On practical problems in precise estimation of 5F-ADB in urine samples

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Received: 25 February 2020 / Accepted: 8 July 2020 / Published online: 31 July 2020
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Abstract

Purpose The purpose of this work is to establish if there are any problems in precise quantitation of methyl 2-[1-(5-fluoropentyl)-1*H*-indazole-3-carboxamido]-3,3-dimethylbutanoate (5F-ADB) in human urine by QuEChERS extraction coupled with gas chromatography–tandem mass spectrometry (GC–MS/MS).

Methods To establish the method for quantifying 5F-ADB in human urine samples, QuEChERS extraction coupled with GC–MS/MS was applied. To elucidate 5F-ADB degradation products, liquid chromatography coupled with linear trap quadrupole-orbitrap hybrid MS (LC–MS) was used.

Results The applied QuEChERS GC–MS/MS procedure appeared to be satisfactory for 5F-ADB estimation in acidic and alkaline urine samples. Its validation parameters were the following: good linearity ($R^2=0.9988$), high detection (limit of detection = 0.33 ng/mL) and quantitation (limit of quantitation = 1.1 ng/mL) sensitivities and satisfactory inter- and intraday precisions (% relative standard deviation below 5.6%). 5F-ADB recovery from acidic urine by QuEChERS procedure was slightly lower than that from urine sample with neutral pH; however, the difference in the recovery was not statistically significant. The recovery of the drug from alkaline urine is extremely low. LC–MS analysis proved the presence of 5F-ADB hydrolysis products in alkaline urine and in alkaline solution of the drug.

Conclusions The presented studies indicate that the validated QuEChERS technique can be successfully used in routine analyses of 5F-ADB in urine. Yet, due to hydrolytical instability of 5F-ADB, the medical diagnosis of the health condition of the patient suspected of 5F-ADB abuse on the basis of the drug concentration in his/her urine may be incorrect, especially when the urine is alkaline.

Keywords 5F-ADB · Drug decomposition · QuEChERS · Urine analysis · GC–MS/MS · LC–MS

Introduction

One of the most rapidly evolving group of new psychoactive substances is the class of synthetic cannabinoids consisting of structurally diverse compounds with the ability to mimic Δ^9 -THC, the active component of cannabis [1]. They were synthesized and studied by in vitro and in vivo methods as early as in the 1990s to better understand how cannabinoid

system receptors work [2]. Over the past few years, special attention has been paid to a new generation of synthetic cannabinoids with 3-carboxamide indazole moiety [3–8]. Among them, 5F-ADB (methyl 2-[1-(5-fluoropentyl)-1*H*-indazole-3-carboxamido]-3,3-dimethylbutanoate) appears to be one of the most potent and dangerous synthetic drugs of abuse [9]. This novel synthetic cannabinoid is characterized by high CB₁ receptor affinity, 289 times higher than that of Δ^9 -THC [10]. It is well known today that 5F-ADB use may have various adverse effects leading to fatal intoxications and motor vehicle collisions: anxiety, psychomotor agitation, tachycardia, nausea, seizures, loss of consciousness, amnesia, hallucinations, psychosis, encephalopathy, myocardial infarction and kidney dysfunctions [9, 11–17]. Acute intoxications with 5F-ADB have been the cause of numerous fatalities [13–17].

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11419-020-00542-x>) contains supplementary material, which is available to authorized users.

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According to the available literature, only a few techniques—protein precipitation, liquid–liquid extraction, solid-phase extraction and QuEChERS (acronym from words Quick, Easy, Cheap, Effective, Rugged and Safe)—are currently employed for analytical procedures of 5F-ADB [11, 13–18]. Protein precipitation and liquid–liquid extraction, the most common sample preparation methods employed for 5F-ADB analysis, ensure relatively fast isolation of the analyte from biological matrices. Unfortunately, their lack of selectivity may cause various difficulties during the estimation of drug concentration when biological samples differing in matrix composition are examined. This is evident when liquid chromatography–mass spectrometry (LC–MS), for which the analytical signal is sensitive to matrix variation, is employed [18]. Moreover, it has been well demonstrated that protein precipitation causes a co-precipitation of analyte, which lowers the accuracy of quantitation [19].

