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A full validated hydrophilic interaction liquid chromatography-tandem mass spectrometric method for the quantification of oxaliplatin in human plasma ultrafiltrates

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1 **Abstract**

2 Oxaliplatin is a platinum agent that is used for treatment of colorectal cancer. A sensitive and
3 selective hydrophilic interaction liquid chromatography-tandem mass spectrometric method for the
4 quantification of oxaliplatin was developed. Human plasma ultrafiltrates were precipitated by
5 acetonitrile containing carboplatin as an internal standard and further diluted with acetonitrile.
6 Chromatographic separation of oxaliplatin and the internal standard was achieved with a column
7 modified with phosphorylcholine and an isocratic mobile phase (acetonitrile/water/acetic acid =
8 90:10:0.1, v/v/v) at the flow rate of 0.2 mL/min. The lower limit of quantification for oxaliplatin was
9 25 ng/mL. The linearity range of the method was from 25 to 5000 ng/mL. The intra-day precision
10 and inter-day precision (RSD) ranged from 0.8 to 6.1%, and the accuracy (RE) was within $\pm 4.5\%$.
11 The extraction recoveries from human plasma ultrafiltrates were 83.6-91.6%, and ion suppression
12 caused by matrix components was 86.7-88.5% at three different levels, respectively. This method
13 was applied to a clinical pharmacokinetic study of oxaliplatin in a cancer patient. The maximum
14 concentration of colorectal cancer patient administered oxaliplatin was 1650 ng/mL.

15
16 **Keywords**

17 Oxaliplatin; Hydrophilic interaction liquid chromatography-tandem mass spectrometry; Plasma
18 ultrafiltrates

1. Introduction

Oxaliplatin (1R, 2R-diaminocyclohexane-oxalatoplatinum (II)) is a third-generation of platinum agent that is used for treatment of colorectal cancer. Oxaliplatin was developed to reduce the nephrotoxicity of cisplatin as the first-generation platinum agents and myelosuppression of carboplatin as the second-generation platinum agents. Platinum agents are activated intracellularly to form reactive platinum complexes that bind to nucleophilic groups producing both inter- and intra-strand crosslinks in DNA, thereby inhibiting DNA replication [1]. The majority of previously untreated metastatic colorectal cancer patients are treated with oxaliplatin and infusional 5-fluorouracil/leucovorin or capecitabine (FOLFOX or XELOX) [2]. It has been reported that the AUC of platinum agents correlates with the degree of adverse events such as thrombopenia [3,4]. Besides, oxaliplatin was one of major organic cation transporter (OCT) 2 substrates. OCT2 is located on the basolateral membrane of the proximal tubular epithelium in the kidney and related to uptake of cationic drugs in the kidney. Burger et al. indicated that high OCT2 expression caused to platinum toxicity [5]. Therefore, therapeutic drug monitoring of platinum agents could be an effective tool to reduce adverse effects. To establish therapeutic drug monitoring of platinum agents, it is necessary to determine the plasma target concentration and to clarify the cause of variation in pharmacokinetics.

Methods using atomic absorption spectrometry [6] and inductively coupled plasma–mass spectrometry [7] have been developed for the determination of oxaliplatin. However, the total quantity of the element platinum is measured by these methods, and the intact form cannot be distinguished from inactive platinum intermediates. For pharmacokinetic investigation, it is necessary to measure intact oxaliplatin. Ficarra et al. reported the HPLC-UV method for measuring intact oxaliplatin [8]. However, the inherent lack of favorable UV absorption properties of these agents has led to poor sensitivity. They reported that the limit of quantification of oxaliplatin was 70 µg/mL [8]. To obtain more sensitivity with UV detection, post-column derivatization techniques have been needed [9,10].

