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Impact of Sample Preparation Methods on LC-MS/MS Analysis of Molecular Targeted Drugs

Yusuke Tanaka[1], Hideaki Yashima, Ph.D.[1], Takuya Araki, Ph.D. [1,2], Koujirou Yamamoto, Ph.D.[1,2]

[1]Department of Clinical Pharmacology and Therapeutics, Gunma University Graduate School of Medicine
[2]Department of Pharmacy, Gunma University Hospital, 3-39-15 Showa-machi, Maebashi 371-8511, Japan

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ABSTRACT

The therapeutic efficacy of molecular targeted drugs (MTD)s and the risk of certain adverse drug effects are closely related to their blood concentrations, highlighting the importance of optimizing dosage based on therapeutic drug monitoring. In this study, we compared four pretreatment methods for the analysis of 15 MTDs by LC-MS/MS: protein precipitation (PPT), solid-phase extraction (SPE) using a reversed-phase (RP) column, SPE using a mixed-mode cation-exchange RP column, and supported liquid extraction (SLE), and evaluated their effects on recovery rates and matrix effects. While PPT showed high recovery rates (>80%) for 8 out of 15 compounds, certain highly polar MTDs exhibited significant peak intensity decreases with repeated analyses, indicating potential issues with ion suppression due to impurities. SPE using a reversed-phase column (HLB) resulted in low recovery rates for 12 out of 15 compounds. In contrast, SPE using an MCX column yielded high recovery rates (>80%) for 14 out of 15 compounds but exhibited substantial matrix effects for 9 out of 15 compounds (matrix factors >2). Addressing these matrix effects required sample dilution, and achieving higher sensitivity would necessitate extensive method adjustments tailored to each compound. SLE demonstrated the most favorable results, with the largest number of compounds showing acceptable recovery rates and minimal matrix effects. In conclusion, these findings, based on standard protocols from product manuals, suggest that while method optimization could improve performance for specific compounds, SLE appears to be the most suitable first-choice pretreatment method for the LC-MS/MS analysis of MTDs due to its balance of recovery and matrix effect control.

Keywords: LC-MS/MS, pretreatment, SPE, PTT, SLE, matrix efficacy

*Corresponding author,
e-mail: tkyaraki@gunma-u.ac.jp T. Araki)

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1. Introduction

Molecular targeted drugs (MTDs) play a pivotal role in the treatment of various malignancies. These agents target key molecular pathways that regulate cancer cell proliferation and survival, and their use has expanded significantly in recent years due to their higher safety and efficacy compared to conventional chemotherapeutic agents. It has been reported that the therapeutic efficacy and the risk of certain adverse effects of MTDs are closely related to their concentrations in blood or tissue, highlighting the importance of dose optimization based on therapeutic drug monitoring (TDM)[1–5].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is widely employed as a highly sensitive and precise method for quantifying MTDs [2–5]. The sensitivity, accuracy and reproducibility of LC-MS/MS analysis are strongly influenced by the sample pretreatment method. With increasing recognition of the significance of intracellular drug concentrations, there is a growing demand for more sensitive and precise analytical methods. Therefore, the optimization of pretreatment methods for biological samples has become increasingly important. Commonly used pretreatment methods include protein precipitation (PPT) and solid-phase extraction (SPE), while the application of supported liquid extraction (SLE) has also been advancing in recent years. Each method has its unique advantages and challenges, requiring careful selection based on the analytes and objectives of the analysis.[6–10]

PPT is widely adopted due to its simplicity and rapid processing. However, as it primarily involves precipitating proteins with organic solvents, many impurities remain in the sample, which can lead to matrix effects. Additionally, high impurity concentrations and the aqueous nature of the solvents often make sample concentration difficult, posing a challenge. SPE, while dependent on the type and performance of the column used, generally offers higher purification efficiency than PPT. However, it may require significant time for optimization, and in some cases, matrix effects with SPE-treated samples can be greater than those with PPT-treated samples. SLE combines the advantages of liquid-liquid extraction, enabling simple and highly reproducible operations. While SLE has already been applied to the analysis of certain MTDs, its performance as a pretreatment method for LC-MS/MS analysis has not been thoroughly investigated.

