Brief Report

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Simultaneous determination of belzutifan and cabozantinib, oral agents for renal cell carcinoma, in human plasma using high-performance liquid chromatography

Yoshito Gando, Takeo Yasu*

Department of Clinical Pharmacodynamics and Toxicology Analytics, Meiji Pharmaceutical University, Tokyo, Japan.

SUMMARY: Belzutifan and cabozantinib are oral drugs for advanced renal cell carcinoma, which display exposure-dependent toxicities. No validated method exists for simultaneously quantifying these drugs in human plasma. In this study, we developed and validated a high-performance liquid chromatography–ultraviolet method for simultaneously quantifying belzutifan and cabozantinib in human plasma. Plasma samples (50 μL) were processed *via* simple protein precipitation. Chromatographic separation on a C18 column was performed isocratically with 0.5% KH_2PO_4 (pH 4.5)/acetonitrile (47/53, v/v) at 1.0 mL/min, with UV detection at 230 nm. Performance characteristics (calibration, precision, accuracy, recovery, stability, selectivity, and interference by concomitant drugs) were systematically evaluated. Linear calibration ranges were 50–4,000 ng/mL for belzutifan and 25–4,000 ng/mL for cabozantinib with excellent linearity ($r^2 \geq 0.9998$). Intra- and interday coefficients of variation were ≤ 5.41% for belzutifan and ≤ 12.25% for cabozantinib. Both analytes were stable under bench-top, short-term, long-term, and freeze–thaw conditions. No interference from endogenous plasma components or six commonly co-administered medications was observed. The newly developed assay for the simultaneous quantification of belzutifan and cabozantinib in human plasma is rapid, requires minimal sample volumes, and demonstrates robust analytical performance.

Keywords: high-performance liquid chromatography–ultraviolet, human plasma concentration, belzutifan; cabozantinib

1. Introduction

Belzutifan and cabozantinib are orally administered anticancer agents indicated for the treatment of advanced renal cell carcinoma (RCC) (*1,2*). Belzutifan is predominantly metabolized by UDP-glucuronosyltransferase (UGT) 2B17 and cytochrome P450 (CYP) 2C19 (*3*). Poor-metabolizer (PM) phenotypes associated with UGT2B17 and CYP2C19 occur at appreciable frequencies in Asian populations, with reported prevalences of approximately 60% and 20%, respectively, among Japanese individuals (*4,5*). Although the predicted prevalence of the dual UGT2B17/CYP2C19 PM phenotype in the United States population is approximately 0.5%, it may be as high as 15% in East Asian populations (*3*). Anemia is a characteristic adverse event associated with belzutifan, with incidences of 82.8% for any grade and 32.5% for grade ≥ 3 (*1*). Exposure–response analyses have demonstrated a significant association between plasma belzutifan concentrations and the incidence of grade ≥ 3 anemia (*6*). In individuals with the dual UGT2B17/CYP2C19 PM phenotype, the area under the concentration–time curve (AUC) of belzutifan is reportedly 3.2-fold higher than those for UGT2B17 extensive metabolizers and CYP2C19 non-PMs, translating into an estimated 13.0% increase in the risk of grade ≥ 3 anemia (*3,6*). Collectively, these observations suggest that monitoring plasma belzutifan concentrations and implementing appropriate dose adjustments may mitigate the risk of severe anemia while preserving therapeutic efficacy.

For cabozantinib, plasma exposure is associated with its efficacy and toxicity, with thresholds of 536.8 and 617.7 ng/mL, respectively (*7*). Moreover, plasma cabozantinib concentrations in Japanese patients have been reported to be approximately 30% higher than those in non-Japanese patients (*8*). In light of these findings, therapeutic drug monitoring (TDM)–guided dose optimization has been recommended for cabozantinib (*9*).

Recently, the efficacy of the combination of belzutifan and cabozantinib was evaluated in a clinical trial, and this combination therapy might become one

of the treatment options for renal cell carcinoma in the future (*10*). Although several analytical methods have been described for the individual quantification of belzutifan or cabozantinib in human plasma (*11-13*), no validated method is available for simultaneous quantification of both the agents. To address this gap, we developed and validated a high-performance liquid chromatography–ultraviolet (HPLC–UV) method for simultaneous measurement of belzutifan and cabozantinib in human plasma in accordance with the U.S. Food and Drug Administration Bioanalytical Method Validation Guidance (*14*).