1 The LC/MS/MS system has been widely used for the determination of drugs because of its high
2 sensitivity and selectivity. Luo et al. reported the LC/ESI-MS method for the determination of the
3 intact oxaliplatin and its biotransformation products in rat plasma [11]. Recently, Zhang et al.
4 reported the LC/MS/MS method for the determination of the intact oxaliplatin in human plasma [12].
5 The sensitivity of their method is comparable with that of atom absorption spectrometry. However,
6 they used a long ODS column (250 mm) to avoid the matrix effect by endogenous impurities.
7 Hydrophilic interaction chromatography (HILIC) using bare silica or polar bonded phase and low
8 aqueous/high organic mobile phase has been shown to be a suitable method for the quantification of
9 polar compounds in biological samples. HILIC is superior separation of polar compounds in
10 biological samples with reversed retention to the traditional reversed-phase liquid chromatography
11 [13-16]. Recently, Hemström et al. and Falta et al. reported the ICP-MS method combined with
12 HILIC for the determination of total platinum concentrations in human plasma ultrafiltrates [17,18].
13 However, the method was not validated, so we considered that their method did not have higher
14 confidence. Their method could determine total platinum concentrations, but that could not
15 distinguish intact oxaliplatin from platinum intermediates which do not have biological activity.
16 Moreover, the method was not applied to the actual patients, who were administered oxaliplatin.

17 In this paper, we describe a HILIC/MS/MS method for the determination of oxaliplatin in
18 human plasma ultrafiltrates and results of application of the method to a pharmacokinetic study in a
19 cancer patient. The sample preparation of our established method employs protein precipitation and
20 dilution with acetonitrile without evaporation step, meaning that not only it needs simple technique
21 but also the exposure of antineoplastic drugs could be avoidable.

22

23 **2. Materials and methods**

24 2.1. *Materials*

25 Oxaliplatin, carboplatin, and acetic acid were purchased from Wako (Osaka, Japan). Purity of
26 oxaliplatin standard was 97.0+%. All other solvents and reagents were of HPLC grade. Human

1 plasma with sodium heparin as the anticoagulant was obtained from Terumo (Tokyo, Japan).

2

3 2.2. Sample preparation

4 2.2.1. Preparation of stock and working solution

5 A stock solution of oxaliplatin was prepared in methanol/water (50:50, v/v) at a concentration of 100
6 $\mu\text{g/mL}$ and was stored at -80°C . As an internal standard solution, 10 $\mu\text{g/mL}$ carboplatin in
7 acetonitrile was stored in the dark at -80°C .

8

9 2.2.2. Preparation of calibration standards and validation samples

10 We obtained human plasma ultrafiltrates using Centrifree[®] Ultrafiltration Devices purchased from
11 Millipore (Tokyo, Japan). Calibration standards of oxaliplatin in human plasma ultrafiltrates were
12 prepared by diluting a stock solution at a concentration of 5000 ng/mL and serially diluting a plasma
13 working solution at concentrations of 25, 50, 100, 200, 500, 1000 and 2000 ng/mL. Quality Control
14 samples (QC) of oxaliplatin in human plasma ultrafiltrates were prepared by diluting human plasma
15 ultrafiltrates working solutions at concentrations of 25, 50, 500 and 5000 ng/mL. All solutions were
16 stored at -80°C .

17

18 2.3. Sample preparation

19 Human plasma ultrafiltrates were precipitated by acetonitrile containing carboplatin as the internal
20 standard. To 40- μL sample volumes, 60 μL internal standard in acetonitrile containing 10000 ng/mL
21 of internal standard was added. The samples were then centrifuged for 10 min at $10,000\times g$. The
22 supernatant was diluted twenty-fold with acetonitrile. Ten μL of solution was injected into the HPLC
23 column.

24

25 2.4. Chromatographic and mass spectrometric conditions

26 The HPLC system consisted of a fully equipped Prominence20A (Shimadzu, Kyoto, Japan).

1 For the analytical column, we used PC HILIC (50 × 2.0 mm i.d., 3 μm, Shiseido, Tokyo, Japan). The
2 column temperature was maintained at 40°C. Mobile phase A consisted of acetonitrile/water/acetic
3 acid (90:10:0.1, v/v/v), and mobile phase B consisted of acetonitrile/water (50:50, v/v). The initial
4 mobile phase composition was 100% mobile phase A, pumped at a flow rate of 0.2 mL/min for 5.0
5 min. From 5.0 to 5.5 min, mobile phase B was increased linearly from 0 to 100%. For removal of
6 insoluble salts and matrix components, this setting was held for 4.5 min. From 10.0 to 10.5 min,
7 mobile phase B was decreased to 0% and kept at that rate until 15.0 min, after which the next
8 sample was injected. From 0 to 5 min, the flow was introduced into a mass spectrometer using a
9 switching valve. The overall run time was 15.0 min.