In this study, we compared four pretreatment methods—PPT, SPE using reversed-phase (RP) columns, SPE using mixed-mode cation exchange RP columns, and SLE—for the analysis of MTDs by LC-MS/MS. We evaluated their recovery ratio and impact on matrix effects. The findings of this study are expected to contribute to the selection of reliable analytical methods for MTDs, thereby advancing therapeutic monitoring and pharmacokinetic research.

2. Methods

2.1 Materials

Abemaciclib and cabozantinib were purchased from LKT Laboratories, Inc. (St. Paul, Minnesota, USA). The metabolites of abemaciclib (M2, M18, M20) were purchased from MedChemExpress (Monmouth Junction, USA). Sunitinib was purchased from LC Laboratories (Woburn, MA, USA), N-desethyl sunitinib was purchased from Toronto Research Chemicals (Toronto, Canada). Asciminib was purchased from

selleck chemicals (Houston, USA). Axitinib was purchased from R&D Systems (Minneapolis, USA). Osimertinib, pazopanib and ponatinib were purchased from ChemScence (Monmouth Junction, USA). Dasatinib and tirabrutinib were purchased from Cayman Chemical (Ann Arbor, USA). Gilteritinib was purchased from BioVision (Milpitas, USA). Human serum pool (P/N. 12181201, lot#. BJ20683A) was purchased from COSMO Bio Co., LTD (Tokyo, Japan). Oasis PRiME HLB 1 cc Vac Cartridge (30 mg) and Oasis PRIME MCX Cart 1cc Vac Cartridge (30 mg) were purchased from Waters. (Milford, USA). ISOLUTE SLE+ 400 µL 96-well plates were purchased from Biotage Japan Ltd. (Tokyo, Japan).

All other reagents were obtained from commercial sources and were LCMS or HPLC-grade or special-grade reagents.

2.2 Sample preparation

The stock solutions of cabozantinib, dasatinib, gilteritinib, Osimertinib, pazopanib, sunitinib, and N-desethyl sunitinib were prepared in methanol at a concentration of 1.0 mg/mL or 0.5 mg/mL. Stock solutions of other compounds were prepared in DMSO at a concentration of 1.0 mg/mL. A mixture of all target compounds, each at a concentration of 1 µg/mL, was prepared in 50% methanol and subsequently diluted to 100 ng/mL in 25% methanol. Stock solutions were stored at -20° C, and mixtures were stored at 4° C under light-protected conditions.

2.3 Sample pretreatment

For the PPT method, an equal volume of acetonitrile was added to the sample, which was then vigorously mixed and centrifuged. The resulting supernatant was collected and two-fold dilution of the supernatant with Milli-Q water used as the sample for analysis.

For RP SPE and mixed-mode cation exchange RP SPE, an Oasis PRiME HLB column (waters) and an Oasis PRIME MCX column (waters) were used, respectively, following the manufacturer's instructions. Briefly, for RP SPE, a sample (100 µL of serum) diluted 10-fold with Milli-Q water (total 1 mL) was applied to the HLB column and washed with 1 mL of 5% methanol. Finally, the sample was eluted with an acetonitrile/methanol (90:10) mixture. The eluate was evaporated to dryness under reduced pressure at room temperature and reconstituted in 100 µL of 25% methanol. For the mixed-mode cation exchange RP SPE, a sample (100 µL of serum) diluted 10-fold with 100 mM ammonium formate and 2% H_3PO_4 (total 1 mL) was applied to the MCX column and washed with 1 mL of methanol. Finally, the sample was eluted with 1 mL of 5% ammonium hydroxide in methanol. The eluate was evaporated to dryness under reduced pressure at room temperature and reconstituted in 100 µL of 25% methanol.

