Simple LC-MS/MS Methods for the Quantitation of Total and Free Aprepitant and its Active N-Dealkylated Metabolites in Human Plasma

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Original Article

Simple LC-MS/MS Methods for the Quantitation of Total and Free Aprepitant and its Active *N*-Dealkylated Metabolites in Human Plasma

Takafumi Naito, PhD[1], Yusuke Suzuki, BSc[1], Kaito Shibata, PhD[1], Junichi Kawakami, PhD[1]

[1]Department of Hospital Pharmacy, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

Corresponding author: Takafumi Naito, Department of Hospital Pharmacy, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, Japan

Phone: +81 53 435 2623

Fax: +81 53 435 2764

E-mail: naitou@hama-med.ac.jp

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Abstract

Background: Aprepitant, an antiemetic selective neurokinin-1 receptor antagonist, is primarily metabolized to active *N*-dealkylated form (ND-AP) and then converted to its carbonyl form (ND-CAP) in humans. This study developed simple liquid chromatography-tandem mass spectrometry methods using electrospray ionization (ESI) for the quantitation of plasma total and free aprepitant and its *N*-dealkylated metabolites and applied it to patient plasma.

Methods: Free aprepitant and ND-AP in plasma were fractionated by centrifugal ultrafiltration. The analytes in plasma or its ultrafiltrated specimen treated by triethylamine/acetonitrile were isocratically separated on a 3-μm octadecylsilyl column with a total run time of 10 minutes and scanned with positive ion ESI.

Results: The calibration curves of total aprepitant, ND-AP, and ND-CAP ranged from 50–2500, 20–1000, and 5–250 ng/mL, respectively, while that of free aprepitant and ND-AP ranged from 2–150 ng/mL. Their intra- and inter-assay accuracy and imprecision values were 93.5–107.7% and 94.6–103.3%, and 2.1–7.5% and 1.0–8.9%, respectively. Aprepitant and its metabolites did not exhibit any matrix effects or instabilities in plasma specimens. In cancer patients receiving oral aprepitant, the plasma concentration ranges of total aprepitant, ND-AP, and ND-CAP, and of free aprepitant and ND-AP were 137–2170, 104–928, 22.4–97.6, 8.11–60.0, and 3.53–56.0 ng/mL, respectively. The median plasma free fraction rates of aprepitant and ND-AP were 4.14% and 4.90%, respectively.

Conclusions: The present methods with acceptable analytical performance can be used to

evaluate total and free aprepitant and its *N*-dealkylated metabolites in patient plasma.

Keywords:

aprepitant; LC-MS/MS; metabolite; human plasma; free fraction

Short Title:

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Introduction

Aprepitant, a selective neurokinin-1 (NK1) receptor antagonist, is commonly used for the prevention of both acute and delayed chemotherapy-induced nausea and vomiting (CINV). Prophylactic treatment of aprepitant in combination with a selective serotonin 3 receptor antagonist and dexamethasone dramatically decreased the incidence of CINV and rescue medication after highly and moderately emetogenic chemotherapy.[1-3] However, concomitant aprepitant could not achieve sufficient relief for CINV in 37% of patients during the overall phases.[1] Moreover, some patients experience aprepitant-related adverse reactions such as fatigue, hiccups, constipation, diarrhea, and anorexia.[4]

Aprepitant is primarily metabolized to the *N*-dealkylated form (ND-AP) without the triazolone group and then converted to its carbonyl form (ND-CAP) in humans (Figure 1).[5,6] The primary elimination pathway of aprepitant is mediated by hepatic cytochrome P450 3A4.[7] These metabolites as well as aprepitant possess high binding affinity to NK1 receptors. Plasma aprepitant and its metabolites must transfer into the brain to obtain the antiemetic effects. After oral administration to ferrets, aprepitant and ND-AP were found in brain as well as in plasma.[8] However, the plasma disposition of aprepitant metabolites including ND-AP and ND-CAP has not been characterized in cancer patients.

Several validated methods have been reported on the quantitation of aprepitant in human plasma using liquid chromatographic (LC) techniques coupled to ultraviolet detection[9] or tandem mass spectrometry (MS/MS).[10-12] These methods do not possess LC or detection

77 conditions for the quantitation of aprepitant metabolites in human plasma. The *N*-dealkylated
78 forms as major metabolites have stronger basicity and higher polarity than the parent drug.
79 Their simultaneous determination together with aprepitant requires complicated sample
80 pretreatments and sufficient LC retention in addition to sufficient sensitivity. One analytical
81 report achieved the simultaneous quantitation of plasma aprepitant and its metabolites using
82 atmospheric pressure chemical ionization (APCI) MS/MS detection.[13] However, MS/MS
83 detection using electrospray ionization (ESI) is more suitable in polar and thermally unstable
84 metabolites and is easy to apply to clinical laboratory practice.

85 Aprepitant binds to a high degree, of more than 90%, to plasma proteins,[5,14] while few
86 reports have been published on quantitative methods for free aprepitant in human plasma. The
87 binding rates of active metabolites, including ND-AP, have not been investigated in cancer
88 patients. Simultaneous quantitation of free aprepitant and its major metabolite in human plasma
89 by highly sensitive MS/MS detection has the potential to explain the individual variations in
90 clinical responses to aprepitant. In the present study, we developed simple LC-MS/MS methods
91 using ESI for the quantitation of total and free aprepitant and its metabolites in human plasma.
92 Additionally, the validated methods were applied to plasma specimens obtained from cancer
93 patients receiving oral aprepitant.

94

95 **Material and methods**

96 **Chemicals and solutions**

Aprepitant, ND-AP, and ND-CAP as analytical standards were purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Diazepam as an internal standard (IS) and triethylamine were obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Stock solutions of analytes were prepared by dissolving analytical standards in methanol. Calibration standard, lower limit of quantitation (LLOQ), and quality control (QC) samples were prepared with drug-free pooled plasma and ultrafiltrated plasma specimens. The calibration standards of total aprepitant, ND-AP, and ND-CAP were within the ranges of 50–2500, 20–1000, and 5–250 ng/mL, respectively, at each of 7 points, while that of free aprepitant and ND-AP were within the ranges of 2–150 ng/mL at each of 7 points. The concentrations of LLOQ and 3 QC samples were 50, 100, 500, and 2000 ng/mL for total aprepitant, 20, 40, 200, and 800 ng/mL for total ND-AP, 5, 10, 50, and 200 ng/mL for total ND-CAP, and 2, 5, 25, and 100 ng/mL for free aprepitant and ND-AP.

