Simultaneous Quantitative Screening for Pain Medications in Serum by High-Performance Liquid Chromatography/Time-of-Flight Mass Spectrometry with Solid-Phase Dispersive Extraction

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Abstract
In this study, solid-phase dispersive extraction (SPDE) was used for serum pretreatment and in the simultaneous analysis of analgesics and adjuvant analgesics (30 types in total) that are usually used as first- and second-choice treatments for pain patients, by liquid chromatography/time-of-flight mass spectrometry (LC/TOF-MS). Examination of the optimum conditions for SPDE using Oasis MCX as the solid-phase gel revealed that the recovery rates for serum samples deproteinized in advance were 49–87%, whereas the recovery rates were as high as 78–112% when deproteinization was not performed. The matrix effect was within ±10% regardless of the presence or absence of deproteinization, and its influence could be suppressed even if deproteinization was not performed. The results indicate that serum deproteinization was unnecessary when SPDE was used for pretreatment. In LC/TOF-MS measurement, gradient elution was carried out using core-shell type column Kinetex C18 (150 mm × 2.1 mm, 1.7 µm) as the LC column and 50 mM ammonium acetate buffer (pH 7.8)/acetonitrile/methanol mixture as the mobile phase. The 30 drugs were well separated, and the limit of quantification was 0.25–10 ng/mL, the correlation coefficients of the calibration curves were higher than 0.998, and the average recoveries ranged from 77.7 to 112.1%. The method would be useful to screen for analgesics and adjuvant analgesics (30 types in total) in serum in the fields of forensic science and emergency medicine.

Keywords: Pain medication; Solid-phase dispersive extraction; LC/TOF-MS; Serum

1. Introduction
Adjuvant analgesics, such as antidepressants, antiepileptic drugs, and antiarrhythmic drugs, are used for pain treatment in addition to typical analgesics, such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids [1]. As the pathology of chronic pain is extremely complex, it is almost impossible to obtain adequate analgesia with a single analgesic, and several drugs need to be properly combined [2]. The combined use of an adjuvant analgesic and a typical analgesic enhances the analgesic effect but also increases the risk of developing new side effects. Many of these drugs also act on the central nervous system and are likely to cause serious addiction and toxicity symptoms if not properly used. When an abuser suspected of using such drugs suffers from acute poisoning, prompt identification of the drug as well as the dosage and timing of administration is essential for treatment. Therefore, in emergency medicine and forensic science, it is required to establish a rapid and highly reliable analysis method for the substance of abuse in biological samples in order to identify and quantify the causative substance of poisoning and abuse.

Several multi-analyte methods that analyze different types of drugs have been published. Conventionally, the simultaneous analysis of multiple pain medications, including NSAIDs [3], benzodiazepines [4], tricyclic antidepressants [5], and antidepressants including metabolites and psychotropic drugs [6-10], has been carried out by liquid chromatography (LC)/time-of-flight mass spectrometry (TOF-MS) or LC/tandem mass spectrometry.
Typical pretreatment methods for drug analysis in blood (serum, plasma) include liquid-liquid extraction using an organic solvent [9,10], solid-phase extraction (SPE) after diluting plasma with purified water or buffer [3,5,6], and denaturation and precipitation of plasma proteins with zinc sulfate solution [7] or organic solvent (acetonitrile) [8] in advance to remove proteins, followed by SPE. However, with regard to these methods, there is concern that purification may be insufficient, which may lead to matrix effects, such as ion suppression and enhancement [7-10], depending on the type of drug.

As previously reported [3,5,6], it is possible to omit the preliminary serum deproteinization step and perform SPE directly. However, the conventional cartridge-type SPE method has some drawbacks, including clogging of the sample solution in the case of a highly viscous biological sample, which results in low recovery [11]. In addition, it is difficult to maintain a constant flow rate for all SPE cartridges when a vacuum-type manifold is used for the conventional cartridge-type SPE. Other pretreatment methods are not suitable for the simultaneous processing of multiple samples.