10 Mass spectrometry was carried out in an API3200 triple quadrupole mass spectrometer
11 (Applied Biosystems, Foster City, CA). Positive ionization electrospray mass spectrometry was
12 performed. For the determination oxaliplatin, the ionspray voltage was set at 5000 V. The turbospray
13 gas (N₂) probe was heated at 500°C. Nitrogen was used as curtain gas, gas 1 and gas 2, and their
14 flows were set to 50, 60 and 80 units, respectively. Unit mass resolution was set in both mass
15 resolving quadrupole Q1 and Q3. The declustering potential (DP) and collision energy (CE) for
16 oxaliplatin were 32 V and 19 eV, and these for internal standard were 46 V and 27 eV, respectively.
17 The transitions of m/z 398 → 306 and m/z 372 → 294 on selected reaction monitoring (SRM) were
18 used for monitoring oxaliplatin and carboplatin, respectively. The dwell time was 500 msec. Data
19 were collected and processed using Analyst 1.4.2 data collection and integration software (Applied
20 Biosystems).

21 22 2.5. Method validation

23 2.5.1. Linearity and lower limit of quantification (LLOQ)

24 For validation, oxaliplatin standards (eight non-zero standards of the analyte, 25, 50, 100, 200, 500,
25 1000, 2000, and 5000 ng/mL for oxaliplatin) were prepared in control human plasma ultrafiltrates
26 and analyzed. Linear regression of ratio of the areas of the analyte and internal standard peaks

1 versus the concentration were weighted by $1/x$ (reciprocal of the concentration). LLOQ was defined
2 as the concentration with a signal-to-noise ratio of at least 10 and acceptable precision and accuracy
3 data (RSD and RE less than 20%).

4

5 2.5.2. Precision and accuracy

6 Intra-day (n=6) and inter-day (n=6) precision and accuracy were investigated at four different levels,
7 50, 500 and 5000 ng/mL, for oxaliplatin. The precision (RSD) was determined on the basis of the
8 coefficient of variation (RSD (%)), and the accuracy was calculated as ((found
9 concentration–theoretical concentration)/theoretical concentration) $\times$ 100 (RE (%)).

10

11 2.5.3. Specificity and selectivity

12 To investigate whether endogenous matrix constituents interfered with the assay, control drug-free
13 plasma ultrafiltrate samples containing neither analyte nor internal standard (double blank) and
14 plasma ultrafiltrate samples containing LLOQ (25 ng/mL) of oxaliplatin and internal standard were
15 prepared. Human plasma ultrafiltrates were prepared from six different donors.

16

17 2.5.4. Stability

18 The stability of oxaliplatin in human plasma ultrafiltrates was examined by analyzing three
19 concentrations (50, 500 and 5000 ng/mL) in triplicate. These samples were stored at -80°C for 4
20 weeks and at 4°C for 4 hours to evaluate long-term and short-term stability, respectively.

21 Freeze-thaw stability was tested following three freeze-thaw cycles (-80°C to 4°C). Stability of the
22 processed samples was assessed by reinjecting the samples after 4 hours in an autosampler (4°C).

23

24 2.5.5. Extraction recovery from human plasma ultrafiltrates and matrix effect

25 Extraction recovery of oxaliplatin from human plasma ultrafiltrates was evaluated at three
26 concentrations by comparing peak areas obtained from samples spiked with oxaliplatin before

1 extraction with those obtained from samples to which oxaliplatin was added after extraction.

2 The matrix effect of components from human plasma ultrafiltrates on oxaliplatin ionization
3 was also evaluated at three concentrations by comparing peak areas obtained from plasma
4 ultrafiltrate samples after extraction with those obtained from samples without matrix components.

5 For infusion experiments, reference standard oxaliplatin (50 $\mu\text{g/mL}$, 0.1 mL/min) was
6 continually infused and then the corresponding matrix samples with (processed samples) or without
7 dilution (only protein precipitation samples) with acetonitrile were injected.

9 2.6. Application to clinical pharmacokinetic study

10 The method was applied to the determination of plasma concentrations of oxaliplatin in a colorectal
11 cancer patient hospitalized in Hokkaido University Hospital. Before treatment, the patient gave
12 written informed consent as approved by the institutional review board. We applied our method to
13 samples obtained from a cancer patient receiving 85 mg/m^2 of oxaliplatin i.v. over 2 hours. The
14 blood samples were collected into sodium heparin-containing tubes at 0, 0.5, 1 and 1.5 h post-dosing.
15 Samples were immediately stored at 4 $^{\circ}\text{C}$, and plasma ultrafiltrates were obtained. The resultant
16 ultrafiltrates were stored at -80°C until use.