QuEChERS, has been considered as a promising combination of liquid–liquid extraction (LLE) and dispersive solid-phase extraction (d-SPE). It is characterized by fast LLE extraction and high SPE selectivity. To our knowledge, there are only two reports describing the use of QuEChERS for 5F-ADB analysis in human tissues and fluids [14, 16]. The first report deals with the applications of QuEChERS for 5F-ADB analysis in blood samples, whereas the second one concerns solid tissues (adipose tissue, heart muscle and others), blood and urine. Although both clearly demonstrate the utility of QuEChERS for 5F-ADB analysis in solid and liquid tissues, it is difficult to conclude unequivocally about the applicability of this sample preparation method for the analysis of 5F-ADB in all types of human specimens as the results concerning the QuEChERS application for 5F-ADB analysis in urine are scarce and uncertain. On the basis of the results in [16], the QuEChERS procedure for 5F-ADB analysis in urine was not validated and its application for 5F-ADB analysis in real urine samples failed in detecting the drug. The 5F-ADB absence in the examined urine samples was explained in [16] by the fact that only a small amount of the analyte in the herbal smoke was inhaled by the donor in a very short period of time. Yet, it is worth noting, that urine, the most easily accessible and not so complex human fluid, may be an inconvenient matrix in the analysis of ionic character drugs due to their possible pH variability.

To increase the extraction efficiency of ionic compounds such as 5F-ADB from biological matrices, their buffering is required. On the other hand, there are literature suggestions [20] about possible 5F-ADB instability of the drug in its solutions of different pH.

The paper presents a validated QuEChERS GC–MS/MS (gas chromatography tandem mass spectrometry) procedure

for screening and quantitation of 5F-ADB in urine samples and discusses possible difficulties concerning its precise quantitation in human urine differing in pH.

Materials and methods

Materials

Acetonitrile (LC grade purity) was obtained from Merck (Darmstadt, Germany). Sodium chloride and anhydrous magnesium sulfate were obtained from POCH (Gliwice, Poland), and Septra C-18E sorbent (50 µm, 65 Å) applied in the QuEChERS protocol was purchased from Phenomenex (Torrance, CA, USA). Deionized water was purified by the Milli-Q system (Millipore Sigma, Bedford, MA, USA).

5-Fluoro-ADB standard and internal standard (IS), 5-fluoro-AMB (methyl 2-[1-(5-fluoropentyl)-1*H*-indazole-3-carboxamido]-3-methylbutanoate) were donated by the forensic laboratory of the Provincial Police Station in Lublin. Working solutions were prepared in methanol and stored in stable conditions at – 20 °C.

Collection and storage of urine samples

The urine samples used for the optimization, calibration and validation studies were obtained from volunteers by means of aseptic urine collection kits (Bene, Szczecin, Poland) and subsequently stored in sterile conditions at – 20 °C.

Methods

QuEChERS procedure

The QuEChERS technique involves the two-step process of extracting the examined analytes from tested materials. The first stage involves classical extraction (LLE or liquid–solid extraction, depending on sample matrix type) using a chosen type of organic extractant (mostly acetonitrile). In this stage, the addition of extraction salts (NaCl and anhydrous MgSO₄) is necessary. The second stage consists of supernatant purification by the d-SAPE using a chosen type of sorbent (mostly primary–secondary amine (PSA) and octadecyl (C-18) sorbents) [21, 22]. To optimize the QuEChERS procedure for 5F-ADB analysis in the urine samples, the influence of the following factors on the drug recovery was examined:

- in the first stage—the amount of NaCl and MgSO₄, and the volume of acetonitrile;
- in the second stage—the amount of C-18 sorbent.

