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Long-term stability of 24 synthetic cannabinoid metabolites spiked into whole blood and urine for up to 168 days, and the comparable study for the 6 metabolites in non-spiked real case specimens stored for several years

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Abstract

Purpose The aim of this study is to investigate the stabilities of the 24 synthetic cannabinoid metabolites (SCMs) in blood and urine at various temperatures from –30 to 37℃ stored for 1 to 168 days. In addition, experiments of stabilities at lower temperatures and for much longer duration have been performed as described below.

Methods The quantification was performed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The blank blood and urine spiked with SCMs and non-spiked real case (authentic) specimens were incubated at 37 ℃ up to 56 days and at 22, 4 or –30℃ up to 168 days. The non-spiked authentic blood and urine specimens were also stored at –30 or –80℃ for 1, 3 or 5 years to investigate stabilities during very long time frames.

Results All the 24 SCMs were much more stable in urine than in blood at 37, 22 or 4℃. All 24 SCMs spiked into blood or urine were stable at –30℃ for up to 168 days. The 6 SCMs in the authentic specimens exhibited long stabilities at –30 or –80℃ for 3 to 5 years. Some tendencies were observed according to the relation between the structures of SCMs and their stabilities.

Conclusions The long-term stabilities of 24 SCMs in spiked samples and those of 6 SCMs in the authentic specimens were examined using LC–MS/MS. SCMs were largely very stable and usable several years after storage at –30 or –80℃.

Keywords Synthetic cannabinoid metabolite; Long-term stability; Blood and urine; Human authentic specimens; Deep-freezing; LC–MS/MS

Introduction

The detection of synthetic cannabinoid metabolites (SCMs) was widely performed to confirm/identify the synthetic cannabinoids (SCs), because the detection of the SCs is usually a difficult task especially in urine. The reasons may be that the amounts of SCs administered are usually low, because their pharmacological actions and toxicities are sufficiently high; the speed of their metabolization is high and/or the excretion of SCs into urine is small due to their high lipophilicity. The short-term temperature stability tests of SCMs in urine were repeatedly performed in the validation of quantification methods

[1-4]. The results were as follows: 24 SCMs spiked into urine were examined after 16 h at room temperature and 3 days at 4℃; and all of them were found to be stable [1]. The 47 SCMs were stable after 24 h at room temperature and 3 days at 4℃ [2]. The 37 SCMs were stable after 14 days at 4℃ and at –20℃ [3]. The 34 SCMs were stable after 3 and 5 days at 25℃; and also stable after 7 and 14 days at 4℃, with the exception of JWH-210 *N*-(5-hydroxyindole), for which a decrease of 25 % was observed [4]. Concerning the stability tests of SCMs in blood, one study was reported [5]; 3 SCMs (one methylbutanoic acid derivative and two dimethylbutanoic acid derivatives) spiked into whole blood were all stable at 22, 4 and –20℃ up to 35 days.

In our study, long-term stabilities of 24 SCMs up to 168 days at 22, 4, or –30℃, and ten-cycle freeze-thaw stability were examined in the spiked both whole blood and urine. In addition, the stabilities of 6 SCMs in blood and urine specimens obtained from three cadavers [6–8] stored at –30 and –80℃ for 1, 3 and 5 years were tested.

In this work, 24 SCMs named M1–M24 as listed in Table 1 were selected to examine their long-term temperature stabilities as well as to find out the relations between the structures and their stabilities. As shown in Fig. 1, the structures of SCMs examined in the present work mostly consisted of heterocyclic core (part I) (indole [M1, M3, M6] and indazole [M17, M19, M21]) with attached group (hydroxypentyl [M1], carboxybutyl [M3], cyclohexylmethyl [M6] and others), lipophilic substituent (part II) (naphthalene [M1], qinoline [M3], amino-3-methyl-oxobutane and its derivatives such as terminal amide [M17], terminal acid [M19], terminal ester [M21] and others) and the linker connecting part I and II (ketone [M1], ester [M3] and amide [M17, M19, M21]).

The concentrations of six SCMs in blood specimens could not been measured in the previous works [6–8], because their quantification method had not been established in our laboratories at that time. Given that the quantification method of 24 SCMs in whole blood using liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been validated in the present work, it has become able to quantify six SCMs in non-spiked (the “non-spiked” is skipped for simplicity hereafter) authentic blood specimens preserved under the same conditions as those of the authentic urine specimens, and their stabilities have also been examined.

Materials and methods

Reference compounds, chemicals and human matrices

The 24 SCMs named from M1 to M24 listed in Table 1 and 1-(5-fluoropentyl)-*N*-(naphthalen-1-yl)-1*H*-indole-3-carboxamide (5F-NNEI) as internal standard (IS), were obtained from Cayman Chemical (Ann Arbor, MI, USA), and their commercial names with formal names from M1 to M24 were as follows; JWH-018 *N*-3-hydroxypentyl {(1-(3-hydroxypentyl)-1*H*-indol-3-yl)(naphthalen-1-yl)methanone}, PB-22 *N*-5-hydroxypentyl {quinolin-8-yl 1-(5-hydroxypentyl)-1*H*-indole-3-carboxylate}, PB-22 *N*-pentanoic acid {5-(3-quinolin-8-yloxy)carbonyl)-1*H*-indol-1-yl)pentanoic acid}, PB-22 3-carboxyindole {1-pentyl-1*H*-indole-3-carboxylic acid}, 5F-PB-22 3-carboxyindole {1-(5-fluoropentyl)-1*H*-indole-3-carboxylic acid}, BB-22 3-carboxyindole {1-(cyclohexylmethyl)-1*H*-indole-3-carboxylic acid}, AB-PINACA *N*-4-hydroxypentyl {*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-hydroxypentyl)-1*H*-indazole-3-carboxamide}, AB-PINACA *N*-pentanoic acid {3-[[[1-(aminocarbonyl)-2-methylpropyl]amino]carbonyl]-1*H*-indazole-1-pentanoic acid}, ADB-PINACA *N*-4-hydroxypentyl {*N*-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-hydroxypentyl)-1*H*-indazole-3-carboxamide}, AB-CHMINACA M1A {(S)-*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-hydroxycyclohexyl)methyl)-1*H*-indazole-3-carboxamide }, AB-CHMINACA M2 {*N*-[[1-(cyclohexylmethyl)-1*H*-indazol-3-yl]carbonyl]-L-valine}, AB-CHMINACA M3A {(1-((4-hydroxycyclohexyl)methyl)-1*H*-indazole-3-carbonyl)-L-valine}, AB-CHMINACA M4 {(1-cyclohexylmethyl)-1*H*-indazole-3-carboxylic acid}, MAB-CHMINACA M1 {*N*-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-((4-hydroxycyclohexyl)methyl)-1*H*-indazole-3-carboxamid e}, MAB-CHMINACA M2 {(S)-2-(1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamido)-3,3-dimethylbutanoic acid}, MAB-CHMINACA M3 {(S)-2-(1-((4-hydoxycyclohexyl)mathyl)-1*H*-indazole-3-carboxamido)-3,3-dimethylbutanoic acid},

