Journal of Chromatography B, 879 (2011) 2162–2170

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of carboplatin in human plasma using HybridSPE-precipitation along with liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 10 December 2010
Accepted 29 May 2011
Available online 13 June 2011

Keywords:
Carboplatin
HybridSPE-precipitation
SCX
Matrix effects
LC–MS/MS
Phospholipids removal

ABSTRACT

The main purpose of this study was to develop and validate a rapid, specific, sensitive, and reliable LC–MS/MS-based bioanalytical method for the determination of carboplatin in human plasma. The optimal chromatographic behavior of carboplatin was achieved on a Biobasic SCX column (50 mm × 2.1 mm, 5 μm) using ion exchange chromatography. The total LC analysis time per injection was 2.6 min with a flow rate of 1.5 mL/min with a gradient elution. Optimization with regard to improving recovery and minimizing matrix effects using HybridSPE-precipitation (HybridSPE-PPT) has been evaluated under various extraction conditions. As a result, sample preparation via HybridSPE-PPT with 1% formic acid in acetonitrile in a 96-well format was applied for method validation and sample analysis and showed acceptable recovery of greater than 25% and negligible matrix effects. The method validation was conducted over the curve range of 2.00–2000 ng/mL using 0.0500 mL of plasma sample. The intra- and inter-day precision and accuracy of the quality control samples at low, medium, and high concentration levels showed ≤4.8% relative standard deviation (RSD) and −13.2 to −3.6% relative errors (RE). The method was successfully applied to determine carboplatin in human plasma samples.

1. Introduction

Carboplatin (cis-diammine (1,1-cyclobutanedicarboxylato)-platinum), as shown in Fig. 1, is a second-generation platinum compound with a wide range of antineoplastic properties similar to cisplatin, yet possessing an improved toxicity profile that has been attributed to its greater chemical stability [1]. It has been extensively used in the treatment of ovarian, head, neck, lung, bladder, and testicular cancer [2,3]. In addition, it is very common to use carboplatin in combination with other anti-cancer drugs such as paclitaxel and eribulin mesylate in order to minimize side effects via reducing dose and improve efficacy [4,5]. Therefore, it is necessary to have a sensitive, specific, and reliable bioanalytical method for the investigation of the clinical pharmacokinetic and pharmacodynamic behavior of carboplatin for studies with low dose carboplatin, especially for long-term pharmacokinetic investigation [6]. For present study, a detection limit of 2.00 ng/mL was expected.

Although there are over a dozen published methods for the determination of carboplatin, most of them are limited by suitable sensitivity, specificity, and through-put. These methods can be classified as either non-specific that only determine the elemental platinum in carboplatin or specific type that selectively detect the intact carboplatin. Among the nonspecific methods, the most widely used is atomic absorption spectrophotometry [7,8]. With regard to selective methods for the determination of free carboplatin, high performance liquid chromatography (HPLC) separation coupled to various detection techniques appears to be the method of choice. Carboplatin has been analyzed using reversed phase [9,10], hydrophilic [1], and strong cation exchange [4] chromatographic conditions. Due to its very polar property, carboplatin has minimal *k* values on most reversed phase columns. Different detection techniques including UV [9,11], electrochemical [12], MS [10,13], ICP-MS [14,15], MS/MS [4] have been applied in the determination of carboplatin. UV detection of carboplatin is limited by its low sensitivity and non-specificity because of the lack of favorable UV absorption properties and the interference of either endogenous compounds or biotransformation products. UV detection usually requires HPLC run times of over 10 min and detection limits of over 50.0 ng/mL [9]. Electrochemical detection of carboplatin also has very limited applicability due to reproducibility issues and reliance on HPLC separation of carboplatin from possible interferences [12]. LC–MS in selected ion monitoring mode offers some degree of detection specificity. However, high background noise and possible matrix effects constrain the limit of detection

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doi:10.1016/j.jchromb.2011.05.057

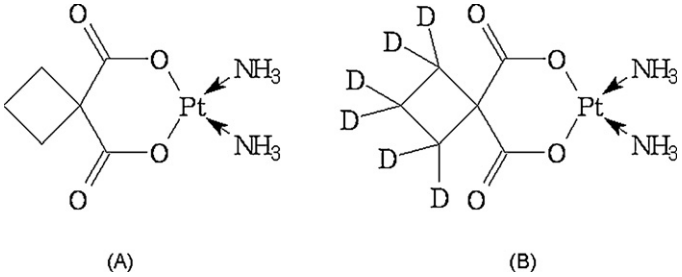

Fig. 1. Chemical structures of carboplatin (A) and carboplatin-d_6 (B).

to 35–70 ng/mL [10,13]. This may be explained by the very polar nature of carboplatin, which makes clean-up of plasma samples by either liquid-liquid extraction (LLE) or solid-phase extraction (SPE) difficult. LC–ICP-MS has demonstrated excellent sensitivity with a detection limit of 130 pg/mL [14]. However, it still requires sufficient chromatographic separation to attain specificity and is not readily available in many bioanalytical labs. LC–MS/MS methods can be very specific and sensitive. A detection limit at 2.00 ng/mL for quantification of carboplatin in human plasma has been reported from a validated LC–MS/MS assay [4]. The total HPLC run time per injection required over 10 min. No representative chromatograms of plasma samples were shown in this report. The dimension of the HPLC column for analysis of carboplatin was not reported as well. In addition, matrix effects were not discussed in this method even though protein precipitation was used for sample preparation. According to another article on determination of oxaliplatin in human plasma with carboplatin as internal standard, significant ionization suppression was detected for both analytes from coeluting endogenous impurities when a short column was used in reversed phase chromatography [16]. Therefore, a LC–MS/MS method for the determination of carboplatin with thorough experimental investigation is needed.

The main purpose of the present study was to develop and validate a high throughput, sensitive, specific, and reliable bioanalytical assay for the determination of carboplatin in human plasma by LC–MS/MS.

