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Determination of main tetrahydrocannabinoids by GC-MS: Impact of protein precipitation by acetonitrile on solid phase extraction of cannabinoids from human serum

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The analysis of Δ^9-tetrahydrocannabinol (Δ^9-THC) and its main metabolites [11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol] in serum is a routine assay in forensic toxicology in the case of drivers influenced by *Cannabis* abuse and in other cases. Analysis of the specimen may involve protein precipitation, although there are authors who do not use this step. In this study we investigated the effect of acetonitrile as protein precipitant added to the serum on the absolute extraction recoveries of the analytes. This is very important not only from a forensic point of view, but also from the aspect of impact of Δ^9-THC therapy. Our results showed that in the case of spiked serum (2 ml), 80–87 % extraction recovery can be achieved if 4 ml of acetonitrile is added before solid phase extraction. The second best result could be reached if no acetonitrile was added (64–73 %). However, in the case of physiological sera of *Cannabis* consumers, no precipitation may be more advantageous in some cases. Matrix effects, which were studied by comparing the detectability and relative intensities of matrix peaks within the corresponding time windows of the analytes, were less influenced if the extraction was achieved with or without acetonitrile.

1. Introduction

Cannabis abuse is still the most prevalent type of substance abuse. In 2012, there were altogether 2845 cases in Hungary positive for *Cannabis* abuse analysed at the National Institute of Forensic Toxicology (NIFT) (total No. of cases: 8093). In addition there were 35 traffic accidents in 2012 in which forensic toxicological analysis of serum samples from the driver, performed at the NIFT, proved the abuse of an illicit drug. Among these, 18 drivers had consumed *Cannabis.* Controlling drivers impaired by *Cannabis* smoking requires blood assays for cannabinoids, in particular, free Δ^9-tetrahydrocannabinol (Δ^9-THC) and its main oxidative metabolites 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-COOH) (Paul et al. 2009). Solid phase extraction of these analytes from human serum is a widespread routine sample preparation method often followed by gas chromatographic-mass spectrometric analysis. Mostly, sorbents showing (at least partly) non-polar sorption mechanism are chosen such as octadecyl (C18) silica. There are methods where no protein precipitation has been made prior to application of the specimen to the C18 sorbent (Moeller et al. 1992; Weller et al. 2000; Steinmeyer et al. 2002; Teske et al. 2002). Other authors suggested protein precipitation, mainly by an organic solvent such as acetonitrile, after which the deproteinized specimen was applied to cartridges (Daldrup 1996; Giroud et al.2001; Nadulski et al. 2005). Both approaches showed convincing validation parameters. However, for improving robustness, it is important to know the effect of protein precipitation by acetonitrile on quantitative results obtained using the assay. In this short study we aimed to compare the impact of protein precipitation on the extraction yields of Δ^9-THC, 11-OH-Δ^9-THC and Δ^9-THC-COOH from human blood serum by use of octadecyl modified silica solid phase extraction cartridges.

2. Investigations, results and discussion

Analytes Δ^9-THC, 11-OH-Δ^9-THC and Δ^9-THC-COOH showed retention times of 6.47, 7.13 and 7.49 min, respectively. All eluted 0.3–0.5 s later than the corresponding deuterated internal standards. In this study we examined the impact of the addition of four different amounts of acetonitrile (including addition of no acetonitrile) to the serum specimens on the extraction yield of the analytes. To compare the extraction yields of the four different extraction methods, analytes were added to aliquots of blank serum. However, corresponding internal standards were added directly prior to evaporation after SPE. We also prepared unextracted reference samples by which the absolute extraction yield could be calculated. In these cases both the mixture of analytes and the mixture of internal standards were added to extracts of blank sera directly prior to evaporation. These samples corresponded to 100 % yield. Fig. 1 shows the correlation between the extraction yields and the amount of acetonitrile applied during the extraction of Δ^9-THC and its metabolites. Standard deviations of the results are highlighted on the plots, related also to the 100 % values.