MAB-CHMINACA M11 {*N*-(1-amino-4-hydroxy-3,3-dimethyl-1-oxobutan-2-yl)-1-((4-hydoxycyclohexyl)methyl)-1*H*-indazole-3-carboxamide}, AB-FUBINACA M2A {4-amino-3-(1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamido)-2-methyl-4-oxobutanoic acid}, AB-FUBINACA M3 {*N*-[[1-[(4-fluorophenyl)methyl]-1*H*-indazol-3-yl]carbonyl)-L-valine}, AB-FUBINACA M4 {1-[(4-fluoropheny)metyl]-1*H*-indazole-3-carboxylic acid}, 5F-AMB M2 {*N*-[[1-(5-hydroxypentyl)-1*H*-indazol-3-yl]-L-valine, methyl ester}, 5F-AMB M3 {*N*-[[1-(4-carboxybutyl)-1*H*-indazol-3-yl]-carbonyl]-L-valine, 1-methyl ester}, 5F-AMB M7 {1-(5-fluoropentyl)-1*H*-indazole-3-carbonyl)-L-valine}, 5F-ADB M7 {(S)-2-(1-(5-fluoropentyl)-1*H*-indazole-3-carboxamido)-3,3-dimethylbutanoic acid}.

β-Glucuronidase-type-H-1 was obtained from Sigma (St. Louis, MO, USA); methanol and acetonitrile suitable for LC–MS, 1-chlorobutane (CB) and other chemicals of analytical grade from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Pure water with a specific resistance of 18 MΩ•cm was used (Millipore, Bedford, MA, USA).

Fresh blood and urine specimens were collected from five healthy subjects under their permission with obtaining informed consent and stored at – 80 ℃. They were thawed and used as blank blood and urine. Blank blood and urine spiked with several amounts of 24 SCMs were used as quality control samples. The authentic specimens obtained from three cadavers autopsied at our department had been stored at – 30 and – 80 ℃ until analyses [6–8]. The drug testings of these authentic specimens were requested officially by judicial authorities.

Standard solutions

Individual stock solutions of 24 SCMs and IS were prepared separately by dissolving appropriate amounts of each compound in acetonitrile at 1 mg/mL and stored at – 30 ℃. The three quantification-range groups a, b, and c were adopted because the largest difference of the peak counts was 200-fold among the 24 SCMs as listed in Table S1. The quantification ranges were 0.03 – 0.6, 0.1 – 2.0 and 0.5 – 10 ng/mL for the groups a, b, and c, respectively. Working calibration samples were prepared at 0, 0.03, 0.06, 0.18, 0.36 and 0.6 ng/mL for a; 0, 0.1, 0.2, 0.6, 1.2 and 2.0 ng/mL for b; and 0, 0.5, 1, 3, 6 and 10 ng/mL for c,

by spiking acetonitrile solution containing suitable amounts of 24 SCMs to 100 μL of blood or urine (*n*= 6 at each concentration). The quality control samples were prepared at 0.03, 0.18 and 0.6 ng/mL for a; 0.1, 0.6 and 2 ng/mL for b; and 0.5, 3 and 10 ng/mL for c by spiking acetonitrile solution containing suitable amounts of 24 SCMs to 100 μL of blood or urine (*n*= 3 at each concentration). As stated above 5F-NNEI at 1 ng/mL in blood or urine was used as IS for the quantification. 5F-NNEI is selected as IS because it is very stable, and its polarity and molecular weight are relatively similar to those of 24 SCMs.

Detection of phase I metabolites

The reference standards of 24 SCMs obtained were all phase I metabolites. In the quantification of the six SCMs in the authentic urine specimens, the glucuronide-hydrolysis was applied to the urine specimens, because the previous quantifications were performed after the hydrolysis, respectively [6–8]. In the quantification of the six SCMs in the authentic blood specimens, the hydrolysis was not applied to the blood specimens, and hence the stabilities of the six SCMs without hydrolysis treatment were examined.

Extraction of 24 SCMs from whole blood and urine

The extraction of 24 SCMs from blood is as follows. To 100 μL of blood (either spiked blood or the authentic blood specimen) in a tube, two stainless beads (diameter 3 mm), 100 μL of water and 100 μL of acetonitrile containing IS (1 ng/mL blood) were added and gently mixed. After adding 0.7 mL of acetonitrile, they were vortex-mixed for 1 min, and centrifuged at 10,000 *g* for 2 min. To the 0.9 mL of supernatant placed in another tube, 0.3 mL of CB and 150 mg of $MgSO_4$ were added, vortex-mixed for 1 min and centrifuged at 10,000 *g* for 2 min. The upper 0.8 mL was placed into another tube and evaporated to dryness using the centrifugal dryer (miVac Duo LV; Genevac Ltd, Ipswich, England). The residue was reconstituted with 150 μL of acetonitrile and the supernatant (5 μL) was used for LC–MS/MS analysis.

