prolab Resources, Madison, WI, USA). A peak, which eluted at 25 min

and had a mass-to-charge ratio (m/z) of 457, was identified as the mono-methionine Pt-dach complex. This was the only peak observed to have the characteristic mass distribution of Pt (based on the Pt natural abundance).

3. Results and discussion

A two-column HPLC (reversed-phase/cation-exchange) separation method has been previously developed for resolving and identifying the biotransformation products of platinum drugs with the dach carrier ligand [7]. This method has been used to characterize the biotransformations of ormaplatin in rat plasma, tissue culture and carcinoma cells [9,10,12]. We used this method to identify the biotransformation products of oxaliplatin formed in rat plasma in vitro (Fig. 2). Oxaliplatin and one of its major biotransformation products Pt(dach)Cl_2 were resolved. However, the rest of major biotransformation products were poorly separated (Fig. 2A, C and E). Even though some of the poorly resolved biotransformation products could be further separated on a cation-exchange column, the time required and the stability of active biotransformation products between the reversed-phase and cation-exchange column were major drawbacks of the two-column HPLC separation method.

With the goal of resolving all of major biotransformation products on a single column, a new reversed-phase elution program was developed as described in Section 2.5 and applied to resolve the biotransformation products of oxaliplatin formed in rat plasma in vitro. A comparison between the previous two-column HPLC method and the newly developed single-column HPLC method shows that the major biotransformation products were well resolved with the single-column HPLC method (Fig. 2B, D and F). In addition to the improved resolution, the single-column HPLC allowed the analysis of biotransformation products to be finished within one or two days, which is much shorter than one to two weeks required by the previous two-column HPLC method. In order to characterize the HPLC chromatograms, standard Pt-dach compounds were generated and characterized with respect to their retention times (t_Rs). The standard compounds were

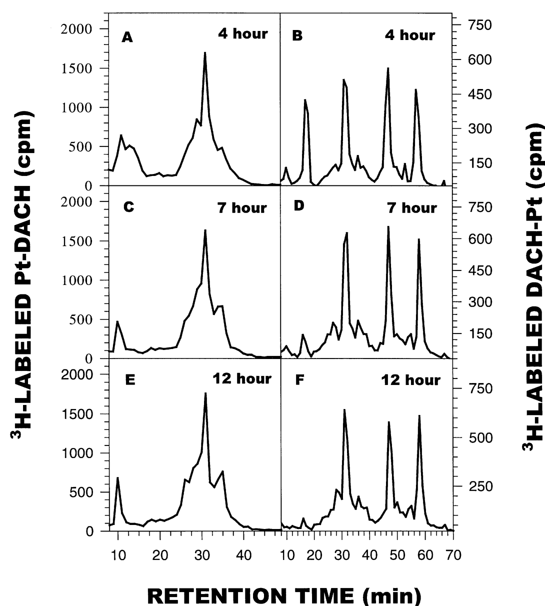

Fig. 2. Comparison between the reversed-phase separation of the two-column HPLC method and the single reversed-phase column HPLC method. ^{3}H-Oxaliplatin (0.05 mM) was incubated with rat plasma at 37°C. At the indicated times, plasma aliquots were diluted 1:20 in cold 10 mM $NaClO_4$ and filtered through an Amicon YMT 30 membrane. The biotransformation products in plasma ultrafiltrates were analyzed by reversed-phase HPLC with either the two-column HPLC elution method (A, C, E) described previously [7] or the single-column HPLC elution method (B, D, E) described in Section 2.5.

generated by individually incubating ^{3}H-oxaliplatin with the major biologically relevant nucleophiles present in the plasma and RBC cytosol (Table 1). The selection of biological nucleophiles used for generating the standard compounds was based in part on previous biotransformation studies of platinum drugs [7–10,12]. The oxaliplatin concentration used in above incubations was 50 μM, which was comparable to the maximum plasma concentration (C_{max}) of oxaliplatin achieved in vivo [22,23]. The concentration of each selected nucleophile approximately equaled its plasma physiological concentration [24]. As previously reported for Pt(dach)(mal) [10], bicarbonate at physiological concentration was found to greatly enhance the reaction of oxaliplatin with biological nucleophiles (Table 2). Thus, bicarbonate was included in all incubations for generating standard compounds. To obtain the t_R values of the

Table 2
The effect of bicarbonate on the reactivity of oxaliplatin with selected biological nucleophiles