18 3. Results and discussion

19 3.1. Mass spectrometry

20 ^{194}Pt , ^{195}Pt , ^{196}Pt and ^{198}Pt are four major natural isotopes of platinum, and their natural abundances
21 are of 32.9, 33.9, 25.3 and 7.2%, respectively. Positive ion full-scan mass spectra (Q1) of oxaliplatin
22 indicated the presence of protonated molecular ions $[\text{M}+\text{H}]^+$ with m/z of 397, 398, 399 and 401,
23 corresponding to Pt isotope abundance ratio. A protonated molecular ion with m/z of 398 was most
24 strongly generated and was used for the precursor ion. The product ion mass spectrum of $[\text{M}+\text{H}]^+$ at
25 m/z of 306 is shown in Fig. 1A. The product ion at m/z of 306 was the most strongly produced and
26 was used for quantitative SRM of oxaliplatin. The proposed fragmentation pattern for oxaliplatin is

presented in Fig. 1A.

Positive ion full-scan mass spectra (Q1) of carboplatin as an internal standard indicated the presence of protonated molecular ions $[M+H]^+$ with m/z of 371, 372, 373 and 375, corresponding to Pt isotope abundance ratio. A protonated molecular ion with m/z of 372 was most strongly generated, and it was used for the precursor ion. The product ion at m/z of 294 was the most strongly produced (Fig. 1B) and was used for quantitative SRM of the internal standard.

3.2. Chromatography

Oxaliplatin and the internal standard are hydrophilic molecules and are consequently poorly retained on reversed-phase chromatography columns such as Inertsil ODS-3 (GL Science, Tokyo, Japan), CACELL PAK MGII (Shiseido, Tokyo, Japan), and TSKgel ODS 100V (TOSOH, Tokyo, Japan). The present method employed HILIC using a column modified with phosphorylcholine. HILIC mode is suitable for polar compounds such as oxaliplatin.

Using the mobile phase described in section 2.4, oxaliplatin and the internal standard were base-line separated. The retention time of oxaliplatin was 2.4 min and that of the internal standard was 4.1 min. We avoided reduction of the signal by ion suppression from impurities in plasma samples by washing the analytical column using mobile phase B.

3.3. Validation

3.3.1. Linearity and lower limit of quantification (LLOQ)

The present method covered a linearity range of 25 to 5000 ng/mL for the concentration of oxaliplatin in plasma ultrafiltrates with a weighting by $1/x$. The correlation coefficient (r) was >0.999 for oxaliplatin determination. A typical standard curve was $y = 0.646x - 0.00802$. The LLOQ for oxaliplatin was 25 ng/mL. This value is comparable with the method by Zhang et al. [12].

3.3.2. Precision and accuracy

1 Intra-day (n=6) and inter-day (n=6) precision and accuracy were investigated at four different levels,
2 25, 50, 500 and 5000 ng/mL, for oxaliplatin. The results are summarized in Table 1. For the
3 determination of oxaliplatin, the intra- and inter-day precisions were less than 6.1% for all
4 concentrations. The accuracies were within $\pm 4.5\%$ for all concentrations. These results suggest that
5 intact oxaliplatin in human plasma can be measured accurately and reproducibly by the present
6 method.

7 8 3.3.3. *Specificity and selectivity*

9 We evaluated the specificity and selectivity of the method. A representative chromatogram of blank
10 human plasma and chromatograms of LLOQ of oxaliplatin spiked in human plasma are shown in
11 Fig. 2A, 2B. There is no interference from an endogenous substance.

12 13 3.3.4. *Stability*

14 The stability of oxaliplatin in human plasma ultrafiltrates is shown in Table 2. We confirmed that
15 oxaliplatin in human plasma ultrafiltrates is stable for 4 weeks at a temperature below -80°C , 4
16 hours at a temperature below 4°C , and after processing. In addition, freeze-thaw stability also had
17 good value. Therefore, we decided to preserve oxaliplatin in human plasma ultrafiltrates at -80°C
18 and thaw them just before measurement.

