Abstract
Therapeutic drug monitoring (TDM) is a clinical practice that designs personalized medication for patients with blood concentrations of the drug. TDM approach is used for many drugs, including immunosuppressant, antifungal, antiarrhythmic, and anti-cancer drugs. Combination therapies are often adopted in TDM. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a useful analytical method in such cases. However, the development of a simultaneous LC-MS/MS analytical method is difficult owing to the differences in MS sensitivity and the therapeutic range of each drug. In order to avoid saturation of the detector, in-source collision induced dissociation (CID) was used to reduce the ion inlet. In this study, we investigated the in-source CID behavior of 13 compounds of drugs and metabolites in TDM practice. As a result, all compounds provided a sharp reduction of ion inlet over the threshold ion guide voltage. In addition, a shift to the higher concentration of the calibration range was observed according to such changes. The intensity and linearity data in this study that all 13 drugs could be analyzed under in-source CID conditions simultaneously. These results might be useful for TDM of combination therapy in clinical practice.

Keywords: LC-MS/MS; In-source CID; TDM; Combination therapy; Calibration curve

1. Introduction
Therapeutic drug monitoring (TDM), which is the medication management approach with the clinical findings such as therapeutic and adverse effects and the drug blood concentration [1,2]. Therefore, TDM is a personalized medicine approach. TDM approaches are adopted for many medications, such as immunosuppressive drugs [3], antibacterial drugs [4], antiarrhythmic drugs [5], antiepileptic drugs [6] and antineuroblastoma drugs in recent years [7-9]. In addition, TDM also is useful for the confirmation of adherence in pharmaceutical cares [6,10] and for assessment of combination therapies. However, the combination therapies often induce drug-drug interaction in such as metabolic enzyme and drug transporter. They might induce the increasing of the blood concentration of drugs and the side effects finally. Accordingly, the simultaneous drug analysis in blood is desired for TDM in clinical practice [10]. As routine analysis methods for TDM, immunoassay [13-15] and LC-MS/MS method [16,17] are generally used in recent. Although, immunoassay methods provide high sensitivity and simple handling procedure, only one analyte could be analyzed every specimen.

*Corresponding author: Masamitsu MAEKAWA
Tel: +81-22-717-7541; Fax: +81-22-717-7545
E-mail: m-maekawa@hosp.tohoku.ac.jp

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Therefore, they could not be applied for the simultaneous analysis. In other, LC-MS/MS analysis methods are used for simultaneous analysis for multiple components such as drugs administered in combination therapy by only one specimen. However, both therapeutic range and the MS/MS linearity need to be considered for the designing of simultaneous analysis using LC-MS/MS. Therapeutic ranges every drug vary based on the difference of pharmacokinetics and pharmacodynamics parameters every drug. In other, the linearity ranges in MS are specified with physico-chemical properties based on the chemical structure. Recently, we proposed the application of the in-source collision induced dissociation (CID) for adjustment of MS/MS ion abundance aimed to taking advantage of LC-MS/MS [18-20]. This strategy shifts the linearity range to high concentration by the reduction of ion inlet via orifice probe and supports the development the simultaneous analysis methods with adjusting the linearity range by orifice voltage. In previous report, we adopted the strategy for the basic endogenous metabolites [18], fluoropyrimidine antineoplastic drugs [19], and the drugs such as non-steroidal anti-inflammatory drugs and diuretics [20]. In this study, we aimed to verify the strategy for major drugs used frequently in TDM practice (Fig. 1). Mycophenolic acid was chosen as a representative immunosuppressant drug (Fig. 1A). Voriconazole and itraconazole are both antifungal drug used for treatment or prevention of fungal infections (Fig. 1B and C). Hydroxyitraconazole is an active metabolite of itraconazole (Fig. 1D). Pilsicainide and cibenzoline are antiarrhythmic drug classified as group class 1c and class 1a of Vaughan-Williams classification, respectively (Fig. 1E and F). Imatinib is the first anticancer drug whose efficacy for TDM has been established and used for chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia (Fig. 1G). In other, pazopanib (Fig. 1H), axitinib (Fig. 1I), sunitinib (Fig. 1J), N-desethyl sunitinib (K), sorafenib (Fig. 1L) are used for treatments of solid tumors such as for renal cell carcinoma, hepatocellular carcinoma and gastrointestinal stromal tumor.