2. Experimental

2.1. Chemical, reagents, materials, and apparatus

The 96-well HybridSPE-PPT plates were obtained from Sigma–Aldrich/Supelco (Bellefonte, PA, USA). Carboplatin with purity of 100% was purchased from Sigma–Aldrich (St. Louis, MO, USA). Carboplatin-d_6 with purity of 97.9% was purchased from BDG synthesis (Wellington, New Zealand). HPLC grade acetonitrile and formic acid (≥96%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Ammonium formate (Certified) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade methanol was received from J.T. Baker (Phillipsburg, NJ, USA). A PURELAB Ultra system from ELGA (Marlow, UK) was used in the laboratory to produce deionized water. Human plasma with sodium heparin as the anticoagulant was obtained from Biochemed (Winchester, VA, USA).

An automated SPE system (Quadra 96, model 96-320) for transferring samples during sample preparation was obtained from Tomtec (Hamden, CT, USA). A 96-well sample concentrator (SPE DRY-96) with a temperature control from Jones Chromatography (Lakewood, CO, USA) was used for evaporating samples.

2.2. Chromatographic conditions

The HPLC system consisted of a solvent delivery system LC-20AD, autosampler SIL-20AC, column oven CTO-20AC, degasser DGU-20A, and controller CBM-20A from Shimadzu (Kyoto, Japan). Chromatographic separation of carboplatin was evaluated on a Biobasic SCX column (50 mm × 2.1 mm, 5 μm) from Thermo Scientific (Bellefonte, PA, USA) for ion exchange chromatography. Several reversed-phase columns including an Aquasil C18 column (50 mm × 2.1 mm, 5 μm) from Thermo Scientific (Bellefonte, PA, USA), a Synergi Polar-RP (50 mm × 2.0 mm, 4 μm) and a Synergi Max-RP column (50 mm × 2.0 mm, 4 μm) from Phenomenex (Torrance, CA, USA) for reversed phase chromatography and a Kromasil silica column (50 mm × 2.1 mm, 5 μm) from Thermo Electron (Bellefonte, PA, USA) for hydrophilic interaction liquid chromatography (HILIC) were also tested during method development. Various mobile phase conditions were evaluated to achieve optimal retention and minimize matrix effects. For method validation and sample analysis, the chromatographic analysis was conducted using gradient elution on a Biobasic SCX column with acetonitrile/water (95/5, v/v) as mobile phase A (MA) and 10 mM ammonium formate in acetonitrile/methanol/water (50/25/25, v/v/v) as mobile phase B (MB). The HPLC program for gradient elution was as follows: 0% of MB (0–0.2 min), from 0% to 90% of MB (0.2–1.2 min), 90% of MB (1.2–1.7 min), from 90% to 0% of MB (1.7–1.8 min), and 0% of MB (1.8–2.6 min). The separation was performed at a flow rate of 1.5 mL/min. The sample injection volume was 5 μL. The column temperature was set at 25 °C. The cycle time between two consecutive injections was approximately 3.0 min.

2.3. Mass spectrometric conditions

An API 5000 triple quadrupole mass spectrometer (MDS-Sciex, Concord, Canada) with turboionspray (TIS) interface was operated in positive ionization mode with multiple reactions monitoring (MRM) for LC–MS/MS analyses. The mass spectrometric parameters were optimized to improve the MRM sensitivity. The instrument parameters for monitoring carboplatin and carboplatin-d_6 were as follows: TIS temperature, 650 °C; TIS voltage, 2000 V; curtain gas (CUR), nitrogen, 25; nebulizing gas (GS1), 50; TIS gas (GS2), 50; collision gas, 6; declustering potential (DP), 60 V; entrance potential (EP), 10 V; collision energy (CE), 22 eV; collision cell exit potential (CXP), 45 V. The following precursor to product ion transitions were used for the MRM of carboplatin and carboplatin-d_6, respectively, m/z 372.1 → 294.0 and m/z 378.1 → 300.0 with dwell times of 125 ms. The mass spectrometer was operated at unit mass resolution for both the first and third quadrupoles.

2.4. Preparation of standard solutions

Stock solutions of carboplatin and carboplatin-d_6 were prepared at 0.400 mg/mL and 0.100 mg/mL, respectively, in methanol/water (50/50, v/v). Intermediate standard solutions at the desired concentration for the preparation of calibration curve and QC samples were made by serial dilution with methanol/water (50/50, v/v) starting from their respective concentrated stock solutions. The intermediate internal standard solution at 100 ng/mL was diluted from the stock solution of carboplatin-d_6 at 0.100 mg/mL with methanol/water (50:50, v/v). These standard solutions were stored in glass vials and kept refrigerated (2–8 °C).

2.5. Preparation of calibration standards and quality control samples

Calibration standards were prepared daily by spiking an appropriate quantity of the intermediate standard solutions into human plasma. Eight calibration standards for carboplatin were prepared at 2.00, 4.00, 10.0, 50.0, 200, 1000, 1600, and 2000 ng/mL. Quality control samples were prepared by spiking an appropriate amount of intermediate standard solutions into human plasma to reach the

desired concentration with non-matrix composition less than 5% of the final volume. Lower limit of quantification QC (LLOQ), low QC (LQC), medium QC (MQC), high QC (HQC), and dilution QC (DQC) were prepared at 2.00 ng/mL, 6.00 ng/mL, 100 ng/mL, 1500 ng/mL, and 10,000 ng/mL, respectively. All QC samples were aliquoted into 1.4 mL polypropylene vials and stored at −20 °C and −70 °C for stability evaluation.