The extraction of SCMs from the spiked urine was the same as that of blood. The extraction of SCMs from the authentic urine specimens was as follows. To 100 μL of urine, two stainless beads (3-mm diameter), 95 μL of 0.1 M sodium acetate buffer (pH 5) and a 5-μL aliquot of β-glucuronidase solution

(5000 unit) was added and incubated at 37 ℃ for 2 h. To the sample, 100 μL of acetonitrile containing IS (1 ng/mL urine) and 0.7 mL of acetonitrile were added, and they were vortex-mixed for 1 min, and centrifuged at 10,000 *g* for 2 min. To the 0.9 mL of supernatant placed in another tube, 0.3 mL of CB and 150 mg of $MgSO_4$ were added, vortex-mixed for 1 min and centrifuged at 10,000 *g* for 2 min. The upper 0.8 mL was placed into another tube and evaporated to dryness using the centrifugal dryer. The residue was reconstituted with 150 μL of acetonitrile and the supernatant (5 μL) was used for LC–MS/MS analysis.

Validation of the quantification method

Quality control samples (n = 3 at each concentration listed) were prepared independently from the calibrator solutions and used for the calculations of accuracies, precisions, recoveries, matrix effects, limits of detection (LODs) and limits of quantification (LOQs), respectively. For the validation of quantification of 24 SCMs spiked at LOQ, 6 x LOQ and 20 x LOQ in blood and urine, we employed the usual matrix-matched calibration method according to Peters et al. [9]. LOD was calculated to be the concentration that gave signal-to-nose (S/N) ratio = 3 using three blank samples.

Instrumental conditions

LC–MS/MS was performed on an Acquity LC instrument (Waters, Milford, MA, USA) connected with an electrospray ionization QTRAP 4000 MS/MS system (AB SCIEX, Framingham, MA, USA) in the positive ion mode. The column and measurement conditions for this instrument were the same as described in [6]. In the selected reaction monitoring (SRM) mode, the collision energy and the transition were 31 V and *m/z* 375.2 → 232.1 for IS, respectively. For the 24 SCMs, the protonated molecular ions, quantifier product ions (QTs) and qualifier product ions (QL1 and QL2) with their collision energies are listed in Table 2.

Stability tests for spiked samples

To examine the temperature stabilities, 100 μL of blood or urine sample in a tube with cap was spiked with 1 μL of acetonitrile solution to make their concentrations to be LOQ or 20 x LOQ, and incubated (*n* = 2, each) under one of the four temperature conditions for each time frame; at 37 ℃ for 1, 2, 4, 7, 14, 28 and 56 days; at 22 or 4℃ for 1, 2, 4, 7, 14, 28, 56, 112 and 168 days; at –30℃ for 14, 28, 56, 112 and 168 days as shown in Table 2. Stability measurements were performed according to three time frames. In each time frame, the incubation started from the longest interval to the shortest one; that is, from the sample on day 7 to the sample on day 1 in frame 1. For the sample on day 0, SCMs were spiked to the blank sample just before the extraction in each frame.

To examine freeze-thaw stability, 100 μL blood or urine sample was spiked with 1 μL of acetonitrile solution containing 24 SCMs to make their concentration to be LOQ or 20 x LOQ (*n* = 2, each), and frozen at –30℃ and thawed at 4℃ for ten cycles.

After above treatments, IS was added, and SCMs with IS was extracted. The remaining amounts of SCMs were compared with those in freshly prepared corresponding sample (day 0) and were expressed in %.

Stability tests for the authentic specimens

The authentic blood or urine specimens preserved at –80℃ were divided into 100 μL aliquot of blood or urine specimens, and they were incubated at 37 ℃ for 4, 7, 14, 28 and 56 days; at 22 or 4℃ for 4, 7, 14, 28, 56, 112 and 168 days; at –30℃ for 112 and 168 days (*n* = 2, each) as listed in Table 2. After these treatments, IS was added at 1 ng/mL in blood or urine, and the remaining amounts of SCMs were compared with those of 100 μL aliquot of blood or urine specimen preserved at –80℃, and then added with IS just before the extraction of the SCMs (day 0).

Results

Validation of the quantification method in blood and urine

The regression equations of the calibration curves for 24 SCMs spiked at 0, LOQ, 2 x LOQ, 6 x LOQ, 12 x LOQ or 20 x LOQ are listed in Table S2 where the correlation coefficients were 0.994 – 0.999 for blood, and Table S3 where the correlation coefficients were 0.992 – 0.998 for urine. The LODs and LOQs were also listed there. The validation of the method was performed for 24 SCMs spike at LOQ, 6 x LOQ and 20 x LOQ. The accuracies and precisions (spiked three times a day or on three different days), recoveries and matrix effects for 24 SCMs in blood samples were assessed and listed in Table S4. The accuracies were 70.8–131 %; precisions not greater than 16.6 %; recoveries 71.7–104 %; and matrix effects 81.8–117 %. The validation data for urine in Tables S5 showed that the accuracies were 75.4–126 %; precisions not greater than 27.2 %; recoveries 75.8–104 %; and matrix effects 75.5–116 %. These results indicated that the present method was acceptable for the quantification of 24 SCMs in blood and urine.

The SRM chromatograms of 24 SCMs spiked at respective LOQ in blood were detected by LC–MS/MS and are shown in Fig. 2. (**a**) Eight SCMs belonging to group a, (**b**) eight SCMs belonging to group b and (**c**) eight SCMs belonging to group c were shown.

Quantification of six SCMs in the authentic blood and urine

The concentrations of six SCMs such as M19, M20 [6], M14, M17 [7] and M10, M12 [8] in authentic urine specimens were reported previously and these were stored at – 30 and – 80 ℃ for about 1, 3 or 5 years, respectively. Therefore, the specimens stored at – 30 and – 80 ℃ were quantified again in the present work. The concentrations of urine preserved at – 30℃ (n = 3) were almost equal to those preserved at – 80℃ (n = 3) as listed in Table 3. The percentage values of the present concentrations in urine preserved at – 80℃ were calculated based on the concentrations reported previously [6–8] and also listed in Table 3, indicating that these SCMs were stable at – 30 and – 80℃ for about 1, 3 or 5 years.