![Chemical structures of analytes](image)

**Fig. 1.** Chemical structures of analytes; mycophenolic acid (A), voriconazole (B), itraconazole (C), hydroxyitraconazole (D), pilsicainide (E), cibenzoline (F), imatinib (G), pazopanib (H), axitinib (I), sunitinib (J), N-desethyl sunitinib (K), sorafenib (L), and sorafenib N-oxide.
Several active metabolites were also selected as analytes (Fig. 1K and M). After the behaviors of in-source CID of analytes are investigated, we demonstrate the development of the simultaneous LC-MS/MS analysis method.

2. Materials and methods

2.1. Chemicals

Mycophenolic acid, hydroxy itraconazole, pazopanib, sunitinib, N-desethyl sunitinib, sorafenib, sorafenib N-oxide, mycophenolic acid-2H3, voriconazole-2H3, itraconazole-2H4, hydroxyitraconazole-2H5, imatinib-2H8, axitinib-2H8, and sorafenib-2H8, were purchased from Toronto Research Chemicals, Inc (Toronto, Canada). Voriconazole was purchased from Tokyo Chemical Industry (Tokyo, Japan). Pilsicainide hydrochloride was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Imatinib was purchased from Cayman Chemical (Ann Arbor, MI, USA). Axitinib was purchased from Selleck Chemicals (Houston, TX, USA). Disopyrimide, ammonium formate, methanol for HPLC analysis grade and formic acid was FUJIFILM WAKO Pure Chemicals (Osaka, Japan). Cibenzoline and dimethylcibenzoline were donated from Astellas Pharma.

Fig. 2. Peak intensities of tested 13 compounds with various Q-array; mycophenolic acid (A), voriconazole (B), itraconazole (C), hydroxyitraconazole (D), pilsicainide (E), cibenzoline (F), imatinib (G), pazopanib (H), axitinib (I), sunitinib (J), N-desethyl sunitinib (K), sorafenib (L), and sorafenib N-oxide (M).
Ultrapure water was prepared with PURELAB ultra apparatus and used for all LC-MS/MS analysis.

2.2. Optimization for SRM conditions

LC-MS-8050 triple quadrupole tandem mass spectrometer (Shimadzu, Kyoto, Japan) and Nexera ultra high performance liquid chromatograph system (Shimadzu) were combined and used as LC-MS/MS system. Nebulizer gas, drying gas, and heating gas were set at 3, 10, and 10 L/min, respectively. Interface temperature, desolvation line, and heat block temperature were set at 300, 250, and 400 ºC, respectively. Interface voltage was set at 4000 V on positive ion mode. CID gas was set at 17 kPa. Ten millimole per liter ammonium formate buffer in water and 10 mmol/L ammonium formate buffer in methanol were used as mobile phase A and B, respectively. All stock solutions of each analyte were prepared in methanol, except for pazopanib prepared in dimethyl sulfoxide. One milligram per milliliter of stock solutions were diluted to 1 μg/mL and used for analysis.

Fig. 3. Linear range of calibration curves every Q-array bias parameter; mycophenolic acid (A), voriconazole (B), itraconazole (C), hydroxyitraconazole (D), pilscainide (E), cibenzoline (F), imatinib (G), pazopanib (H), axitinib (I), sunitinib (J), N-desethyl sunitinib (K), sorafenib (L), and sorafenib N-oxide (M), respectively.
MS/MS optimization. One microgram per milliliter of working solutions was injected into LC-MS/MS and the selected reaction monitoring (SRM) parameters were optimized.

2.3. Change of ion abundance accompanied with ion guide voltage

One microliter of working solutions at 10 ng/mL were analyzed with optimized SRM conditions in Section 2.2. The Q-array bias in SRM analyses were changed from 0 to 180 V and the peak intensities were plotted against the voltages. Triplicate analyses were performed for every voltage in all analytes.

2.4. Shifting of linear range of calibration curves

Eleven working mixture were prepared by mixing and diluting each stock solutions: 0.1, 0.3, 1.0, 3.0, 10, 30, 100, 300, 1000, 3000, and 10000 ng/mL in acetonitrile (named level 1 to 11, respectively). All internal standard (IS) solutions were prepared at 100 ng/mL concentrations. The same volume of the working solution and IS solution were mixed as samples, and 2 μL of the mixtures were injected. Triplicate analyses were performed at every level and the peak area ratio of analytes to the ISs were calculated. The results in Table 1. Optimized collision energy (CE) values ranged from 17 V to 37 V. Internal standards such as isotopic labeled compound and analogues of the analytes showed the similar result as well. And the results were adopted to the following experiments about ion reduction using in-source CID.