The following QuEChERS procedure was established as optimal:

1. introducing 10 μL of internal standard solution (1 $\mu\text{g}/\text{mL}$) to 350 μL of urine sample and thoroughly mixing with 80 mg of NaCl and 150 mg of MgSO_4 for 1 min,
2. adding 650 μL of cold acetonitrile (-20°C) to the obtained mixture and shaking by a vortex mixer for 5 min,
3. centrifuging the obtained extraction mixture ($18,600\times g$) and collecting 550 μL of the supernatant,
4. adding 12.5 mg of the C-18 sorbent to the supernatant and mechanical mixing for 1 min,
5. centrifuging and transferring the obtained supernatant to autosampler vials for analysis by GC–MS/MS.

The QuEChERS procedure appeared to be optimal also for the estimation of 5F-ADB in both the acidified and alkaline urine samples. Those sample types were obtained by adding buffer solution (35 μL) to samples of natural urine (350 μL). Two molar phosphoric buffers, pH 4.0 or 8.5 were used for this purpose.

GC–MS/MS measurements

Qualitative and quantitative analyses of 5F-ADB were conducted using a gas chromatograph hyphenated with a triple quadrupole tandem mass spectrometer (GCMS-TQ8040; Shimadzu, Kyoto, Japan) equipped with a ZB5-MSi fused-silica capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μm film thickness; Phenomenex). Helium (grade 5.0) as carrier gas and argon (grade 5.0) as collision gas were used. Column flow was 1.56 mL/min, and 1 μL of the sample was injected by an AOC-20i + s type autosampler (Shimadzu). The injector was working in high pressure mode (250.0 kPa for 1.5 min; column flow at initial temperature was 4.90 mL/min) at the temperature of 320°C ; the ion source temperature was 220°C .

For qualitative purposes the full scan mode with range 40–550 m/z was employed and for quantitative analyses the MRM mode (multiple reaction monitoring) was used.

Optimization of MRM transitions for MS/MS detection

The chemical structures and mass spectrums of 5F-ADB and 5F-AMB (internal standard) are shown in Fig. 1a, b. Quantitative analysis of 5F-ADB with a GC–MS/MS instrument was operated in the MRM mode. For this reason, the drug fragmentation pathways for 5F-ADB and 5F-AMB were established and energies for MRM transitions were optimized. The fragmentation pathways of 5F-ADB and 5F-AMB are shown in Fig. 2a, b. To find the optimal collision energies (CE) for MRM transitions, the standard

solutions of 5F-ADB and 5F-AMB were used. Five characteristic MRM transitions for each compound were monitored. The collision cell was operated at 5, 7, 10, 12, 15, 17, 20, 22, 25 and 27 eV. The results of those experiments are summarized in Fig. S1a, b (supplementary material) and Table S1 (supplementary material). Three MRM transitions of the highest intensity were selected for further experiments:

- 377 > 321 (CE = 7 eV), 321 > 289 (CE = 7 eV) and 289 > 233 (CE = 12 eV) for 5F-ADB,
- 363 > 321 (CE = 7 eV), 321 > 289 (CE = 7 eV) and 289 > 233 (CE = 12 eV) for 5F-AMB.

Estimation of 5F-ADB stability in acidic and alkaline environmental

To confirm the literature suggestions about 5F-ADB instability in acidic and alkaline environments [20], solutions of the drug in the following solvents were prepared:

- acetonitrile/water (10/1 v/v),
- acetonitrile/phosphoric buffer (2 M, pH 4.0) (10/1 v/v),
- acetonitrile/phosphoric buffer (2 M, pH 8.5) (10/1 v/v) and

All those solvents were subsequently incubated at room temperature for 3 h and then examined by LC–MS.

LC–MS analysis was also applied for the investigation of 5F-ADB solution in urine of pH 8.5. The last matrix was prepared in an artificial way by adding a small volume of phosphoric buffer (2 M, pH 10.5) to natural urine. Before LC–MS analysis, the urine drug solution was incubated at room temperature for 1 h.