An ISOLUTE SLE+ 400 µL 96-well plate (SLE array plate) (Biotage, Uppsala, Sweden) was used for SLE pretreatment. The processes were optimized for each compound according to the manufacturer's instruction. Briefly, a mixture of 100 µL of blank serum, 10 µL of standard solution mixture or 50% methanol, and 290 µL of Milli-Q water or 1% aqueous ammonia was applied to SLE plate. After a 5-minute incubation, 900 µL of methyltert-butyl ether (MTBE) or ethyl-acetate was added as the elution buffer. After another 5-minute wait, an additional 900 µL of same elution buffer was applied. The collected eluate was evaporated to dryness under reduced pressure at room temperature. The residue was reconstituted in 100 µL of 25%

methanol.

2.4 Detection of MTDs by LC-MS/MS

A tandem quadrupole mass spectrometer was used to detect MTDs. Xevo-TQ (Waters, Milford, USA) equipped with an electrospray ionization (ESI) source in positive ionization mode was operated with the following ionization parameters: capillary voltage, 3.0 kV; desolvation temperature, 500° C; source temperature, 150° C; desolvation gas flow, 950 L/h; cone gas flow, 50 L/h. The target m/z, cone voltages, and collision energy for each compound are shown in Table 1. Liquid chromatography (LC) was performed using an ACQUITY UPLC® system (Waters) equipped with an ACQUITY UPLC BEH® C18 separation column (2.1 mm × 50 mm, 1.7 μm) (Waters). The LC conditions were as follows: column temperature, 40° C; mobile phase, 10 mM ammonium formate in Milli-Q water (A) and acetonitrile (B); flow rate, 0.3 mL/min; and gradient program, 30% to 90% B over 5.0 minutes, 90% to 30% B over 0.5 minutes, followed by 30% B for 1.5 minutes. The temperature in the autosampler was set at 10° C, and the injection volume was set at 5 μL.

2.5 Assay validation

The extraction recoveries were determined by comparing the peak area of each compound in pretreated samples with that of standard mixture of compounds spiked into pretreated blank serum at the same concentrations.

The matrix effects were determined by calculating the ratio of peak areas of each compound in pretreated blank serum to those prepared in the mobile phase under initial conditions at the same concentrations.

3. Results

3.1 Detection of MTDs

The chromatograms of the MTDs mixture were shown in Figure 1, and their retention times are summarized in Table 1.

Table 1. Instrument settings of Molecular target drugs

compound	transition	Cone energy (V)	Collision energy (V)	retention time (min)
abemaciclib	507.2/245.0	28	62	1.68
M2	479.2/245.0	30	56	1.33
M18	495.2/337.1	30	48	0.84
M20	523.2/337.1	28	46	1.04
asciminib	450.1/183.3	42	60	2.74
axitinib	387.2/356.1	30	22	2.12
cabozantinib	502.1/307.1	52	56	3.42
dasatinib	488.2/161.1	48	56	1.77
gilteritinib	553.5/436.4	40	34	1.32
osimertinib	500.2/72.0	24	24	2.55
pazopanib	438.3/341.4	50	44	2.01
ponatinib	533.1/127.0	30	76	2.70
sunitinib	399.4/283.2	30	30	1.55
N-desethyl sunitinib	371.4/283.2	22	22	1.14
tirabrutinib	455.3/320.2	40	32	2.50