The concentrations of the six SCMs in the authentic blood specimens could not been quantified in the previous works [6–8], but the specimens were stored at – 30 and – 80℃. However, their concentrations could be quantified using the validated method in the present work. The concentrations of blood preserved at – 30℃ (n = 3) were almost equal to those preserved at – 80℃ (n = 3) as listed in Table 3.

Results of stability tests of 24 SCMs spiked into blood and urine and those of six SCMs in the authentic blood and urine

The results of temperature stability of spiked 24 SCMs were listed in Table 4. The samples spiked at either LOQ (n = 2) or 20 x LOQ (n = 2) showed completely the same stability tendencies in either blood or urine, and hence the table listed the results based on the total four samples. Comparing the stability in blood with that in urine, all the SCMs were less stable in blood than in urine. The numbers of stable SCMs (>80 %) at 37 ℃ on day 56, at 22 ℃ on day 168 and 4 ℃ on day 168 were 3, 3 and 5 in blood (Table 4, left) whereas those were 19, 19 and 23 in urine (Table 4, right), respectively. All the SCMs were stable in both blood and urine at – 30 ℃ on day 168 (data not shown).

All the SCMs were stable in both blood and urine after 10 freeze-thaw cycles between – 30 ℃ and 4 ℃ (data not shown).

Concerning the relations between the chemical structure of SCMs and stabilities, following tendencies were observed in both blood and urine; the most fragile two metabolites were M21 (5F-AMB M2) and M22 (5F-AMB M3) having terminal ester moieties at the side chain of part II, and secondary fragile two metabolites were M2 (PB-22 5-hydroxypenthyl) and M3 (PB-22 pentanoic acid) that have ester linker connecting indole group and quinoline group. In blood samples two 3-carboxyindazole metabolites, such as M13 (AB-CHMINACA M4) and M20 (AB-FUBINACA M4) were more fragile than three 3-carboxyindole metabolites such as M4 (PB-22 3-carboxyindole), M5 (5F-PB-22 3-carboxyindole) and M6 (BB-22 3-carboxyindole).

The stabilities of six SCMs in authentic blood and urine specimens were examined under the same temperature conditions as those for spiked blood and urine samples, and compared as listed in Table 5. The six SCMs in authentic blood and urine specimens were stable at – 30 ℃ on day 168 (data not shown).

Discussion

In this article, we have examined the long-term stabilities of 24 SCMs in blood or urine under the

temperature conditions at – 30 to 37 ℃. In addition, using the authentic human specimens stored in deep freezer (– 80℃) for 1 to 5 years, much longer-term stabilities of the SCMs were studied.

No appreciable difference was observed between the stability of SCMs at the concentrations of LOQ and that of 20 x LOQ. Twenty times concentration difference of SCMs did not affect the degradation reactions of SCMs.

Although the instability was observed at 4 ℃ to some extent, all the 24 SCMs in spiked fresh blood or urine and the authentic blood and urine were stable at – 30℃ for 168 days in the present study (Tables 4, 5). Therefore, freezing may be an important method to avoid the degradation of SCMs. In the authentic specimens, the concentrations of blood and urine preserved at – 30℃ (n = 3) were almost identical with those preserved at – 80℃ (n = 3) for 1, 3 and 5 years (Table 3).

There were several reports on the stabilities of SCs themselves in human urine [10], oral fluid [11], serum [12] and blood [5]. In the study of 5F-ADB in urine [10], the mean recoveries of 5F-ADB were 87, 93 and 26 % from the urine at pH 4.0, 6.9 and 8.5, respectively, indicating that the hydrolysis of ester and amide groups occurs easier in alkaline than in acidic environments. The pH of urine in the blank samples and authentic specimens were all 6 – 7 in the present study.

In the report of SCs in oral fluid [11], highly lipophilic SCs such as JWH-018 and MAM-2201 in oral fluid samples adsorbed to the surface of polypropylene containers when stored at 25 ℃ for 3 days, leading to considerable drug loss, but JWH-200 having hydrophilic morpholine moiety showed no drug loss. In the present study polypropylene tubes (Eppendorf AG, Hamburg, Germany) were used, but M1 (JWH-018 *N*-3-hydroxypentyl) was quite stable as listed in Table 4, and other SCMs especially in urine did not show the appreciable tendency of adsorption to polypropylene. The reason may be that the present target compounds were metabolites that were more hydrophilic than SCs themselves.

As listed in Table 4 (left), M21 (5F-AMB M2) and M22 (5F-AMB M3) having terminal ester moieties were unstable within 1 day at 37 ℃ in blood. FUB-AMB and 5F-ADB having terminal ester moiety were also reported to be unstable within 1 day at 22 ℃ in blood as shown in Fig. 2 of [5]. In the stability tests of SCs in serum [12], however, FUB-AMB, 5F-AMB and AMB having terminal ester moiety were also stable at 20 ℃ until day 7 in serum as reported in the Table 4 of [12], and nearly the same or higher stability at 22 and 37 ℃ until day 7 was observed as to M21 and M22 in the present urine samples as listed

in Table 4 (right). Comparing the above stabilities in blood, serum and urine, following order of stability as to matrix substances can be proposed: blood << serum < urine, indicating the strong enzymatic and chemical degradations in blood.

In the present study, the second fragile two metabolites both in blood and urine were M2 (PB-22 5-hydroxypentyl) and M3 (PB-22 pentanoic acid) that have an ester linker connecting the indole and the quinoline. That is, the order of stability is M21, M22 (amide linker having terminal ester moiety in part II) < M2, M3 (ester linker connecting indole and quinoline). The same order of stability was also reported for SCs themselves in serum [12]: AMB, 5F-AMB, FUB-AMB (amide linker having terminal ester moiety in part II) < PB-22, 5F-PB-22 (ester linker connecting indole and quinoline). Because the esters of M21, M22, M2 and M3 were easily changed to the corresponding acid compounds, all the produced acids were not included in the present 24 SCMs.