Sample pretreatment

Free aprepitant and ND-AP were fractionated from plasma specimens using an ultrafiltration device (Centrifree, Merck Millipore Ltd., Billerica, MA, USA) for 30 minutes at 2000 × *g* at 37°C. Six-hundred microliters of acetonitrile, 100 μL of IS solution (100 ng/mL in acetonitrile), and 50 μL of triethylamine were added to 100 μL of plasma or an ultrafiltrated plasma specimen. The samples were mixed well and centrifuged at 18,000 × *g* at 4°C, and then 700 μL of the centrifugal supernatant was evaporated to dryness using a concentrator. The residues

were reconstituted with 150 μL of mobile phase and its centrifugal supernatants were filtrated before injection into the LC system.

Chromatographic conditions

The LC system consisted of a Nexera X2 LC system (Shimadzu Corporation, Kyoto, Japan). Analytes in pretreated samples were separated using a 3-μm particle size octadecylsilyl (C18) column (TSKgel ODS-100V, 75 mm length × 2.0 mm inner diameter, Tosoh, Tokyo, Japan) maintained at 40°C. The mobile phase consisted of methanol and 5 mM ammonium acetate at pH 4.5 adjusted with acetic acid (7:3, v/v). The flow rate in the LC was 0.2 mL/min. The sample storage temperature of the autoinjector was 4°C, and the volume injected into the LC system was 10 μL.

Mass spectrometer conditions

The triple quadrupole tandem mass spectrometer (LCMS8050, Shimadzu Corporation) was operated using LabSolutions LCMS ver. 5.91 software (Shimadzu Corporation). The ion transitions were scanned in the positive ion multiple reaction monitoring mode with a dwell time of 250 milliseconds for each compound: aprepitant, *m/z*, 535.1/179.1; ND-AP, 438.3/180.1; ND-CAP, 452.3/151.0; and IS, 285.1/154.1 (Figure 2). A high positive voltage of 3.0 kV was applied to the ion spray at 300°C. The flow rates of drying gas, nebulizer gas, and heating gas were set at 10 L/min, 3 L/min, and 10 L/min, respectively. The collision-induced

dissociation gas pressure was 270 kPa. Collision energies, entrance potential, and collision cell exit potential were set at -29, -22, and -19 volts for aprepitant, -17, -18, and -19 volts for ND-AP, -53, -14, and -10 volts for ND-CAP, and -27, -12, and -15 volts for IS, respectively.

Analytical performance

Analytical performance in the present methods was evaluated according to US Food and Drug Administration Guidance.[15] The selectivity of the present methods was assessed by observation of the MS/MS chromatograms of plasma and ultrafiltrated plasma specimens in 6 aprepitant-free cancer patients. Calibration curves were obtained by plotting the measured peak area ratios of aprepitant, ND-AP, and ND-CAP to IS with the 1/x weighted least squares regression method. The linearities of total aprepitant, ND-AP, and ND-CAP were evaluated at concentration ranges of 50–2500, 20–1000, and 5–250 ng/mL, respectively, while that of free aprepitant and ND-AP were evaluated at a concentration range of 2–150 ng/mL. For sensitivity, the LLOQs were confirmed to test whether their coefficients of accuracy (% error) and imprecision (% relative standard deviation (RSD)) were within 20% (n = 6). The LLOQs were set as the practical values in clinical settings. Extraction recovery rates were determined by comparing the peak area of the analytes extracted from each of the 3 QC samples with that of the analytes spiked in plasma specimen extracts (n = 3). Matrix factors were calculated by comparing the peak areas of analytes spiked in extracts from analyte-free plasma specimens (6 lots) with that of analytes spiked in mobile phase. Carry-over was evaluated by measuring the

157 analyte-free specimens after measurement of the upper limit of quantification (ULOQ) samples
158 of the calibration curve (n = 3). The variations in plasma free aprepitant and ND-AP at 3 QC
159 levels (n = 6) after the ultrafiltration of plasma specimens were also evaluated. The intra- and
160 inter-assay accuracy and imprecision were evaluated with 6 replicates at 3 QC levels for a single
161 assay and 6 assays for 4 days, respectively. Acceptance criteria for the intra- and inter-assay
162 accuracy at 3 QC levels (n = 6) were within 15% of the target value, while those for the intra-
163 and inter-assay imprecision at 3 QC levels (n = 6) were within 15% of RSD. The dilutability
164 was assessed by comparing the peak area ratios of plasma specimens of ULOQ with that of
165 samples prepared by dilution using each matrix of its 5-fold concentrated samples (n = 3).
166
167 **Stability of analytes**
168 The stability of analytes in each stock solution for 3 months was evaluated by anteroposterior
169 comparisons (n = 3). Short-term stability in plasma and ultrafiltrated plasma specimens at 4°C
170 (n = 3 for each of 3 QC) and room temperature (n = 3 for each of 3 QC) were evaluated by
171 comparing peak areas after 24 hours of storage with initial peak area. Long-term stability in
172 plasma and its ultrafiltrated specimens at -80°C was determined after one month (n = 3 for each
173 of 3 QC). The analytical stability of analytes after reconstitution with mobile phase was
174 evaluated by comparing peak areas after 24 hours of storage at 4°C with initial peak area (n =
175 3 for each of 3 QC). The freeze-thawing stability of analytes in plasma and the ultrafiltrated
176 plasma specimens was evaluated by comparing peak areas after 2 freeze-thaw cycles with initial

177 peak area (n = 3 for each of 3 QC).

178

179 **Clinical application**

180 The study protocol complied with the Declaration of Helsinki and its later amendments and was
181 approved by the Ethics Committee of Hamamatsu University School of Medicine (17-102).
182 Each patient received information about the scientific aim of the study and provided written
183 informed consent. Twenty head and neck cancer patients receiving oral aprepitant (Emend
184 Capsules, Ono Pharmaceutical Co., Ltd., Osaka) at a dose of 125 mg on day 1 and 80 mg on
185 days 2 and 3 in combination with a selective serotonin 3 receptor antagonist and dexamethasone
186 for the prevention of CINV at Hamamatsu University Hospital were enrolled. All enrolled
187 patients received cisplatin-based chemotherapy. Patients with severe liver or renal dysfunction
188 were excluded from the study. Blood samples were drawn into tubes containing
189 ethylenediaminetetraacetic acid dipotassium salts at 24 hours after oral aprepitant
190 administration on day 3. The blood samples were centrifuged at 1670 × g at 4°C for 10 minutes,
191 and then an aliquot of plasma was immediately ultrafiltrated using a Centrifree device. These
192 samples were stored at -80°C until sample pretreatment.