### Table 1. Optimized SRM conditions.

| Compound                      | Q1 (m/z) | CE (V) | Q3 (m/z) | IS                  |
|-------------------------------|----------|--------|----------|---------------------|
| Mycophenolic acid             | 321      | 23     | 207      | Mycophenolic acid-2H3 |
| Voriconazole                  | 351      | 17     | 127      | Voriconazole-2H1    |
| Itraconazole                  | 705      | 37     | 392      | Itraconazole-2H4    |
| Hydroyx-itraconazole          | 721      | 36     | 392      | Hydroyx-itraconazole-2H4 |
| Pilsicainide                  | 273      | 24     | 110      | Disopyramide        |
| Cibenzoline                   | 263      | 36     | 115      | Dimethylcibenzoline |
| Imatinib                      | 494      | 28     | 394      | Imatinib-2H8        |
| Pazopanib                     | 438      | 30     | 357      | Imatinib-2H8        |
| Axitinib                      | 387      | 21     | 356      | Axitinib-2H1        |
| Sunitinib                     | 399      | 22     | 326      | Axitinib-2H1        |
| N-desethyl sunitinib          | 371      | 18     | 326      | Axitinib-2H1        |
| Sorafenib                     | 465      | 25     | 270      | Sorafenib-2H1       |
| Sorafenib N-oxide             | 481      | 27     | 286      | Sorafenib-2H1       |
| Mycophenolic acid-2H3         | 324      | 23     | 210      | -                   |
| Voriconazole-2H1              | 353      | 17     | 284      | -                   |
| Itraconazole-2H4              | 709      | 37     | 396      | -                   |
| Hydroxy-itraconazole-2H4      | 725      | 36     | 396      | -                   |
| Disopyramide                  | 340      | 20     | 239      | -                   |
| Dimethylcibenzoline           | 291      | 33     | 129      | -                   |
| Imatinib-2H8                  | 502      | 28     | 394      | -                   |
| Axitinib-2H1                  | 390      | 21     | 356      | -                   |
| Sorafenib-2H3                 | 468      | 25     | 273      | -                   |

3. Results and discussion

3.1. SRM parameters

In this study, we investigated the application of ion reduction strategy using in-source CID aiming for 13 compounds containing drugs and the metabolites used in TDM practice frequently. First, we optimized the SRM conditions with flow injection analysis and summarized the results in Table 1. Optimized collision energy (CE) values ranged from 17 V to 37 V. Internal standards such as isotopic labeled compound and analogues of the analytes showed the similar result as well. And the results were adopted to the following experiments about ion reduction using in-source CID.

3.2. Reduction of ion inlet by in-source CID

Next, we investigated the ion abundance of the analytes with in-source CID conditions. We changed the Q-array bias parameter, which is the ion guide voltage set at right after the orifice, and tried the ion reduction to quadrupoles by in-source CID. Mycophenolic acid provided the 23 V of CE with maximum intensity (Table 1). However, the compound was stable up to 50 V of Q-array bias, the ion intensity gradually decreased after 50 V (Fig. 2B). As compared with voriconazole, itraconazole is stable against the increase in Q-array voltage and the optimum value of CE is also high. The ion reduction of this compound was observed up to 120 V (Fig. 2C). Hydroxy itraconazole, which is a metabolite of itraconazole, showed the result similar to itraconazole (Fig. 2D). Pilsicainide, which has “cain” as a stem structure, showed high MS sensitivity. The peak intensity greatly reduced at an ion guide voltage above 80 V (Fig. 2E). In the case of cibenzoline, a decrease in peak intensity was
observed over 100 V (Fig. 2F). As Imatinib, that is molecule targeted therapeutic anticancer drug, the peak intensity decreased gradually over 100 V (Fig. 2G). Pazopanib was stable in in-source CID, and the peak intensity decreased over 120 V (Fig. 2H). Axitinib is easier to cleave than other drugs such as imatinib and pazopanib, and the cleavage was observed from about 80 V (Fig. 2I). Both sunitinib and its metabolite were easy to cleavage than the other molecule targeted therapeutic drugs, and the peak intensities decreased over 70 V (Fig. 2J and K). As sorafenib, the ion reduction was observed over 100 V similar to imatinib, which has a common partial structure (Fig. 2L). The metabolite, sorafenib N-oxide also showed a similar result (Fig. 2M).