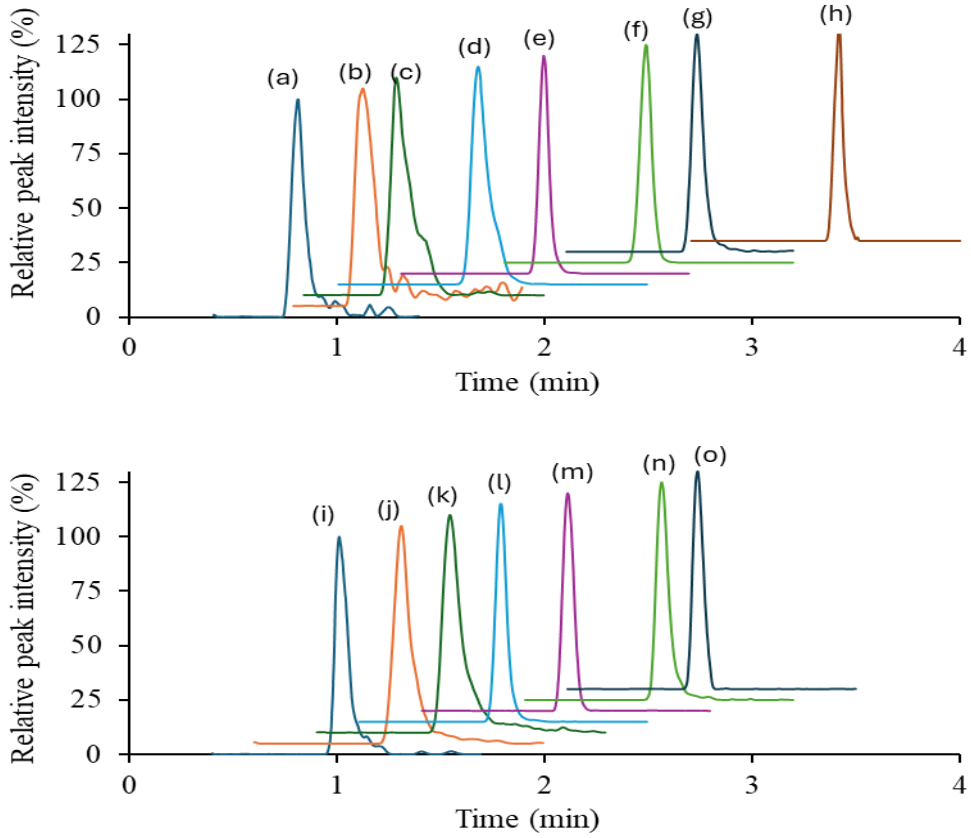

Figure 1. Typical chromatograms of molecular targeted drugs in human serum.
(a) M18, (b) N-desethyl sunitinib, (c) gilteritinib, (d) abemaciclib, (e) pazopanib, (f) tirabrutinib, (g) ponatinib, (h) cabozantinib, (i) M20, (j) M2, (k) sunitinib, (l) dasatinib, (m) axitinib, (n) osimertinib, (o) asciminib

3.2 Conditions in pretreatment of SLE pretreatment

For gilteritinib and pazopanib, 1% aqueous ammonia was used for dilution, and ethyl acetate served as the elution buffer. For osimertinib, Milli-Q water was used for dilution, and TBME was used as the elution buffer. For all other compounds, 1% aqueous ammonia was used for dilution, and TBME was employed as the elution buffer.

3.3 Recovery rate

The recovery rates of the target compounds are presented in Figure 2. Using PPT treatment, the recovery rates for abemaciclib, asciminib, cabozantinib, dasatinib, osimertinib, pazopanib, ponatinib, and tirabrutinib exceeded 80%. However, for other compounds, quantification was impossible due to a dramatic decrease in peak intensity with each measurement. When HLB treatment was applied, the recovery rates of asciminib, axitinib and tirabrutinib exceeded 80%. In contrast, using MCX treatment, recovery rates of abemaciclib, M2, M18, M20, asciminib, axitinib, cabozantinib, dasatinib, gilteritinib, pazopanib, ponatinib, sunitinib, N-desethyl sunitinib, and tirabrutinib exceeded 80%. Similarly, SLE treatment yielded recovery rates exceeding 80% for M20, asciminib, axitinib, cabozantinib, gilteritinib, pazopanib, ponatinib, sunitinib, N-desethyl sunitinib, and tirabrutinib.