2.6. Sample preparation

A volume of 50.0 μL of each calibration standard, QC sample, incurred sample, and blank matrix control sample were aliquoted into individual wells in a 96-well plate. Next, 25.0 μL of the intermediate internal standard solution of carboplatin-d_6 at 100 ng/mL were added to individual wells containing samples with the exception of the blank control samples, to which 25.0 μL of methanol/water (50/50, v/v) were added. After that, 50.0 μL of water was added to all samples during method validation and sample analysis. Different volumes of water (0 μL, 25.0 μL, and 75.0 μL) were also evaluated during method development. Then 300 μL of 1% formic acid in acetonitrile was added to each sample during method validation and sample analysis. Other organic solvents including acetonitrile and methanol were also evaluated during method development. Vortexing at high speed for approximately 5 min was applied to mix the sample and to precipitate proteins. The 96-well plate with the samples was centrifuged at 1640 × *g* for approximately 5 min. After centrifugation, 300 μL of the supernatant was transferred to a clean 96-well plate for PPT or to a 96-well HybridSPE-PPT plate using an automated SPE system. Conventional PPT was evaluated for comparison purpose during method development by evaporating 300 μL of supernatant to dryness using a 96-well sample concentrator (SPE DRY-96) set at 50 °C for about 15 min. For the HybridSPE-PPT, 300 μL of supernatant was filtered through a 96-well HybridSPE-PPT plate under adjustable positive pressure using a Speedisk 96. Thereafter, the filtrate was evaporated to dryness using a 96-well sample concentrator (SPE DRY-96) set at 50 °C for about 15 min. The resulting dry residues from either PPT or HybridSPE-PPT were reconstituted in 150 μL of acetonitrile/water (95/5, v/v) for LC–MS/MS analysis.

2.7. Data analysis

Sciex Analyst software (version 1.5.1) was used for the data acquisition and analysis. The calibration curves (analyte peak area/IS peak area for *Y*-axis and analyte concentration/IS concentration for *X*-axis) were obtained based upon the least square linear regression fit ($y = mx + b$) with a weighting factor of $1/x^2$. The coefficient of determination (r^2) was set as >0.98 for the acceptance criteria of the calibration curves. The accuracy and precision were required to be within100 ± 15% of the nominal concentration and ≤15% RSD, respectively, for LQC, MQC, HQC, and DQC samples while the accuracy and precision were required to be within 100 ± 20% of the nominal concentration and ≤20% RSD for LLOQ samples in the intra-batch and inter-batch assay.

2.8. Method validation

Carboplatin was validated over the range of 2.00 ng/mL and 2000 ng/mL for an LC–MS/MS assay. Matrix effect, specificity, sensitivity, carryover, linearity, precision, accuracy, dilution integrity, and stability were evaluated during method validation. The matrix effect was determined by calculating matrix factor (MF), which was obtained as a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions by post-extraction spiking analyte at the LQC level into blank plasma extracts and blank water extracts. The specificity was assessed by testing 6 lots of blank plasma extracts for the presence or absence of interference with and without spiking other potential anti-cancer drugs including gemcitabine and oxaliplatin at the 5-fold upper limit of quantification (ULOQ) level. In addition, the specificity was also evaluated by quantification of LQC samples of carboplatin spiked with either gemcitabine or oxaliplatin at the 5-fold ULOQ level. Sensitivity of the analyte was determined by calculating the signal to noise ratio of LLOQ samples. Carryover of analytes was also evaluated by analyzing blank plasma extract samples immediately after an ULOQ sample or HQC sample. The linearity of the calibration curve was evaluated as described in Section 2.7.

The precision and accuracy of the method were assessed by the analyses of three separate batches of human plasma samples. Each batch consisted of one set of calibration standards (eight concentration levels) and six replicates of QC samples at each of LLOQ, LQC, MQC, and HQC levels. Dilution integrity was evaluated by a 10-fold dilution of the DQC sample with blank plasma prior to extraction in one of the three batches. The short-term matrix stability was evaluated in one of the three validation batches in which the LQC and HQC samples were subjected to five freeze–thaw cycles (freeze–thaw stability) or exposed to room temperature (∼22 °C) for approximately 24 h (room temperature stability) prior to extraction. Long-term stability was evaluated in two additional batches after LQC and HQC samples were stored at approximately −20 °C and −70 °C for 40 and 116 days. Freshly prepared calibration standards were utilized for each of the stability evaluations.

3. Results and discussion

3.1. Chromatographic separation

Most assays for determination of carboplatin have been conducted on ODS columns although these columns showed a minimal capacity factor for carboplatin [10,13,16]. For instance, carboplatin showed a capacity factor of 0.83 even with elution at 100% mobile phase A of water [13]. It has also been reported that carboplatin was analyzed on a Spherisorb silica column in HILIC mode with good retention for a LC–UV study [1]. The reasons why carboplatin was analyzed mainly on reversed phase columns instead of silica column were not well documented in the reports. More recently, it has been reported that carboplatin in human plasma was quantified using a Spherisorb S5 SCX column [4], the column size was not described in this article.

For the present study, different types of HPLC columns were evaluated using various chromatographic conditions. In reversed phase chromatography, several commonly used HPLC columns for the analysis of polar analytes were evaluated for the separation of carboplatin using 0.1% formic acid in water as MA and 0.1% formic acid in acetonitrile as MB at a flow rate of 1.0 mL/min. As shown in Fig. 2, a capacity factor of less than 1, indicating poor retention, was obtained from all these columns even at 100% MA. Next, a Biobasic SCX column was evaluated using various chromatographic conditions described in Section 2.2. In contrast to reversed phase chromatography, good retention was obtained on this SCX column under chromatographic condition for method validation. In addition, an over 10-fold increase in sensitivity was achieved due to the higher ionization efficiency for electrospray ionization with mobile phase conditions of high organic content. Typical chromatograms of a blank plasma, LLOQ QC, and incurred plasma samples are shown in Fig. 3. Similar retention and sensitivity were obtained from a Kromasil silica column under HILIC mode. However, it usually takes a longer time to equilibrate the column for the analysis of carboplatin under the mobile phase conditions that we evaluated. Therefore, a