193

194 **Results**

195 **Selectivity and separation**

196 In the LC-MS/MS chromatograms, no peaks interfering with analytes were observed in plasma

197 or its ultrafiltrated specimens for any of the aprepitant-free cancer patients. Figure 3 and Figure
198 S1 show the LC-MS/MS chromatograms of aprepitant and its metabolites in human plasma and
199 ultrafiltrated plasma specimens. The chromatographic peaks of aprepitant, ND-AP, ND-CAP,
200 and IS were observed at 5.9, 5.0, 5.2, and 2.7 minutes, respectively.
201
202 **Calibration curves and sensitivity**
203 The calibration curves of total aprepitant, ND-AP, and ND-CAP were linear over the
204 concentration ranges of 50–2500, 20–1000, and 5–250 ng/mL, respectively, while that of free
205 aprepitant and ND-AP were linear over the concentration range of 2–150 ng/mL. Their
206 calibration curves had correlation coefficients of more than 0.99. The LLOQs of total
207 aprepitant, ND-AP, and ND-CAP, and of free aprepitant and ND-AP were set at 50, 20, 5, 2,
208 and 2 ng/mL, respectively. Their accuracy and imprecision values for LLOQs were 94.3–
209 108.2% and 2.2–7.5%, respectively (Table S1).
210
211 **Extraction recovery, matrix effects, and carry-over**
212 Extraction recovery ranges of total aprepitant, ND-AP, and ND-CAP were 94.9–104.1%, 93.4–
213 101.3%, and 95.0–104.5%, while that of free aprepitant and ND-AP were 91.5–104.6% and
214 95.4–104.5%, respectively. Matrix factor ranges of total aprepitant, ND-AP, and ND-CAP, and
215 of free aprepitant and ND-AP were 94.8–106.2%, 91.9–101.8%, 96.8–102.0%, 93.4–104.2%,
216 and 94.9–101.3%, respectively. No sharp peak that had a signal-to-noise ratio value higher than

3 was observed in the analyte-free plasma specimens after measurement of the ULOQ samples. With respect to the variation in ultrafiltration procedure, the RSDs in plasma free aprepitant and ND-AP after ultrafiltration were 3.7–6.0% and 3.1–9.1%, respectively.

Assay accuracy and imprecision

Table 1 presents the assay accuracy and imprecision parameters of total and free aprepitant and its metabolites in human plasma. The intra- and inter-assay accuracy ranged from 99.8–107.7% and 97.5–101.6% for total aprepitant, 93.5–98.9% and 99.4–100.2% for total ND-AP, 99.5–105.3% and 95.8–100.6% for total ND-CAP, 93.5–103.7% and 97.6–102.6% for free aprepitant, and 94.7–104.0% and 94.6–103.3% for free ND-AP, respectively. Their corresponding intra- and inter-assay imprecision ranged from 2.1–4.0% and 1.2–4.4%, 2.2–4.5% and 1.0–3.5%, 3.4–5.8% and 1.9–4.3%, 3.5–5.3% and 1.9–7.1%, and 3.1–7.5% and 2.4–8.9%, respectively. The peak area ratios with respect to the dilution effects ranged from 93.3–105.5% for all the analytes.

Stability

Stock solutions of aprepitant and its metabolites, and IS were stable at 4°C for up to 3 months (% of initial value, 97.7–103.9%). Aprepitant, ND-AP, and ND-CAP in plasma samples were stable at room temperature (% of initial value, 95.9–107.7%) and at 4°C (93.4–103.3%) for up to 24 hours, while aprepitant and ND-AP in ultrafiltrated specimens were also stable at room

temperature (88.6–97.9%) and at 4°C (93.0–103.1%) for up to 24 hours. At -80°C for up to 1 month, the amount of aprepitant and its metabolites in plasma and its ultrafiltrated specimens did not change (% of initial value, 89.7–100.4%). After sample pretreatment, aprepitant and its metabolites, and IS in mobile phase were stable at 4°C for up to 24 hours (% of initial value, 92.0–107.7%). The results of the 2-cycle freeze-thaw test indicated little loss of aprepitant and its metabolites in plasma and its ultrafiltrated specimens (% of initial value, 92.1–102.1%).

Clinical application

Figure 4 shows the plasma concentrations of total and free aprepitant and its metabolites in 20 head and neck cancer patients co-treated with a selective serotonin 3 receptor antagonist and dexamethasone. The plasma concentration ranges of total aprepitant, ND-AP, and ND-CAP were 137–2170, 104–928, and 22.4–97.6 ng/mL, respectively. In contrast, the plasma concentrations of free aprepitant and ND-AP ranged from 8.11–60.0 and 3.53–56.0 ng/mL, respectively. Since all enrolled patients had less than 1 ng/mL of free ND-CAP in their plasma, free ND-CAP in patient plasma was not detectable using the present method. The plasma concentrations of total and free aprepitant and its metabolites were within the range of each calibration curve. The median (range) free fraction rates of aprepitant and ND-AP were 4.14% (2.67–8.73%) and 4.90% (1.27–12.9%), respectively.

Discussion

257 This study established simple LC-MS/MS methods using ESI for the quantitation of total and
258 free aprepitant and its active *N*-dealkylated metabolites in human plasma. The analytes in
259 plasma specimens treated by basic organic solvents were isocratically separated on a 3-μm C18
260 column with a total run time of 10 minutes, and scanned using positive ion ESI. The present
261 methods have acceptable analytical performance according to recent guidance.[15] To the best of
262 our knowledge, this is first analytical report to quantify free aprepitant and its *N*-dealkylated
263 metabolite in human plasma using ESI-MS/MS detection.