19 20 3.3.5. *Extraction recovery from human plasma ultrafiltrates and matrix effect*

21 The extraction recoveries from human plasma ultrafiltrates were 83.6-91.6% (Table 3). Ion
22 suppression caused by the matrix components is shown in Fig. 3. Because of the matrix components,
23 ionization of oxaliplatin was suppressed to 50% (Fig. 3 (B)); however, by diluting plasma with
24 acetonitrile, ionization of oxaliplatin recovered to nearly 90% (Fig. 3 (C)). The dilution of matrix
25 components with acetonitrile was possible to remove the effect of matrix components and improve
26 the ionization of the oxaliplatin. However, we confirmed that more than twenty times dilution with

acetonitrile caused to decline of sensitivity due to high dilution ratio. Thus, we thought that this sample preparation was best condition for our method.

3.4. Application to a clinical pharmacokinetic study

The plasma intact oxaliplatin concentration versus time curve in a colorectal cancer patient after infusion for 2 hours is shown in Fig. 4. Plasma concentration of oxaliplatin at 2 hours after infusion was less than the LLOQ. The chromatogram of oxaliplatin in plasma ultrafiltrates taken from a patient at the end of oxaliplatin fusion is shown in Fig. 2D. The maximum concentration was 1650 ng/mL. This result agrees with past report [12], indicating the applicability of this method to pharmacokinetic study of oxaliplatin.

4. Conclusion

We have developed a sensitive and selective hydrophilic interaction liquid chromatography-tandem mass spectrometric method for the quantification of oxaliplatin in human plasma. Using samples diluted with acetonitrile, we succeeded in reducing ion suppression caused by the matrix effect. Our method does not need an evaporation step for sample preparation, which makes a brief sample preparation and a minimal occupational exposure. Validation demonstrated that this method was high levels of sensitivity and selectivity and sufficient precision and accuracy. The method was applied to a clinical pharmacokinetic study of oxaliplatin in a cancer patient.

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1 **Figure legends**

2 Figure 1 Product ion mass spectra of oxaliplatin (A) and internal standard (B) and proposed
3 fragmentation patterns.

4
5 Figure 2 Representative chromatograms of blank plasma (A) and LLOQ of oxaliplatin (25 ng/mL)
6 (B) and an internal standard (C) spiked into human plasma ultrafiltrates and the
7 chromatogram of oxaliplatin in plasma ultrafiltrates taken from a patient at the end of
8 oxaliplatin infusion (D).

9
10 Figure 3 Matrix effect of plasma matrix components on oxaliplatin ionization. Arrow indicates the
11 retention time of oxaliplatin.
12 Reference standard oxaliplatin (50 µg/mL, 0.1 mL/min) (black line), injection of plasma
13 extract without diluting with acetonitrile (blue line), and injection of plasma extract
14 diluted with acetonitrile (red line).

15
16 Figure 4 Intact oxaliplatin concentration in plasma ultrafiltrates versus time curve in a colorectal
17 cancer patient after infusion for 2 hours.

Table 1 Precision and accuracy of the quantification of oxaliplatin in human plasma ultrafiltrates

Analyte	Concentration (ng/mL)	Intra-day (n=6)			Inter-day (n=6)		
		Found (ng/mL)	RSD (%)	RE (%)	Found (ng/mL)	RSD (%)	RE (%)
Oxaliplatin	25	24.5±1.3	5.40	-1.93	25.1±1.5	6.07	0.27
	50	49.7±2.8	5.54	-0.70	50.2±1.5	2.95	0.40
	500	493±14	2.76	-1.50	501±18.7	3.73	0.17
	5000	4770±147	3.08	-4.53	5000±378	0.75	0.07