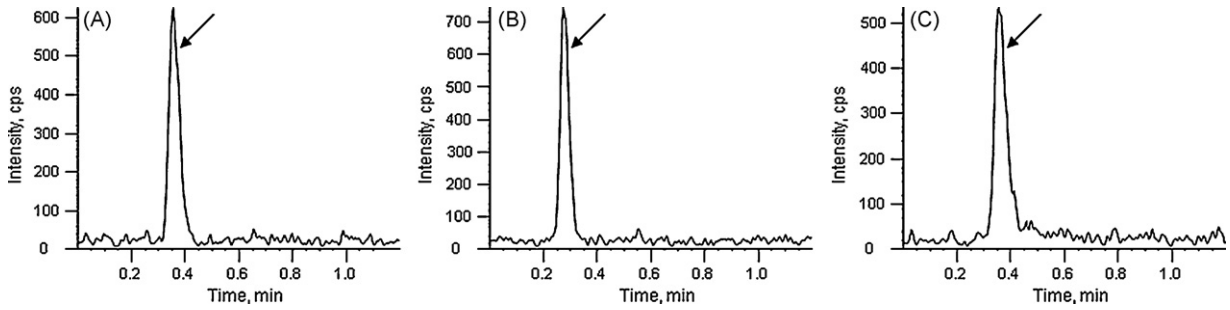

Fig. 2. Representative LC–MS/MS chromatograms of carboplatin neat solution at 1.50 ng/mL analyzed on a (A) Aquasil C18 column (50 mm × 2.1 mm, 5 μm), (B) Synergi Polar-RP (50 mm × 2.0 mm, 4 μm), and (C) Synergi Max-RP (50 mm × 2.0 mm, 4 μm) under isocratic elution with 0.1% formic acid in water. Carboplatin was monitored at *m/z* 372.1 → 294.0. *Note*: Arrow indicates chromatographic peak of carboplatin.

Biobasic SCX column was chosen for method validation and sample analysis.

3.2. Matrix effects and recovery

Significant matrix effects were reported but not quantified in LC–MS and LC–MS/MS assays for the analyses of carboplatin in biological samples without adequate sample clean-up [10,16]. Minimizing matrix effects via SPE has been very challenging since carboplatin does not retain on most solid-phase materials (i.e., C_2, C_{18}, CN, NH_2, and SAX) and very low recovery was obtained [10]. Even though adequate recovery was reported to be obtained from Bond Elut SCX cartridges, they were very sensitive to the washing and elution volume [10]. Therefore, a compromise between reduction of matrix effect and an increased recovery was necessary to achieve sufficient sensitivity for MS detection.

For our experiments, PPT and different SPE products were evaluated for achieving sufficient recovery and minimal matrix effects for carboplatin. The recovery from the extraction was obtained by comparing the average absolute peak areas of the analyte extracted from three replicates of low level QC samples prepared in plasma with those of blank plasma post-extraction fortified with neat analyte solution at the same concentration as the LQC. However, the loss sample volume of about 120 μL on the sorbents from 300 μL of supernatant loaded was not compensated for the HybridSPE-PPT extraction. If PPT was used for sample preparation, sufficient sensitivity at the LLOQ level could not be achieved due to severe matrix effects. Even at the LQC level, the signal-to-noise ratio was less than 5 (shown in Fig. 4(A)). Therefore, SPE must be used for sample clean-up to eliminate the severe matrix effects. Less than 2% recovery for carboplatin was obtained for samples prepared using reversed phase SPE on Oasis HLB 96-well plate from Waters (Milford, MA, USA) even if it was only washed with water. Ionization suppression was very significant if no wash was applied prior to eluting analytes. Similarly, a less than 2% recovery was obtained using ion exchange chromatography-based SPE including Oasis MCX 96-well plates from Waters (Milford, MA, USA), Strata-X-C from Phenomenex (Torrance, CA, USA), and Evolute WCX from Biotage (Uppsala, Sweden). As a result, sufficient sensitivity could not be achieved at the LLOQ level using PPT and SPE with several types of commonly used SPE sorbents.

Further exploration of sample clean-up technologies was needed to develop a successful LC–MS/MS assay for the determination of carboplatin in plasma samples. Recently, HybridSPE-PPT has been reported as a new technique for the elimination of matrix effects via efficient removal of phospholipids and for protein precipitation while remaining non-selective towards a broad range of basic, neutral, and acidic compounds [17,18]. The mechanism underlying the phospholipids removal is based on selective Lewis acid–base interactions between the proprietary zirconium-coated silica sorbents and the phosphate moieties of phospholipids. The present study evaluated this new HybridSPE-PPT technique for sample preparation of carboplatin from human plasma. Various extraction conditions were investigated to achieve minimum matrix effects and maximum recovery for carboplatin.

Both in-well and off-line protein precipitation can be used with a HybridSPE-PPT 96-well plate. To compare the matrix effects from phospholipids removal using HybridSPE-PPT with those from PPT, off-line protein precipitation was applied for this study. Filtering through a HybridSPE-PPT plate is the only difference in sample preparation procedures between samples obtained from PPT only and those obtained from HybridSPE-PPT as described in Section 2.6. Different precipitating agents including acetonitrile, 1% formic acid in acetonitrile, and methanol were evaluated and compared in terms of the recovery of carboplatin and matrix effects (Table 1). Additional amounts of water at various volumes were added to optimize recovery immediately prior to adding the precipitating agents (see Section 2.6).

As shown in Table 1, severe matrix effects were observed for carboplatin in plasma samples prepared using PPT under all extraction conditions evaluated. The severe matrix effects resulted in significantly suppressed, and therefore very low, analyte responses. The

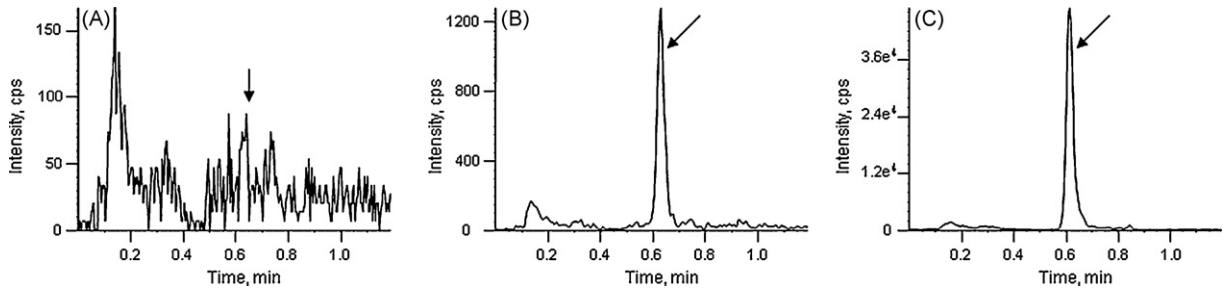

Fig. 3. Representative LC–MS/MS chromatograms of extract from (A) blank plasma; (B) plasma sample spiked with carboplatin at 2.00 ng/mL (LLOQ); (C) incurred plasma sample with a measured concentration at 101 ng/mL. Carboplatin was monitored at *m/z* 372.1 → 294.0. *Note*: Arrow indicates chromatographic peak of carboplatin.