264 The present sample pretreatment employed acetonitrile/triethylamine solution with
265 volatility to remove plasma protein. Aprepitant is a basic compound with an acid dissociation
266 constant of 9.7 and its *N*-dealkylated metabolites with a secondary amine have stronger
267 basicity.[5,16] Acetonitrile solution not including triethylamine cannot fully extract the *N*-
268 dealkylated metabolites from plasma protein owing to its strong basicity. Earlier sample
269 pretreatments employed liquid-liquid extraction using methyl *tert*-butyl ether and pH 9.8
270 carbonate buffer.[10,13] For sample pretreatments, a small volume of acetonitrile/triethylamine
271 solution can be easily evaporated in a short time. The present pretreatment has simplicity and
272 less environmental loading, and so can be applied to clinical laboratory practice.

273 The present LC separation used a 3-μm particle size C18 column with a mobile phase
274 consisting of 70% methanol containing 5 mM ammonium acetate at pH 4.5. Earlier methods
275 employed an octasilyl column coupled to APCI MS/MS detection.[10,13] Our mobile phase is
276 optimized for highly sensitive ESI MS/MS detection of polar metabolites. In the present LC

277 conditions, the use of a C18 column (TSKgel ODS-100V) with its lower ligand density (15%
278 carbon content) raised the signal intensity for polar metabolites without a deterioration of LC
279 retention and an extreme increase of the methanol ratio in mobile phase. Moreover, the mobile
280 phase at pH 4.5 had maximum sensitivity for analytes in the range of pH 3–7 with good
281 separation. Although the present methods need a total run time of 10 minutes for simultaneous
282 quantitation of four analytes, this run time would be acceptable for clinical laboratory practice.
283 Aprepitant and its *N*-dealkylated metabolites hardly adsorbed to the sample reservoir
284 and filtrate cup of a Centrifree. In ultrafiltration procedure, the variation in plasma free
285 aprepitant and ND-AP were less than 6.0% and 9.1%, respectively. We considered that the
286 plasma free aprepitant and its metabolites were not underestimated and not varied by
287 ultrafiltration procedure. Aprepitant and its metabolites and IS did not exhibit any matrix effects
288 in 6 lot plasma specimens in this study. The present study employed diazepam as an IS instead
289 of stable isotope-labeled aprepitant and its metabolites. Diazepam, which shows sufficient
290 extraction recovery rate from plasma specimens, has been shown to be less susceptible to the
291 matrix effect in ESI MS/MS detection[17,18] and exhibited no interference with aprepitant and its
292 metabolites in our methods. Although the stable isotope-labeled compound of aprepitant can be
293 obtained from the foreign suppliers, that of *N*-dealkylated metabolites are not commercially
294 available. Additionally, the stable isotope-labeled compounds are expensive. In contrast,
295 diazepam is easily obtained from several suppliers in many countries. Thus, diazepam as an IS
296 was suitable for the quantitation of total and free aprepitant and its metabolites in human plasma

297 and is applicable to clinical laboratory practice from the viewpoint of availability. The analytical
298 performance data for the present method also met the criteria of a recent international
299 guidance.[15]

300 The plasma concentration ranges of total aprepitant, ND-AP, and ND-CAP at 24 hours
301 after oral aprepitant administration on day 3 were 137–2170, 104–928, and 22.4–97.6 ng/mL,
302 respectively, in cancer patients. Their concentrations in the present methods were not
303 significantly different from that in earlier studies,[9-13] although their study protocols were not
304 identical. In contrast, the plasma concentration of free aprepitant and ND-AP ranged from 8.11–
305 60.0 and 3.53–56.0 ng/mL, respectively. This is first report to present plasma free aprepitant
306 and ND-AP in cancer patients receiving oral aprepitant. Their plasma total and free
307 concentrations were within the range of each calibration curve.

308 Our methods determined the free aprepitant and ND-AP levels in human plasma and
309 found their median plasma free fraction rates were 4.1% and 4.9%, respectively. ND-AP, which
310 has binding affinity to the NK1 receptor,[7] was reported to be highly distributed in brain
311 compared with aprepitant after oral administration in ferrets.[8] Additionally, plasma free ND-AP
312 showed a large interindividual variation in our study population. Monitoring of free ND-AP as
313 well as free aprepitant may be needed to obtain antiemetic efficacy and to avoid adverse
314 reactions. Our analytical method was able to detect 1 ng/mL of free ND-CAP in plasma.
315 However, the enrolled patients had much lower plasma free ND-CAP levels than free aprepitant
316 and ND-AP. Although free ND-CAP was not detectable in patient plasma, it is very likely that

317 ND-CAP as well as ND-AP bind highly to plasma proteins based on our data.

318 This study collected the blood samples on day 4, which is at 24 hours after oral
319 aprepitant administration on day 3. Cancer patients commonly receive oral aprepitant at a dose
320 of 125 mg on day 1 and 80 mg on days 2 and 3. Oral aprepitant can be additionally administered
321 until day 5 for the adult patients with insufficient efficacy. The plasma total and free
322 concentrations of aprepitant and its *N*-dealkylated metabolites on day 4 may explain the efficacy
323 and safety of aprepitant for 3-day treatment in cancer patients. Based on the plasma
324 concentrations on day 4, the administration period or dose of oral aprepitant can be potentially
325 adjusted in next cycles of chemotherapy.

326 The present methods have a few limitations which should be pointed out. First, our
327 methods did not present the permissible range of serum albumin from the viewpoint of plasma
328 protein binding. Aprepitant predominantly binds to albumin in human serum.[5,14] Advanced
329 cancer patients tend to have cachexia and its related hypoalbuminemia.[19,20] This study
330 population had a serum albumin range of 31–43 g/L. Further analyses with a large number of
331 patients would confirm the permissible range of serum albumin. Second, this study enrolled
332 cancer patients without liver dysfunction. The plasma concentration of total aprepitant in
333 patients with moderate renal dysfunction was comparable to that in patients without liver
334 dysfunction.[5,21] In our methods, the 5-fold dilution procedure is acceptable for the quantitation
335 of high plasma total and free concentrations of aprepitant and its metabolites. The present
336 analytical methods are potentially suitable to a broad range of clinical laboratory practices.

Third, the present study employed diazepam as an IS instead of stable isotope-labeled aprepitant and its metabolites. The methods cannot apply to the patients co-treated with diazepam. However, oral aprepitant is rarely combined with diazepam in clinical settings. For patients receiving diazepam, the easily available compounds such as tolvaptan and bortezomib can be used as an alternative IS (Figure S2). These compounds have properties of less matrix effect, high extraction recovery, and similar polarity to diazepam.[17,22]

Conclusions

These simple LC-MS/MS methods using ESI and with an acceptable degree of analytical performance can be used to evaluate total and free aprepitant and its *N*-dealkylated metabolites in patient plasma.