LC–MS measurements

The analyses of 5F-ADB degradation products were performed using an UPLC (ultra-performance liquid chromatography) chromatograph (UltiMate 3000, Dionex, Sunnyvale, CA, USA) coupled with a linear trap quadrupole-orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA) containing Gemini C18 column (100 $\times$ 4.6 mm internal diameter, 3 μm particle size; Phenomenex, Torrance, CA, USA). LC–MS conditions were as follows: elution mode: linear gradient (increase from 5 to 95% of B for 60 min, followed by 10 min of isocratic elution with 95% of B). Mobile phase component A—25 mM formic acid in distilled water, mobile phase component B—25 mM formic acid

Fig. 1 Chemical structures and mass spectra of **a** 5F-ADB and **b** 5F-AMB (internal standard)

in acetonitrile; flow rate: 0.4 mL/min; ionization mode: ESI positive (spray voltage at 3.5 kV); sheath, auxiliary and sweep gas: nitrogen (grade 4.0) of arbitrary units 40, 10 and 10, respectively; capillary temperature: 320 °C; full scan range of HRMS (high resolution mass spectrometry) analysis: 100–2000 m/z .

Method validation and statistical analysis

For the quantitative analysis of 5F-ADB, the internal standard calibration method was employed. The specificity of the method was evaluated by testing blank samples of urine from volunteers for the absence of 5F-ADB and 5F-AMB.

The linearity of the assay was calculated by the least square method and expressed as the coefficient of

Fig. 2 Fragmentation pathways of **a** 5F-ADB and **b** 5F-AMB estimated by gas chromatography–tandem mass spectrometry

determination (R^2). Calibration plots were prepared using the blank urine samples spiked with 5F-ADB at the concentration levels of 0.5, 1, 2.5, 5, 10, 20 ng/mL and a fixed concentration of the internal standard. Each solution was prepared in triplicate.

To determine the limit of detection (LOD) and the limit of quantitation (LOQ), the urine samples were spiked with the analytical reference standard. The LOD and LOQ were considered as signal-to-noise ratios of 3 and 10, respectively.

The method recovery was determined using blank urine samples spiked with 5F-ADB and 5F-AMB at three different concentration levels (1, 2.5 and 5 ng/mL). To find out whether there was a significant difference between the recoveries at individual analyte concentration levels, the one-way analysis of variance test (ANOVA) was performed.

The obtained data prove that the applied analytical conditions are suitable for both qualitative and quantitative analysis of 5F-ADB.

Results

In recent years, GC–MS/MS instruments are becoming widespread in analytical laboratories in the world. Although LC–MS is a method with potentially greater applicability, and a sample preparation before analysis is often simpler, the connection of the gas chromatograph with the tandem mass spectrometer is very often used for testing complex mixtures, due to its higher specificity and sensitivity. GC–MS/

MS systems are cheaper than those of LC–MS/MS and LC–QTOF–MS instruments, and at the same time allowing for notable improvements in the signal to noise as well as the dynamic range, resulting in better performance in terms of sensitivity, precision and accuracy. That is why, the GC–MS/MS analysis seems to be the first-choice technique in determining the concentration level of xenobiotics (such as 5F-ADB) in biological fluids. To obtain the most accurate analysis results, the fragmentation pathways of the analyte and the collision energies for MRM transitions should be examined and optimized. Figure 1a, b shows the chemical structures and mass spectrums of 5F-ADB and 5F-AMB (internal standard). The fragmentation pathways and collision energies optimization for the mentioned compounds are summarized in Fig. 2a, b, Fig. S1a, b (supplementary material) and Table S1 (supplementary material).

An exemplary MRM chromatogram of urine sample containing 10 ng/mL of 5F-ADB and 5F-AMB after QuEChERS procedure is presented in Fig. 3a, b.