Nucleophiles	Plasma concentration[a] (m*M*)	Reactivity (%)[b]	
		$NaHCO_3(+)$	$NaHCO_3(-)$
Water	55 000	13.2	< 1
Chloride	108	67.8	64.6
Glutathione	0.05	69.0	33.9
Cysteine	0.05	56.2	25.2
Methionine	0.05	59.5	45.3
Serine	0.19	10.4	< 1
Threonine	0.40	26.7	12.6

[a] The values were obtained from Altman [24].
[b] Reactivity of nucleophile was defined the percentage of oxaliplatin consumed after a 24-h incubation, which was performed with 50 μ*M* ^{3}H-oxaliplatin and each nucleophile at the plasma physiological concentration in buffer containing 5 m*M* NaH_2PO_4, pH 7.4 in the absence or presence of 24 m*M* $NaHCO_3$.

corresponding standard compounds, the incubation mixtures were analyzed on the reversed-phase HPLC column under the same conditions as the plasma samples (Fig. 3 and Table 1).

In this HPLC separation system, oxaliplatin has a t_R of 18 min and its major biotransformation products in rat plasma have retention times of 32 min, 48 min and 58 min (Fig. 2). Minor biotransformation products are observed with retention times of 13 min, 28 min, 36 min, 53 min and 68 min. Based on the extent of reaction in 24-h incubations of oxaliplatin with the selected nucleophiles (Table 1) and retention times of the major biotransformation products formed, (Fig. 3 and Table 1), the major biotransformation products of oxaliplatin are likely to be Pt(dach)$(Cys)_2$, Pt(dach)(Met) and free dach. The peaks corresponding to these Pt complexes were clearly resolved from other possible biotransformation products. Some of minor biotransformation products were not clearly resolved. For example, both Pt(dach)(Ser) and Pt(dach)(Thr) had a retention time of 39 min; Pt(dach)(H_2O)Cl, Pt(dach)(citrate) and Pt(dach)(lactate) had a retention time of 35–36 min; and Pt(dach)(Lys), Pt(dach)(Asp), Pt(dach)(urea), Pt(dach)(citrulline) and Pt(dach)(creatinine) all had retention times of 65 min. The peak at 68 min could contain both Pt(dach)$(H_2O)_2$ and a small amount of free dach. However, this peak also represents the final methanol wash and probably contains additional unknown minor biotransformation products. Because all of these possible biotransformation products accounted for only a minor

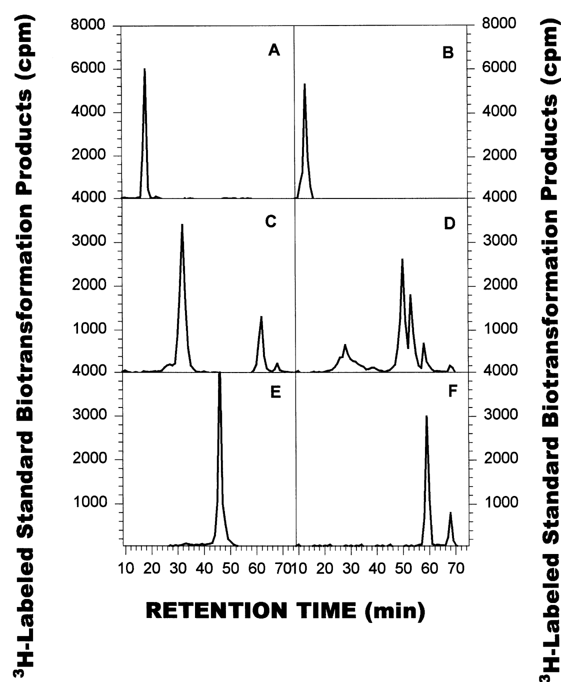

Fig. 3. HPLC separation of Pt-dach standards on the single reversed-phase column. The preparation protocol of Pt-dach standards and the HPLC elution program are described in Sections 2.4 and 2.5. Among these standard compounds, ^{3}H-oxaliplatin, ^{3}H-Pt(dach)Cl_2 and free ^{3}H-dach were authentic standards, and rest of the standards were generated by incubating ^{3}H-oxaliplatin (0.05 m*M*) with the corresponding nucleophiles (0.1 m*M*). (A) Oxaliplatin; (B) Pt(dach)Cl_2; (C) Pt(dach)(Cys) and Pt(dach)$(Cys)_2$; (D) Pt(dach)(GSH) and Pt(dach)$(GSH)_2$; (E) Pt(dach)(Met); (F) dach.

portion of the total amount of biotransformation products formed in the incubation of oxaliplatin with rat plasma, no further attempt was made to resolve these complexes.