We have developed a novel extraction method called the solid-phase dispersive extraction (SPDE) method [4] in order to overcome some of the drawbacks of the conventional cartridge-type SPE method. SPDE can be used for infectious and/or chemically hazardous samples because the SPDE operation is performed in a closed system. SPDE was applied to the analyses of benzodiazepine psychotropic drugs in serum and urine [4] as well as the analysis of synthetic cannabinoids [12,13], which are the main components of so-called dangerous drugs. In addition, SPDE was used as a pretreatment method for the analysis of vancomycin targeted for therapeutic drug monitoring, and it was reported that SPDE could be applied without prior serum deproteinization [11].

In accordance with the 2007 International Association for the Study of Pain (IASP) Guidelines for the treatment of neuropathic pain [14]; in this study, we examined drugs for first- and second-choice treatments except strong opioids. According to data provided by Japan Poison Information Center, hypnotic sedatives, antidepressants, and antiepileptic agents are widely used, in addition to antipyretic analgesics and anti-inflammatory agents, which are the main analgesics [15]. Therefore, we focused on 30 types of analgesics and adjuvant analgesics, namely, moderate opioid analgesics (tramadol, pentazocine), NSAIDs (ethenzamide), antidepressants (amoxapine, imipramine, amitriptyline, clomipramine, desipramine, nortriptyline, doxepin, dosulepin, maprotiline, mianserin, trazodone, fluvoxamine, paroxetine, sertraline, fluoxetine, citalopram, milnacipran, duloxetine, venlafaxine, mirtazapine, reboxetine, bupropion, moclobemide), antiepileptic drugs (carbamazepine), and antiarrhythmic drugs (lidocaine, mexiletine, flecainide). We also investigated whether it was possible to omit the serum deproteinization step as a pretreatment method prior to SPDE. Furthermore, we attempted to improve pretreatment operability and the recovery rate. In addition, a simultaneous analysis method using an LC/TOF-MS, which can capture mass spectrum information by continuous scan measurement, was developed to screen for 30 drugs that have a possibility of being administered to chronic pain patients.

2. Experimental

2.1. Materials and reagents

Tramadol, moclobemide, reboxetine, imipramine, and dosulepin were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Ethenzamide, milnacipran, venlafaxine, flecainide, bupropion, fluoxetine, and sertraline were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Carbamazepine, citalopram, desipramine, paroxetine, maprotiline, doxepin, duloxetine, nortriptyline, amoxapine, fluvoxamine, mirtazapine, trazodone, amitriptyline, and mianserin were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Lidocaine and mexiletine were purchased from U.S. Pharmacopeial Convention (North Bethesda, MD, USA). Pentazocine was purchased from ASKA Pharmaceutical Co., Ltd. (Tokyo, Japan). Each standard was dissolved in methanol to make a 1000 μg/mL standard stock solution. Working standard solutions were then prepared from each standard stock solution by dilution with water.

Acetonitrile and methanol (both HPLC grade); formic acid, acetic acid, ammonium formate, ammonium acetate (all special grade (> 97%)); 25% aqueous ammonia solution (analytical grade); and leucine-enkephalin (biochemical grade) were purchased from FUJIFILM Wako Pure Chemical Corporation. Lyophilized serum Consera (No. 1, 3 mL volume × 25 pieces; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) was used as control serum. An Oasis MCX 6 cc Vac Cartridge (150 mg, particle size 30 μm; Waters Co., Milford, MA, USA) was used. After removing the solid-phase gel from the cartridge, the gel was used for SPDE. @Roka and Captube for centrifugation filter units were purchased from Frontier Science Co., Ltd. (Ishikari, Japan). The MCX gel was conditioned with 1 mL of methanol and 2% aqueous formic acid solution prior to use, and was then suspended at the concentration of 100
mg/mL in 2% aqueous formic acid solution. Ultrapure water was produced by a Milli-Q Gradient A10 system equipped with an EDS-PAK polisher (Merck Ltd., Darmstadt, Germany). All other chemicals were of special grade.