Comparing indazole compounds and indole compounds in the present study, the instability of indazole compounds as compared to indole compounds was observed in blood; stability: M13, M20 (3-carboxyindazole) < M4, M5, M6 (3-carboxyindole). Such stability tendency was also reported for SCs themselves in serum [12]: NPB-22, 3CAF, 5F-SDB-005 (indazole compounds) < FUB-PB-22, PB-22, 5F-PB-22 (indole compounds), and in blood [5]: 5F-ADB (indazole compound) < 5F-MDMB-PICA (indole compound).

The effect of terminal OH at the pentyl chain was compared with terminal COOH; M2 and M21 were selected as the compounds having OH, and M3 and M22 were selected as the compounds having COOH in Table 1. The stability order was M2 < M3 at 37, 22 and 4 ℃ in blood as listed in Table 4 (left), and the stability order was also M21 < M22 at three temperatures in blood, although the difference was small. It means that the stability of terminal COOH compounds is higher than that of OH compounds.

To compare the difference between dimethyl (isopropyl) moiety and trimethyl (*t*-butyl) moiety; M7, M10, M11, M12 and M23 were selected as the compounds having dimethyl moieties and M9, M14, M15, M16 and M24, as those having trimethyl moieties, but obvious stability difference between them was not observed.

M17 was found to be unstable following to M21, M22 and M2, as listed in the Table 4. Because M17

was produced after the hydroxylation at the lipophilic methyl moiety of M14, the hydroxylation there seemed to further decrease the stability of M17.

Some of above findings between the stabilities and the structures of SCMs were merely the hypotheses because the number of SCMs, 24, were insufficient, but some of them may be true because the same tendency was also observed in other studies [5,12].

The stabilities of six SCMs in the authentic blood and urine were compared with those in the spiked blood and urine as listed in Table 5. The order of stability among the six SCMs in the authentic blood (M17<M20, M14<M10, M12, M19) and that of six SCMs in spiked blood were nearly the same. However, the days that the instability appeared in the authentic blood were longer than those in the spiked blood concerning most of six SCMs. That is, in the case of M17 at 37℃, the elapsed day in the authentic blood was day 14 whereas that in spiked blood was day 7. This may indicate that the degradation activity in the authentic blood is lower than that in the spiked blood, because the authentic blood specimens had been preserved for long time and hence its degeneration activity was decreased partially at the beginning of this stability test. The stabilities of six SCMs in the authentic urine were almost the same as those in the spiked urine.

Conclusions

In the present study, stabilities of 24 SCMs in whole blood and urine matrices were examined by storing them at 37, 22, 4 and – 30℃ for long time, and they were stable until 168 days at – 30 ℃ in both matrices. Furthermore, the ultra-long-term stabilities of six SCMs in blood and urine matrices obtained at autopsies were studied by storing them at – 30 and – 80℃ for 1 to 5 years. The six SCMs in the authentic specimens showed nearly the same values between at – 30 and – 80℃ even after 5-year storage. These values in urine were almost equal to those obtained soon after the autopsies [6– 8]. By the present study, it has become clear that SCMs in blood and urine matrices are extremely stable under deep-freeze conditions for a long time. These results have promising potential that many kinds of SCMs can be collected widely without any decomposition during a long time period by deep-freeze, which may enable the search of new

pharmacological actions of SCMs for drug discovery, and clarification of the relation between structure and activity/toxicity. Of course, it is practically very useful for reproduction and/or confirmation of the past SC poisoning cases.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11419…..

Declarations

Conflict of interest Authors have no financial or other relations that could lead to a conflict of interest.

Ethical approval Informed consent was obtained from all participants included in the study, who supplied about 15 mL each of blood and/or 40 mL each of urine for use of blank samples. The analysis of toxic substances from a cadaver was permitted by judicial authorities and supported by official documentation.

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Figure captions

Fig. 1 Structures of representative synthetic cannabinoid metabolites (SCMs)

Fig. 2 Selected reaction monitoring chromatograms by liquid chromatography–tandem mass spectrometry for the detection of 24 SCMs at the concentrations of their limit of quantification. (**a**) Eight SCMs belonging to group a, (**b**) eight SCMs belonging to group b and (**c**) eight SCMs belonging to group c

Table 1 Structures of synthetic cannabinoid metabolites (SCMs): names of the SCMs, linker (L) connecting between part I (with attached group) and part II, and the sites (L, I and/or II) of the biotransformation from each parent synthetic cannabinoid