2. Materials and Methods

2.1. Reagents and chemicals

Belzutifan was obtained from MedChemExpress (Monmouth Junction, NJ, USA). Cabozantinib and ibrutinib (internal standard, IS) were obtained from Toronto Research Chemicals Inc. (Ontario, Canada) (Figure 1). HPLC-grade acetonitrile, methanol, distilled water (Kanto Chemical Co., Inc., Tokyo, Japan), dimethyl sulfoxide (Fujifilm Wako, Osaka, Japan), and KH_2PO_4 (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) were used to prepare the HPLC mobile phase and stock/working solutions. Human plasma (pooled and individual) and ethylenediaminetetraacetic acid (EDTA)-2Na were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan).

2.2. Stock and working standard solutions

Stock solutions containing 1 mg/mL belzutifan and cabozantinib were prepared in acetonitrile. Stock solutions of IS were prepared in methanol at 1 mg/mL. Working solutions of belzutifan (250, 500, 2,500, 5,000, 10,000, and 20,000 ng/mL) and cabozantinib (125, 250, 2,500, 5,000, 10,000, and 20,000 ng/mL) were prepared by dilution with acetonitrile. The IS was further diluted with methanol to a final concentration of 2,500 ng/mL. All working solutions were stored at − 60°C.

2.3. Equipment and chromatographic conditions

All analyses were performed on an HPLC system (Jasco, Tokyo, Japan) equipped with PU-4180 pumps, a UV-4075 detector, and an AS-4550 autosampler. Chromatographic separation was achieved using a Capcell Pak C18 MG II reversed-phase column (250 × 4.6 mm i.d., 5 μm; Osaka Soda, Tokyo, Japan). The mobile phase consisted of 0.5% potassium dihydrogen phosphate (KH_2PO_4, pH 4.5) and acetonitrile (47/53, v/v), delivered at a flow rate of 1.0 mL/min, and analytes were detected at 230 nm. The column and autosampler temperatures were maintained at room temperature (22°C). The run time was 10.5 min.

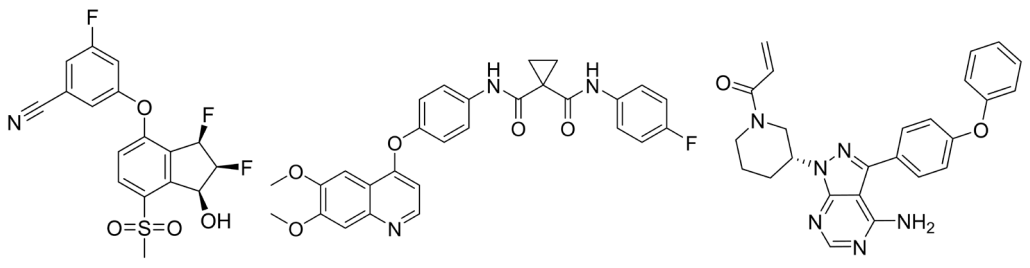
2.4. Internal standard (IS) selection

Ibrutinib, a Bruton's tyrosine kinase inhibitor (TKI) that is primarily used for the treatment of chronic lymphocytic leukemia, was selected as the IS, as it is unlikely to be co-administered in patients with RCC (*15*). In analytical methods for cabozantinib, TKIs are frequently used as ISs (*12,13*). Accordingly, we evaluated five TKIs (brigatinib, tirabrutinib, pirtobrutinib, erlotinib, and nilotinib) as potential IS candidates. However, their retention times (2.4, 5.4, 5.8, 6.0, and 8.9 min, respectively) overlapped with those of plasma matrix components or the analytes of interest, rendering them unsuitable for use as ISs. Ibrutinib provided appropriate chromatographic separation without interference.

2.5. Preparation of samples

Prior to analyses, human plasma and working solutions were thawed and vortexed. Briefly, blank plasma (50 μL) was spiked with 10 μL of belzutifan and 10 μL of cabozantinib and mixed by vortexing for 5 s, followed by the addition of 10 μL of IS and 120 μL of methanol chilled to − 60°C (total volume 200 μL). The mixture was vortexed for 1 min and centrifuged at 15,000 × *g* for 10 min at 4°C. Thereafter, 30 μL of the resulting supernatant was directly injected into the HPLC system for analysis.