Validation is the most important part of method development which confirms its acceptability for analytical purposes. To estimate the analytical utility of the described QuEChERS GC–MS/MS method, the assay was validated by several experimental parameters such as linearity, selectivity, accuracy, precision, LOD and LOQ—see Table 1. As can be seen, the method exhibit good linearity, low LOD, high recovery, good precision and accuracy. Moreover, the amount of purified extract equals several hundred μ L; therefore, if necessary, it is possible to significantly concentrate

Fig. 3 Multiple reaction monitoring chromatograms showing peaks of **a** 5F-ADB and **b** 5F-AMB for a urine QuEChERS extract

Table 1 Validation data for the analysis of 5F-ADB in human urine by QuEChERS extraction coupled with gas chromatography–tandem mass spectrometry (GC–MS/MS)

Tested parameter			
Linearity (R^2)	0.9989		
LOD (ng/mL)	0.33		
LOQ (ng/mL)	1.09		
	Parameter estimated at analyte concentration equal		
	1 ng/mL	2.5 ng/mL	5 ng/mL
Intraday precision (% RSD)	4.51	4.48	4.46
Interday precision (%RSD)	5.62	5.61	5.57
Intraday accuracy (%)	96.9	97.2	97.4
Interday accuracy (%)	96.1	96.4	96.6
Recovery of 5F-ADB estimated using optimal QuEChERS conditions at different analyte concentrations (1/2.5/5 ng/mL) and different pH of urine samples			
Recovery*	85%/87%/88% (pH 4.0)	92%/94%/94% (pH 6.9)	24%/26%/27% (pH 8.5)

R^2 coefficient of determination, *RSD* relative standard deviation, *LOD* limit of detection, *LOQ* limit of quantification

the sample, thereby raising the detection sensitivity, by, e.g., evaporating it in a stream of nitrogen. Due to the relatively low volatility of 5F-ADB, the analyte will not leave the sample together with the solvent. Concerning the matrix effect (ME) no significant differences were found between the slopes. The estimated value of ME equal to 0 led to the conclusion that the presented method was not subjected to any matrix effect. Analyzing the chromatograms from QuEChERS blank samples extracts no peaks of the examined

analytes and/or their significant interferences were found, what confirms the high selectivity of the described method.

As mentioned in “[Introduction](#)”, the extraction efficiency of 5F-ADB from biological fluids depends on their pH. Table 1 represent QuEChERS recovery of 5F-ADB from urine buffered with phosphoric buffer (pH 4.0), natural urine (pH 6.9) and urine buffered with phosphoric buffer (pH 8.5).

Figure S2 (supplementary material) presents LC–MS chromatograms of 5F-ADB dissolved in: (a) acetonitrile/

water (10:1 v/v), (b) acetonitrile/phosphoric buffer (pH 4.0) (10:1 v/v), (c) acetonitrile/phosphoric buffer (pH 8.5) (10:1 v/v) and (d) natural urine with phosphoric buffer to obtain pH 8.5. The HRMS data corresponding to the compounds represented by individual peaks from LC–MS chromatograms are gathered in Table 2.

Discussion

As it is already known [23], the extraction efficiency of organic compounds of ionic character from water sample, like urine, to an organic solvent strongly depends on the sample pH. This also applies to polar organic molecules of ionic character such as 5F-ADB. Considering the results of 5F-ADB analysis in urine with the application of QuEChERS, three aspects should be taken into account:

- possible variation of urine pH,
- physicochemical character of 5F-ADB molecule,
- hydrophobicity/polarity of acetonitrile.

Normal urine pH is assumed to be around 6.5, yet many examples show that pH of this body fluid depends on the diet, health state etc. and can range between 4.5 and 8.5 [24].

5F-ADB is a polar molecule which, due to the presence of indazole and amide groups, exhibits basic properties. In consequence, this compound in water solution can exist in two form, neutral and protonated. The transformation degree of 5F-ADB into a given form and, hence, its extraction degree to organic solvent depends on pH of the drug solution. Unfortunately, there is no accurate data in the literature on the pK_a value of this analyte. For these reasons, it was decided to estimate the recovery of 5F-ADB by QuEChERS from urine after its slight acidification and alkalization. As results from Table 1, the recovery of 5F-ADB by QuEChERS from acidified urine is slightly smaller than that from natural urine (pH of urine applied in experiments equaled 6.9), yet the difference is statistically insignificant. In the

case of alkaline urine, the recovery of 5F-ADB by QuEChERS is very low (below 30%). At the first sight, the observed drop (about 62%) in the 5F-ADB recovery resulting from the increase of urine pH looks to be understandable as the applied extrahent, acetonitrile, is a relatively polar solvent and 5F-ADB in urine of pH 8.5 exists mainly in non-protonated form, less polar than protonated one. However, that the change in the polarity of 5F-ADB molecules resulting from the change of their protonation does not justify such significant drop of.