Name of metabolite	Linker	Part I (attached group)	Part II	Site: biotransformation
M1 (JWH 018 *N*-3-hydroxypentyl)	Ketone	Indole (3-hydroxypentyl)	Naphthalene	I: hydroxylation
M2 (PB-22 *N*-5-hydroxypentyl)	Ester	Indole (5-hydroxypentyl)	Quinoline	I: hydroxylation
M3 (PB-22 *N*-petanoic acid)	Ester	Indole (4-carboxybutyl)	Quinoline	I: carboxylation
M4 (PB-22 3-carboxyindole)	_[a]	Indole (pentyl)	_	L: ester hydrolysis
M5 (5F-PB-22 3-carboxyindole)	_	Indole (5F-pentyl)	_	L: ester hydrolysis
M6 (BB-22 3-carboxyindole)	_	Indole (cyclohexylmethyl)	_	L: ester hydrolysis
M7 (AB-PINACA *N*-4-hydroxypentyl)	Amide	Indazole (4-hydroxypentyl)	AB	I: hydroxylation
M8 (AB-PINACA *N*-petanoic acid)	Amide	Indazole (4-carboxybutyl)	AB	I: carboxylation
M9 (ADB-PINACA *N*-4-hydroxypentyl)	Amide	Indazole (4-hydroxypentyl)	ADB	I: hydroxylation
M10 (AB-CHMINACA M1A)	Amide	Indazole (4-hydroxy cyclohexylmethyl)	AB	I: hydroxylation
M11 (AB-CHMINACA M2)	Amide	Indazole (cyclohexylmethyl)	MBA	II: amide hydrolysis
M12 (AB-CHMINACA M3A)	Amide	Indazole (4-hydroxy cyclohexylmethyl)	MBA	I: hydroxylation, II: Amide hydrolysis
M13 (AB-CHMINACA M4)	_	Indazole (cyclohexylmethyl)	_	L: Amide hydrolysis
M14 (MAB-CHMINACA M1)	Amide	Indazole (4-hydroxy cyclohexylmethyl)	ADB	I: hydroxylation
M15 (MAB-CHMINACA M2)	Amide	Indazole (cyclohexylmethyl)	DBA	II: amide hydrolysis
M16 (MAB-CHMINACA M3)	Amide	Indazole (4-hydroxy cyclohexylmethyl)	DBA	I: aydroxylation, II: amide hydrolysis
M17 (MAB-CHMINACA M11)	Amide	Indazole (4-hydroxy cyclohexylmethyl)	AHB	I: hydroxylation, II: hydroxylation
M18 (AB-FUBINACA M2A)	Amide	Indazole (fluorobenzylmethyl)	ABA	II: carboxylation
M19 (AB-FUBINACA M3)	Amide	Indazole (fluorobenzymethyl)	MBA	II: amide hydrolysis
M20 (AB-FUBINACA M4)	_	Indazole (fluorobenzymethyl)	_	L: amide hydrolysis
M21 (5F-AMB M2)	Amide	Indazole (5-hydroxypentyl)	MMB	I: defluorination+hydroxylation
M22 (5F-AMB M3)	Amide	Indazole (4-carboxybutyl)	MMB	I: defluorination+carboxylation
M23 (5F-AMB M7)	Amide	Indazole (5F-pentyl)	MBA	II: ester hydrolysis
M24 (5F-ADB M7)	Amide	Indazole (5F-pentyl)	DBA	II: ester hydrolysis

AB 1-amino-3-methyl-1-oxobutane, *ADB* 1-amino-3,3-dimethyl-1-oxobutane, *MBA* 3-methylbutanoic acid, *DBA* 3,3-dimethylbutanoic acid, *AHB* 1-amino-4-hydroxy-3,3-dimethyl-1-oxobutane, *ABA* 4-amino-2-methyl-4-oxobutanoic acid, *MMB* methyl 3-methylbutanoate

_[a] Part II is not linked (see Fig. 1)

Table 2 Each number of samples prepared for temperature stability tests. Measurements were performed according to three time frames

Time frame	Spiked blood or urine samples					Authentic blood or urine specimens				
Day(s)	Control (100 %)	37 ℃	22 ℃	4 ℃	– 30 ℃	Control (100 %)	37 ℃	22 ℃	4 ℃	– 30 ℃
Frame 1										
0	4					2				
1		4	4	4	_[a]		_	_	_	_
2		4	4	4	_		_	_	_	_
4		4	4	4	_		2	2	2	_
7		4	4	4	_		2	2	2	_
Frame 2										
0	4					2				
14		4	4	4	4		2	2	2	_
28		4	4	4	4		2	2	2	_
56		4	4	4	4		2	2	2	_
Frame 3										
0	4					2				
112		_	4	4	4		_	2	2	2
168		_	4	4	4		_	2	2	2
Total numbers	12	28	36	36	20	6	10	14	14	4

Day 0 in the spiked samples means that the standard SCMs were spiked into the samples just before the extraction, and day 0 in authentic specimens means that the specimen was preserved at – 80 ℃

The sample number 4 means that 2 samples were spiked with SCMs at LOQ and 2 samples were at 20 x LOQ, respectively

_[a] Not determined

Table 3 Concentrations of six SCMs in authentic specimens (ng/mL) and the percentage values of the present concentrations in urine stored at – 80°C based on the concentrations reported previously [6–8]

Metabolite		M10	M12	M14	M17	M19	M20
Time frame (year(s))		5	5	3	3	1	1
Blood	– 30°C	3.83±0.39	9.31±0.77	1.76±0.08	0.625±0.041	0.134±0.012	0.520±0.018
	– 80°C	3.80±0.52	9.68±1.08	1.62±0.13	0.563±0.041	0.137±0.002	0.518±0.052
Urine	– 30°C	42.0±3.1	46.9±3.1	2.17±0.20	10.0±1.2	0.250±0.031	14.0±1.3
	– 80°C	50.3±3.7 (95.3 %)	47.5±1.4 (115 %)	2.07±0.17 (95.4 %)	10.7±0.8 (105 %)	0.256±0.035 (113 %)	13.4±0.7 (93.1 %)

Table 4 Stabilities of 24 metabolites at 37, 22 and 4℃ in blood and urine (%)

Analyte	Blood							Urine			
	Day(s)							Day(s)			
37 ℃	1	2	4	7	14	28	56	1 – 7	14	28	56
M21	22	3	0	0	0	0	0	>80	20	3	0
M22	42	5	3	0	0	0	0	>80	28	5	3
M2	55	20	19	7	7	6	0	>80	75	53	18
M17	102	95	85	44	16	8	0	>80	36	5	0
M3	100	105	101	65	42	33	8	>80	55	30	12
M18	98	104	100	53	41	44	14	>80	>80	>80	>80
M7	102	88	103	95	70	40	30	>80	>80	>80	>80
M8	103	101	95	98	55	39	13	>80	>80	>80	>80
M12	100	98	87	85	49	36	15	>80	>80	>80	>80
M20	95	97	100	93	75	48	11	>80	>80	>80	>80
M9	105	101	97	85	83	55	34	>80	>80	>80	>80
M13	102	100	103	98	87	58	52	>80	>80	>80	>80
M14	99	101	105	89	100	60	32	>80	>80	>80	>80
M15	103	105	100	91	83	49	40	>80	>80	>80	>80
M16	101	102	93	90	87	70	44	>80	>80	>80	>80
M24	91	103	100	87	101	75	52	>80	>80	>80	>80
M5	104	99	103	88	104	101	60	>80	>80	>80	>80
M10	98	95	104	100	89	83	29	>80	>80	>80	>80
M11	101	104	97	104	102	89	77	>80	>80	>80	>80
M19	105	102	100	93	102	82	35	>80	>80	>80	>80
M23	97	101	105	96	93	82	31	>80	>80	>80	>80
M1	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
M4	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
M6	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80

