Introduction

Poisoning associated with substances of abuse, such as stimulants, narcotics, and psychotropic drugs, and drug overdose suicides remain major social problems. When a patient arrives at a hospital due to acute drug intoxication, it is important to identify the drug or toxicant to determine the cause of the poisoning quickly. We are currently optimizing the analytical conditions for toxicants in serum obtained from critical care patients and are constructing a toxicological analysis screening system using accurate mass high-resolution mass spectrometry (MS) on a Q-Exactive instrument.

Particular attention must be paid to the analysis of synthetic cannabinoids (SCs) containing positional isomers. SCs are easy to access, have a high potential for abuse, and are an ongoing health problem worldwide, especially among young people. New substances constantly appear on the market, which is continuously changing because manufacturers try to sidestep laws by changing the compounds in their mixtures. Legally regulated SCs and future designer drugs are created by making minor positional modifications to pre-existing analogs, and thus compounds with minor structural differences must be isolated and identified accurately. For iodo-benzoylindole derivatives of SCs, only specific isomers are currently the target of legal control, and it is necessary to establish an analytical method for accurately identifying positional isomers. In this study, we synthesized a series of 57 designer drugs and developed a screening method for identifying halogen positional isomers on the phenyl ring of benzoylindole derivative SCs in serum. Analytical methods using the Discovery F5 pentafluorophenyl column gave the best selectivity and retention of the positional isomer analytes. Some of the meta and para iodo-substituted SCs were eluted at similar retention times and were difficult to separate by liquid chromatography (LC). However, they were identified via the relative abundance of the two product ions in the collision-induced dissociation reaction using LC-hybrid quadrupole/orbitrap high-resolution mass spectrometry. Our synthesized halogen-substituted positional isomer SC library and method for differentiating positional isomers of halogenated benzoylindole SC derivatives could provide an indispensable analysis tool for identifying illegal drugs in serum of drug users.

Keywords Synthetic cannabinoid, positional isomers, screening library, orbitrap, LC-MS/MS

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using gas chromatography (GC)-electron ionization (EI)-MS spectra. For example, differences in fragmentation patterns of the GC-EI-MS/MS spectra can distinguish the positional isomers of naphthoylindole-based SCs, the regio- and stereoisomers of phenethylamines and phenylalkylamines, and the fluoro positional isomers on the phenyl ring of indazole-based SCs (AB-FUBINACA). The dimethoxybenzoylindole isomers can be differentiated by a combination of EI-MS and FT-IR spectra. Liquid chromatography (LC)-electrospray ionization (ESI)-triple quadrupole MS has been used to perform positional isomer differentiation in AB-FUBINACA by measuring the abundance of product ions and to differentiate the fluoro positional isomers on the phenyl ring in 1-fluorobenzyl-3-naphthoyl-indoles (FUB-JWH-018). Eckberg et al. used two-dimensional LC to separate the fluoro positional isomer on the hexyl carbon chain of JWH-019 (i.e., N-(2-fluorohexyl), N-(4-fluorohexyl), N-(5-fluorohexyl), and N-(6-fluorohexyl)). Although methods have been reported for identifying many SCs with fluoro positional isomers, other halogen positional isomers of halogenated benzoylindole isomers, have not been investigated.

We have focused on benzoylindole derivative SCs, formed by the addition of a halogen to the phenyl ring (e.g., AM-679 and AM-694), because only specific isomers are legally controlled. AM-679 was among the first derivatives of 3-(2-iodobenzoyl)indole identified as having cannabinoid receptor affinity. Despite having only modest affinity, AM-679 was subsequently used as a starting point for developing several more specialized cannabinoid ligands, such as the potent CB1 agonists, AM-694 (referred to as C5F1-I here), and the selective CB2 agonist, AM-1241. In cannabinoid ligands, such as the potent CB1 agonists, AM-694 (referred to as C5F1-I here), and the selective CB2 agonist, AM-1241, the fluoro positional isomers on the phenyl ring of indazole-based SCs (AB-FUBINACA). The dimethoxybenzoylindole isomers can be differentiated by a combination of EI-MS and FT-IR spectra. Liquid chromatography (LC)-electrospray ionization (ESI)-triple quadrupole MS has been used to perform positional isomer differentiation in AB-FUBINACA by measuring the abundance of product ions and to differentiate the fluoro positional isomers on the phenyl ring in 1-fluorobenzyl-3-naphthoyl-indoles (FUB-JWH-018). Eckberg et al. used two-dimensional LC to separate the fluoro positional isomer on the hexyl carbon chain of JWH-019 (i.e., N-(2-fluorohexyl), N-(4-fluorohexyl), N-(5-fluorohexyl), and N-(6-fluorohexyl)). Although methods have been reported for identifying many SCs with fluoro positional isomers, other halogen positional isomers of halogenated benzoylindole isomers, have not been investigated.

