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Short Communication

Identification and quantification of metabolites of AB-CHMINACA in a urine specimen of an abuser

Amin Wurita, Koutaro Hasegawa *, Kayoko Minakata, Kunio Gonmori, Hideki Nozawa, Itaru Yamagishi, Osamu Suzuki, Kanako Watanabe

Department of Legal Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

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ABSTRACT

We experienced an autopsy case in which the cause of death was judged as poisoning by multiple new psychoactive substances, including AB-CHMINACA, 5-fluoro-AMB and diphenidine [Forensic Toxicol. 33 (2015): 45–53]. Although unchanged AB-CHMINACA could be detected from 8 solid tissues, it could neither be detected from blood nor urine specimens. In this article, we obtained eight kinds of reference standards of AB-CHMINACA metabolites from a commercial source. The AB-CHMINACA metabolites from the urine specimen of the abuser were extracted by a modified QuEChERS method and analyzed by liquid chromatography–tandem mass spectrometry before and after hydrolysis with β-glucuronidase. Among the eight AB-CHMINACA metabolites tested, only 2 metabolites could be identified in the urine specimen of the deceased. After hydrolysis with β-glucuronidase, the concentrations of the two metabolites were not increased, suggesting that the metabolites were not in the conjugated forms. The metabolites detected were 4-hydroxycyclohexylmethyl AB-CHMINACA (M1), followed by *N*-[[1-(cyclohexylmethyl)-1*H*-indazol-3-yl]carbonyl]-L-valine (M3). Their concentrations were 52.8 ± 3.44 and 41.3 ± 5.04 ng/ml (n = 10) for M1 and M3, respectively. Although there is one preceding report showing the estimations of metabolism of AB-CHMINACA without reference standards, this is the first report dealing with exact identification using reference standards, and quantification of M1 and M3 in an authentic urine specimen.

1. Introduction

In recent years, various types of synthetic cannabinoids [1–4] and cathinone derivatives [5–8] have become widely distributed, and are now causing social problems throughout many parts of the world. The newly emerging drugs seem to have become more potent than the previous ones, and sometimes cause deaths [9–15]. Nowadays, thanks to the rapid designation within one month of newly emerged synthetic cannabinoids and cathinone derivatives as drugs under regulation by Pharmaceutical Affairs Law, the epidemic abuse of the drugs seems to be being suppressed to some extent in Japan; most of dubious head shops selling such drugs of abuse have been closed. However, the trade of such drugs via the Internet remains active.

To disclose the abuse of the regulated synthetic cannabinoid(s), the collection of urine specimens rather than blood specimens is being used by the police in view of humanitarianism. As reported by our group [15,16], the unchanged synthetic cannabinoids could be detected from solid tissues, but not from urine specimens. To disclose the abuse of synthetic cannabinoid(s) using urine specimens, it is essential to identify and quantify the predominant metabolite(s) in human urine [17]. As to AB-CHMINACA, only one report [18] has appeared; they studied in vitro metabolism of AB-CHMINACA using human liver microsomes, followed by the estimation of metabolites by liquid chromatography–time-of-flig ht-mass spectrometry. They also tested one human urine specimen obtained from AB-CHMINACA user, but could not quantify the metabolites. Although their study [18] seems excellent and sufficiently detailed, they did not use the reference standards of the metabolite, making their final identification and quantification difficult.

In the present study, we obtained eight kinds of reference standards of AB-CHMINACA metabolites now commercially available, and have finally identified and quantified two predominant metabolites of AB-CHMINACA in an authentic human urine specimen obtained from an abuser.

* Corresponding author.
E-mail address: 07484771@hama-med.ac.jp (K. Hasegawa).

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2. Materials and methods

2.1. Materials

AB-CHMINACA, (*S*)-*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-((4-hydroxycyclohexyl)methyl)-1*H*-indazole-3-carboxamide (M1), (*S*)-*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-((3-hydroxycyclohexyl)methyl)-1*H*-indazole-3-carboxamide (M2), *N*-[[1-(cyclohexylmethyl)-1*H*-indazol-3-yl]carbonyl]-L-valine (M3), [1-((4-hydroxycyclohexyl)methyl)-1*H*-indazole-3-carbonyl]-L-valine (M4), 1-(cyclohexylmethyl)-1*H*-indazole-3-carboxylic acid (M5), 1-((4-hydroxycyclohexyl)methyl)-1*H*-indazole-3-carboxylic acid (M6), 4-amino-3-(1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamido)-2-methyl-4-oxobutanoic acid (M7), and 2-(1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamido)-3-methylsuccinic acid (M8) were purchased from Cayman Chemical (Ann Arbor, MI, USA); β-glucuronidase from *Helix pomatia* type H-1 (3,015,000 units/g) from Sigma–Aldrich (St. Louis, MO, USA); two kinds of QuEChERS dispersive-solid-phase extraction (SPE) centrifuge tubes with caps (2-ml capacity), one of which contained 25 mg of primary-secondary amine (PSA), 25 mg of end-capped octadecylsilane (C_{18EC}) and 150 mg of magnesium sulfate, and the other of which contained 25 mg of C_{18EC} and 150 mg of magnesium sulfate without PSA, and Captiva ND Lipids cartridges (3-ml capacity) from Agilent (Santa Clara, CA, USA). Other common chemicals used were of the highest purity commercially available.