In order to confirm the stoichiometry of the major biotransformation products formed, oxaliplatin (50 μ*M*) was incubated with methionine, cysteine and glutathione at both equimolar and higher concentrations for 24 h. The incubation mixtures were subsequently analyzed by reversed-phase HPLC. When oxaliplatin was incubated with methionine, a single peak with a retention time of 48 min was observed at both equimolar and 10-fold higher concentrations of methionine (Fig. 3E). An authentic mono-methionine Pt-dach standard synthesized by Wyrick and Chaney [21] was also eluted as a single peak at 48 min (data not shown). This suggested that the mono-methionine and bis-methionine Pt-dach were unable to be resolved by the reversed-phase column as we have reported previously with two-column HPLC system [7]. At the physiological plasma concentration of methionine [24], it is likely that only the mono-methionine Pt-dach complex can form. This was confirmed by LC–MS. In this study, oxaliplatin (50 μ*M*) was incubated with rat plasma for 24 h at 37°C, and plasma ultrafiltrates were subjected to reversed-phase HPLC. The resolved peak fractions (47–49 min) were pooled and subjected to LC–MS. Of the signals containing the characteristic mass distribution of Pt, only the mono-methionine Pt-dach complex, (m/z=457) was observed (Fig. 4). This study confirmed that the peak at 48 min observed in plasma samples contained only Pt(dach)(Met).

When oxaliplatin was incubated with cysteine at an equimolar concentration, a peak with a retention time of 62 min was observed (Fig. 5A). When incubated with 10-fold higher concentrations of cysteine, two peaks with retention times of 32 min and 62 min were observed (data not shown). These two peaks were further characterized by incubation of ^{3}H-oxaliplatin with ^{35}S-cysteine at a 10:1 ratio of cysteine to oxaliplatin to double label the products formed. The incubation conditions were the same as those used for incubating ^{3}H-oxaliplatin with unlabeled nucleophiles described above. HPLC analysis of the incubation mixture showed that the peak at 32 min contained both ^{35}S and ^{3}H with a molar ratio of ~2:1 and the peak at 62 min contained both ^{35}S and ^{3}H with a molar ratio of ~1:1 (Fig. 5B). This suggested that the peak at 32 min most likely represented a bis-cysteine Pt-dach complex, Pt(dach)(Cys)$_2$, and the peak at 62 min most likely represented a mono-cysteine complex, Pt(dach)(Cys).

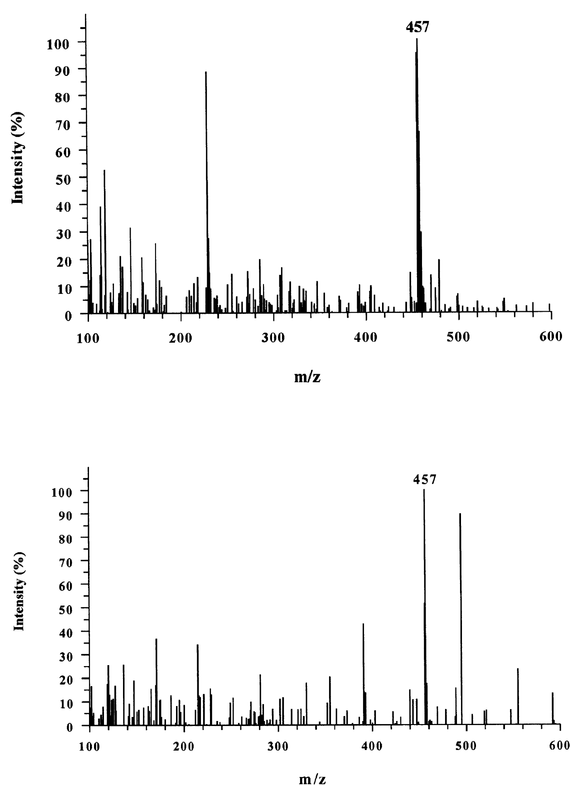

Fig. 4. Mass spectra of the Pt(dach)(Met) standard (the upper panel) and the "peak at 48 min" obtained from HPLC analysis of rat plasma ultrafiltrates (the lower panel). Unlabeled oxaliplatin was incubated with rat plasma for 24 h at 37°C and plasma ultrafiltrates were analyzed by reversed-phase HPLC as described in Section 2.5. The peak fractions 47–49 were pooled and concentrated with a Speed-Vac. The obtained samples were analyzed by coupled LC–ESI-MS on a Finnigan MAT 4000 quadrapole mass spectrometer as described in Section 2.7.