The results show that all analytes could be extracted from spiked serum with an extraction recovery of 64–73 % even if no protein precipitant was added. Adding 2 ml of acetonitrile dramatically decreased the relative signals of the analytes (extraction recovery

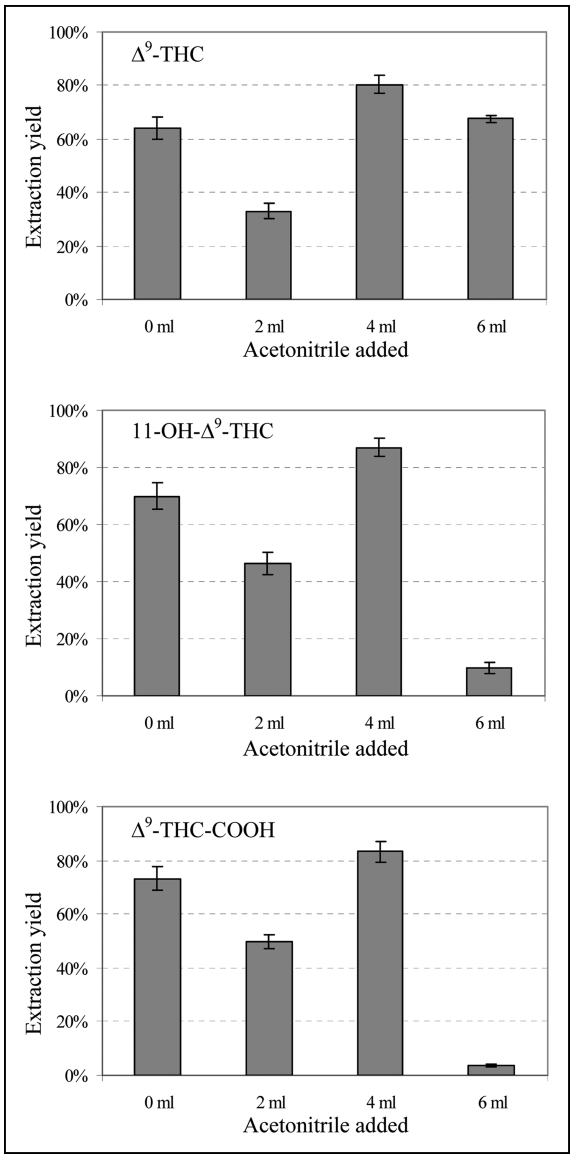

Fig. 1: Impact of acetonitrile addition on the absolute extraction yield of Δ^9-THC, 11-OH-Δ^9-THC and Δ^9-THC-COOH from spiked human serum. Analyte concentration: 45 ng/ml (N = 4).

range 33-50 %). Analyte losses probably occurred because the analytes are partly bound to the proteins of the sample and even partial protein precipitation resulted in their removal from the serum. Doubling the amount of acetonitrile from 2 mL to 4 mL, which meant that the ratio of acetonitrile in the sample before application to the SPE column increased from about 1:4 (*v/v*) to 2:3 (*v/v*), resulted in increased extraction recovery (80–87 %). This was the maximum value within the frame of this study. This high yield could be explained if, besides protein precipitation, acetonitrile acted as an effective extraction solvent for the analytes at this volumetric ratio. Adding 6 ml acetonitrile to the serum and thus increasing its ratio to 3:2 (*v/v*) resulted in much lower recoveries. This was probably due to the decreased retention of the analytes on the extraction columns at this high percentage of acetonitrile, which means that the solvent strength of the sample should be low enough in order to avoid losses. There was also a significant difference between Δ^9-THC and its metabolites if 6 ml acetonitrile was used. Δ^9-THC was less affected by an increase in solvent strength since it is the most nonpolar of the three analytes.

Absolute recoveries could not be calculated when physiological sera of three *Cannabis* users were compared. However, differences in extraction yields between adding no acetonitrile and the assay with addition of 4 ml of acetonitrile were studied. Despite the results with spiked serum the addition of acetonitrile was not always advantageous regarding extraction yield in the case of physiological sera. In some cases adding no acetonitrile resulted in higher yields, while in others precipitation caused the same. We found the most significant differences in extraction efficiency for 11-OH-Δ^9-THC and Δ^9-THC-COOH. Factors influencing the different behaviors of physiological sera need further studies.