2.6. Calibration curves and quantitation



Belzutifan Cabozantinib Ibrutinib

Figure 1. Chemical structures of belzutifan (left), cabozantinib (center), and ibrutinib (right).

Accuracy and linearity were assessed by analyzing a series of calibration standards for belzutifan (50, 100, 500, 1,000, 2,000, and 4,000 ng/mL) and cabozantinib (25, 50, 500, 1,000, 2,000, and 4,000 ng/mL). Intra- and inter-day precision and accuracy were evaluated by replicate analyses of five sets of plasma samples spiked with six concentrations of belzutifan and cabozantinib within the same day or over five consecutive days. Analytical validation procedures were performed in accordance with the recommendations of the FDA Guidance for the Validation of Bioanalytical Methods (*14*).

2.7. Stability studies

Stability was evaluated at 50, 1,000, and 4,000 ng/mL for belzutifan and at 25, 1,000, and 4,000 ng/mL for cabozantinib. To evaluate bench-top, short-term, long-term, and freeze–thaw stability, samples were maintained at room temperature (22°C) for 6 h, stored at 4°C for 24 h, stored at −60°C for 3 months, and subjected to three cycles of freezing at −60°C or below and thawing at room temperature, respectively. Stability was evaluated by analyzing these samples against calibration curves prepared from freshly spiked analytes, and the measured concentrations were compared with the nominal values.

2.8. Recovery

The recovery of belzutifan and cabozantinib was evaluated in five replicates of plasma samples spiked with six concentrations of each analyte and 50 µL of normal saline to determine the recovery ratio (%). The average recovery of each analyte (expressed as a percentage) was calculated by dividing the recovered amount by the original amount and multiplying by 100.

2.9. Selectivity and specificity

Selectivity was evaluated using seven different commercially available lots of blank human plasma without the addition of belzutifan, cabozantinib, or IS. In a previous study, we investigated the top 10 medications most frequently co-administered with cabozantinib in clinical practice in Japan, namely levothyroxine, amlodipine, ursodeoxycholic acid, acetaminophen, magnesium oxide, vonoprazan, esomeprazole, furosemide, febuxostat, and nifedipine (*13*). Six frequently co-administered medications (Table 1) were evaluated under identical analytical conditions to assess potential interference, defined as the presence of peaks co-eluting with belzutifan, cabozantinib, or the IS at a resolution of < 1.5.

2.10. Ethical considerations

This study used commercially available pooled and individual human plasma samples (Cosmo Bio Co., Ltd., Tokyo, Japan; catalog No. 12271450 and 12271440), which was supplied without donor identifiers or individual-level clinical information and was used solely as an *in vitro* assay matrix. In accordance with the Ethical Guidelines for Medical and Biological Research Involving Human Subjects in Japan and the institutional policy of Meiji Pharmaceutical University, this study was considered outside the scope of ethics committee review because it used only a generally available commercial research reagent.

3. Results and Discussion

3.1. Chromatography, selectivity, and specificity

Representative chromatograms of blank human plasma samples are shown in Figure 2a. The retention times of belzutifan, cabozantinib, and the IS were 6.1, 9.5, and 8.3 min, respectively. The chromatographic resolutions of belzutifan and cabozantinib from the IS were 9.4 and 4.0, respectively. No interference from endogenous plasma components was observed at these retention times (Figures 2b and 2c).

The selectivity and specificity of the method were further evaluated using plasma matrices obtained from seven different commercially available lots. No endogenous interference with belzutifan, cabozantinib, or the IS was detected. In addition, six concomitant medications frequently co-administered in oncology practice were evaluated under the same chromatographic conditions, and none interfered with the retention times of belzutifan, cabozantinib, or the IS (Table 1). These

Table 1. Medications included in the specificity evaluation

Medications	Retention time (min)	Rs *vs*. belzutifan peak	Rs *vs*. cabozantinib peak	Rs *vs*. IS peak
Acetaminophen	3.6	8.7	13.7	12.2
Amlodipine	3.8	11.0	17.2	15.4
Esomeprazole	4.6	11.1	18.7	16.7
Febuxostat	6.4	2.8	10.5	8.2
Furosemide	4.1	11.5	18.1	16.4
Levothyroxine	3.8	12.2	18.6	16.7

Medications that are used concomitantly with belzutifan or cabozantinib in clinical practice were analyzed under the conditions used for the present method and did not interfere with belzutifan, cabozantinib, or the IS. Rs: resolution.

findings support the robustness and clinical applicability of the assay in settings where polypharmacy is common. Selection of the IS was carefully considered during method development. Several TKIs were initially evaluated; however, multiple candidates showed overlap with matrix-derived peaks or insufficient chromatographic separation from the analytes. Ibrutinib was ultimately selected as the IS because of its favorable chromatographic behavior and the low likelihood of co-administration with belzutifan and cabozantinib in patients with RCC.