We have focused on benzoylindole derivative SCs, formed by the addition of a halogen to the phenyl ring (e.g., AM-679 and AM-694), because only specific isomers are legally controlled. AM-679 was among the first derivatives of 3-(2-iodobenzoyl)indole identified as having cannabinoid receptor affinity. Despite having only modest affinity, AM-679 was subsequently used as a starting point for developing several more specialized cannabinoid ligands, such as the potent CB1 agonists, AM-694 (referred to as C5F1-I here), and the selective CB2 agonist, AM-1241. In previous studies, we synthesized a series of new cannabinoid derivatives with various halogen positional isomers, and investigated their physicochemical properties based on variable temperature NMR, and we showed that the position of the halogen substituent affected the conformation.

In this study, we developed a screening method to identify the halogen positional isomers on the phenyl ring of benzoylindole derivative SCs, such as AM-679 (referred to as C5-I here) and AM-694 (referred to as C5F1-I here), using LC-hybrid quadrupole/orbitrap high-resolution MS. Our method for differentiating positional isomers of halogenated benzoylindole SCs confirms the potential utility of the accurate identification of legally regulated SCs and new designer drugs that are likely to appear in the future.

### Experimental

**Reagents and chemicals**

LC-MS grade formic acid, acetonitrile, and methanol were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Deionized water was produced by a Milli-Q system and process the data. Human serum was collected from volunteer donors in France, Germany, the USA, and other countries in accordance with applicable ethical standards. The anonymity of the donors was protected.

**Synthesis**

SCs were prepared by a reported method (Scheme 1). Acylation of indole with various benzoyl chlorides in the presence of Et2Cl yielded benzoyl 2 at the 3-position. Then, compounds 3 were N-alkylated using sodium hydride as a base to obtain SCs 4 in good yields. The structures and purity of the SCs were confirmed by 'H-NMR, 13C-NMR, and ESI-MS. The structures of all compounds and yields are shown in Table 1. Under the Pharmaceutical and Medical Device Act, these compounds, including designated substances and their derivatives, were tightly controlled in locked storage and were carefully handled for research purposes only. Detailed spectral data for the synthesized compounds are not disclosed to prevent misuse, including unnecessary synthesis.

**Analytical method**

Data were acquired on a mass spectrometer (Q Exactive, Thermo Fisher Scientific, Waltham, MA) coupled to a high-performance LC system (UltiMate 3000 RSLC, Thermo Fisher Scientific). Xcalibur 2.1 and Thermo LTQ Tune Plus 2.5.5 software (Thermo Fisher Scientific) were used to control the system and process the data.

First, the chromatographic performance of the following analytical columns was tested: a 100 × 2.1 mm, 2.6 μm dp Kinetex C18 column (Phenomenex, Torrance, CA); a 100 × 2.1 mm, 2.7 μm dp Raptor Biphenyl column (Restek, Bellefonte, PA); and a 150 × 2.1 mm, 3.0 μm dp Discovery F5 column (Supelco, Bellefonte, PA). During method development, we tested water-methanol and water-acetonitrile eluent systems with the addition of 0.1% formic acid under generic gradient conditions (50-100% organic). The optimal separation conditions were as follows: Discovery F5 column; column temperature, 35°C; and water-methanol gradient. To prevent degradation of the samples, the autosampler temperature was set at 10°C. The mobile phase initially consisted of 0.1% formic acid in water (eluent A)-0.1% formic acid in methanol (eluent B) (50:50, v/v). The flow rate was 200 μL/min. The following gradient elution profile was used: eluent A-eluent B 50:50 (v/v) to 0:100 (v/v) over 10 min, hold for 2 min, and 0:100 (v/v) to 50:50 (v/v) over 0.01 min. The system was then re-equilibrated for 5 min, giving a total cycle time of 17 min. The injection volume was 5 μL. The needle wash step used acetonitrile-water (50:50, v/v).