The urine specimen was the same one as described previously [16], which was collected at autopsy and stored at −80 °C until analysis. In this specimen, unchanged AB-CHMINACA was below the lower limit of quantitation (<1 ng/ml), while the drug could be detected from solid tissues (7.55–38.9 ng/g) [16]. As the blank urine specimen, it was self-sampled by the author.

2.2. Extraction procedure for the urine specimen

A 1.0-ml volume of urine was mixed with 100 ng of 4-amino-3-(1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamido)-2-methyl-4-oxobutanoic acid (M7) dissolved in 10 μl of acetonitrile as internal standard (IS), and shaken gently in a test tube. Six 0.1-ml portions were taken from the above mixture, and were prepared with and without the different amounts (1–10 ng) of each target metabolite dissolved in 1.0 ml acetonitrile, and shaken gently for the standard addition method of quantification. The each mixture was decanted into the QuEChERS dispersive SPE centrifuge tube without PSA, vortexed for 30 s, and centrifuged at 10,000 rpm for 2 min. The upper acetonitrile layer was passed through a Captiva ND Lipids cartridge. A 3.5-μl aliquot of the eluate was then analyzed by liquid chromatography–tandem mass spectrometry (LC–MS-MS).

For measurements of product ion mass spectra, the final eluate was concentrated 100-fold before injection into the LC–MS-MS system.

When the enzymatic hydrolysis of a urine specimen was used, the following procedure was conducted just before the above procedure. A 1.0-ml volume of the urine specimen was mixed with 100 μl of 2 M sodium acetate buffer (pH 4.5), 50 μl of aqueous solution of β-glucuronidase containing 10,000 units of activity and 100 ng of IS (M7) dissolved in 50 μl of acetonitrile. The mixture solution was incubated at 60 °C for 1 h. Six 0.12-ml portions were taken from the above incubation mixture, and were prepared with and without the different amounts (1–10 ng) of each target metabolite dissolved in 1.0 ml acetonitrile. The following procedure was exactly the same as described above.

2.3. Standard addition method

Although the standard addition method is frequently used for analysis with atomic absorption spectroscopy to overcome matrix effects [19], it is not popular in the field of forensic toxicology. We began using the standard addition method in our laboratories in a study analyzing ethylene glycol and propylene glycol in whole blood specimens collected from non-occupational and healthy subjects [20]. The standard addition method was very useful for overcoming the matrix effects and different recovery rates. The details of the procedure and calculation of the results were described in our previous report [21]. Recently, another group in USA used the standard addition method for the study on post-mortem distribution of 25I-NBOMe in body fluids and solid tissues of a cadaver [22].

2.4. Matrix effects and recovery rates

Although the standard addition method employed can overcome the matrix effects and recovery rates, the readers seem very interested in the matrix effects and recovery rates of AB-CHMINACA metabolites under our extraction conditions. The detailed procedure and calculation method are the same as described in the previous report [21].

2.5. LC–MS-MS conditions

The LC conditions were almost the same as described in our previous study on 5-fluoro-ADB [13] except that the interval of gradient elution was shortened from 15 min to 10 min in the present experiments. For the selected reaction monitoring (SRM) by the tandem MS, the ion transitions were: *m/z* 373 → 257 for the AB-CHMINACA metabolite M1, *m/z* 358 → 241 for the AB-CHMINACA metabolite M3, and *m/z* 387 → 241 for IS (compound M7); fragmentor voltage and collision energy were: 120 and 9 V, respectively, for metabolite M1, 120 and 17 V for metabolite M3, and 120 and 21 V for IS. Other MS conditions were the same as described previously [13].

3. Results and discussion

3.1. Preliminary experimental results

First of all, the product ion mass spectra for the reference standards of eight AB-CHMINACA metabolites obtained were recorded by colliding each protonated molecular cation, which gave each suitable qualifier ion(s) to be used for testing the presence of metabolite(s) in the authentic urine specimen obtained from an abuser (data not shown). Without the hydrolysis with glucuronidase, two peaks at 6.00 and 9.04 min of retention times appeared by SRM, suggesting the presence of 4-hydroxycyclohexylmethyl metabolite (M1) and a metabolite with carboxylic acid at the terminal of the 3-carbonylamide chain (M3) in the authentic urine (Fig. 1). The peaks by SRM did not appear for the rest 6 metabolites (data not shown).

After the hydrolysis with β-glucuronidase, the results were almost the same as those without hydrolysis; no peaks appeared by SRM for the 6 metabolites other than M1 and M3. Furthermore, any increase in peak area was observed for M1 and M3. Peak area ratios of the extract after hydrolysis to that without hydrolysis were 0.98 and 0.93 for M1 and M3, respectively. Therefore, the hydrolysis procedure was found not necessary for analysis of M1 and M3 in the present authentic urine specimen.

It is essential to use IS for accurate quantification of compound(s) in biological specimens. IS should be similar to target