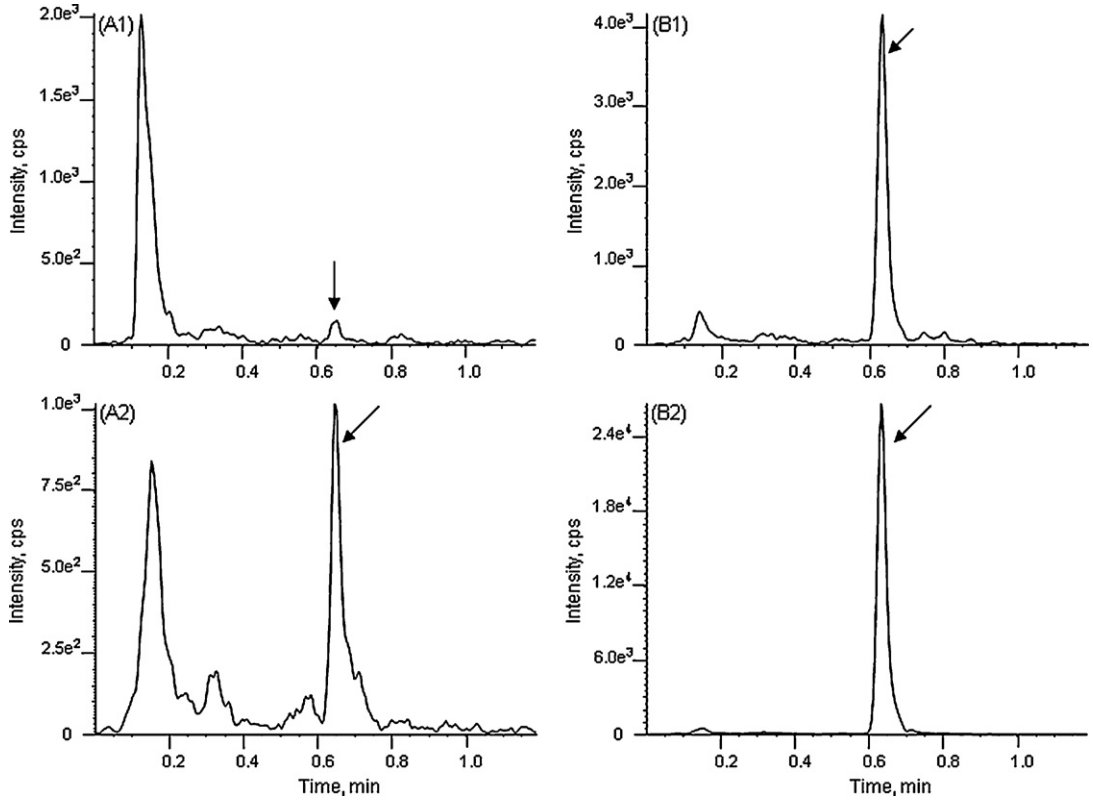

Fig. 4. Representative LC–MS/MS chromatograms of LQC extract obtained from PPT (A1 and A2) and from HybridSPE-PPT (B1 and B2) by monitoring carboplatin at *m/z* 372.1 → 294.0 (A1 and B1) and carboplatin-d_6 at *m/z* 378.1 → 300.0 (A2 and B2). *Note*: Arrow indicates chromatographic peak of carboplatin and its internal standard.

Table 1
Results of matrix effect of carboplatin under various extraction conditions using protein precipitation and HybridSPE-precipitation.

	$Sample^{A1}$	$Sample^{B1}$	$Sample^{C1}$	$Sample^{A2}$	$Sample^{B2}$	$Sample^{C2}$	$Sample^{A3}$	$Sample^{B3}$	$Sample^{C3}$	$Sample^{A4}$	$Sample^{B4}$	$Sample^{C4}$
Protein precipitation												
Matrix factor	0.012	0.014	0.009	0.012	0.014	0.008	0.011	0.015	0.008	0.011	0.021	0.037
RSD (%)	8.3	6.0	8.1	14.4	3.8	6.7	17.9	17.8	10.4	6.2	15.4	7.4
I.S. normalized matrix factor	0.73	0.72	0.76	0.76	0.75	0.71	0.76	0.76	0.73	0.80	0.78	0.99
RSD (%)	5.8	7.4	9.7	9.9	2.0	15.0	14.3	17.1	5.4	10.0	13.0	3.8
HybridSPE-precipitation												
Matrix factor	1.01	1.01	1.15	0.97	0.92	1.00	1.00	0.92	0.97	0.89	0.53	0.94
RSD (%)	3.1	2.8	5.3	5.1	9.3	2.7	5.6	1.6	2.0	3.6	6.1	2.3
I.S. normalized matrix factor	0.98	1.04	0.99	0.96	0.99	1.00	0.99	1.00	1.01	0.98	1.12	1.00
RSD (%)	1.0	4.1	0.8	2.4	0.5	2.1	2.4	1.9	0.9	1.7	2.2	3.0

Note: Letters A–C represent organic solvents used for protein precipitation in the order of acetonitrile, 1% formic acid in acetonitrile, and methanol; numbers 1–4 represent the amount of water added in the step immediate before addition of organic solvent in the order of 0 μL, 25 μL, 50 μL, and 75 μL under various extraction conditions.

