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Nonvolatile salt-free stabilizer for the quantification of polar imipenem and cilastatin in human plasma using hydrophilic interaction chromatography/quadrupole mass spectrometry with contamination sensitive off-axis electrospray

Zhe-Yi Hu^{a,*}, Bradley A. Boucher^a, S. Casey Laizure^a, Vanessa L. Herring^a, Robert B. Parker^a, and William L. Hickerson^b

^aUniversity of Tennessee Health Science Center, College of Pharmacy, Department of Clinical Pharmacy, Memphis, TN 38163, USA

^bUniversity of Tennessee Health Science Center, Department of Plastic Surgery, Firefighters Burn Center, Memphis, TN 38103, USA

Abstract

A hydrophilic interaction chromatography/mass spectrometry (HILIC-MS)-based assay for imipenem (IMP) and cilastatin (CIL) was recently reported. This orthogonal electrospray ion source-based (ORS) assay utilized nonvolatile salt (unremovable) to stabilize IMI in plasma. Unfortunately, this method was not applicable to conventional MS with off-axis spray (OAS-MS) because MS sensitivity was rapidly deteriorated by the nonvolatile salt. Therefore, we aimed to find a nonvolatile salt- and ion suppression-free approach to stabilize and measure the analytes in plasma using OAS-MS. Acetonitrile and methanol were tested to stabilize the analytes in the plasma samples. The recoveries, matrix effects and stabilities of the analytes in the stabilizer treated samples were studied. The variations in MS signal intensities were used as the indicator of the assay ruggedness. The results show that a mixture of methanol and acetonitrile (1:1) is best for the storage and measurement of IMP and CIL in human plasma. Utilization of this precipitant not only blocked the hydrolysis of the analytes in plasma but also resulted in an ion suppression-free, fast (120 s per sample) and sensitive detection. The sensitivity obtained using the less sensitive OAS-MS (API3000, 4 pg on column) is much greater than that of the published ORS-MS-based assay (API4000, 77 pg on column). The ruggedness of the assay was demonstrated by its constant MS signal intensity. In conclusion, an improved HILIC/MS-based assay for IMP and CIL was established. The approach presented here provides a simple solution to the challenge of analyzing hydrolytically unstable β -lactam antibiotics in biological samples.

Keywords

imipenem; cilastatin; hydrophilic interaction chromatography/quadrupole mass spectrometry; off-axis electrospray; pharmacokinetics

Introduction

A combination of the carbapenem antibiotic imipenem (IMP, Fig. 1) and the renal dehydropeptidase inhibitor cilastatin (CIL, Fig. 1) has for many years been used as a potent

*Correspondence to: Zhe-Yi Hu, University of Tennessee Health Science Center, College of Pharmacy, Department of Clinical Pharmacy, 881 Madison Ave., Room 328, Memphis, TN 38163, USA. zhu13@uthsc.edu.

antibacterial combination for the treatment of serious infections.^[1] The historical methods for the quantification of IMP and/or CIL in plasma are based on high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection.^[2–5]

More recently, a hydrophilic interaction chromatography/mass spectrometry (HILIC-MS)-based assay for IMP and CIL was reported.^[6] This HILIC-MS-based assay is much more rapid and sensitive than those based on HPLC-UV. For all the reported assays, plasma samples must be stabilized by an equal volume of stabilizer to avoid IMP loss by hydrolysis, which occurs even in the frozen state.^[2,3] The stabilizer includes a high amount of nonvolatile salt (1 M, e.g. 2-(N-morpholino)ethanesulfonic buffer, MES) and ethylene glycol (50%). Unfortunately, the sample preparation method (protein precipitation) of the MS assay is unable to remove the salt from the injection solution. In our preliminary study, we observed the MS signal intensity decreased by more than 10 times after injecting about 30 protein precipitated samples, and the sampling orifice was visually contaminated. Under the optimized HILIC condition, introducing the MES salt into the MS ion source cannot be completely avoided because IMP, CIL and MES acid have similar octanol–water partition coefficient ($\log P$ of IMP = -3.9 ; CIL = -1.3 ; MES = -2.5) (DrugBank, <http://www.drugbank.ca/>) and thus, similar retention times.^[7]