3.2. Calibration curve and quantitation

Calibration curves for belzutifan and cabozantinib were constructed using the least-squares method. For belzutifan, the six-point calibration curve was expressed as $y = 0.002x - 0.0035$ ($r^2 = 0.9999$) and was linear over a concentration range of 50–4,000 ng/mL. For cabozantinib, the six-point calibration curve was expressed as $y = 0.0027x + 0.0559$ ($r^2 = 0.9998$) and was linear over a concentration range of 25–4,000 ng/mL, where y represents the peak height ratio of the analyte to the IS and x represents the analyte concentration (ng/mL). The calibration ranges established in the present study sufficiently cover clinically observed plasma concentrations. Reported steady-state plasma concentrations in clinical trials are 214–1,305 ng/mL for belzutifan and 383–1,148 ng/mL for cabozantinib (*3,16*). Therefore, the validated ranges are considered appropriate for TDM and pharmacokinetic evaluation in patients receiving these agents. Compared with previously reported HPLC–UV methods for the individual quantification of belzutifan or cabozantinib, the present assay provides comparable or broader calibration ranges while requiring only 50 µL of plasma. The use of a simple protein-precipitation procedure will also enable rapid sample preparation suitable for routine clinical analysis. The characteristics of previously reported analytical methods and the present method are summarized in Table 2. For belzutifan, the present assay provides a wider calibration range and lower plasma volume requirement than previously reported HPLC–UV methods (*11-13*), although with a modestly longer run time. For cabozantinib, the present method achieved a comparable calibration range with a substantially shorter run time than that reported by Maruyama *et al.* (*12*) and a lower limit of quantification than that reported by Yasu *et al.* (*13*). Collectively, these findings indicate that the present method maintains analytical performance comparable to or better than those of previously reported HPLC–UV assays while enabling simultaneous quantification of both analytes.

3.3. Precision, accuracy, and recovery

The intra- and inter-day coefficients of variation (CVs), accuracies, and recoveries for belzutifan and

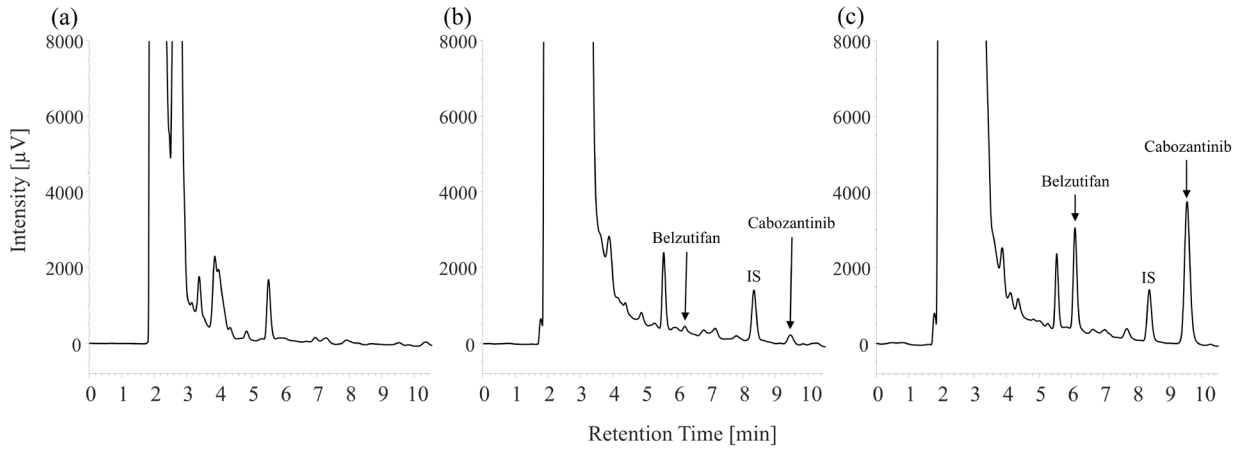


Figure 2. Chromatograms of (a) blank plasma sample, (b) plasma sample containing belzutifan 50 ng/mL (limit of quantitation (LOQ)) and cabozantinib 25 ng/mL (LOQ), and (c) plasma sample containing belzutifan 1,000 ng/mL and cabozantinib 1,000 ng/mL.