Finally, differences in chromatographic background were compared between adding no acetonitrile and the assay with addition of 4 ml of acetonitrile. Co-eluting and other interfering matrix peaks were more or less the same in the two methods (Fig. 2). Nevertheless their relative intensities were different in some instances. It is obvious that the current separation is not optimized (e.g. in the case of Δ^9-THC there were significant co-eluting matrix peaks with fragment ions of m/z 386 and 343) but actually we intended only to compare interfering matrix peaks.

3. Experimental

3.1. Chemicals

Reference standards of (–)-Δ^9-THC, (±)-11-hydroxy-Δ^9-THC, (–)-11-nor-9-carboxy-Δ^9-THC, (–)-Δ^9-THC-D_3, (±)-11-hydroxy-Δ^9-THC-D_3 and (±)-11-nor-9-carboxy-Δ^9-THC-D_3 were provided by Cerilliant® (Round Rock, TX). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Sigma-Aldrich (Saint Louis, MO). Other chemicals were of analytical grade. ISOLUTE® C18 500 mg/10 ml solid phase extraction columns were provided by Biotage (Uppsala, Sweden).

3.2. Specimens and extraction

Blank human serum used for experiments was provided by a transfusion centre and was previously screened for cannabinoids. For preparation of spiked specimens 2 ml of blank human serum were spiked at concentrations of 3 and 45 ng/ml with the 2 µg/ml mixed methanolic solution containing Δ^9-THC, 11-OH-Δ^9-THC and Δ^9-THC-COOH. After spiking, the samples were allowed to stand for 15 min. Four samples were extracted in parallel for studying extraction yields and matrix background. Serum samples of three *Cannabis* consumers provided by police authorities were analyzed as duplicates or triplicates.

Either 2, 4 or 6 ml of acetonitrile were added to serum samples or else addition of acetonitrile was omitted. Samples were mixed thoroughly and let stand for 15 min. The samples were then centrifuged (3000 r.p.m., 10 min). The supernatants were separated and complemented by adding 50 mM aqueous phosphoric acid solution in corresponding amounts (8, 6, 4 or 2 ml to the sera to which were added no acetonitrile, or 2, 4, 6 ml of acetonitrile, respectively). SPE cartridges were conditioned by adding 4 ml of acetonitrile and 4 ml of acetonitrile—50 mM phosphoric acid mixture (at the appropriate volumetric ratio, 0:1, 1:4, 2:3 or 3:2, *v/v*). Supernatants were applied to columns and passed through the column dropwise; vacuum was used if necessary (ca. 70–80 mm Hg). Columns were first rinsed with the same acetonitrile—50 mM phosphoric acid mixture which was used for conditioning, then with distilled water. The cartridges were dried under full vacuum for 30 min. Analytes were eluted with *n*-hexane—ethyl acetate (2:1, *v/v*) and the eluates were evaporated under a mild stream of N_2 at room temperature. For the studies of extraction yields, eluates were spiked with 15 µl of mixed deuterated internal standard solution containing Δ^9-THC-D_3, 11-OH-Δ^9-THC-D_3 and Δ^9-THC-COOH-D_3 in methanol (5 µg/ml) before evaporation. For trimethylsilylation of the residues 100 µl of MSTFA were added and incubated at 80 °C for 30 min.

3.3. Gas chromatographic–mass spectrometric (GC/MS) analysis

Analyses were performed using a Shimadzu GC-2010 Plus gas chromatograph-mass spectrometer equipped with GCMSsolution software. The gas chromatographic parameters were as follows: capillary column: VF-DA, 12 m length, 0.2 mm inner diameter and 0.33 µm film thickness (Agilent Technologies, Santa Clara, CA). Injector temperature: 280 °C, oven initial temperature: 90 °C, hold time: 1 min, rate: 30 °/min, final temperature: 290 °C (13 min). Carrier gas: He (6.0), starting pressure: 150 kPa (constant linear velocity), splitless time: 0.4 min, split ratio: 20, interface temperature: 280 °C, ion source temperature: 280 °C, ionization: EI at 70 eV.

