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In vivo detection of the new psychoactive substance AM-694 and its metabolites

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ABSTRACT

AM-694 or 1-(5-fluoropentyl)-3-(2-iodobenzoyl)indole is a synthetic cannabinoid that acts as a selective and a powerful agonist for CB1 receptor, inducing cannabinoid-like effects (euphoria, sedation, hallucinations and anxiety). Its spread, like for other synthetic cannabinoids, has increased in recent years and many web sources freely supply these kinds of new drugs. It can be taken by smoking or through oral consumption. A 25-years-old man was hospitalized at the local hospital following a major trauma after ingestion of alcohol and an unknown pill. Urine and blood samples were sent to our Forensic Toxicology Division to investigate on possible substance abuse. A general unknown screening of biological samples, extracted by liquid–liquid extraction (ethylacetate and dichloromethane) in basic, acidic and neutral conditions, was achieved to verify the presence of drugs of abuse and/or their metabolites, both in gas chromatography-mass spectrometry (GC–MS) and liquid chromatography-tandem mass spectrometry (LC–MS/MS). For the quantification of AM-694, urine was extracted by solid phase extraction (SPE) on a Bond Elut Certify cartridge; an acidic hydrolysis (HCl 30%, 95 °C, 60 min) was necessary before liquid–liquid extraction of metabolites. For the detection of benzodiazepines and their metabolites, an enzymatic hydrolysis was applied (β-glucuronidase, pH 4.5, 50 °C, 18 h). Quantification of AM-694 (internal standard AM-2201), midazolam and α-hydroxymidazolam (internal standard halazepam) were performed by LC–MS/MS analysis in multiple reaction monitoring ($[M+H]^+$: *m/z* 436→190, 272, AM-694; *m/z* 360→155, 127, AM-2201; *m/z* 326→291, 223, midazolam; *m/z* 342→168, 203, α-hydroxymidazolam; *m/z* 353→241, 222, halazepam). The general unknown screening revealed the presence of AM-694 (urine sample) and benzodiazepines (urine and blood). The concentration of AM-694, obtained by LC–MS/MS, was 0.084 μg/L. Midazolam and α-hydroxymidazolam were detected in urine (0.97 and 74.58 μg/L, respectively) and in blood (34.84 and 23.15 μg/L, respectively). Qualitative information about the AM-694 metabolites was obtained by LC–MS/MS in selected-ion monitoring for the putative $[M+H]^+$ ions: *m/z* 448, carboxylated metabolite; *m/z* 434, defluorinated metabolite; quantification was not possible since reference standards are not available. Our report is the first case of detection of AM-694 and its metabolites in human biological fluids in Italy. For this reason, this case constitutes a first worrisome alarm about the spread of this substance.

1. Introduction

In recent years, many new psychoactive substances (NPS) have been synthetically produced and commercialized. These substances, well known as "designer drugs", "legal highs", "herbal highs", or "bath salts", are mainly marketed to teens and young adults since they are relatively inexpensive, easily available on the Internet and are not detectable by routine drug screening tests. The two main classes of NPS are the synthetic cathinones and the synthetic cannabinoids (SCs), [1,2]. In particular, SCs refer to compounds that act as cannabinoid receptor agonists and mimic the effect of Δ^9-tetrahydrocannabinol (THC), inducing, among others, analgesia, catalepsy, hypomobility and hypothermia [3–5]. These molecules were originally developed for potential medical purposes and to study cannabinoid receptors [6–9]. Indeed, most of them possess a four to five times improved binding affinity to the cannabinoid CB1 receptor than the THC, reporting a higher onset of anxiety, paranoia, tachycardia, irritability, hallucination, numbness, seizures, hypertension and slurred speech [4,10–12]. More than 500 SCs have appeared in the market and their use has

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increased in the last years [13]. Each new structural modification of the basic SC structure may produce different effects, keep the new substances in an ambiguous legal status and make hard their detection [14]. Notwithstanding, a large number of analytical methods recently published [15–21], cases of detection of SCs are still rare [22]. In our Forensic Toxicology Unit, two validated screening methods for SCs (23 parent compounds) and synthetic cathinones (16 parent compounds), are routinely applied to cases of intoxication and previously allowed us to detect two cases of consumption of JWH-073 (a SC, Fig. 1) and MDPV (a synthetic cathinone) [23,24] that were reported to the Italian Early Warning System and Rapid Response to Drug programme (NEWS). In this paper, we describe for the first time in Italy the report of an intoxication where the SC AM-694 (Fig. 1) and its main metabolites, the hydrolytically defluorinated metabolite, M1 and the carboxylated metabolite, M2, [25] (Fig. 2) were identified in biological fluids (urine) by LC–MS/MS. AM-694 or 1-(5-fluoropentyl)-3-(2-iodobenzoyl)indole is an aminoalkylindole. It was synthesized in 2008 [26] for CB1 receptor studies because of its high selectivity toward this receptor (Ki = 0.08 nM) [27] but AM-694 recreational use is increasing. It is available as white powder, in pills, in solution or sprayed on herbs and its administration can be by smoking or through oral consumption.