Table 2. Comparison of present and previous methods of measuring plasma belzutifan and cabozantinib concentrations using HPLC-UV

	Present method	Kolli *et al.* (*11*)	Maruyama *et al.* (*12*)	Yasu *et al.* (*13*)
Analysis time (min)	10.5	5	18	8
Required plasma volume (µL)	50	75	100	50
Target agent	Belzutifan and cabozantinib	Belzutifan	Cabozantinib	Cabozantinib
Calibration range (ng/mL)	Belzutifan 50–4,000, Cabozantinib 25–4,000	75–3,000	25–4,000	50–5,000

cabozantinib are summarized in Table 3. For belzutifan, all intra- and inter-day CVs were below 5.41%. The intra-day accuracy ranged from −6.50% to 5.12%, whereas the inter-day accuracy ranged from −1.86% to 4.99%. For cabozantinib, all intra- and inter-day CVs were below 12.25%, with intra- and inter-day accuracies ranging from −5.47% to 0.96% and −1.55% to 4.95%, respectively.

These validation results satisfied the acceptance criteria defined in the U.S. FDA bioanalytical method validation guidelines (*14*), demonstrating adequate reproducibility and reliability for quantitative analysis. Both belzutifan and cabozantinib exhibit exposure-dependent toxicities, and clinical studies evaluating the combination of belzutifan and cabozantinib have recently been conducted. This combination therapy may become a treatment option for RCC in the future. Despite this increasing clinical relevance, no analytical method for simultaneous quantification of these agents in human plasma has previously been reported. Therefore, the validated precision and accuracy achieved in the present study support the potential utility of this assay for TDM applications in routine laboratory settings.

3.4. Stability

Stability studies were conducted under conditions relevant to the handling and storage of clinical samples. Belzutifan and cabozantinib in human plasma were stable for 6 h at room temperature (22°C), for 24 h at 4°C, for 3 months at −60°C, and after three freeze–thaw cycles. The results are summarized in Table 4. These findings indicate that the present assay is sufficiently robust for routine clinical use, including sample transport, temporary refrigerated storage, long-term frozen storage, and repeated freeze–thaw handling during reanalysis procedures.

3.5. Clinical applicability and potential utility for TDM

We established and validated the first HPLC–UV method capable of simultaneously quantifying belzutifan and cabozantinib in human plasma. Because HPLC–UV systems are widely available in hospital laboratories, the developed assay may provide a practical and accessible platform for TDM implementation in routine clinical practice. This method may be particularly useful in patients with RCC complicated by hepatic and/or renal impairment. Although belzutifan exposure is reportedly unaffected by mild hepatic dysfunction and mild-to-moderate renal dysfunction (*3*), cabozantinib exposure

Table 3. Intra- and inter-day precision and accuracy results for belzutifan and cabozantinib in human plasma using the proposed HPLC method (*n*=5)

Added belzutifan concentration (ng/mL)	Intra-day (*n* = 5)			Inter-day (*n* = 5)			Recovery (%)
	Detected (ng/mL) mean ± SD	CV (%)	Accuracy (%)	Detected (ng/mL) mean ± SD	CV (%)	Accuracy (%)	
50	52.56 ± 2.84	5.41	5.12	52.23 ± 1.90	3.64	4.46	93.08%
100	95.70 ± 4.46	4.66	−4.30	104.99 ± 4.57	4.36	4.99	90.65%
500	467.48 ± 6.56	1.40	−6.50	492.51 ± 11.40	2.31	−1.50	95.54%
1000	948.14 ± 19.40	2.05	−5.19	997.28 ± 19.56	1.96	−0.27	99.52%
2000	1919.93 ± 10.72	0.56	−4.00	1969.00 ± 48.19	2.45	−1.55	101.17%
4000	3792.91 ± 100.38	2.65	−5.18	3925.42 ± 90.76	2.31	−1.86	100.10%
Added cabozantinib concentration (ng/mL)	Detected (ng/mL) mean ± SD	CV (%)	Accuracy (%)	Detected (ng/mL) mean ± SD	CV (%)	Accuracy (%)	Recovery (%)
25	24.07 ± 0.99	4.13	−3.70	25.66 ± 3.14	12.25	2.63	103.41%
50	50.48 ± 0.80	1.59	0.96	52.48 ± 1.73	3.30	4.95	106.76%
500	472.65 ± 18.60	3.94	−5.47	494.24 ± 1.73	0.35	−1.15	102.67%
1000	959.47 ± 29.57	3.08	−4.05	997.72 ± 26.16	2.62	−0.23	97.24%
2000	1928.01 ± 48.21	2.50	−3.60	1972.51 ± 69.00	3.50	−1.37	95.20%
4000	3846.98 ± 110.19	2.86	−3.83	3937.80 ± 158.42	4.02	−1.55	96.11%