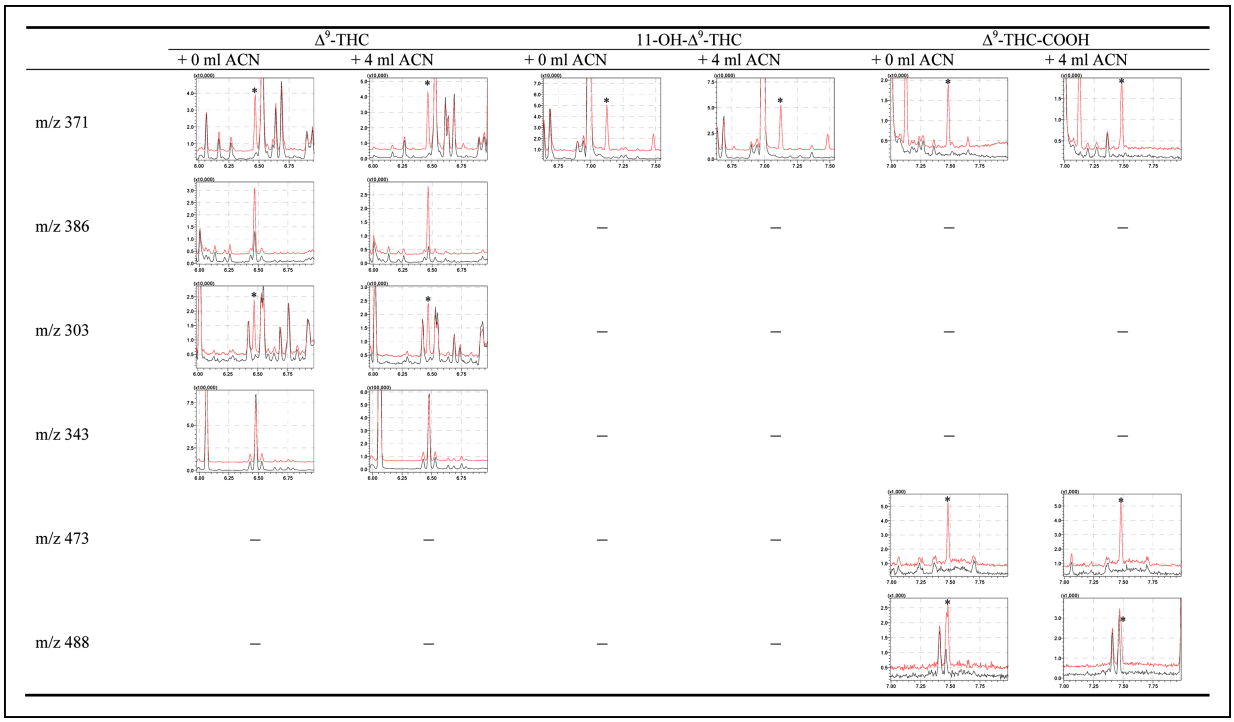

Fig. 2: Quantitation and qualifier fragment ion chromatograms of trimethylsilylated Δ^9-THC, 11-OH-Δ^9-THC and Δ^9-THC-COOH within the corresponding time windows after SPE extraction of spiked serum (concentration: 3 ng/ml each) versus matrix background of blank serum. Matrix peaks after applying no acetonitrile and 4 ml of acetonitrile are compared. Black chromatogram: blank serum, red chromatogram: spiked serum. Asterisks: analytes.

Compounds were analysed in SIM mode monitoring the following fragment ions: m/z 303, 343, 371, 386 (Δ^9-THC), m/z 371 (11-OH-Δ^9-THC), m/z 371, 473, 488 (Δ^9-THC-COOH) 306, 374, 389 (Δ^9-THC-D_3), m/z 374 (11-OH-Δ^9-THC-D_3), m/z 374, 476, 491 (Δ^9-THC-COOH-D_3) with event time of 0.3 sec (underlined: target m/z).

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