	Day(s)									Day(s)					
22℃	1	2	4	7	14	28	56	112	168	1 – 7	14	28	56	112	168
M21	67	55	38	6	3	0	0	0	0	>80	69	55	10	0	0
M22	90	86	60	5	3	0	0	0	0	>80	84	60	28	19	0
M2	89	86	59	30	12	5	4	0	0	>80	101	102	85	60	5
M17	101	95	90	75	50	9	0	0	0	>80	64	10	4	0	0
M3	91	95	103	93	56	44	33	22	15	>80	103	96	89	70	40
M18	104	89	96	72	53	46	33	23	16	>80	>80	>80	>80	>80	>80
M7	101	102	89	86	100	60	43	30	27	>80	>80	>80	>80	>80	>80
M8	92	88	100	87	69	55	34	23	16	>80	>80	>80	>80	>80	>80
M12	98	89	101	100	89	59	45	18	13	>80	>80	>80	>80	>80	>80
M20	96	101	89	100	99	63	43	29	20	>80	>80	>80	>80	>80	>80
M9	105	102	96	100	90	85	42	15	16	>80	>80	>80	>80	>80	>80
M13	103	100	98	103	85	55	52	16	13	>80	>80	>80	>80	>80	>80
M14	91	107	103	98	101	88	66	56	32	>80	>80	>80	>80	>80	>80
M15	93	105	103	95	85	52	47	40	6	>80	>80	>80	>80	>80	>80
M16	102	97	100	99	83	47	42	27	19	>80	>80	>80	>80	>80	>80
M24	95	102	88	89	100	87	57	50	32	>80	>80	>80	>80	>80	>80
M5	93	100	105	101	100	89	88	66	34	>80	>80	>80	>80	>80	>80
M10	98	100	95	110	103	95	74	30	22	>80	>80	>80	>80	>80	>80
M11	91	103	102	92	103	100	61	48	28	>80	>80	>80	>80	>80	>80
M19	103	105	97	101	103	100	75	50	41	>80	>80	>80	>80	>80	>80
M23	105	101	89	104	97	88	60	43	31	>80	>80	>80	>80	>80	>80
M1	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
M4	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
M6	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80

Table 4 (continued)

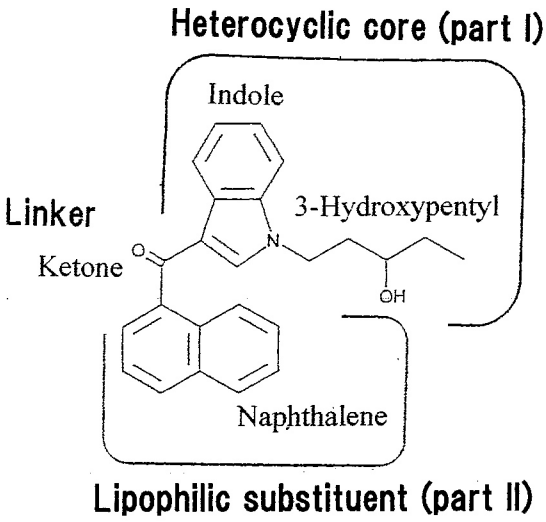
Analyte	**Blood**						**Urine**		
	Day(s)						Day(s)		
4 ℃	1 – 7	14	28	56	112	168	1 – 56	112	168
M21	>80	20	7	0	0	0	>80	>80	>80
M22	>80	25	5	3	0	0	>80	>80	>80
M2	>80	85	19	7	7	6	>80	>80	>80
M17	>80	100	87	90	54	25	>80	53	30
M3	>80	103	89	93	70	60	>80	>80	>80
M18	>80	101	100	97	24	15	>80	>80	>80
M7	>80	97	103	88	50	36	>80	>80	>80
M8	>80	100	100	100	62	56	>80	>80	>80
M12	>80	105	88	98	44	28	>80	>80	>80
M20	>80	100	104	100	75	57	>80	>80	>80
M9	>80	100	101	99	63	55	>80	>80	>80
M13	>80	103	89	85	50	24	>80	>80	>80
M14	>80	92	103	84	75	60	>80	>80	>80
M15	>80	102	101	90	83	51	>80	>80	>80
M16	>80	104	97	101	41	30	>80	>80	>80
M24	>80	88	103	101	74	51	>80	>80	>80
M10	>80	102	100	83	38	30	>80	>80	>80
M19	>80	103	98	100	67	52	>80	>80	>80
M23	>80	97	105	102	77	63	>80	>80	>80
M1	>80	>80	>80	>80	>80	>80	>80	>80	>80
M4	>80	>80	>80	>80	>80	>80	>80	>80	>80
M5	>80	>80	>80	>80	>80	>80	>80	>80	>80
M6	>80	>80	>80	>80	>80	>80	>80	>80	>80
M11	>80	>80	>80	>80	>80	>80	>80	>80	>80

>80: Stability was higher than 80 %

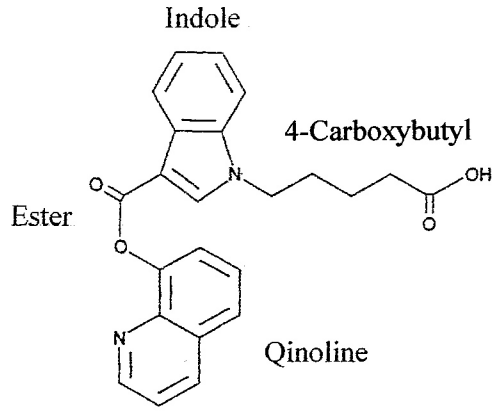
Table 5 Comparison of stabilities (%) of six SCMs in authentic blood and urine with those of six SCMs in spiked blood and urine according to the elapsed days under different temperature conditions