Table 4. Stability analysis

Stability test conditions	Ratio of the plasma belzutifan concentration to the spiked value (%)			Ratio of the plasma cabozantinib concentration to the spiked value (%)		
	50 ng/mL (mean ± S.D.)	1,000 ng/mL (mean ± S.D.)	4,000 ng/mL (mean ± S.D.)	25 ng/mL (mean ± S.D.)	1,000 ng/mL (mean ± S.D.)	4,000 ng/mL (mean ± S.D.)
Benchtop storage (22°C, 6 h)	98.56 ± 5.55	101.33 ± 0.24	101.63 ± 0.85	87.48 ± 6.48	97.80 ± 0.61	99.40 ± 1.32
Short-term storage (4°C, 24 h)	91.71 ± 12.76	90.59 ± 2.10	95.44 ± 1.89	98.30 ± 7.99	102.25 ± 1.83	101.08 ± 0.69
Long-term storage (−60°C, 3 months)	94.27 ± 13.08	97.54 ± 1.65	97.49 ± 2.93	96.27 ± 11.53	98.56 ± 1.60	98.05 ± 3.86
Freeze–thaw, three cycles (−60°C to room temperature)	103.01 ± 4.38	97.59 ± 1.56	95.31 ± 1.80	88.69 ± 7.03	96.97 ± 0.93	97.79 ± 1.50

increases in such patients, with the AUC elevated by 6–81% (*17*). In addition, cabozantinib has a prolonged elimination half-life of approximately 99 h and requires approximately 15 days to reach steady state (*18*). Consideration of these pharmacokinetic characteristics may support individualized dosing strategies and extended dosing intervals aimed at reducing toxicity and treatment costs (*19*). Belzutifan exposure is also influenced by genetic polymorphisms. In UGT2B17/CYP2C19 dual poor metabolizers, the elimination half-life is prolonged by 14.3 h compared with that in UGT2B17 extensive metabolizers and CYP2C19 non-poor metabolizers (*3*). Therefore, TDM of belzutifan may facilitate optimization of systemic exposure and individualized dose adjustment according to genetic background.

The present method has several implementation-oriented advantages. First, the assay was established using cost-efficient and widely available HPLC–UV instrumentation while meeting FDA bioanalytical validation criteria, thereby lowering the barrier to clinical adoption. Second, the workflow relies on a simple protein-precipitation procedure using chilled methanol and requires only 50 μL of plasma, resulting in low per-sample costs and minimal hands-on time. Third, the absence of interference from commonly co-administered medications supports applicability in oncology settings where polypharmacy is common. Collectively, these features suggest that the assay has strong potential for integration into routine clinical workflows and multicenter implementation.

This study has certain limitations. First, validation was performed exclusively using spiked plasma samples, and patient-derived clinical samples were not evaluated. Additional studies using actual patient samples, including specimens with high and low drug concentrations, are warranted to further confirm clinical applicability. Second, comparison with LC–MS/MS methods, which remain the gold standard for drug quantification, was not performed. Future studies incorporating cross-platform comparisons, such as Bland–Altman analysis, may help confirm analytical agreement and facilitate broader implementation of the method.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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**Address correspondence to:*
Takeo Yasu, Department of Clinical Pharmacodynamics and Toxicology Analytics, Meiji Pharmaceutical University, 2-522-1, Noshio, Kiyose, Tokyo 204-8588, Japan.
E-mail: yasutakeo@my-pharm.ac.jp

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