2. Case presentation

A 25-year-old man was brought, following a major trauma, at a local hospital in an altered status (agitation, hallucination, anxiety and paranoia were described by health-care professionals). The subject declared the ingestion of alcohol and an unknown pill. Urine and blood specimens were collected 6 and 9 h after the patient was admitted to the hospital, respectively. The biological samples were then sent to the Forensic Toxicology laboratory for analyses.

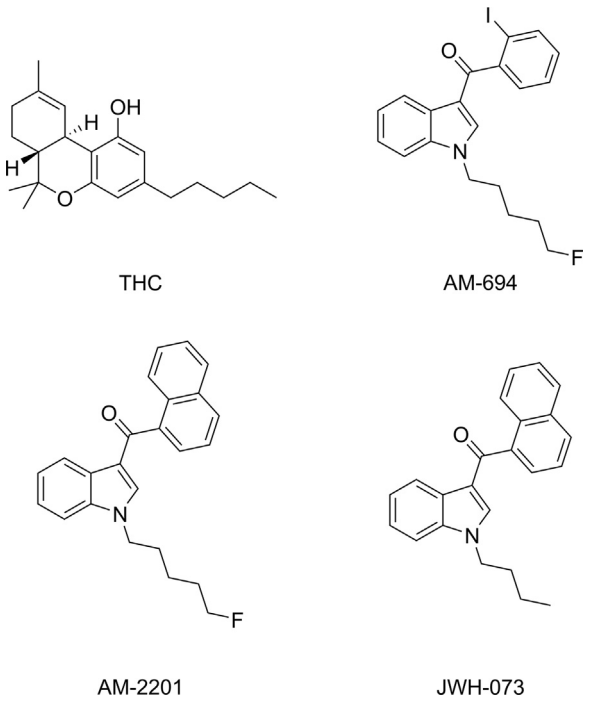

Fig. 1. Chemical structures of Δ^9-tetrahydrocannabinol and of the synthetic cannabinoids AM-694, AM-2201 and JWH-073.

3. Material and methods

3.1. Chemicals

Chloroform (CH_3Cl), dichloromethane (DCM), 2-butanol, β-glucuronidase enzyme (type HP-2 from *Helix Pomatia*, 152,900 units/mL) and LC-MS CHROMASOLV® methanol (MeOH) were purchased by Sigma–Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl), ammonium hydroxide (NH_4OH), glacial acetic acid were from J.T. Baker (Deventer, Holland). Sodium hydroxide and isopropanol were provided by Panreac Quimica S.L.U. (Castellar del Vallès, Spain). AM-694 and AM-2201 (internal standard, IS) were provided by the Italian National Health Institute. Benzodiazepine (3-hydroxyflunitrazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, α-hydroxyalprazolam, α-hydroxymidazolam, alprazolam, bromazepam, brotizolam, clonazepam, chlordiazepoxide, delorazepam, diazepam, flunitrazepam, halazepam, ketazolam, lorazepam, lormetazepam, midazolam, nitrazepam, nordiazepam, oxazepam, pinazepam, prazepam, temazepam, triazolam) standards (BDZs) were purchased from Lipomed Inc. (Cambridge, MA, USA). All standards were diluted to the appropriate concentration with MeOH. Sterile water for injection (H_2O) was obtained from B. Braun (Milano, Italy).

3.2. EMIT® Immunoassay Screening Test

Urine sample was firstly analyzed with an EMIT® Siemens VIVA-E drug testing system (Siemens, Newark DE) in order to investigate the common drugs of abuse: cocaine, opiates, cannabinoids, amphetamines, barbiturates, methadone and benzodiazepines according to the manufacturer's instructions.

3.3. General Unknown Screening (GUS) in Gas Chromatography–Mass Spectrometry (GC–MS)

Urine or blood samples (1 mL) were extracted with 3 mL of CH_3Cl in basic (200 μL of NaOH 40%), acidic (200 μL of HCl 36%) and neutral conditions (200 μL of H_2O). The organic phases were reduced to about 100 μL under a gentle stream of nitrogen (N_2) and analyzed as such by GC–-MS., following the Society of Forensic Toxicology/American Academy Forensic Sciences (SOFT/AAFS) Guidelines for "general unknowns" [28].

3.4. Blood alcohol content (BAC)

BAC was determined by the standard procedure in our Laboratory [29]. Briefly, 1 mL of blood were added with 2-butanol (internal standard) in a vial for headspace analysis and loaded in a headspace sampler Agilent 7697A Headspace (Agilent Technologies, Palo Alto, CA, USA); the oven was set at 60 °C. After 30 min, the gas was injected in an Agilent 7890B GC system (Agilent Technologies) equipped with a flame ionization detector. The column was an Alltech Superox II, 10 m length, 0.54 mm i.d. and 1.2 μm film thickness (Alltech Associates INC., Deerfield, IL, USA). Chromatographic run was carried out at 60 °C for 4.5 min.

3.5. Screening for synthetic cathinones

To 1 mL of urine or blood were added 3 mL of CH_3Cl and, extracted for 1 min by agitation and centrifuged at 4500 rpm for 10 min; the organic phase was dried under a gentle stream of N_2 at 40 °C; the residue was dissolved in 100 μL of MeOH. An aliquot of 7 μL were injected in the LC–MS/MS system.

3.6. Screening for synthetic cannabinoids

Urine and blood samples (1 mL) were added with 2 mL of H_2O, 2 mL of phosphate buffer (pH 6) and then extracted by means of