Figure 1

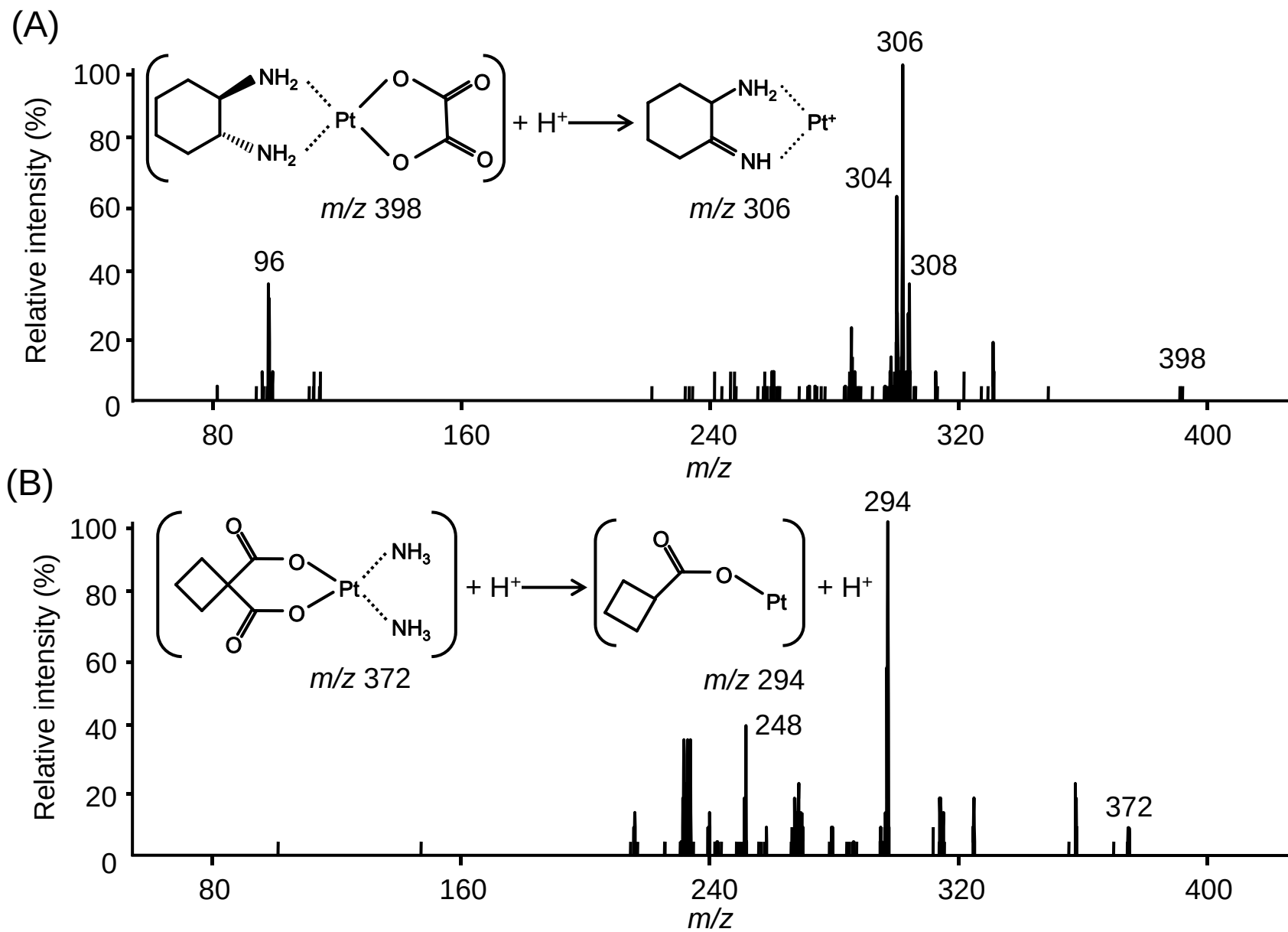


Figure 2

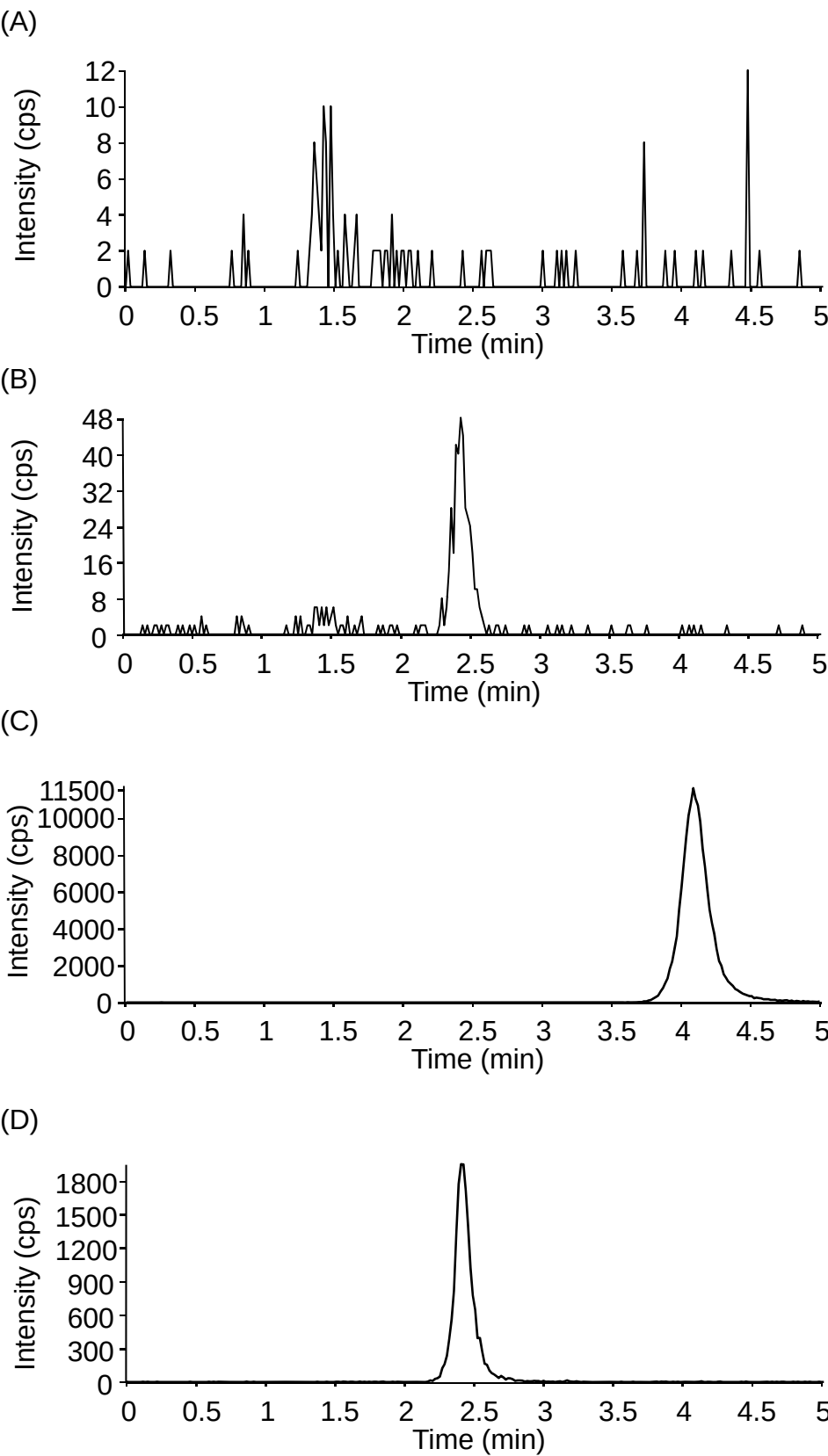