2.2. Apparatus and operating conditions

An Alliance HT 2795 HPLC system equipped with an LCT Premier XE TOF-MS (Waters Corporation) was used. LC separation was performed with a Kinetex C18 column (150 mm × 2.1 mm, 1.7 µm; Phenomenex Inc., Torrance, CA, USA). Column temperature was maintained at 40°C. The mobile phase was a mixture of (A) 50 mmol/L ammonium acetate buffered solution (pH 7.8), (B) acetonitrile, and (C) methanol, and was delivered in the gradient elution mode (Table 1) at the flow rate of 0.5 mL/min. A 5 µL aliquot of the sample was injected into the system.

Table 1. Gradient conditions for LC mobile phase.

| Time (min) | 0    | 20   | 61   | 62   | 90   |
|------------|------|------|------|------|------|
| A) 50 mmol/L Ammonium acetate buffer (pH 7.8) | 10   | 10   | 10   | 10   | 10   |
| B) Acetonitrile | 25   | 0    | 0    | 40   | 40   |
| C) Methanol | 0    | 40   | 55   | 0    | 0    |
| D) Water   | 65   | 50   | 35   | 50   | 50   |

The optimum working parameters for TOF-MS were as follows: ionization: electrospray ionization (ESI) positive mode, capillary voltage: 2500 V, cone voltage: 2500 V, desolvation temperature: 350°C, source temperature: 120°C, desolvation gas flow (N2): 800 L/h, and cone gas flow (N2): 50 L/h. The leucine-enkephalin solution (400 ng/mL; dissolved in 0.1% formic acid/50% acetonitrile) was delivered at the flow rate of 5 µL/min. Mass accuracy was maintained using Lock–Spray with the leucine-enkephalin [M+H]+ ion, m/z = 556.2771, as the lock mass. The resolution was at least 10,000 as calculated by using the full width at half-maximum method. The monitoring ions of the 30 drugs are shown in Fig. 1 together with the mass chromatograms of the standards added to serum samples. The proton adduct of each compound was used as the monitoring ion.

2.4. Serum sample pretreatment protocol with deproteinization procedure

Five hundred microliters of serum and 500 µL of acetonitrile were added, and the mixture was vortexed for 1 minute and then centrifuged (3000 × g, 5 min). The supernatant was nitrogen-purged at 40°C, and the residue was redissolved in 500 µL of 2% aqueous formic acid solution, followed by SPDE treatment similar to that described in the Section 2.3.

3. Results and discussion

3.1. Optimization of LC conditions

The optimum mobile phase for LC was examined, and the optimum capillary voltage and cone voltage for TOF-MS were determined.

3.1.1. Mobile phase

As for the mobile phase, five aqueous solvents, namely, acetonitrile, methanol, aqueous formic acid solution, aqueous ammonium formate solution, and aqueous ammonium acetate solution, were examined to determine the optimal solvent for the Phenomenex Kinetex C18 column. As a result, because both sensitivity and separation were good when aqueous ammonium acetate solution was used, we decided to create gradient conditions using aqueous ammonium acetate solution in combination with acetonitrile and/or methanol. The concentration and pH of aqueous ammonium acetate solution, the type of eluate (acetonitrile, methanol), and their mixing ratio were examined. As a result, the best separation was obtained under the gradient conditions listed in Table 1 for a 50 mmol/L ammonium acetate buffer (pH 7.8)/acetonitrile/methanol mixture.
Fig. 1. Typical chromatograms of target compounds in serum samples spiked with 100 ng/mL standard.

Fig. 2. Effect of capillary voltage for ionization of target compounds in LC/TOF-MS analysis.

Fig. 3. Effect of cone voltage for ionization of target compounds in LC/TOF-MS analysis.
Fig. 4. Comparison of recoveries of target compounds between Oasis® HLB and MCX. The order of the bars matches the order from the top of the legend.