5F-ADB extraction efficiency from alkaline urine, the more so that acetonitrile, being polar, exhibits also weak hydrophobic character.

As mentioned in Introduction, there are unproved suggestions [20] about possible instability of 5F-ADB in its solutions of different pH. For this reason it was decided to estimate 5F-ADB stability examining the composition of three drug solutions differing in pH, after their 3 h incubation at room temperature. LC–MS analysis revealed the presence of a four other compounds (in addition to 5F-ADB) in the acidic and alkaline drug solutions. The HRMS data for individual 5F-ADB degradation products, their identification and structures are given in Table 2 and in Fig. S2 (supplementary material). As the compounds were not confirmed by NMR analysis, their identification should be treated tentatively. However, this identification is very likely as the formed compounds are hydrolysis products of ester and amide groups in 5F-ADB molecule. The elemental analysis and a very low difference in the exact mass between the theoretical and the experimental mass (Δ ppm) for individual 5F-ADB degradation products and the assumed 5F-ADB hydrolysis products proves the correctness of the proposed identification. As results from Ref. [25], the hydrolysis of ester and amide groups occurs easier in alkaline than in acidic environments. This fact is confirmed by data in Fig. S2 showing much higher concentrations of 5F-ADB degradation products in the alkaline solution than in the acidic one. Hence, in the light of the presented data, the very low extraction efficiency of 5F-ADB from alkaline urine is

Table 2 HRMS data for 5F-ADB degradation products

Peak no.	Compound	Elemental composition	Theoretical mass [M+H] ⁺ (Da)	Experimental mass [M+H] ⁺ (Da)	Δ mDa	Δ ppm
1	(2S)-2-Amino-3,3-dimethylbutanoic acid	C ₆ H ₁₃ NO ₂	132.10245	132.10242	− 0.03	0.23
2	Methyl(2S)-2-amino-3,3-dimethylbutanoate	C ₇ H ₁₅ NO ₂	146.11810	146.11822	0.12	0.82
3	1-(5-fluoropentyl)-1H-indazole-3-carboxylic acid	C ₁₃ H ₁₅ FN ₂ O ₂	251.11958	251.11967	0.09	0.36
4	2-(1-(5-fluoropentyl)-1H-indazole-3-carboxamide)-3,3-dimethylbutanoic acid	C ₁₉ H ₂₆ FN ₃ O ₃	364.20366	364.20361	− 0.05	0.14
5	Methyl 2-[1-(5-fluoropentyl)-1H-indazole-3-carboxamido]-3,3-dimethylbutanoate (5F-ADB)	C ₂₀ H ₂₈ FN ₃ O ₃	378.21930	378.21939	0.09	0.24

probably ascribed to the effect of partial degradation of the drug to its hydrolysis products. The chromatogram (d) in Fig. S2 corresponds to natural urine of pH 8.5 incubated for 1 h at room temperature after spiking with 5F-ADB. As can be seen, the same 5F-ADB degradation products are formed in the alkaline biomatrix.

Conclusions

The presented studies prove that the validated QuEChERS technique can be successfully used in routine analyses of 5F-ADB in urine. It shows that connecting the health state of the patient who abused 5F-ADB with the drug concentration in his/her urine can lead to wrong medical diagnosis of patients with alkaline urine. It is especially important in investigation of the cases of acute intoxications by 5F-ADB due to the drug's evident hydrolytical instability in alkaline environment.

Compliance with ethical standards

Conflict of interest None of the authors have any potential conflict of interest (financial or otherwise) associated with this research manuscript.

Ethical approval Samples of blank urine were collected from volunteers after obtaining informed consent from each of them. This article does not contain any studies performed on animals by any of the authors.

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