Glutathione was also incubated with oxaliplatin at equimolar and higher concentrations. HPLC analysis showed two major peaks with retention times of 50 min and 53 min and a minor peak with a retention time of 58 min when oxaliplatin was incubated with glutathione at an equimolar concentration (Fig. 6A). When incubated with 10-fold higher concentrations of glutathione, a broader peak appeared with a

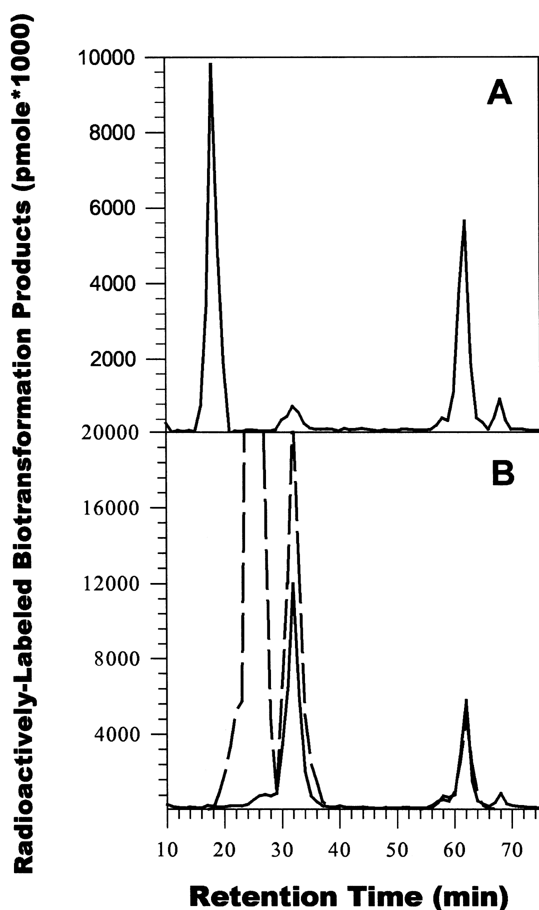

Fig. 5. Determination of the stoichiometry of the dach-Pt–cysteine complexes. ^{3}H-Oxaliplatin was incubated with cysteine at either equimolar or higher concentration for 24 h. The incubation conditions and HPLC analysis of generated standard biotransformation products were described in Section 2.5. (A) ^{3}H-oxaliplatin (0.05 m*M*, $1.1\cdot10^{5}$ cpm/nmol) and non-radioactively labeled cysteine were incubated at a molar ratio of 1:1; (B) ^{3}H-oxaliplatin (0.05 m*M*, $2.6\cdot10^{5}$ cpm/nmol) and ^{35}S-cysteine (0.5 m*M*, $1.1\cdot10^{5}$ cpm/nmol) were incubated at a molar ratio of 1:10. (—) ^{3}H; (- - -) ^{35}S.

