Abstract
The combination of methotrexate (MTX) and non-steroidal anti-inflammatory drugs (NSAIDs), which is frequently used for rheumatoid arthritis (RA) treatment, courses of adverse events. To prevent these, simultaneous monitoring of these compounds is available. Therefore, we developed a new method for MTX and NSAIDs such as loxoprofen (LP), meloxicam (MX), lornoxicam (LX), diclofenac (DF) and celecoxib (CX) determination using HPLC with simple pretreatment of human serum sample. The separation of MTX and 5 NSAIDs was performed on C6-Phenyl column with gradient elution using 10 mmol/L ammonium acetate aqueous solution and methanol, and achieved within 25 min of analytical runtime. Calibration curves using standards showed good linearity ($r^2$=0.9998) in the range of 0.02-500 pmol/10 μL injection for MTX, 10-500 pmol for LP, 0.2-500 pmol for MX and DF, 0.05-500 pmol for LX and 2-500 pmol for CX. The detection limits of the proposed method were at least less than 96.0 fmol, and repeatability was less than 4.62 RSD%. In addition, acceptable precision of less than 10.19 RSD% and recovery of more than 54.4% of the method were obtained because the peaks of MTX and NSAIDs could be well-separated from those of interferings in serum. Therefore, the proposed method might be useful to avoid occurring adverse events, and confirm the curative effect of MTX and NSAIDs.

Keywords: Methotrexate; NSAIDs; HPLC; Simultaneous assay

1. Introduction
Rheumatoid arthritis (RA) is an autoimmune disease that is mainly characterized by polyarticular synovitis as main lesion, and it often complicates a symptom besides the joint. Current RA treatment involves the control of the symptoms such as the reduction of the joint destruction. Common medicines for RA include conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs), biological disease-modifying anti-rheumatic drugs (bDMARDs), and steroids. For the RA treatment, early use of csDMARDs is recommended [1].

Methotrexate (MTX) belongs to the csDMARDs and is more effective compared to other csDMARDs. MTX is highly effective in reducing bone destruction, faster recovery, and reduces the incidence of myocardial infarction [2-4]. Therefore, MTX is the preferred choice for RA treatment [5,6]. There is an evidence of the synergistic effect of MTX [7]. However, MTX often causes side effects such as hepatic disorder, myelosuppression, and nausea. A decrease in dosage or complete discontinuation is therefore recommended upon remission [8,9]. Evidence from Japan indicates that even in small doses, MTX can cause hepatic disorder and myelosuppression [10]. MTX still remains an important drug in RA treatment despite the side effects, which can be resolved by combining it with bDMARDs [11,12].

Non-steroidal anti-inflammatory drugs (NSAIDs) are often used for symptomatic treatment of RA to reduce inflammation and pain. Although NSAIDs improve the quality of life of the RA patients, the side effect of gastrointestinal dysfunction is observed in about 3-15% of the cases [13]. This side effect is reduced by using

*Corresponding author: Mitsuhiro WADA
Tel & Fax: +81-836-39-9120
E-mail: m-wada@rs.socu.ac.jp

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selective the cyclooxygenase-2 (COX-2) inhibitor among NSAIDs [14]. Even if a selective COX-2 inhibitor is used, the risk of the gastrointestinal dysfunction could not be prevented completely. Moreover, it has been reported that drug-drug interaction with increase of the blood concentration of the drugs is occurred and caused adverse events such as myelophthisis or gastrointestinal dysfunction when NSAIDs are used in combination with MTX [15]. Therefore, it is useful to measure the blood concentration of MTX and NSAIDs simultaneously to prevent the adverse events and confirm the therapeutic effects.

However, to our best knowledge, there is no method for the simultaneous determination of MTX and NSAIDs. Until now, the assays for MTX include commonly HPLC with UV detection with solid-phase extraction (SPE) [16,17], HPLC with online photolysis fluorescence detection (FL) [18], liquid chromatography-tandem mass spectrometry (LC-MS/MS) with liquid-liquid extraction (LLE) [19], or LC-MS/MS with column switching [20]. The assays for NSAIDs include commonly HPLC with UV detection [21-23], LC-MS/MS with micro solid-phase extraction (MSPE) [24]. The LC-MS/MS assays have high sensitivity, but they have limitations such as requirement of special instrument.

In this study, we developed a simultaneous assay method for MTX and 5 NSAIDs. The NSAIDs in this study such as loxoprofen (LP), meloxicam (MX), lornoxicam (LX), diclofenac (DF), and celecoxib (CX) are selective COX-2 inhibitors which are used for RA treatment. Therefore, it is useful to measure the blood concentration of MTX and NSAIDs simultaneously to prevent the adverse events and confirm the therapeutic effects.

2. Materials and methods
2.1. Reagents and chemicals

LX and CX were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). MTX, LP, MX, and DF were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human serum used was purchased from Cosmo Bio (Tokyo, Japan). All solvents and reagents were of the Guaranteed Reagent (GR) grade or higher.