I.S. normalized matrix factor is much lower than 1.0, suggesting that even stable isotopically labeled I.S. may not completely compensate for the severe matrix effects for analytes in plasma samples obtained from PPT. In contrast, the use of HybridSPE-PPT led to minimum matrix effects for carboplatin as evidenced by the matrix factor of approximately 1.0 under nearly all of the evaluated extraction conditions. In one instance, a relatively lower matrix factor of 0.53 was observed when adding 75 μL extra amount of water immediately before the protein precipitating agent–1% formic acid, while it was compensated by I.S. normalization ($Sample^{B4}$). Similarly, different types and pH levels of precipitating agents and their ratios of aqueous phase to organic phase are tremendously important for the recovery of carboplatin. As shown in Table 2, methanol is a better precipitating agent than acetonitrile due to its much higher recovery. Methanol is a more polar solvent than acetonitrile so that it inhibits any potential secondary HILIC interactions between the analyte and HybridSPE silica surface. 1% formic acid in acetonitrile showed better recovery than acetonitrile and that can be explained by the low pH environment which neutralizes residual silanol activity on the silica surface thereby eliminating

Table 2
Results of recovery of carboplatin under various extraction conditions using HybridSPE-precipitation.

	$Sample^{A1}$	$Sample^{B1}$	$Sample^{C1}$	$Sample^{A2}$	$Sample^{B2}$	$Sample^{C2}$	$Sample^{A3}$	$Sample^{B3}$	$Sample^{C3}$	$Sample^{A4}$	$Sample^{B4}$	$Sample^{C4}$
Recovery (%)	0.1	3.5	1.1	4.6	10.8	7.1	10.2	26.7	14.0	15.1	32.4	21.1
RSD (%)	120.0	6.6	60.2	27.0	9.7	14.2	11.5	7.1	18.7	8.9	7.8	25.8

Note: Letters A–C represent organic solvents used for protein precipitation in the order of acetonitrile, 1% formic acid in acetonitrile, and methanol; numbers 1–4 represent the amount of water added in the step immediate before addition of organic solvent in the order of 0 μL, 25 μL, 50 μL, and 75 μL under various extraction conditions.

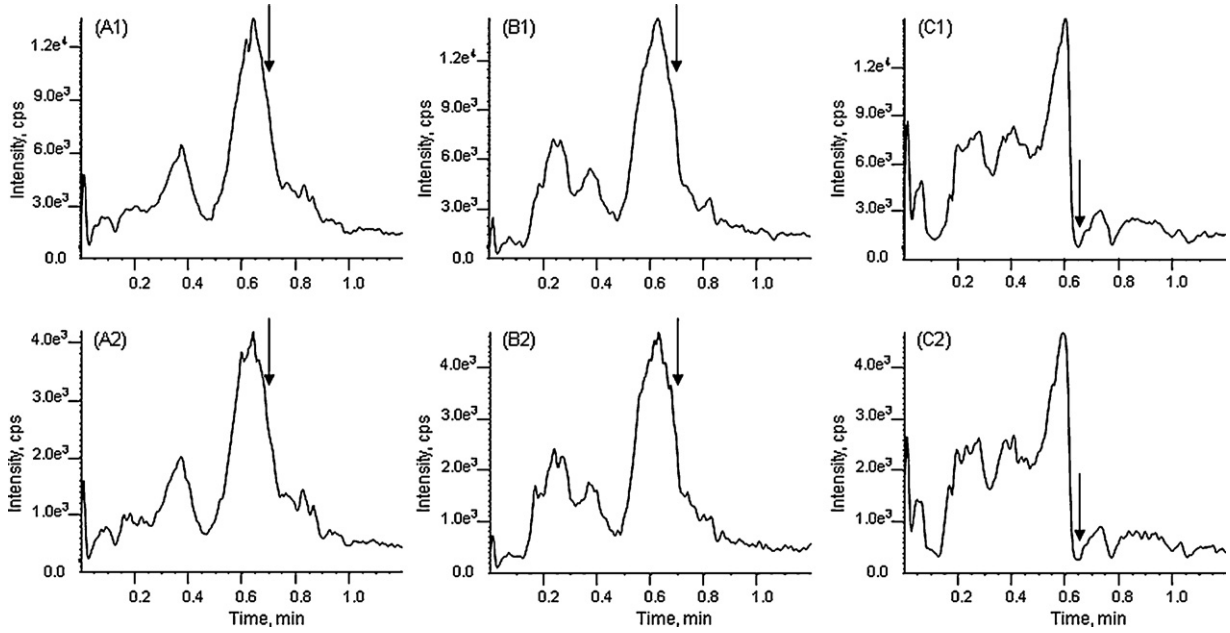

Fig. 5. Representative LC–MS/MS chromatograms of extracted reagent blank using HybridSPE-PPT (A1 and A2), extracted blank plasma using HybridSPE-PPT (B1 and B2), and extracted blank plasma using PPT (C1 and C2) under post-column infusion of a neat solution of carboplatin at 30 ng/mL and carboplatin-d_6 at 10 ng/mL by monitoring carboplatin at m/z 372.1 → 294.0 (A1, B1, and C1) and carboplatin-d_6 at m/z 378.1 → 300.0 (A2, B2, and C2). *Note*: Arrow indicates retention time of carboplatin and its internal standard.

secondary cation exchange interaction with carboplatin. Adding additional amounts of water (0 μL/25 μL/50 μL/75 μL) immediately before adding precipitating agents can significantly improve the recovery (Table 1), which might contribute to the reduction of HILIC interactions. Volumes greater than 75 μL of additional amount of water were not evaluated because insufficient protein precipitation was noticed during method development. Based on our experimental results as greater amounts of water were added, higher recoveries of carboplatin were achieved. However, this trend of increasing recovery slowed when additional amounts of water increased from 50 μL to 75 μL. Although the reasons that adding additional amounts of water or adjusting the ratios of the aqueous to organic phase can impact the matrix effects and recovery of carboplatin is not fully understood from this study, reduction of HILIC interactions between the analyte and the HybridSPE silica surface may contribute to this. Solubility issues can be excluded because concentrations higher than ULOQ of carboplatin can be dissolved in high organic content solutions of acetonitrile/water (v/v, 95:5).

To increase recovery and minimize matrix effects, 1% formic acid in acetonitrile as the precipitating agent and an additional 50 μL of water added immediately prior to adding the precipitating solvent were selected for method validation and sample analysis. At this optimal condition, greater than 25% recovery and a matrix factor close to 1.0 were obtained (Tables 1 and 2). If needed, the recovery can be further improved by adding solvents to wash out the residue analyte in the sorbents (~120 μL). Since excellent sensitivity was already achieved at the LLOQ level (Fig. 3) under the selected method validation conditions, further optimization of recovery was not conducted.