Figure 3

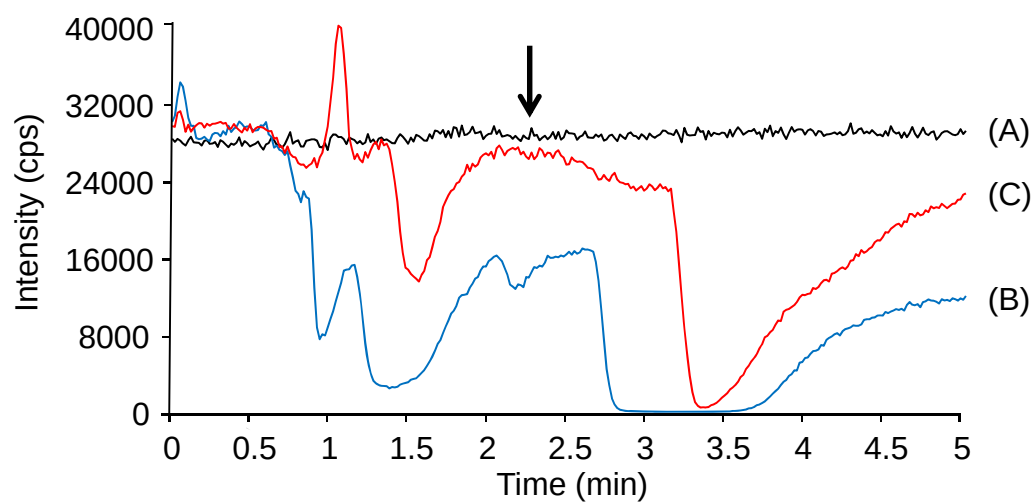


Figure 4