The different assay performance between the reported assay and our preliminary study could be attributed to the different electrospray ion (ESI) sources used (Fig. 2). An orthogonal ESI source (ORS, Turbo VTM) was used in the reported assay whereas an off-axis ESI source (OAS, Turbo ion sprayTM) was used in our study. The mechanism of the ESI process has been discussed in detail elsewhere.^[8] In brief, the ESI process consists of the following phases: 1) formation of charge carrying droplets from LC effluent; 2) drastic reduction of droplet size by continuous solvent evaporation and repeated droplet fissions; 3) release of ions from highly charged microdroplets into the gas phase; 4) transport of free gas phase ions into the mass analyzer. The ORS has more contamination resistance and ionization efficiency than OAS due to its improved design in heat transfer and gas dynamics (Fig. 2).

Although OAS-MS is less contamination resistant and sensitive than ORS-MS, OAS-MS is one of the most widely used MS techniques due to its low cost and lower consumption of nitrogen gas, as well as adequate sensitivity for most applications. This report describes the first nonvolatile salt- and matrix effects-free approach for the sensitive measurement of IMP and CIL in plasma. The approach presented here provides a simple solution to the challenge of analyzing hydrolytically unstable β -lactam antibiotics in biological samples using OAS-MS.

Experimental

Chemicals

Imipenem (IMP), cilastatin (CIL), oseltamivir (OS), oseltamivir carboxylate (OSC), and clopidogrel carboxylate (CLC) were the products of Toronto Research Chemicals Inc. (North York, ON, Canada) (structures are shown in Fig. 1). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). LC-MS-grade formic acid and ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade water was prepared with an in-house Milli-Q Advantage A10 Ultrapure water purification system (Bedford, MA, USA).

Chromatographic and mass spectrometric conditions

The LC-MS/MS system consisted of an Agilent 1100 series liquid chromatograph (Santa Clara, CA) interfaced via a TurboIon SprayTM source with a triple-quadrupole mass spectrometer, i.e. an AB SCIEX API 3000 mass spectrometer (Toronto, Canada).

The LC separation was achieved on a 5.0 μm Waters Atlantis HILIC silica column (50 mm $\times$ 2.1 mm I.D.; Milford, MA) at 24°C. Mobile phases were water, containing 0.01% (v/v) formic acid and 5.0 mM ammonium formate, for A and acetonitrile/water, 95:5 (v/v), modified with the same electrolytes, for B. An isocratic chromatographic method was used. 80% B from 0.0 to 2.0 min.

Ionization conditions were optimized to maximize generation of the singly protonated species and to produce the characteristic product ions for the test compounds. The precursor-product ion pairs used for multiple reaction monitoring (MRM) of IMP, CIL, OS, OSC, and CLC were m/z 300 $\rightarrow$ 142, 359 $\rightarrow$ 97, 313 $\rightarrow$ 225, 285 $\rightarrow$ 138, and 308 $\rightarrow$ 198, respectively. The parameters of the ESI source were optimized under the actual chromatographic conditions. The LC eluent was introduced to the ESI source at a flow rate of 0.40 mL/min over the period of 0.5–1.5 min.

Three internal standard candidates (IS), OS, OSC, and CLC, were tested for the quantification of IMP and CIL. The calibration standards were prepared using blank plasma samples spiked with IMP and CIL, the concentrations ranges of which were 0.2–50 $\mu\text{g/mL}$. The calibration curves were constructed using weighted (1/X) linear regressions of the analyte/IS peak area ratios (Y) against the corresponding nominal concentrations of the analytes (X, $\mu\text{g/mL}$).