Matrix effects were also evaluated by post-column infusion of a neat solution of carboplatin at 30.0 ng/mL and carboplatin-d_6 at 10.0 ng/mL while injecting extracted blank plasma samples obtained from PPT and HybridSPE-PPT. As shown in Fig. 5, a blank plasma sample obtained from HybridSPE-PPT demonstrated similar degree of matrix effects to that of the reagent blank. However, a significant ion suppression zone at the retention time of carboplatin and its I.S. was observed from the extracted blank plasma sample using PPT. Furthermore, six major phospholipids and total phospholipids in plasma samples were monitored using their characteristic MRM transitions (see Fig. 6), however, only three out of these six major phospholipids with similar retention to carboplatin are shown in this figure. As indicated by the peak intensity, greater than 99% of the three major phospholipids were removed by using HybridSPE-PPT under method validation conditions. Although the monitoring of total phospholipids via m/z 184 → 184 is not very specific, the major peaks around the retention zone of carboplatin were significantly reduced through the use of HybridSPE-PPT. Correlating the information with regard to matrix effects in Table 1 and in Fig. 6 suggests that removal of phospholipids via HybridSPE-PPT can significantly reduce matrix effects for carboplatin. Therefore, HybridSPE-PPT may have broad application in eliminating matrix effects for very polar analytes such as carboplatin. Optimization of extraction conditions will be needed to improve the recovery for analytes that do not extract well.

3.3. Linearity, sensitivity, carryover, and specificity

Linearity was assessed based on the average of eight calibrators analyzed in three separate batches. Acceptable linearity was achieved in the range of 2.00–2000 ng/mL. The coefficient of determination (r^2) was greater than 0.994 in all validation batches. The back-calculated results for all calibration standards showed ≤5.7% RSD for carboplatin in the three validation batches.

Assay sensitivity was determined by the analysis of LLOQ samples (n = 6) in three separate validation batches. A signal-to-noise ratio (S/N) of approximately 20 was obtained at the LLOQ level (shown in Fig. 3). Acceptable precision of 4.6% RSD and accuracy of −12.0% RE were obtained for inter-day assays (Table 3).

No peaks around the same retention time of carboplatin were observed in the chromatogram of the blank plasma extract immediately after the ULOQ or HQC sample. As a result, carryover from previous concentrated samples was determined to be negligible.

Under current LC–MS/MS and sample preparation conditions, no obvious interference peaks were observed in the chromato-

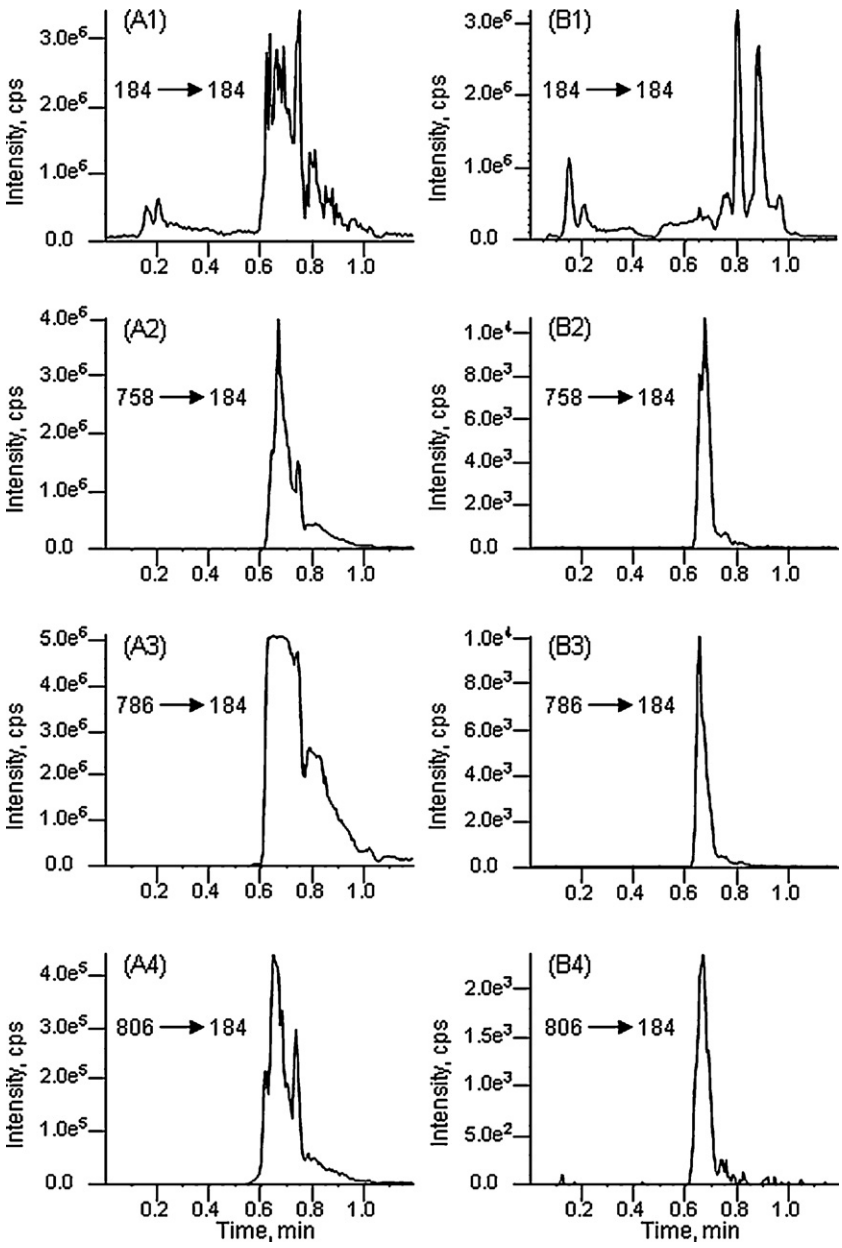

Fig. 6. Representative LC–MS/MS chromatograms of LQC extract obtained from PPT (A1–A4) and from HybridSPE-PPT (B1–B4) by monitoring total phospholipids at *m/z* 184 → 184 in top chromatograms (A1 and B1) and three major phospholipids at *m/z* 758 → 184, 786 → 184, and 806 → 184, respectively.

graphic region of carboplatin and its internal standard from extracts of 6 lots of blank plasma with or without spiking gemcitabine or oxaliplatin at 5-fold ULOQ level. In addition, no impact on quantification of carboplatin in LQC samples spiked with either gemcitabine or oxaliplatin at 5-fold ULOQ level was observed (data not shown). These experimental results indicated that the present LC–MS/MS method is very specific for the determination of carboplatin in human plasma and can be used to analyze carboplatin when coadministered with either gemcitabine or oxaliplatin.