2.2. Instruments

Shimadzu ultra-fast liquid chromatography (UFLC) Prominence system (Kyoto, Japan), consisting of a CBM-20A system controller, two LC-20AD pumps, a DGU-20A5R online degasser, an SPD-20A UV/VIS detector, a Rheodyne 7725i manual sample injector and a Sugai U-620 column oven (Wakayama, Japan) was used. Detection wavelength were changed from 300 nm to 222 nm for 9 min, to 360 nm for 1.2 min, to 270 nm for 3.3 min, and to 250 nm for 1.5 min. The collected data were analyzed using a Shimadzu Lab Solutions LC software (v. 1.21) and the peak heights were estimated using the base-to-base method. The Gemini C6-Phenyl 110Å column packed with 3 μm particles (150×3 mm I.D., Phenomenex Inc., Torrance, CA, USA) was used as separation column. Mobile phases, 10 mmol/L ammonium acetate aqueous solution (A) and methanol (B) were used. Gradient profiling was a linear gradient elution from A/B (73:27) to A/B (73:27) for 5 min, from A/B (73:27) to A/B (45:55) for 0.1 min, from A/B (45:55) to A/B (30:70) for 8.9 min, from A/B (30:70) to A/B (20:80) for 0.5 min, from A/B (20:80) to A/B (20:80) for 3.5 min, from A/B (20:80) to A/B (5:95) for 0.1 min, from A/B (5:95) to A/B (5:95) for 1.9 min, and from A/B (5:95) to A/B (73:27) for 0.1 min. The flow rate of the mobile phase, the column temperature, and the injection volume were set at 0.35 mL/min, 35°C, and 10 μL, respectively.

2.3. Standard solutions

A stock solution of 1 mmol/L MX, LX, and CX were prepared using acetonitrile, and the solutions of DF and LP was solved with methanol. Standard sample solutions for the calibration curve were prepared using 35% methanol aqueous solution.

2.4. Sample preparation

Human serum containing 10 μmol/L of MTX and 5 NSAIDs was prepared as a control sample. To 200 μL of serum, acetonitrile containing 5% acetic acid (200 μL) was added and mixed-well. After centrifugation at 5,000 × g for 10 min, whole supernatant was collected. The supernatant was evaporated to dryness, and dissolved in 200 μL of 35% methanol aqueous solution. A portion of sample (10 μL) was injected into HPLC. Human serum without drugs was subjected to the same treatment and used as blank.

2.5. Method validation

Calibration curves of MTX and NSAIDs were prepared with known concentration of standard (0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 50, 100, 200 and 500 pmol for MTX, 10, 20, 50, 100, 200 and 500 pmol for LP, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 and 500 pmol for MX and DF, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 50, 100, 200 and 500 pmol for LX and 2, 5, 10, 20, 50, 100, 200 and 500 pmol for CX). The detection limit (DL) was defined as the concentration equal to a signal-to-noise ratio (S/N) of 3. Repeatability of the proposed method using 100 pmol/10 μL of standard sample was expressed as relative standard deviation (RSD) for five-multiple measurements. Recovery of the method was indicated as the peak area ratio of control sample to the blank.
standard solution. Precision (for intra-day assays) were evaluated using control sample (n=5).

3 Results and discussion

3.1. LC separations of MTX and NSAIDs

MTX has two pKa values (4.84 and 5.51), thus two peaks were observed in the chromatogram with the octadeyl-silyl (ODS) HPLC column [25]. The target drugs in this study have partial plane structure (Fig. 1). To separate these drugs, we used the Gemini C6-Phenyl 110Å column, which has selectivity of aromatic compounds by π-π interaction.

In general, the elution order of substrates by ODS column depended on their hydrophobicity (logP value). The logP value of MTX, LP, MX, LX, DF and CX were -1.85, 0.82, 3.43, 2.62, 4.51, and 3.53, respectively [26-31]. However, the elution order of analytes by C6-Phenyl column did not correspond to their logP values. Therefore, it was expected that retention mechanism such as π-π interaction other than hydrophobic interaction might influence the elution order in this study. Owing to the selectivity of the separation column, sample pretreatment could be completed using simple deproteinization. Next, effects of organic solvents such as acetonitrile, methanol and isopropanol on the separation of compounds were examined for the deproteinization of serum. Since the separation of compounds from methanol and isopropanol treatment were affected with interfering peaks from serum, acetonitrile was selected. The recovery of MTX, LP, MX, LX, DF and CX deproteinized with acetonitrile were 50.7, 71.4, 65.5, 54.3, 36.0 and 71.9%, respectively. To improve the recovery, acid (= acetic acid) or base (= triethylamine) was added to acetonitrile, since recovery of MTX and DF were insufficient. The recovery of most compounds increased by spiking acetic acid, but that of CX decreased greatly with increasing the concentration of acetic acid. On the other hand, the recovery of MTX, LP, and DF decreased with increasing the concentration of triethylamine. Finally, acetonitrile spiked with 5% acetic acid was selected as the deproteinization condition (data was not shown). To determine the drugs sensitively, absorption spectrum of each drugs were measured. As results, detection wavelengths at 300, 222, 360, 270, and 250 nm for MTX, LP, MX, LX, DF, and CX were set, respectively.