Assessment of mobile phase modifier

The effect of the ammonium formate concentration (0, 5, 10, 20 mM) in the mobile phase on the signal intensity and peak width of the tested compounds was studied. In order to facilitate the formation of protonated species for the test compounds, a low concentration (0.01%, v/v) of formic acid was added to the mobile phases based on the results of our earlier reports.^[9,10]

Stock solutions of IMP and CIL

IMP (5.0 mg/mL) and CIL (2.5 mg/mL) stock solutions were prepared in dimethyl sulfoxide (DMSO) and acetonitrile/water (1:1, v/v), respectively. The stock solutions were aliquoted and stored at -70°C (50 μL per tube). Aliquots were thawed once and then discarded; refreezing was not allowed. Long term stability of stock solutions was tested for 1 month.

Selection of the nonvolatile salt-free stabilizer and assay validation

Acetonitrile and an acetonitrile-methanol mixture (1:1) were tested as the potential stabilizers for IMP in plasma. 10 μL of plasma were precipitated with 90 μL of stabilizer. After centrifugation for 5.0 min at 16,000 $\times g$, 10 μL of the resulting supernatant were further diluted by 10-fold using acetonitrile-methanol mixture (1:1) containing three internal standards (100–400 ng/mL). Two μL the diluted supernatant (injection solution) was injected into the HILIC-MS/MS for analysis. In order to identify whether the human plasma components generate matrix effects on the ESI-based measurement of the analytes, a post-extraction spike method was employed, which assessed the sample absolute and relative matrix effects. The extraction recoveries were compared between different stabilizers.

The optimal stabilizer was utilized in the stability studies. The stabilities of the analytes in the plasma extracts (stabilizer treated samples) were examined at 2°C (autosampler) and -70°C freezer (long term storage) for 10 hours and 1 month, respectively. The freeze-thaw stability was tested for two cycles. In order to determine how soon the study subjects samples should be stabilized after blood collection, the stabilities of the analytes in the plasma at 24°C were also tested. Blood stability was not performed because imipenem was reported to be stable for up to 8 h in blood at 4°C.^[11] Matrix effect, recovery, stability, and

accuracy and precision (within-run and between-run) studies were tested at three different levels of the analytes (0.21, 1.85, and 16.7 $\mu\text{g/mL}$).

Comparison of the traditional stabilizer and nonvolatile salt-free stabilizer

Blank plasma spiked with IMP and CIL (1.85 $\mu\text{g/mL}$) were pretreated with traditional stabilizer (plasma:stabilizer = 1:1) or the nonvolatile salt-free stabilizer (plasma:stabilizer = 1:9). The resulting samples were further diluted 10-fold with the acetonitrile-methanol mixture (1:1). Then the plasma extracts were consecutively injected into the HILIC-MS/MS. Variations of the MRM signal intensities of IMP and CIL were monitored during the consecutive injection.

Comparison of three different internal standards

The three internal standards candidates were selected because they have similar ionization group and molecular weight as IMP and CIL. The best internal standard was selected based on the matrix effects, retention times, and variation of MS signal intensity.

Application to human pharmacokinetic study

To demonstrate its applicability, the newly developed analytical method was applied to quantify the plasma concentration-time profile of IMP and CIL in a burn patient receiving high-volume hemofiltration. This study was approved by the University of Tennessee Health Science Center Institutional Review Board and written informed consent was obtained before participation. Serial blood samples (0, 0.25, 0.5, 1, 2, 4, and 6 h) were collected after intravenous dosing of 1 g IMP and CIL. The blood samples were immediately transported on ice to the laboratory. The plasma samples were separated (centrifuged at 3000 $\times$ g, 4 $^{\circ}\text{C}$ for 5 min), and 10 μL of plasma was precipitated with 90 μL of stabilizer within 0.5 h after blood collection. As the stabilizer is toxic and flammable, the use of stabilizer was only allowed in the laboratory. After centrifugation for 5.0 min at 16,000 $\times$ g (4 $^{\circ}\text{C}$), 90 μL of the resulting supernatant was transferred to another tube and then kept frozen at 70 $^{\circ}\text{C}$ until analysis.

Assay reproducibility for the incurred samples

Two measurements (separated by a 1-month period) were conducted for the same set of patient samples. The ratios of the concentrations obtained by the second measurement to those determined by the first measurement were used to indicate the assay reproducibility. The range of 85% to 115% was used as the acceptance criterion.