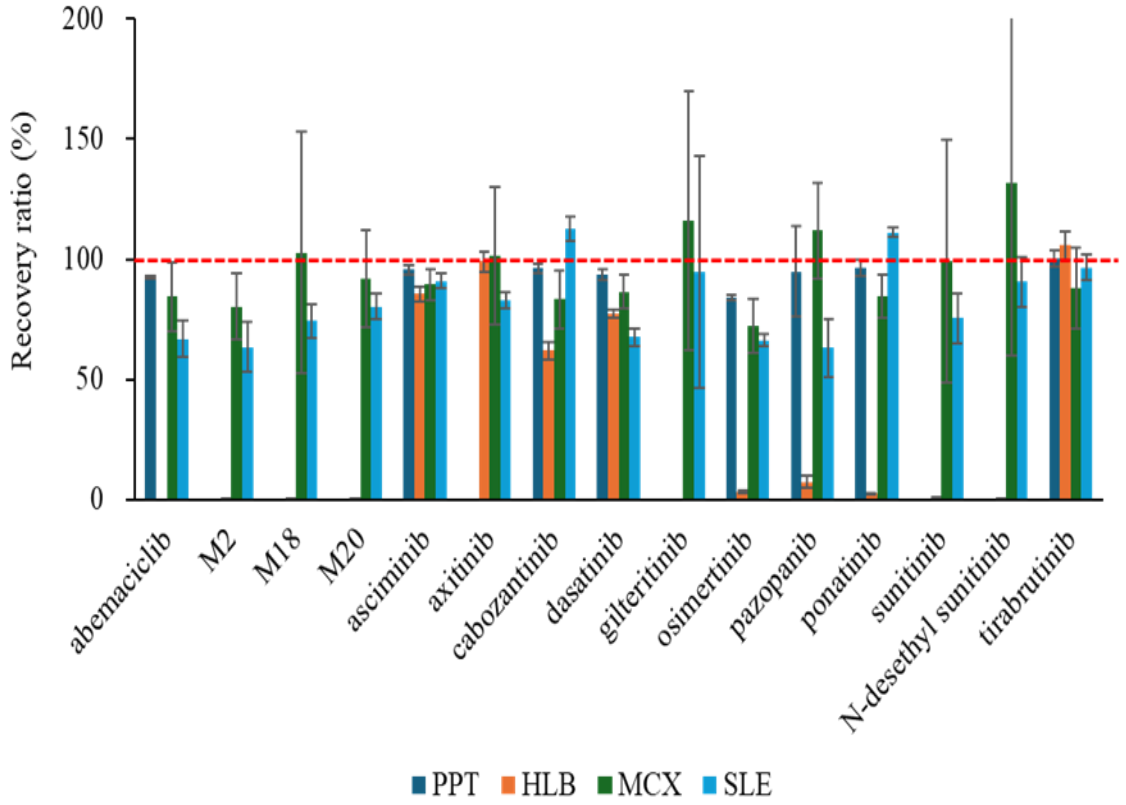

Figure 2. Recovery rates.
PPT; protein precipitation, SLE; supported liquid extraction

3.3 matrix efficacy

The matrix factors are shown in Figure 3. Using PPT treatment, matrix coefficients for asciminib and dasatinib were within 1.00 ± 0.15. However, the coefficients for abemaciclib, cabozantinib, and ponatinib were <0.8, and those for osimertinib, and pazopanib were >1.2, with the matrix factor for osimertinib being particularly large at approximately 14.5. For other compounds, quantification was impossible due to a dramatic decrease in peak intensity with each measurement. Applying HLB treatment, the matrix coefficient for abemaciclib, M2 and ponatinib were within 1.00 ± 0.15, whereas those for M20, asciminib, axitinib, cabozantinib, osimertinib, pazopanib, sunitinib, N-desethyl sunitinib, and tirabrutinib exceeded 2. When MCX treatment was used, no compound exhibited a matrix coefficient within 1.00 ± 0.15. The coefficient for M2, M18, axitinib, cabozantinib, gilteritinib, osimertinib, pazopanib, sunitinib, and tirabrutinib exceeded 2, with osimertinib showing an exceptionally high matrix factor of approximately 11.5. Using SLE treatment, the matrix coefficients for abemaciclib, M18, M20, axitinib, dasatinib, osimertinib, pazopanib, tirabrutinib were within 1.00 ± 0.15. Furthermore, the coefficients for abemaciclib, M18, M20, asciminib, axitinib, dasatinib, osimertinib, pazopanib, sunitinib, N-desethyl sunitinib, and tirabrutinib were within 1.00 ± 0.20. No compounds exhibited a matrix coefficient below 0.5 or above 2.