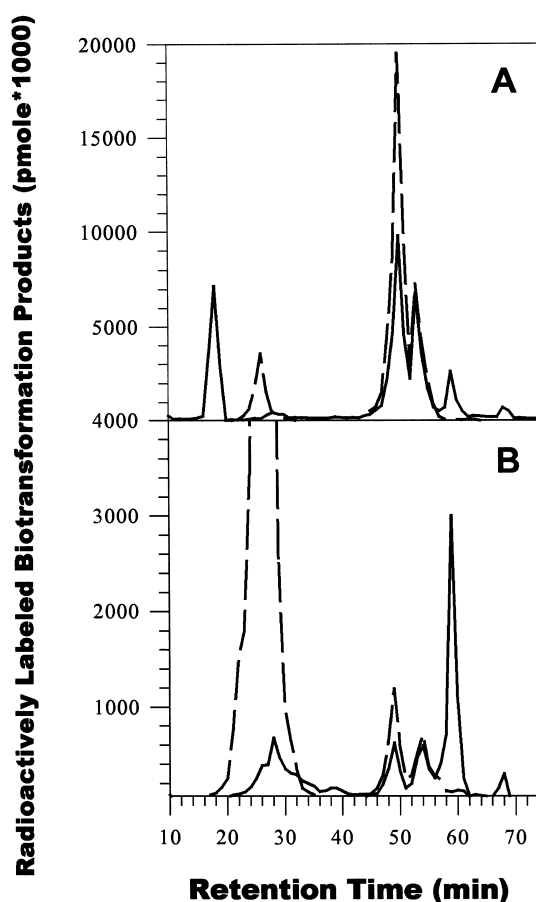

Fig. 6. Determination of the stoichiometry of the dach-Pt–glutathione complexes. ^{3}H-Oxaliplatin was incubated with glutathione at either equimolar or higher concentration for 24 h. The incubation conditions and HPLC analysis of generated standard biotransformation products were described in Section 2.5. (A) ^{3}H-oxaliplatin (0.05 m*M*, $1.1\cdot10^{5}$ cpm/nmol) and ^{35}S-glutathione (0.05 m*M*, $1.1\cdot10^{5}$ cpm/nmol) were incubated at a molar ratio of 1:1; (B) ^{3}H-oxaliplatin (0.05 m*M*, $2.6\cdot10^{5}$ cpm/nmol) and ^{35}S-glutathione (0.5 m*M*, $1.1\cdot10^{5}$ cpm/nmol) were incubated at a molar ratio of 1:10. (—) ^{3}H; (- - -) ^{35}S.

retention time of 28 min and the height of the peak at 58 min was significantly increased (Fig. 6B). These peaks were also further explored using the double isotope labeling method (^{3}H-oxaliplatin and ^{35}S-GSH) as described above. No ^{35}S-GSH was detected in the peak at 58 min, which indicated that glutathione was not associated the peak at 58 min. The molar ratios of ^{35}S and ^{3}H for the peaks at 50 min and 53 min were ~2:1 and 1:1, which suggested that the peak at 50 min likely contained a bis-glutathione Pt-dach complex Pt(dach)$(GSH)_2$, and the peak at 53 min contained a mono-glutathione complex Pt(dach)(GSH). Because the retention of ^{35}S-GSH itself was around 28 min, the stoichiometry of Pt(dach)(GSH) complex in the peak at 28 min could not be accurately determined. However, since the height of the peak at 28 min was increased with increasing glutathione concentration in the incuba-

tion with oxaliplatin, this peak probably also contained a bis-glutathione Pt-dach complex, which is presumably an isomer of the $Pt(dach)(GSH)_2$ in the peak at 50 min. An authentic mono-glutathione Pt-dach standard Pt(dach)(GSH) was analyzed under the same conditions and only one peak was observed at 53 min. These results further confirmed that the peak at 53 min likely represented the mono-glutathione Pt-dach.

The free dach ligand has a retention time of 58 min in our HPLC separation system. When ^{3}H-oxaliplatin was incubated with ^{35}S-glutathione, no incorporation of ^{35}S-glutathione was observed for the peak at 58 min. Therefore, the peak at 58 min likely contained mainly the free dach ligand, which is produced by trans-labilization of the dach carrier ligand by the sulfhydryl group of glutathione [25]. In addition, when oxaliplatin was incubated with rat plasma, Pt contents were determined for the pooled fractions with retention times of 12–13 min, 17–18 min, 28–30 min, 31–32 min, 35–36 min, 39–40 min, 53–54 min, 57–58 min, and 67–68 min (data not shown). No Pt was detected in the peak fractions with retention time of 57–58 min, further confirming that the peak at 58 min contained only free dach when oxaliplatin was incubated rat plasma. The ratio of ^{3}H to Pt was ~1:1 for rest of pooled peak fractions. This showed that the dach carrier ligand was still bound to Pt for the rest of the biotransformation products formed from oxaliplatin in rat plasma.

The trans-labilization of the carrier ligand when platinum (II) complexes react with sulfur-containing nucleophiles is well documented [25]. However, in the incubations that were performed to determine the stoichiometry of the Pt-dach biotransformation products (Figs. 3, 5 and 6), it was apparent that glutathione caused much greater trans-labilization of the dach carrier ligand than either cysteine or methionine. This can best be seen when one compares extent of trans-labilization and extent of reaction with oxaliplatin caused by all three nucleophiles at different molar ratios (Fig. 7). The mechanism for the greater trans-labilization caused by glutathione is not known, but it is likely due to the fact that the glutathione molecule has multiple reactive centers which are ideally positioned to directly displace the carrier ligand, subsequently forming tridentate or even tetradentate glutathione–Pt complexes. This interpretation is further supported by the fact that the extent of trans-labilization of the dach carrier ligand by glutathione (Fig. 7B) is directly proportional to the amount of glutathione–Pt-dach complexes formed (Fig. 7A) rather than to the molar ratio of glutathione to oxaliplatin in the reaction mixture.