The mass spectrometer was operated in full MS/data-dependent-tandem mass spectrometry (dd-MS²) (TOP5) positive mode. The settings for the full MS scan were as follows: mass resolution, 70000 fwhm; automatic gain control (AGC) target,
Method validation

The method was validated by establishing linearity, intra- and inter-day assay precision, limits of detection, and recoveries. The quality control samples were assayed in sets of six replicates to evaluate the intra- and inter-day assay precision. Linearity was evaluated by the coefficient of determination ($r^2$). Recovery rate was evaluated by spiking control human serum with 8 ng/mL of each standard in triplicate and by calculating the % relative standard deviation (RSD) and % recovery for each SC.

Results and Discussion

Liquid chromatography

The LC parameters were optimized for the screening analysis to obtain the best separation and peak intensities of the SCs containing positional isomers with similar structures (Table 1). Because there are many studies of SC analysis with C18 columns (see review in Ref. 18), initially, a Kinetex C18 column (100 × 2.1 mm, 2.7 μm) with acetonitrile and water was used. The mobile phase was also optimized for a gradient of acetonitrile or methanol. However, most of the *meta* and *para* halogenated derivatives were difficult to separate (Table S1, Supporting Information). With the other C18 columns, the *meta* and *para* isomers still coeluted (data not shown), which may have resulted from the minimal difference in interaction strength with the C18 stationary phases because of their high conformational similarity.

Next, we used a Raptor Biphenyl column (150 × 2.1 mm, 3 μm) for separating the positional isomers. Biphenyl columns combine the performance of a traditional alkyl phase (C8 or C18) with that of a phenyl phase, which is frequently used in clinical and forensic applications. Biphenyl columns exhibit the highest aromatic selectivity of phenyl columns, such as phenyl-hexyls. However, the biphenyl column did not separate most of the *meta* and *para* isomers in the acetonitrile mobile phase,
span of the retention time distribution provided sufficient time for the dd-MS² experiment between full MS scans. Selectivity was based on high resolution, ions of interest could be separated from background matrix using EICs with the ±5 ppm mass tolerance, and almost no interfering peaks from serum were observed.

Identification by the MS patterns

The Q-Orbitrap acquired full MS scan data and performed a three-step NCE (collision energy [CE]) of 10, 30, and 60 eV) dd-MS² fragmentation on the precursor ion. All fragments created in the three steps were collected sequentially and obtained by single-scan detection. According to the observed accurate mass fragments, we assigned the formulae for iodo-substituted SCs (Table S2) and other halogenated SCs (-F, -Cl, and -Br; Table S3).

The MS cleavage pattern of the benzoyl group depended on the type of halogen substituent (Scheme 2). For example, for iodo substituent derivative C5-I, the fragment ions at \( m/z \) 418 were used as the precursor ions for MS/MS analysis. The base peak was \( m/z \) 231 arising from a benzoyl ring moiety. The product ion at \( m/z \) 231 produced the fragment ions at \( m/z \) 203 by consecutive losses of a molecule of carbon monoxide (-28 Da). The product ion at \( m/z \) 203 gained a water molecule (+18 Da) upon storage in the ion trap to yield the ion at \( m/z \) 221 via an acylium ion intermediate. This fragmentation scheme in the collision cell was reported by Beuck et al. The ion at \( m/z \) 221 corresponded to a nominal loss of 10 Da (-C/+2H) from \( m/z \) 231. The loss of an iodo radical gave the ion at \( m/z \) 94. The loss of a halogen radical from aromatic systems is often observed in ESI-MS/MS experiments. The probability of the formation of a radical ion increases in the order F < Cl < Br < I with decreasing bond dissociation energy. Among the SCs containing F or Cl substituents in this study, no fragment ions at \( m/z \) 94 and 76 formed via loss of a halogen radical from an aromatic ring were observed (Scheme 2, Table S3). Common fragment ions at \( m/z \) 231, 203, 221, 94, and 76 were observed in the product ions of C0-I, C1-I, C2-I, C4-I, C5-I, C5F-I, and CSF3-I, and the length of the tail part of the SCs did not affect the MS patterns. Low relative intensities of product ions at \( m/z \) 221 and 203 were observed for the ortho isomers (Table S2). The retention properties of these isomers were also similar (Fig. 1); sufficient chromatographic separation could not be achieved between the C5-m-I and C5-p-I isomers of AM-679, or the CSF1-m-I and CSF1-p-I isomers of AM-694, or the CSF3-m-I and CSF3-p-I isomers. Thus, further analysis was performed for these isomers.