Analyte	**Authentic blood** Days							**Spiked blood** Day(s)						
37 ℃	4	7	14	28	56			1 – 4	7	14	28	56		
M17	98	86	63	15	0			>80	44	16	8	0		
M20	103	93	99	66	30			>80	93	75	48	11		
M14	92	99	100	67	50			>80	89	100	60	32		
M10	108	88	89	98	70			>80	100	89	83	29		
M12	105	96	103	87	60			>80	85	49	36	15		
M19	95	91	88	82	70			>80	93	102	82	35		
22℃	4	7	14	28	56	112	168	1 – 4	7	14	28	56	112	168
M17	99	92	84	58	26	0	0	>80	75	50	9	0	0	0
M20	93	98	86	96	72	39	25	>80	100	99	63	43	29	20
M14	109	103	101	100	62	56	32	>80	98	101	88	66	56	32
M10	101	94	90	86	100	75	44	>80	110	103	95	74	30	22
M12	93	100	87	99	89	70	50	>80	100	89	59	45	18	13
M19	98	109	88	87	86	71	66	>80	101	103	100	75	50	41
4 ℃					4 – 56	112	168					4 – 56	112	168
M17					>80	70	45					>80	54	25
M20					>80	72	50					>80	75	57
M14					>80	73	55					>80	75	60
M10					>80	75	57					>80	38	30
M12					>80	88	60					>80	44	28
M19					>80	78	66					>80	67	52
Analyte	**Authentic urine** Days							**Spiked urine** Day(s)						
37 ℃	4 – 7		14	28	56			1 –7		14	28	56		
M17	>80		55	10	0			>80		36	5	0		
M20	>80		>80	>80	>80			>80		>80	>80	>80		
M14	>80		>80	>80	>80			>80		>80	>80	>80		
M10	>80		>80	>80	>80			>80		>80	>80	>80		
M12	>80		>80	>80	>80			>80		>80	>80	>80		
M19	>80		>80	>80	>80			>80		>80	>80	>80		
22℃	4 – 7		14	28	56	112	168	1 – 7		14	28	56	112	168
M17	>80		75	54	9	0	0	>80		64	10	4	0	0
M20	>80		>80	>80	>80	>80	>80	>80		>80	>80	>80	>80	>80
M14	>80		>80	>80	>80	>80	>80	>80		>80	>80	>80	>80	>80
M10	>80		>80	>80	>80	>80	>80	>80		>80	>80	>80	>80	>80
M12	>80		>80	>80	>80	>80	>80	>80		>80	>80	>80	>80	>80
M19	>80		>80	>80	>80	>80	>80	>80		>80	>80	>80	>80	>80
4 ℃					4 – 56	112	168					4 – 56	112	168
M17					>80	85	63					>80	53	30
M20					>80	>80	>80					>80	>80	>80
M14					>80	>80	>80					>80	>80	>80
M10					>80	>80	>80					>80	>80	>80
M12					>80	>80	>80					>80	>80	>80
M19					>80	>80	>80					>80	>80	>80

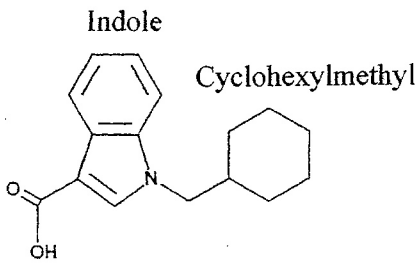
>80: Stabily was higher than 80 %



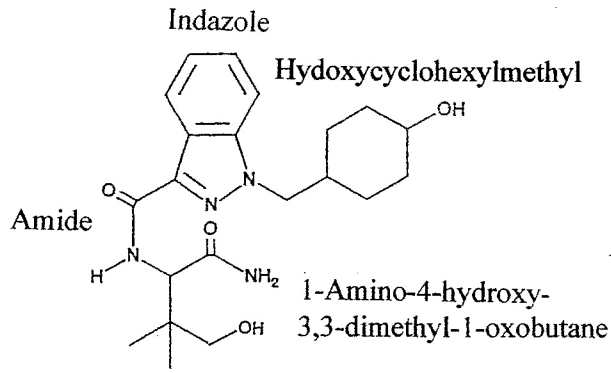
M1 (JWH-018 *N*-3-hydroxypentyl)



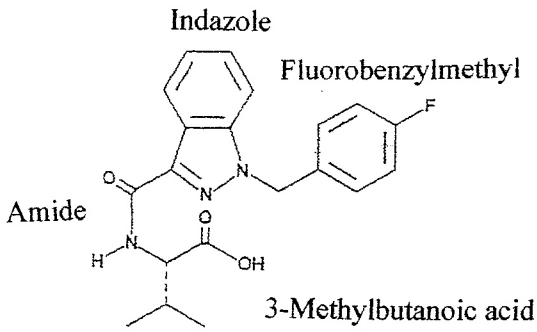
M3 (PB-22 *N*-pentanoic acid)



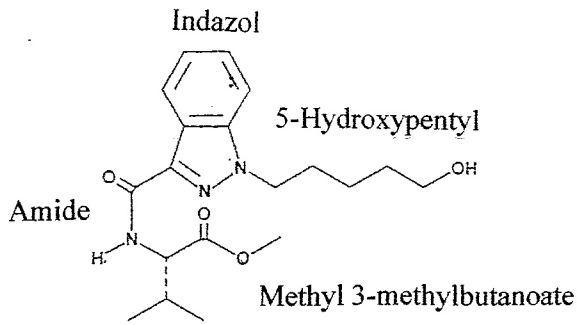
M6 (BB-22 3-carboxyindole)



M17 (MAB-CHMINACA M11)



M19 (AB-FUBINACA M3)



M21 (5F-AMB M2)

Figure2

Click here to access/download;Figure;Fig 2.pdf