Fig. 5. Comparison of recoveries with and without deproteinization, prior to SPDE. The order of the bars matches the order from the top of the legend.
3.1.2. Optimization of TOF-MS operating conditions

TOF-MS was performed in the ESI positive ion mode because all of the 30 drugs tested were ionized in the positive ion mode. The optimum capillary voltage in the range of 500 to 3000 V and the optimum cone voltage in the range of 10 to 70 V were examined. As a result, maximum peak intensities were obtained with the combination of 500 V capillary voltage (Fig. 2) and 50 V cone voltage (Fig. 3) for most of the targeted drugs. On the other hand, depending on the drug tested, precision mass error was also found to be affected by capillary voltage. The precision mass error fluctuation was large (-35 to 20 ppm) in the capillary voltage range of 500 to 1500 V, whereas precision mass error fluctuation for all drugs was within -20 to 5 ppm in the capillary voltage range of 2000 to 3000 V. Therefore, we adopted 2000 V as the optimum capillary voltage. The mass chromatograms of standard solutions (100 ng/mL) added to serum, which were measured under the optimized conditions, are shown in Fig. 1.

3.2. Optimization of SPDE method

3.2.1. Solid-phase gel

The type and amount of solid-phase gel were investigated to optimize the conditions for SPDE. Oasis HLB and MCX solid-phase gels were tested because most of the target drugs are basic compounds with amino groups. The drugs were sufficiently retained by both Oasis® HLB and MCX. Although Oasis MCX showed good recoveries (80–100%), HLB showed ion enhancement, which resulted in higher than 100% recoveries (140–160%) for ethenzamide, carbamazepine, bupropion, doxepin, mirtazapine, dosulepin, amitriptyline, sertraline, and mianserin (Fig. 4). It was speculated that the contaminants were not completely removed. Therefore, Oasis MCX was adopted as the solid-phase gel for SPDE.

Subsequently, the optimal amount of solid-phase gel (MCX) for the recovery of the drugs by SPDE was investigated. As a result, the average recoveries (and relative standard deviations) for the 30 drugs subjected to SPDE using 1, 2.5, 5, 10, 20, and 30 mg of MCX were as follows: 59.2% (12.6%), 82.9% (7.7%), 92.9% (4.0%), 93.4% (6.1%), 93.2% (9.3%), and 92.2% (8.6%), respectively. Therefore, 5 mg was adopted as the optimal amount.

3.2.2. SPDE operating conditions

As most of the drugs to be measured are basic, the pH of the measurement sample was adjusted by adding 2% formic acid solution to dissociate the amino groups of the drug to be measured, which resulted in the enhancement of mutual ion exchange interaction between the solid phase and the target compound. Next, the optimal volume of the eluent and the number of elutions were examined. We investigated the case of elution with 1,000 µL once and the case of elution with 500 µL of methanol solution containing 5% ammonia once or twice. The recovery rate was highest when the elution was performed twice with 500 µL of methanol solution containing 5% ammonia. With regard to sertraline, when methanol solution containing 5% ammonia was injected as is, peak splitting was observed. The peak shape was improved when 2% aqueous acetic acid solution was added to neutralize the eluate. Therefore, in preparing the sample solution, it was decided to add 2% aqueous acetic acid solution to neutralize the eluate.