Results and discussion

Chromatographic and mass spectrometric conditions

Although a higher ammonium formate concentration in the mobile phase resulted in a sharper peak for IMP, lower signal intensities were observed for both analytes. Balancing the peak shape and sensitivity, 5 mM ammonium formate in 80% acetonitrile was selected, and the total run time under this isocratic condition was 120 s per injection. The chromatographic retention times of IMP, CIL, OS (IS1), OSC (IS2), and CLC (IS3) were 79, 56, 43, 66, and 35 s, respectively (Fig. 3).

Selection of the nonvolatile salt-free stabilizer

Initially, acetonitrile was tested as the stabilizer and precipitant for the plasma samples. Unfortunately, the extraction recoveries for both analytes were lower than 50% when acetonitrile was used. Further study showed that a methanol and acetonitrile mixture (1:1) yielded satisfactory extraction recoveries (Table 2).

The stability results for IMP and CIL under various conditions are shown in Table 1. The analytes are stable in plasma for 2 h, suggesting that the plasma samples should be stabilized within 2 h after blood collection and plasma separation. Once the plasma samples were stabilized by the 10-fold dilution with methanol and acetonitrile mixture, the plasma extracts can be stored in a 70°C freezer for at least 1 month. Two cycles of freeze-thaw did not cause any loss of the analytes. The analytes in the injected solution (plasma extracts diluted 10-fold by methanol and acetonitrile mixture) were also stable in the autosampler for at least 10 h, indicating that almost 300 samples can be analyzed in a single batch.

The post-extraction spike-based assessment indicated that the absolute matrix effects of plasma components on ESI-based measurement of IMP, CIL, OS (IS1), OSC (IS2), and CLC (IS3) were 114–127%, 112–118%, 109%, 95%, and 117%, respectively (Table 2). Although a small signal enhancement was observed for IMP and CIL, it is compensated for by the matrix-matched calibration samples because there were minimal relative matrix effects (Table 2).

Comparison of the traditional stabilizer and nonvolatile salt-free stabilizer

Variations of the MRM signal intensities of the analytes using traditional or nonvolatile salt-free stabilizer are shown in Fig. 4. The signal intensity decreased by more than 10 times after injection of 30 samples treated with traditional stabilizer, and the sampling orifice was visually contaminated. Only thorough cleaning of the ion source and the first quadrupole could the initial intensities be reproduced. We believe this was caused by the MES salt contained in the traditional stabilizer. Under the optimized chromatographic condition, introducing the salt into the MS ion source cannot be completely avoided because IMP, CIL and MES acid have similar octanol–water partition coefficient ($\log P$ of IMP = -3.9 ; CIL = -1.3 ; MES = -2.5), which is the key predictor of the retention time of the analyte on a HILIC system.^[7] In contrast to traditional stabilizer, the signal intensity was constant (90–110%) after injection of 100 plasma samples treated with nonvolatile salt-free stabilizer (Fig. 4).

Comparison of three different internal standards

Based on the matrix effects, retention times, and stability of MRM signal intensity, OSC was selected as the best internal standard for the quantification of IMP and CIL. OSC has a similar retention time to those of IMP and CIL. The matrix effects were minimal and the MS signal intensity was very stable (coefficients of variation <5%) among all the samples (including pharmacokinetic study samples, calibration samples, and quality control samples).

Assay validation

The calibration curves for quantification of IMP and CIL in human plasma samples showed a good linear relationship, ranging up to 50 $\mu\text{g/mL}$ with correlation coefficients >0.99 . The LLOQ of a 10 μL initial sample size was 0.2 $\mu\text{g/mL}$ for both analytes (Fig. 3). The ratio of signal to noise for LLOQ was about 16 and 12 for IMP and CIL, respectively (Fig. 3). The on-column sensitivity was 4 pg. Although a less sensitive OAS-MS (API3000) was utilized in the current assay, the on-column sensitivity of the current assay was almost 20-times more sensitive than the published ORS-MS-based assay (API4000, 77 pg). The sensitivity of the present assay can be attributed to three sensitivity-improving approaches: 1) lower electrolyte concentration in the mobile phase resulted in higher signal intensity; 2) use of salt-free stabilizer resulted in ion-suppression free assay; 3) methanol-acetonitrile mixture yielded higher extraction recoveries.