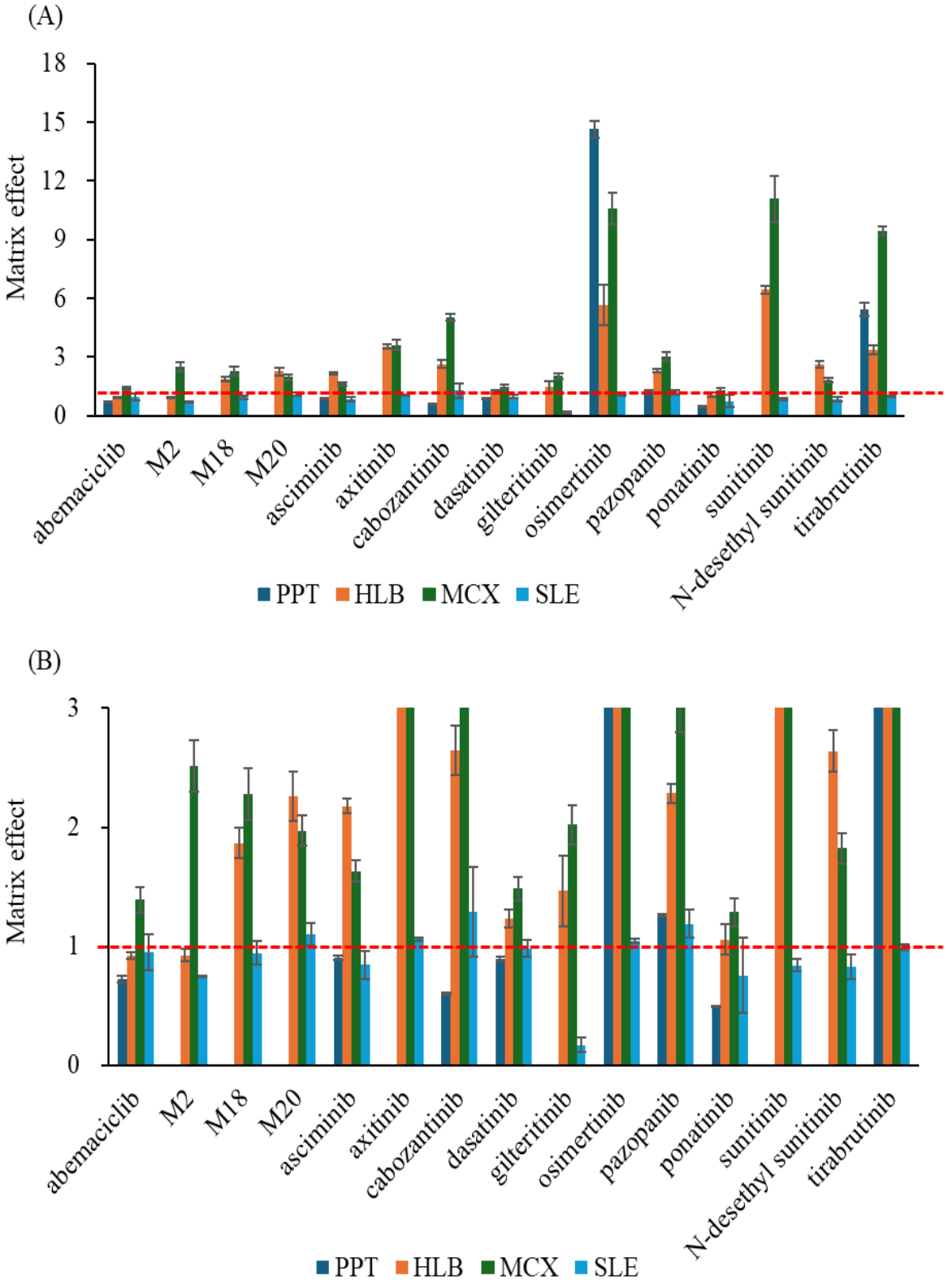

Figure 2. Recovery rates.
PPT; protein precipitation, SLE; supported liquid extraction

4. Discussion and Conclusion

In this study, we evaluated the impact of different pretreatment methods on the recovery rates and matrix effects of molecular targeted drugs (MTDs) using LC-MS/MS analysis.

Using PPT treatment, the risk of losing target compounds is low unless co-precipitation with proteins occurs. Consequently, eight out of 15 compounds achieved recovery rates exceeding 80%. However, for M2, M18, M20, axitinib, gilteritinib, sunitinib, N-desethyl sunitinib, and tirabrutinib, the peak intensity decreased dramatically with repeated measurements, making quantification impossible. This phenomenon is likely due to impurities in the samples obtained from PPT treatment, which accumulated on the MS interface and reduced ionization efficiency. Notably, this trend was not observed for less polar compounds within the same MTD category. These findings suggest that highly polar MTDs are more susceptible to contamination effects on the MS interface, necessitating caution when using PPT treatment for their analysis.

When using HLB column, which are considered versatile for SPE, the recovery rates for MTDs were generally low. Only three out of 15 compounds achieved recovery rates exceeding 80%. Additionally, nine compounds showed matrix factors exceeding 2. Although the recovery rate for dasatinib was slightly below 80%, it could potentially be improved by adjusting the elution solvent. However, for most MTDs, HLB column may not be suitable due to suboptimal recovery rates and significant matrix effects.

MCX treatment demonstrated superior recovery rates, with 14 compounds achieving recovery rates exceeding 80%, compared to HLB. However, the matrix effects were pronounced, with nine compounds exhibiting matrix factors exceeding 2, and the maximum matrix factor reaching approximately 11. These significant matrix effects could negatively impact analytical accuracy, necessitating sample dilution. Conversely, sample concentration is challenging, making MCX treatment unsuitable for analyses requiring higher sensitivity. This result aligns with the findings of Turkovic *et al.