3.3. Shifting of linear range of calibration curves

Next, calibration curves were prepared based on the data in the previous experiment, and we tried shifting the calibration curves to higher concentrations. In mycophenolic acid, a linearity shift towards a higher concentration occurred with a threshold around 60 V in which the change of the peak intensity occurred (Fig. 3A). Voriconazole provided also the almost same result as mycophenolic acid, and a linearity shift occurred from about 50 V of Q-array bias (Fig. 3B). However, itraconazole, which the peak intensity decreased over 120 V, also shifted to high concentration from the voltage. At the same time, it was impossible to hold the linearity on the lower concentration (Fig. 3C). Hydroxy itraconazole showed a lower MS sensitivity than other compounds, and a linearity of calibration curve could be hold over 1 ng/mL concentration. In addition, the intensity decrease remarkably exceeding 120 V of Q-array bias, the range of calibration curve narrowed with the decrease of the peak intensity on the lower concentrations (Fig. 3D). Pilscainide showed a result similar to voriconazole, with a shifting to high concentrations over 60 V of Q-array bias. Cibenzoline, which has a relatively rigid structure, shifted to higher concentrations over 120 V like itraconazole (Fig. 3F). Imatinib also showed the results similar to itraconazole and cibenzoline (Fig. 3G). Pazopanib showed a slightly unstable behavior up to 50 V, however it showed the behavior of a calibration curve based on the obtained data (Fig. 3H). In the case of axitinib, a shift from about 80 V to high concentration was observed, and calibration curve was kept to prepare even at higher Q-array bias (Fig. 3I). Sunitinib and the metabolite, N-desethyl sunitinib showed a gradual shift from 90 V or 70 V to higher concentration (Fig. 3J and K). Sorafenib showed a similar shift appearance as imatinib (Fig. 3L). Sorafenib N-oxide kept a linear range up to higher concentration from 0 V. However, even if it was changed to higher voltage, the narrowing the linear range in lower concentrations was observed (Fig. 3M).

3.4. Simultaneous analysis of the 13 test compounds

Finally, the simultaneous LC-MS/MS analysis of the 13 drugs was performed. The in-source CID parameters were simulated as shown in Table 2 so that the peak intensity was similar to the test. Typical chromatogram of simultaneous analysis of 13 compounds under the conditions of combination optimized SRM and the in-source CID was shown in Fig. 4. As a result, the peak intensity of the data presented in the present SRM conditions was within 5 times the range. Both sunitinib and the metabolite were detected separately for the isomerization between Z- and E-form similar to the literatures [21,22]. As a result, it is suggested the dataset about in-source CID in this study is valuable basic data for development of simultaneous analysis methods in TDM of combination therapy. Furthermore, in the future, it is expected to apply for the development of analysis methods for combination therapy used in clinical practice.

| Compound                     | Q-array bias (V) |
|------------------------------|------------------|
| Mycophenolic acid            | 10               |
| Voriconazole                 | 10               |
| Itraconazole                 | 10               |
| Hydroxy itraconazole         | 40               |
| Pilscainide                  | 0                |
| Cibenzoline                  | 0                |
| Imatinib                     | 5                |
| Pazopanib                    | 5                |
| Axitinib                     | 0                |
| Sunitinib                    | 0                |
| N-desethyl sunitinib         | 0                |
| Sorafenib                    | 2                |
| Sorafenib N-oxide            | 10               |
| Mycophenolic acid-2H+        | 10               |
| Voriconazole-2H+             | 10               |
| Itraconazole-2H+             | 10               |
| Hydroxy itraconazole-2H+     | 40               |
| Disopyramide                 | 0                |
| Dimethylcibenzoline          | 0                |
| Imatinib-2H+                 | 5                |
| Axitinib-2H+                 | 0                |
| Sorafenib-2H+                | 2                |

4. Conclusion

In this study, we investigated the reduction of ion inlet using in-source CID and the shifting the linear range of the calibration curve aimed to the development of simultaneous analysis methods for TDM of combination therapy used in clinical practice. As a result, it was suggested that the principle could be applied to all 13 compound containing drugs and the metabolites which have different therapeutic effects and have different chemical structures successfully. Therefore, it is possible to easily adjust the amount of ion inlet to mass spectrometer by using this principle, and develop a simultaneous analysis method even for a
combination therapy consisting of a significantly different therapeutic concentration range. In the future, it is expected to be applied to the development of simultaneous analysis methods for TDM of combination therapy actually used in the clinical practice based on the principle in this study. Furthermore, the application of this approach could effort rapid and accurate personalized medicine using mass spectrometry customized by this strategy.

Conflict of interest
The authors declare no conflicts of interest, financial or otherwise.

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