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427 **Figure legends**

428 Figure 1. Possible metabolic pathways of aprepitant in humans.[5,6]

429 ND-AP, *N*-dealkylated aprepitant; and ND-CAP, *N*-dealkylated carbonylaprepitant

430

431 Figure 2. Mass spectra and mass-to-charge (*m/z*) of aprepitant and its *N*-dealkylated
432 metabolites.

433 (A) Aprepitant, (B) *N*-dealkylated aprepitant (ND-AP), (C) *N*-dealkylated carbonylaprepitant
434 (ND-CAP), and (D) diazepam as an internal standard

435 Aprepitant, *m/z*, 535.1/179.1; ND-AP, 438.3/180.1; ND-CAP, 452.3/151.0; and diazepam,
436 285.1/154.1

437

438 Figure 3. LC-MS/MS chromatograms of aprepitant and its *N*-dealkylated metabolites in human
439 plasma and its ultrafiltrated specimens.

440 (A) Drug-free plasma, (B) drug-free plasma spiked with 1000 ng/mL aprepitant, 400 ng/mL *N*-
441 dealkylated aprepitant (ND-AP), and 100 ng/mL *N*-dealkylated carbonylaprepitant (ND-CAP),
442 (C) a plasma specimen obtained from a head and neck cancer patient receiving oral aprepitant
443 at 24 hours after dosing of 125 mg on day 1 and 80 mg on days 2 and 3, (D) drug-free plasma
444 ultrafiltrated specimen, (E) drug-free plasma ultrafiltrated specimen spiked with 50 ng/mL
445 aprepitant and 50 ng/mL ND-AP, and (F) a plasma ultrafiltrated specimen obtained from patient
446 in (C) above. (A'), (B'), and (C') of Figure S1 are enlarged views of (A), (B), and (B) of Figure

447 3, respectively.

448 (1) Aprepitant, (2) ND-AP, (3) ND-CAP, and (4) diazepam as an internal standard

449

450 Figure 4. Plasma concentrations of total and free aprepitant and its *N*-dealkylated metabolites

451 in 20 head and neck cancer patients receiving an oral aprepitant dose of 125 mg on day 1 and

452 80 mg on days 2 and 3. Blood samples were collected at 24 hours after oral aprepitant

453 administration on day 3.

454 Plasma concentrations of (A) total aprepitant, (B) total *N*-dealkylated aprepitant (ND-AP), (C)

455 total *N*-dealkylated carbonylaprepitant (ND-CAP), (D) free aprepitant, and (E) free ND-AP

Table 1. Intra- and inter-assay accuracy and imprecision values of total and free aprepitant and its *N*-dealkylated metabolites in human plasma

Analyte	Theoretical value (ng/mL)	Intra-assay (n = 6)		Inter-assay (n = 6)	
		Accuracy (%)	Imprecision, RSD (%)	Accuracy (%)	Imprecision, RSD (%)
Total aprepitant	100	107.7	2.3	97.8	4.4
	500	101.6	2.1	97.5	2.4
	2000	99.8	4.0	101.6	1.2
Total ND-AP	40	93.5	4.5	100.1	3.5
	200	98.9	2.7	99.4	1.7
	800	98.3	2.2	100.2	1.0
Total ND-CAP	10	105.3	5.8	100.6	4.3
	50	102.9	4.4	95.8	2.1
	200	99.5	3.4	100.4	1.9
Free aprepitant	5	103.7	4.2	102.6	2.6
	25	93.5	3.5	99.6	7.1
	100	99.9	5.3	97.6	1.9
Free ND-AP	5	103.8	7.5	103.3	8.9
	25	94.7	4.8	94.6	2.4
	100	104.0	3.1	100.0	3.3

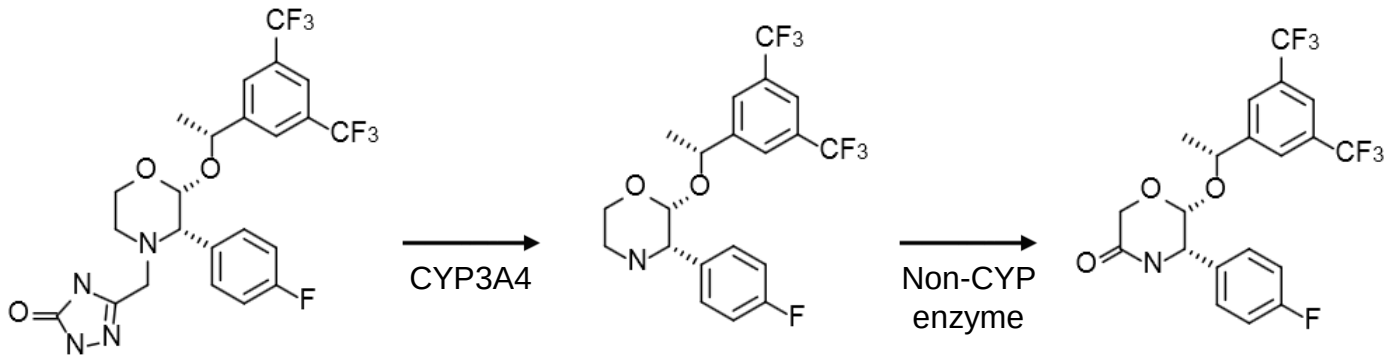
RSD, relative standard deviation; ND-AP, *N*-dealkylated aprepitant; and ND-CAP, *N*-dealkylated carbonylaprepitant

Table S1. Intra- and inter-assay accuracy and imprecision values for lower limit of quantitation of total and free aprepitant and its *N*-dealkylated metabolites in human plasma

Analyte	Theoretical value (ng/mL)	Intra-assay (n = 6)		Inter-assay (n = 6)	
		Accuracy (%)	Imprecision, RSD (%)	Accuracy (%)	Imprecision, RSD (%)
Total aprepitant	50	102.3	6.3	106.5	5.4
Total ND-AP	20	106.3	7.1	97.4	6.2
Total ND-CAP	5	95.2	4.2	108.2	5.6
Free aprepitant	2	105.9	7.5	97.3	5.3
Free ND-AP	2	94.3	2.2	107.5	4.6

RSD, relative standard deviation; ND-AP, *N*-dealkylated aprepitant; and ND-CAP, *N*-dealkylated carbonylaprepitant