*, who reported that samples prepared with MCX treatment had greater matrix coefficients compared to PPT treatment in the analysis of anastrozole [11]. On the other hand, there are reports of reduced matrix effects by modifying protocols to optimize for compound-specific characteristics [12]. Therefore, while MCX treatment offers high recovery rates for many MTDs, it requires careful optimization of conditions tailored to individual compounds to mitigate matrix effects.

In contrast, SLE demonstrated superior performance in both recovery rates and matrix effects compared to other pretreatment methods. Recovery rates exceeded 80% for 10 compounds, and the remaining compounds also achieved recovery rates of over 60%. While some method modifications may be required to improve the recovery rates of compounds that did not reach 80%, matrix effects were generally negligible for most compounds. Therefore, minor adjustments, such as increasing the volume of organic solvents, are likely sufficient to address these issues. These results suggest that recovery rates could be further improved with minimal modifications. Thus, SLE is a promising first-choice pretreatment method for MTDs analysis using LC-MS/MS.

In conclusion, while HLB and

MCX can be utilized under specific conditions, they may require extensive method modifications and optimization, limiting their applicability for high-precision analysis of a broad range of MTDs. On the other hand, SLE exhibits excellent characteristics in terms of both recovery rates and matrix effects, making it a promising pretreatment method capable of meeting the increasing demand for high-precision and high-sensitivity analysis. Future studies should focus on further optimizing SLE-based analytical methods and exploring their applicability to other analytical targets.

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