3.2. Method validation

Using the above conditions, MTX and 5 NSAIDs could be detected each as a single peak within 25 min of analytical runtime (Fig. 2). Retention times of MTX, LP, MX, LX, DF and CX were 5.8, 9.8, 10.5, 11.5, 14.3, and 17.6 min, respectively. The linearity of calibration curves, DLs and repeatability using a standard mixture of MTX and 5 NSAIDs are summarized in Table 1. The calibration curves of the drugs are given good linearity with correlation coefficient \(r^2\) of 0.9998 in the range of at least 10.0-500 pmol/injection volume (10 µL). The DLs (S/N=3) of MTX, LP, MX, LX, DF and CX were found to be 1.09, 51.7, 10.0, 2.03, 6.86 and 96.0 fmol, respectively. The MTX could be detected 55 fmol in urine by HPLC-UV [16], and 1.1 fmol in serum by LC-MS/MS [20]. The DLs of LP and DF in urine [21] and environmental water [24] were 609 fmol and 507 fmol by HPLC-UV, and 0.4 fmol and 0.7 fmol by LC-MS/MS, respectively. Our method wasn’t applied to actual sample, but it was enough sensitive to determine analytes in patient’s sample [16,21]. Additionally, in previous report, MTX concentrations in rheumatic patients’ sera were ranging from 287.8 to 1,164 nmol/L (correspond to 2,878-11,640 fmol) [32]. The LP concentrations in rheumatic patients’ plasmas were ranging from 0.61-2.45 µg/mL (correspond to 2.48×10^4-9.95×10^4 fmol) [33]. The DF concentrations in rheumatic patients’ sera were ranging from 45.6-119.7 ng/mL (correspond to 1,540-4,042 fmol) [34]. These finding indicate that the proposed method has sufficient sensitivity to measure the blood concentration of RA patients on therapy. The RSD (n=5) of repeatability was less than 4.62%.

Figure 3 shows the chromatogram for human serum spiked with standard. The preferable peaks from drugs indicate good separation power. The recovery of MTX, LP, MX, LX, DF and CX was 56.8, 82.0, 80.6, 59.5, 78.5 and 54.4%, respectively. The deproteinization condition selected in this study could be applied to the combination of MTX and any NSAIDs without additional modification.
However, in the case of MTX and LP, LX, MX or DF, using acetonitrile containing 10% acetic acid for deproteinization might give higher recovery (69.6, 103.9, 104.6, 90.9 and 78.9%). The precision was 6.16, 10.19, 3.80, 6.89, 7.43 and 6.44 RSD%, respectively (n=5) (Table 1).

![Fig. 2. Chromatogram of standard mixture containing 0.1 mmol/L MTX and NSAIDs. A portion (10 μL) of a standard solution (100 pmol each on column) was applied to the HPLC system.](image)

### Table 1. Validation of the method for determination of MTX and NSAIDs.

| Compound | Retention time (min) | Dynamic range (pmol per 10 μL injection) | Linearity | DL (fmol) | Repeatability (RSD %, n=5) | Recovery (RSD %, n=5) | Precision (RSD %, n=5) |
|----------|----------------------|----------------------------------------|-----------|----------|------------------------|----------------------|---------------------|
| MTX      | 5.8                  | 0.020 - 500                            | 0.9999    | 1.09     | 0.59                   | 59.8                 | 6.16                |
| LP       | 9.8                  | 10.0 - 500                             | 1.0000    | 51.7     | 3.07                   | 82.0                 | 10.19               |
| MX       | 10.5                 | 0.2 - 500                              | 0.9999    | 10.0     | 4.62                   | 80.6                 | 3.80                |
| LX       | 11.5                 | 0.05 - 500                             | 0.9999    | 2.03     | 0.92                   | 59.5                 | 6.89                |
| DF       | 14.3                 | 0.2 - 500                              | 0.9998    | 6.86     | 0.85                   | 78.5                 | 7.43                |
| CX       | 17.6                 | 2.0 - 500                              | 0.9999    | 96.0     | 3.46                   | 54.4                 | 6.44                |

a) Defined as the amount per injection volume (10 μL) giving a signal-to-noise ratio of 3.
b) Relative standard deviation of absorbance intensity; 100 pmol per 10 μL injection.
c) Control sample (100 pmol per 10 μL injection) were used.

![Fig. 3. Chromatograms of human serum and that spiked with drugs. A portion (10 μL) of serum and that spiked with a standard solution (100 pmol each on column) was applied to the HPLC system.](image)
4. Conclusion
In this study, we developed a simple simultaneous assay for MTX and NSAIDs by HPLC. By using the Gemini C6-Phenyl column, good separation of the compounds within 25 min of analytical runtime was achieved. The linearity, DL, and repeatability of the method are acceptable to analyze serum sample. On analyzing the commercial human serum preferable peak resolution, recovery range, and precision levels were achieved. Our method would be suitable for addressing the side effects of MTX by combining with NSAIDs, leading to a simple therapy with a high therapeutic index.

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