Figure 1



Aprepitant ND-AP ND-CAP

Figure 2

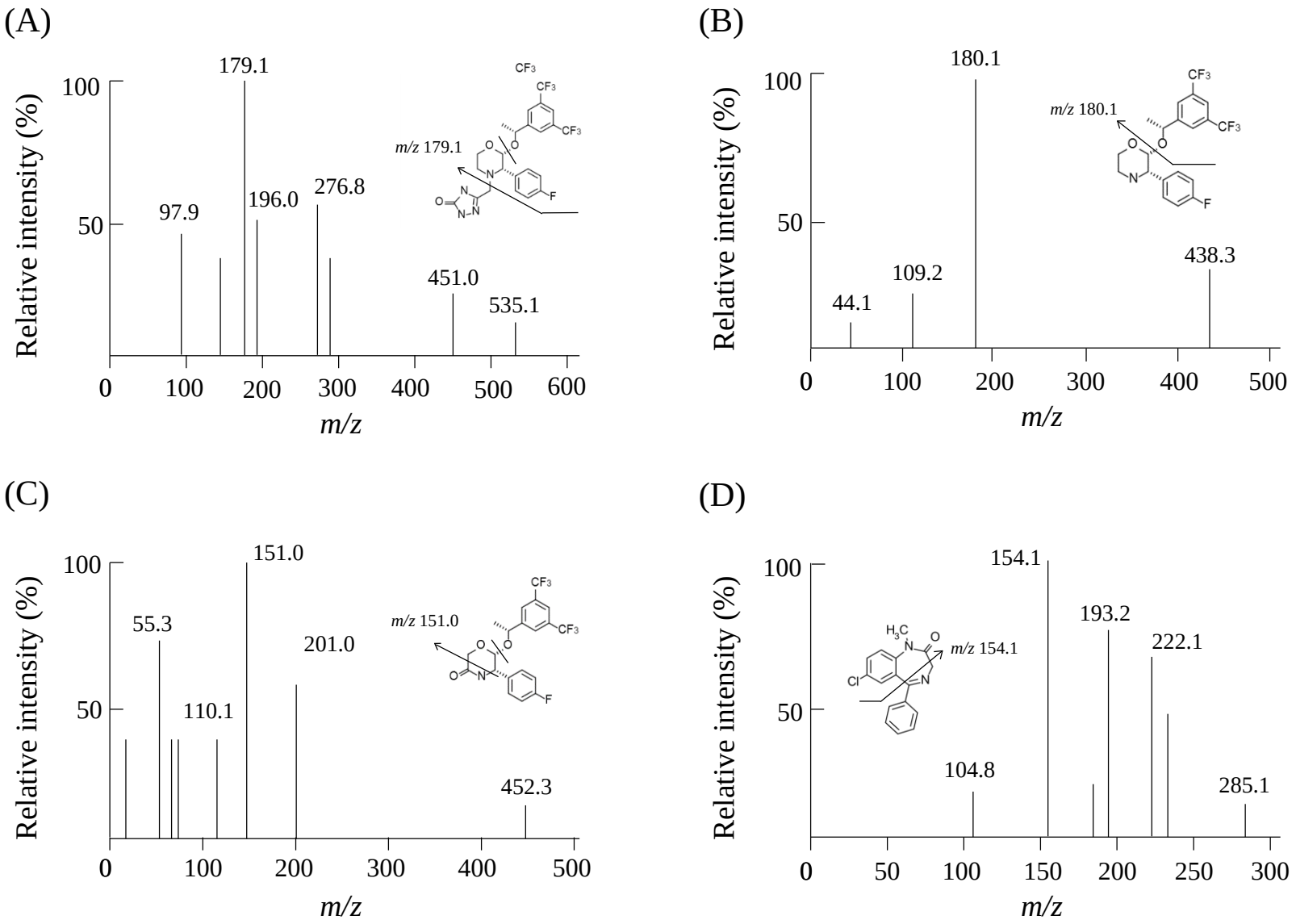

(A)
Relative intensity (%)
100
50
0
179.1
97.9
196.0
276.8
451.0
535.1
100
200
300
400
500
600
m/z
m/z 179.1
(B)
Relative intensity (%)
100
50
0
180.1
44.1
109.2
438.3
100
200
300
400
500
m/z
m/z 180.1
(C)
Relative intensity (%)
100
50
0
151.0
55.3
110.1
201.0
452.3
100
200
300
400
500
m/z
m/z 151.0
(D)
Relative intensity (%)
100
50
0
154.1
193.2
222.1
104.8
285.1
50
100
150
200
250
300
m/z
m/z 154.1