The within-run ($n = 3$) and between-run ($n = 3$) precision of the assay were quite good for both analytes, i.e., 1–9% and 4–10%, respectively, while the assay accuracy was also satisfactory, 94–102% and 92–106%, respectively (Table 3).

Application to human pharmacokinetic study

After assay validation, the newly developed bioanalytical method was applied to the pharmacokinetic study of IMP and CIL in a burn patient. As shown in Fig. 5, both IMP and CIL were measurable in all plasma samples up to 6 h. The plasma concentration-time data enabled us to calculate the pharmacokinetic parameters reflecting the systemic exposure and elimination properties of IMP and CIL in the burn patient.

Assay reproducibility for the incurred samples

The results of assay reproducibility are shown in Fig. 6. The ratios of the concentrations obtained by the second measurement to those determined by the first measurement were in the range of 97–115% and 86–111% for IMP and CIL, respectively.

Conclusions

The HILIC-OAS-MS-based method described in the present study enables simple, fast, sensitive, and robust determination of IMP and CIL in a small volume of human plasma (10 μ L). The utilization of nonvolatile salt-free stabilizer not only blocked the hydrolysis of analytes in plasma, but also resulted in an ion suppression-free and sensitive detection. To the best of our knowledge, the present method offers the highest sensitivity (4 pg on column) and shortest running time (120 s) for bioanalytical assay of IMP and CIL. The approach described here can likely be extended to the measurement of other hydrolytically unstable β -lactam antibiotics in biological samples. The present study also provides an example on how to transfer an assay from ORS-MS to the less sensitive and contamination resistant OAS-MS without sacrificing assay sensitivity and ruggedness. This is of practical importance because OAS-MS is still one of the most widely used MS techniques due to its low cost, lower consumption of nitrogen gas, as well as adequate sensitivity for most applications.

Acknowledgments

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Figure 1.

Chemical structures of imipenem (IMP) and cilastatin (CIL), as well as those of the internal standard candidates, oseltamivir (OS), oseltamivir carboxylate (OSC), and clopidogrel carboxylate (CLC).

Figure 2. Schematics of the off-axis electrospray ion (ESI) source (TurboIon Spray™) and orthogonal ESI source (Turbo V™). The orthogonal ESI source has greater contamination resistance and ionization efficiency than the off-axis ESI source due to its improved design in heat transfer and gas dynamics.

Figure 3.

LC-MS/MS chromatograms of blank human plasma (left panel); blank human plasma spiked with imipenem (IMP, 10.2 $\mu\text{g/mL}$), cilastatin (CIL, LLOQ 0.2 $\mu\text{g/mL}$), and internal standard (IS) oseltamivir carboxylate (400 ng/ml) (middle panel); and patient plasma collected after intravenous dosing of IMP and CIL (right panel).

Figure 4. Change of the HILIC-MS/MS signal intensities of imipenem (IMP, open cycle) and cilastatin (CIL, closed cycle) after consecutive injection of plasma extracts (1.85 $\mu\text{g/mL}$), which were pretreated with traditional stabilizer or the nonvolatile salt-free stabilizer.

Figure 5. Plasma concentration-time profile of imipenem (IMP, open circle) and cilastatin (CIL, solid circle) after an intravenous dosing of 1.0 g IMP and CIL to a burn patient receiving high-volume hemofiltration.

Figure 6.

Assay reproducibility for the quantification of imipenem (IMP) and cilastatin (CIL) in patient samples. Two measurements (separated by a 1-month period) were conducted for the same set of patient samples. The dotted red lines represent 85–115% ranges of the assay reproducibility.