3.4. Precision and accuracy

The precision and accuracy of the method were determined by analyzing QC samples at low, medium, and high levels. As shown in Table 3, these results indicated that good precision and accuracy can be achieved for this assay under the current method validation conditions.

3.5. Dilution integrity

As shown in Table 3, the results demonstrated that samples with a concentration greater than the upper limit of the standard curve could be quantified with reliable precision and accuracy after being appropriately diluted with blank matrix.

3.6. Stability

No stability issue was observed from any short-term stability experiments and from long-term stability experiment at −70 °C while stability was an issue for QCs stored at −20 °C (Table 4). These results from long-term stability experiments were consistent with those reported by DesJardins et al. [4]. Therefore, sample storage conditions are critical to ensure accurate determination of carboplatin in plasma samples.

Table 3
Precision and accuracy of carboplatin.

	LLOQ (2.00 ng/mL)	LQC (6.00 ng/mL)	MQC (100 ng/mL)	HQC (1500 ng/mL)	DQC (10,000 ng/mL)
Day 1					
N	6	6	6	6	6
Mean	1.73	5.21	94.3	1348	11,285
RSD (%)	5.0	3.9	3.6	2.0	1.2
RE (%)	−13.5	−13.2	−5.7	−10.1	12.9
Day 2					
N	6	6	6	6	
Mean	1.79	5.23	96.4	1396	
RSD (%)	2.9	4.8	2.0	2.5	
RE (%)	−10.5	−12.8	−3.6	−6.9	
Day 3					
N	6	6	6	6	
Mean	1.72	5.33	94.9	1386	
RSD (%)	3.6	2.9	2.8	4.1	
RE (%)	−14.0	−11.2	−5.1	−7.6	
Inter-day					
N	18	18	18	18	
Mean	1.76	5.26	95.2	1377	
RSD (%)	4.6	3.8	2.9	3.2	
RE (%)	−12.0	−12.3	−4.8	−8.2	

Table 4
Room temperature, freeze/thaw/, and long-term stability of carboplatin.

	LQC (6.00 ng/mL)	HQC (1500 ng/mL)
Room temperature stability for 24 h, N = 6		
Mean	5.20	1383
RSD (%)	5.4	2.4
RE (%)	−13.3	−7.8
Stability for storage at −20 °C and five freeze–thaw cycles, N = 6		
Mean	5.61	1412
RSD (%)	2.2	4.7
RE (%)	−6.5	−5.9
Stability for storage at −70 °C and five freeze–thaw cycles, N = 6		
Mean	5.86	1497
RSD (%)	4.6	2.7
RE (%)	−2.4	−0.4
Storage at −20 °C for 40 days		
Mean	3.84	1041
RSD (%)	4.1	6.9
RE (%)	−36.0	−30.6
Storage at −70 °C for 40 days		
Mean	5.61	1442
RSD (%)	6.5	1.9
RE (%)	−6.5	−3.9
Storage at −20 °C for 116 days		
Mean	2.09	612
RSD (%)	5.9	4.3
RE (%)	−65.2	−59.2
Storage at −70 °C for 116 days		
Mean	5.36	1440
RSD (%)	4.3	7.8
RE (%)	−10.7	−4.1

3.7. Application of the method

This method was applied to incurred human plasma sample analysis of carboplatin for a small study with 35 samples. A representative chromatogram of an incurred sample with a measured concentration at 101 ng/mL is shown in Fig. 3, indicating similar chromatographic behavior to QCs. Four samples were chosen for incurred sample reanalysis (ISR). The differences in concentrations between the ISR and the initial values were calculated as less than 10%. Therefore, this validated method can be reliably used for future incurred sample analysis.

4. Conclusion

The current chromatographic conditions for method validation provide good retention, peak shape, and sensitivity for the analysis of carboplatin in human plasma for the present study. The use of HybridSPE-PPT has been discussed in detail for the first time with regard to improving recovery and minimizing matrix effects for determination of carboplatin in human plasma. This study demonstrated the capability of HybridSPE-PPT to effectively remove matrix effects for determination of carboplatin, a very polar analyte not readily retained by commonly used SPE products and difficult to detect due to the presence of severe matrix effects. This also suggests its broad application for analyses of very polar analytes. Another advantage of the HybridSPE-PPT methods is that, unlike other SPE methods, preconditioning the cartridges or plates is not needed. Also, both in-well and off-line precipitation give HybridSPE-PPT more flexibility in application. Although it may need optimization regarding recovery improvement for some analytes, the use of HybridSPE-PPT 96-well plates is very simple and fast. Combining the efficiency of sample preparation using HybridSPE-PPT with the short LC run time enables the present method for high through-put sample analysis. In conclusion, a rapid, specific, sensitive, and reliable LC–MS/MS based bioanalytical method has been successfully developed and validated to determine carboplatin in human plasma. This method demonstrated reproducible chromatographic and statistical results in terms of precision and accuracy during method validation. It has also been successfully applied in incurred sample analysis.

Acknowledgements

The authors thank Mary Ballweg at Covance Laboratories, Inc., Madison, Wisconsin, for her help in obtaining some references cited in this manuscript and thank Glenn Hanson for his advice in conducting this study. The authors also want to thank Xiaoning Lu at Supelco for his advice in using HybridSPE-PPT.

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