Figure 3

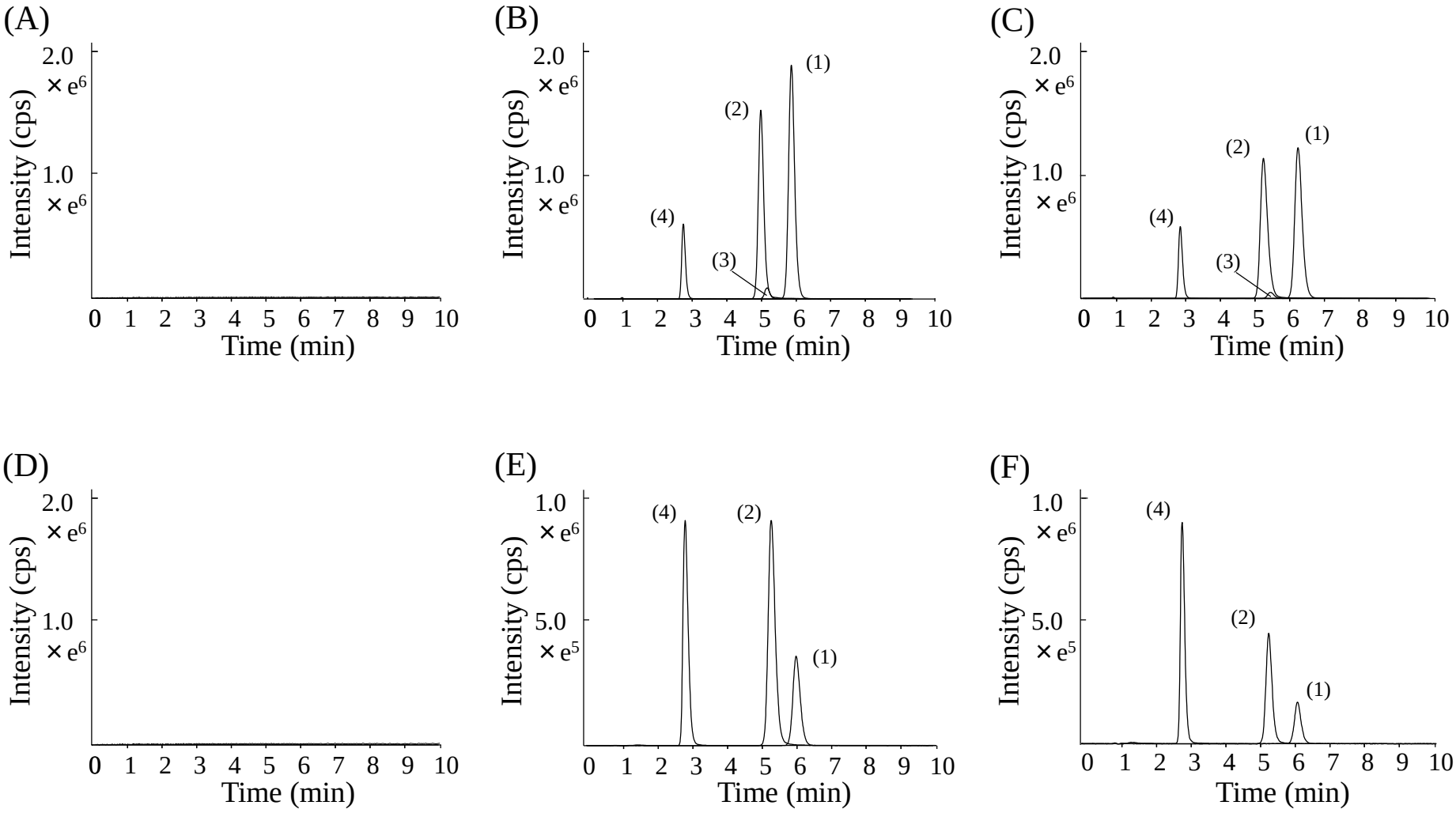
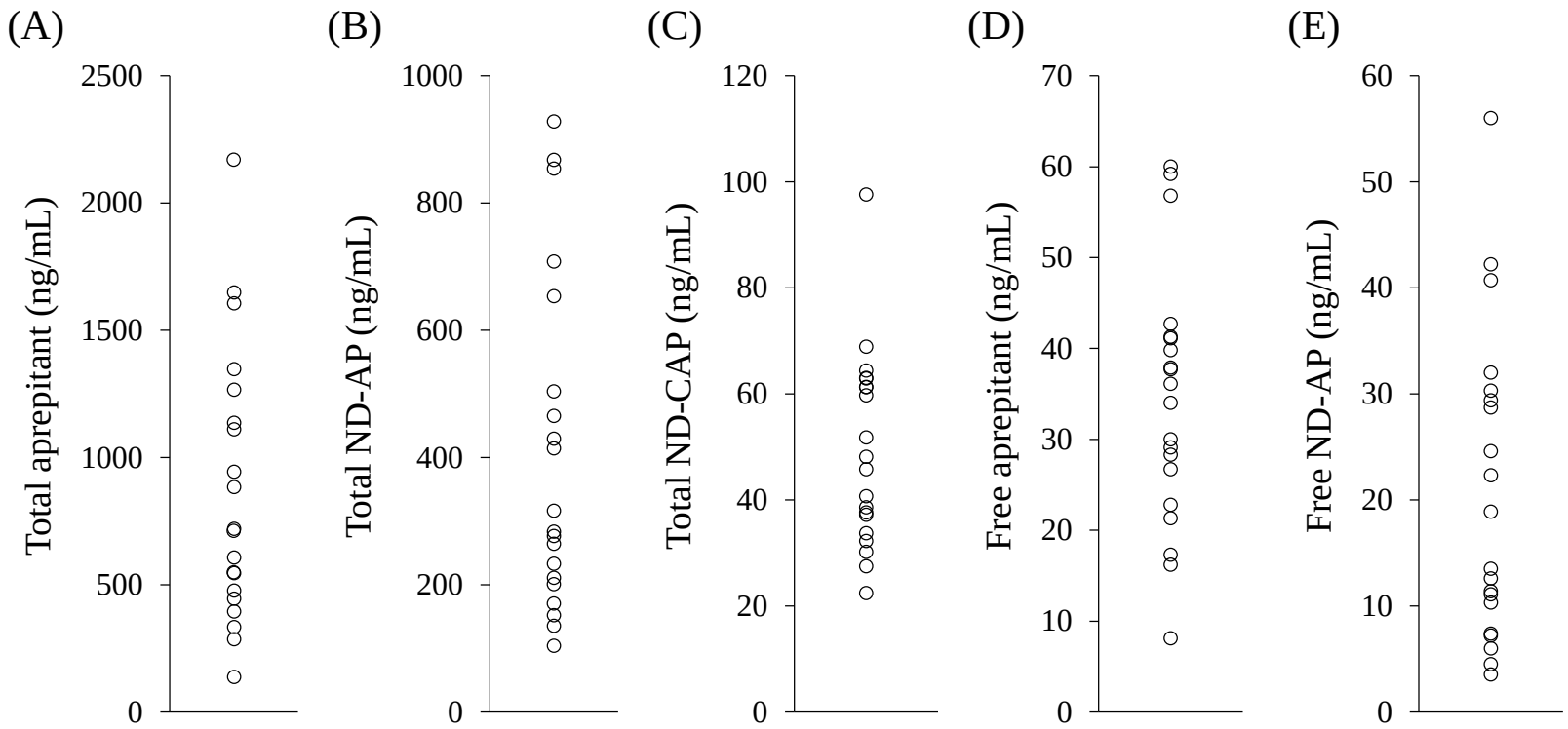


Figure 4


(A)
Total aprepitant (ng/mL)
2500
2000
1500
1000
500
0
(B)
Total ND-AP (ng/mL)
1000
800
600
400
200
0
(C)
Total ND-CAP (ng/mL)
120
100
80
60
40
20
0
(D)
Free aprepitant (ng/mL)
70
60
50
40
30
20
10
0
(E)
Free ND-AP (ng/mL)
60
50
40
30
20
10
0