Differentiation by MS/MS

According to Murakami et al. and Chikumoto et al., the fluoro positional isomers of SCs can be differentiated by the relative abundance of the two product ions in the collision-induced dissociation reaction using LC-MS. Based on these studies, we examined whether the iodo positional isomers of SCs can be differentiated in serum according to the difference in the relative abundances of product ions. To find the optimum CE for distinguishing positional meta and para isomers of C5-I by the product ion spectrum, the CE was changed from 10 to 60 eV in increments of 10 eV (Fig. 2). Fragmentation increased with CE, and \( m/z \) 231 appeared as a base peak at above CE 20 eV (Fig. 2c). The production of product ions \( m/z \) 221 and 94 increased at above CE 40 eV, and the difference in the relative intensity between meta and para isomers was greatest at CE 50 eV (Figs. 2a and 2b). The product ion spectra for the precursor ion at \( m/z \) 418 with CE of 50 eV are shown in Fig. 3a,
Due to the orientation of the halogen substituents, CO loss and $\text{H}_2\text{O}$ gain tended to occur more easily in the meta isomer than in the para and ortho isomers, and the product ions at $m/z$ 221 and 94 were also more easily generated from the meta isomer. Similarly, in other compounds with a halogen (F, Cl, or Br) substituent, the relative abundance ratio of the product ion formed by 10 Da neutral loss via an acylium ion intermediate determined with CE...
50 eV was greater for the meta isomer than for the para isomer (Fig. S1). The differences in relative intensity abundance were also seen with other SCs that had other carbon chain tails, such as C0-1, C1-1, C2-1, C4-1, C5F1-1, and C5F3-1.

Welch’s t-test was performed to determine the statistical differences between the abundances of the fragment ions of the isomers. For each fragment ion, the mean relative intensities at m/z 221 for the meta and para isomers were compared and showed small p-values at p < 0.001. The reproducibility test confirmed the high reproducibility of the relative intensity ratios in any iodinated SCs at a CE of 50 eV and at NCEs of 10, 30, and 60 eV.

The regioisomeric identification by the MS/MS pattern proved that the method was effective for iodo substituents in addition to fluoro substituents. These results will be useful for differentiation analysis of positional isomer SCs with iodine substituents, such as AM-2233 and AM-1241, and new designer drugs.

**Developed method validation**

Validation data for our method are shown in Table S4. The linearity of the 57 compounds that were used to spike human control serum were evaluated over a concentration range of 0.03 – 40 ng/mL (n = 6). Linear regression was applied by plotting the peak area responses of each compound. The R² values were between 0.9941 and 0.9996. Limits of detection calculated at a signal-to-noise ratio of 3.3 were between 0.001 and 0.01 ng/mL for all samples regardless of the presence of matrix. Recoveries were 81.5 – 130.4% and RSDs were 0.2 – 8.2%. The precisions of the method were evaluated for each compound at 8 ng/mL in triplicate by spiking the control serum matrix with the analyte standard. Intra-assay precision was 0.2 – 4.4% RSD and the inter-assay precision was 0.3 – 6.6% RSD. The concentrations of general SCs in serum have been reported as 0.1 – 190 ng/mL in poisoning cases. In fatal cases, the concentrations were 0.1 – 199 ng/mL for JWH-018, 0.1 – 68.3 ng/mL for JWH-073, 12 ng/mL for AM-2201, and 1.1 – 1.5 ng/mL for 5F-PB-22. To validate our screening method, it would be necessary to measure serum samples from actual drug users. The limits of quantification achieved in this screening method for SCs are similar to or lower than those in other studies.

**Conclusions**

Our method positively identified SCs with various halogen positional isomers in serum by differentiating them with a combination of retention time and fragmentation pattern (MS/MS). Among the iodo-substituted benzyl SC derivatives, only the ortho positional isomers, such as AM-679 and AM-694, are legally controlled, and good separation patterns from the meta and para isomers were obtained by using a pentafluorophenyl column. Although AM-679 isomers C5-m-I and C5-p-I, and AM-694 isomers C5F3-m-I and C5F3-p-I were eluted within 1 s of each other and had the same accurate mass, they could be identified by their MS/MS fragmentation patterns. MS/MS spectra distinguished meta and para positional isomers based on the differences in the relative abundance of the product ion assigned as C6H6IO⁺ (m/z 221). Our method for differentiating halogenated positional isomers SCs may provide an indispensable analysis tool for identifying illegal drugs and adulterated products.

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**Supporting Information**

The supporting information contains retention times, mean relative intensity, product ion spectra, and method validation for all SCs. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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