Table 1

Stability of imipenem (IMP) and cilastatin (CIL) in various conditions (n = 3)

Nominal concentration (μg/mL)	Stability (%) for different conditions ^a					
	2 h in plasma at 24°C	10 h in injected solutions at 2°C (autosampler)	In extracts (stabilized plasma) at -70°C			Freeze-thaw stability in extracts ^b
			7 days	14 days	30 days	
IMP						
0.2	99 ± 6	92 ± 8	105 ± 3	85 ± 3	105 ± 10	88 ± 5
1.85	94 ± 6	94 ± 2	95 ± 4	96 ± 5	106 ± 7	85 ± 1
16.7	93 ± 5	94 ± 5	101 ± 2	106 ± 7	113 ± 8	99 ± 4
5000 ^c	NA	NA	NA	NA	89 ± 5	NA
CIL						
0.2	90 ± 4	106 ± 9	107 ± 7	93 ± 8	97 ± 11	109 ± 8
1.85	103 ± 1	109 ± 1	90 ± 7	93 ± 6	100 ± 1	93 ± 5
16.7	99 ± 1	111 ± 2	96 ± 6	105 ± 4	107 ± 1	113 ± 2
2500 ^c	NA	NA	NA	NA	92 ± 6	NA

^aStability = concentrations before storage/concentrations after storage. Mean and SD values are shown.

^bFreeze-thaw stability was tested for 2 cycles.

^cFor the stability test of stock solutions only.

NA, not applicable

Table 2

Matrix effects (ME) and recoveries of imipenem (IMP), cilastatin (CIL) and internal standard candidates oseltamivir (OS), oseltamivir carboxylate (OSC), and clopidogrel carboxylate (CLC) in human plasma ($n = 3^a$).

Compounds	Retention time (s)	Concentration ($\mu\text{g/mL}$)	Absolute ME (%) ^b	Relative ME (SD, %) ^c	Recovery (mean $\pm$ SD, %)
IMP	79	0.2	127	1	90 $\pm$ 5
		1.85	114	8	94 $\pm$ 5
		16.7	118	6	89 $\pm$ 2
CIL	56	50	117	6	99 $\pm$ 9
		0.2	116	5	93 $\pm$ 4
		1.85	118	6	91 $\pm$ 6
		16.7	112	9	96 $\pm$ 8
		50	116	4	101 $\pm$ 7
OS (IS1)	43	0.2	109	9	NA
OSC (IS2)	66	0.4	95	5	NA
CLC (IS3)	35	0.1	117	5	NA

^aPlasma from 3 different human subjects.

^bDeviation of the mean peak areas of set 2 versus those of set 1 was used to indicate the possibility of ionization suppression or enhancement for the analytes; this is called an absolute ME. In set 1, the tested analytes were added to a matrix component-free solvent (water:precipitant = 1:99). In set 2, the tested analytes were added to a matrix solvent, which was prepared using the protein precipitation method (human plasma: precipitant = 1:9, and the resulting supernatant was further diluted by 10-fold). The details of the protein precipitation method are shown in the method section.

^cThe standard deviation (SD) of the absolute ME was used to indicate the relative ME.
NA, not applicable.

Table 3

Accuracy and precision (RSD) for assay of imipenem (IMP) and cilastatin (CIL) in human plasma

Nominal concentration (µg/mL)	Within-run (<i>n</i> = 3)	Accuracy	Between-run (<i>n</i> = 3)	Accuracy
	Measured concentration (µg/mL) mean ± SD (RSD)		Measured concentration (µg/mL) mean ± SD (RSD)	
IMP				
0.2	0.21 ± 0.01 (5%)	102%	0.22 ± 0.02 (8%)	106%
1.85	1.74 ± 0.15 (9%)	94%	1.85 ± 0.14 (8%)	100%
16.7	16.5 ± 0.4 (2%)	99%	16.4 ± 0.7 (4%)	98%
CIL				
0.2	0.21 ± 0.02 (9%)	101%	0.20 ± 0.02 (10%)	99%
1.85	1.83 ± 0.05 (3%)	99%	1.70 ± 0.11 (6%)	92%
16.7	16.5 ± 0.2 (1%)	99%	15.3 ± 1.0 (6%)	92%