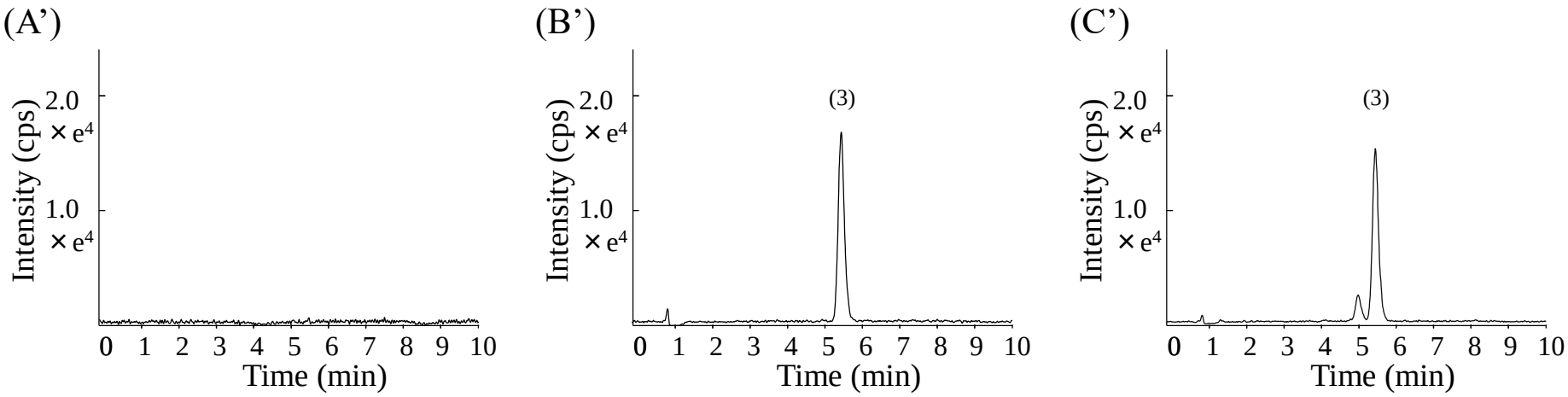


Figure S1. LC-MS/MS chromatograms of total *N*-dealkylated carbonylaprepitant (ND-CAP) (3) in human plasma.
(A'), (B'), and (C') are enlarged views of (A), (B), and (B) of Figure 3, respectively.
(A') Drug-free plasma, (B') drug-free plasma spiked with 100 ng/mL ND-CAP, (C') a plasma specimen obtained from a head and neck cancer patient receiving oral aprepitant at 24 hours after dosing of 125 mg on day 1 and 80 mg on days 2 and 3

Figure S2

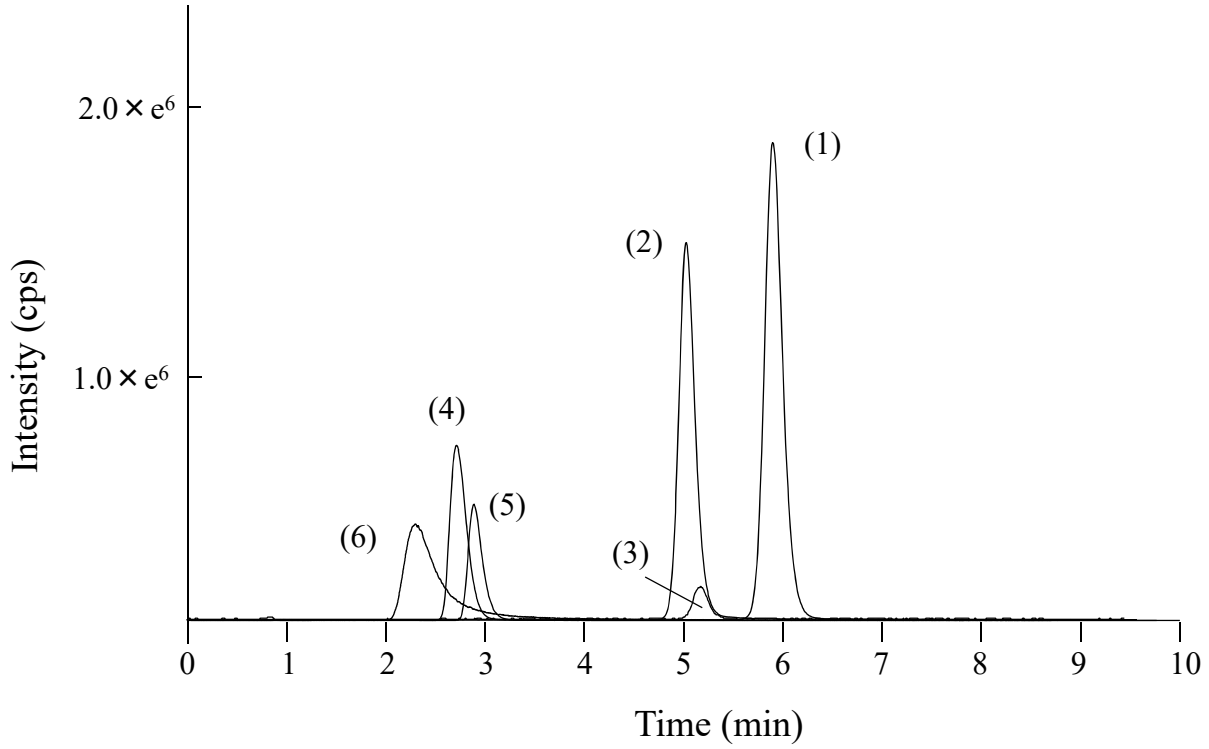


Figure S2. LC-MS/MS chromatogram of the proposed alternative internal standards in human plasma.
Drug-free plasma spiked with 1000 ng/mL aprepitant, 400 ng/mL *N*-dealkylated aprepitant (ND-AP), and 100 ng/mL *N*-dealkylated carbonylaprepitant (ND-CAP), 100 ng/mL diazepam as an internal standard, and 10 ng/mL tolvaptan and 500 ng/mL bortezomib as proposed alternative internal standards
(1) Aprepitant, (2) ND-AP, (3) ND-CAP, (4) diazepam, (5) tolvaptan, and (6) bortezomib
Tolvaptan and bortezomib were monitored by the respective transitions of *m/z* 448.9 to 119.1 and 367.0 to 226.0. Tolvaptan may be more suitable as an alternative internal standard based on peak shape and retention time.