Table 2. Method validation for the LC/TOF-MS analysis

| Compound         | LOD (ng/mL) | LOQ (ng/mL) | Linear range (ng/mL) | Correlation coefficient (r) |
|------------------|-------------|-------------|----------------------|-----------------------------|
| Ethenzamide      | 0.5         | 2.5         | 5-100                | 0.999                       |
| Tramadol         | 1           | 5           | 5-500                | 0.998                       |
| Moclobemide      | 0.25        | 0.5         | 0.5-100              | 0.999                       |
| Mexiletine       | 1           | 5           | 10-100               | 0.999                       |
| Milnacipran      | 0.5         | 2.5         | 5-100                | 0.999                       |
| Venlafaxine      | 0.25        | 1           | 1-500                | 0.999                       |
| Carbazamazine    | 0.1         | 0.25        | 1-50                 | 0.999                       |
| Pentazocine      | 0.5         | 1           | 5-100                | 0.999                       |
| Flecainide       | 0.1         | 0.5         | 5-100                | 0.999                       |
| Citalopram       | 0.25        | 1           | 5-100                | 0.999                       |
| Lidocaine        | 0.25        | 1           | 5-100                | 0.999                       |
| Reboxetine       | 0.25        | 1           | 5-100                | 0.999                       |
| Bupropion        | 2.5         | 5           | 5-100                | 0.999                       |
| Desipramine      | 0.5         | 1           | 5-100                | 0.999                       |
| Paroxetine       | 0.5         | 5           | 5-100                | 0.999                       |
| Dexamipin        | 0.5         | 5           | 5-100                | 0.999                       |
| Maprotiline      | 0.25        | 1           | 5-100                | 0.999                       |
| Duloxetine       | 5           | 10-500      | 10-500               | 0.999                       |
| Nortriptyline    | 0.5         | 5           | 5-100                | 0.999                       |
| Amoxapine        | 0.25        | 1           | 5-100                | 0.999                       |
| Fluvoxamine      | 0.5         | 1           | 5-100                | 0.999                       |
| Mirtazapine      | 0.1         | 0.5         | 5-100                | 0.999                       |
| Fluoxetine       | 0.25        | 1           | 5-100                | 0.999                       |
| Trazodone        | 0.1         | 0.25        | 5-100                | 0.999                       |
| Dosulepin        | 0.5         | 1           | 5-100                | 0.999                       |
| Imipramine       | 0.5         | 1           | 5-100                | 0.999                       |
| Amitriptyline    | 0.25        | 1           | 5-100                | 0.999                       |
| Sertraline       | 2.5         | 5           | 5-100                | 0.999                       |
| Clomipramine     | 0.5         | 1           | 5-100                | 0.999                       |
| Mianserin        | 0.25        | 0.5         | 5-100                | 0.999                       |

3.3. Examination of necessity of deproteinization

We compared the recovery rate and the matrix effect for the case of SPDE after adding acetonitrile to serum sample and deproteinization, with those for the case of SPDE of serum sample diluted with purified water without deproteinization. The recovery rates were 49–87% when SPDE was performed after deproteinization, and 78–113%
when only SPDE was performed. The results indicated that the latter condition was desirable (Fig. 5). We also confirmed that the matrix effect was within ±10% regardless of deproteinization or lack thereof, and that the influence of the matrix effect could be suppressed even if deproteinization was not performed. It is well known that most of the tricyclic antidepressants are strongly protein-bound in blood [5]. However, SPDE may disrupt drug-protein interactions and therefore, the previous deproteinization step may be omitted. As typical examples of the chromatograms measured by this method, Fig. 1 shows the chromatograms for serum samples to which a standard solution was added so that the final concentration in the serum samples was 100 ng/mL.

3.4. Method validation of the proposed SPDE-LC method

The limit of detection (LOD; S/N ≥ 3) and the limit of quantification (LOQ; S/N > 10) of the 30 types of analgesics and adjuvant analgesics were in the range of 0.1 to 5 ng/mL and 0.25 to 10 ng/mL, respectively. The correlation coefficients of the calibration curves were higher than 0.998 (Table 2).

For serum samples, the average recoveries (n = 6) of the 30 drugs spiked at 100 ng/mL ranged from 77.7% to 112.1% (RSD: 1.6%–9.7%) (Table 3). These results suggest that the method can be applied to actual sample analysis.

4. Conclusions

A simple and rapid simultaneous quantitative screening method for serum analgesics and adjuvant analgesics (30 types in total) using LC/TOF-MS was developed. When SPDE was used as the pretreatment method, good recovery rates were obtained even if the preliminary deproteinization was not performed, thus simplifying the operation. As for method validation, the LOD and the LOQ of the 30 types of analgesics and adjuvant analgesics were in the range of 0.1 to 5 ng/mL and 0.25 to 10 ng/mL, respectively. Furthermore, the average recoveries of the 30 types of drugs spiked at 100 ng/mL were 77.7%–112.1% (RSD: 1.6%–9.7%).

From these results, we suggest that this method would be useful for identifying poisonous substances in forensic science and emergency medicine.

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Ethical approval

This article does not contain any studies on human participants or animals performed by any of the authors.

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