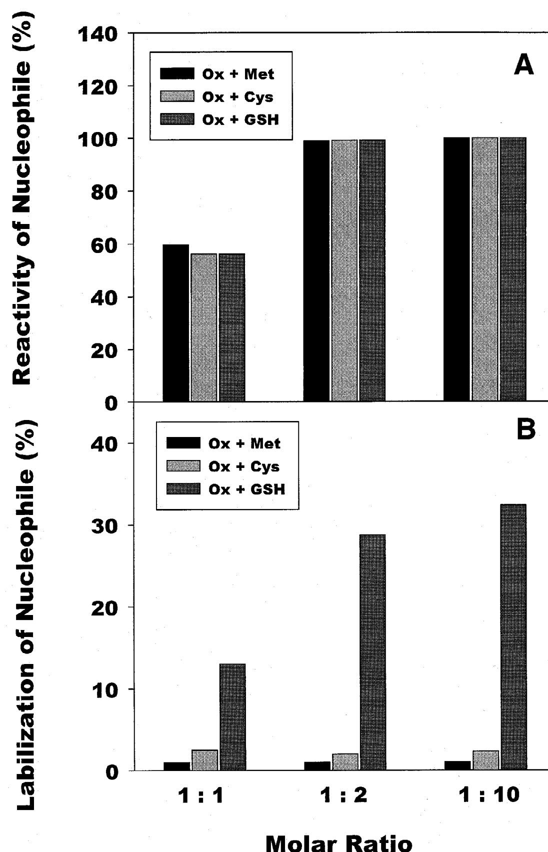

Fig. 7. Reactivity and trans-labilization effect of methionine, cysteine, and glutathione. ^{3}H-Oxaliplatin (0.05 m*M*) was incubated with methionine, cysteine and glutathione at 1:1, 1:2, and 1:10 molar ratios for 24 h under standard incubation conditions and the reaction products were analyzed by HPLC as described in Section 2.5. Reactivity of each nucleophile (panel A) was defined the percentage of oxaliplatin consumed in the 24-h incubation. The trans-labilization effect (panel B) was defined as the percentage of free dach produced in the same incubation.

Our current study has identified Pt(dach)(Met) and $Pt(dach)(Cys)_2$ as the major stable biotransformation products of oxaliplatin formed in rat plasma in vitro. Previous studies have identified the methionine

platinum complex as one of the major cisplatin biotransformation products formed in rat plasma [3] and both the cysteine and methionine platinum complexes as major ormaplatin biotransformation products in rat plasma [12]. However, the stoichiometry of the major Pt(dach)(Cys) biotransformation product has not been established previously. The formation of these biotransformation products is consistent with the reactivity of the corresponding nucleophiles (Cys and Met) at physiological plasma concentrations (Table 1). Our study has shown that at plasma physiological concentrations glutathione has comparable activity with cysteine and methionine with respect to the displacement of oxaliplatin leaving ligand in vitro (Table 1). However, glutathione forms multiple complexes which are resolved into several individual peaks by reversed-phase HPLC. Therefore, no single glutathione–Pt-dach complex is observed at concentrations approaching those of the Pt(dach)(Met) or $Pt(dach)(Cys)_2$ complexes when oxaliplatin was incubated with rat plasma. Similarly, chloride ion at the plasma physiological concentration has a comparable reactivity to methionine, cysteine, and glutathione (Table 1). However, $Pt(dach)Cl_2$ is only formed transiently in the plasma. The chloro ligands of $Pt(dach)Cl_2$ are known to be easily displaced by biological nucleophiles such as methionine, cysteine and glutathione as well as protein sulfhydryl. Therefore, the transient appearance of $Pt(dach)Cl_2$ observed is likely due to its high reactivity.

4. Conclusions

In summary, we have developed a novel HPLC method utilizing a single reversed-phase column and have applied this newly developed HPLC method to identify the major biotransformation products of oxaliplatin formed in rat plasma in vitro. This newly developed HPLC technique will allow us to readily identify and quantitate individual biotransformation products of oxaliplatin formed in rat blood in vivo, which will lead to a detailed characterization of pharmacokinetics of oxaliplatin. By combining the pharmacokinetics and toxicological evaluations of oxaliplatin and its biotransformation products, we should gain a much more complete understanding of the chemical reactions which lead to the characteristic toxicity and efficacy of oxaliplatin and other Pt-dach compounds.

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