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Quantitation of tetrahydrocannabinol in hair using immunoassay and liquid chromatography with tandem mass spectrometric detection

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A quantitative analytical procedure for the determination of Δ^9-tetrahydrocannabinol (THC) in hair has been developed and validated using liquid chromatography with tandem mass spectral detection (LC-MS/MS). Specimens that were determined as containing cannabinoids following immunoassay testing were quantified using solid-phase extraction followed by liquid chromatographic separation and tandem mass spectral detection in positive electrospray ionization mode. For confirmation, two transitions were monitored and one ratio determined. Samples being reported as positive were required to have both transitions present, the ratio of quantifying transition to qualifying transition being within 20% of that determined from known calibration standards. The limit of quantitation and the limit of detection was 10 pg/mg. The percentage recovery of the THC from hair at 20 pg/mg was 56% and a matrix effect of the hair showed an ion suppression percentage of −51%. The immunochemical screening method was performed following a rapid aqueous extraction, requiring only 10 mg of hair; the confirmatory procedure required 20 mg of hair. The methods were applied to proficiency specimens from the Society of Hair Testing, which had been received in August 2008.

Keywords: hair; marijuana; ELISA; LC-MS/MS

Introduction

Marijuana is a commonly used illicit drug throughout the world, the active constituent being Δ^9-tetrahydrocannabinol (THC). Various countries are including hair cut-off concentrations in laboratory certification recommendations as a guideline for testing levels. The Society of Hair Testing (SoHT) in 2004 recommended 100 pg/mg of THC as both an immunochemical assay cut-off concentration and a limit of quantification using mass spectral detection.[1] The European Workplace Drug Testing Society (EWDTS) is considering similar levels and in Germany the proposed detection limit for THC in hair when assessing re-granting of driving licenses is 20 pg/mg. It must be noted, however, that the detection of THC alone does not prove cannabis consumption. In order to prove actual use of marijuana as opposed to potential exposure to the drug, the metabolite, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCA) must be detected in hair. The concentration of THCA in hair is extremely low, requiring either two-dimensional GC with chemical ionization mass spectrometric detection (GC-GC/MS), or gas chromatography with tandem mass spectrometric detection (GC-MS/MS) for adequate detection.[2,3]

Musshoff and Madea recently addressed the scientific demands for validation of hair analysis with an extensive discussion of the issues associated with this unique matrix.[4] Of the various published procedures for the determination of THC and its metabolites in hair, most use GC/MS or GC-MS/MS instrumentation. While there were several publications using liquid chromatography with tandem mass spectrometry (LC-MS/MS) for hair analysis of wide ranges of drugs, none included THC as an analyte.[5–7] In fact, no publications employing the detection of THC in hair using LC-MS/MS were located. This article reports the use of LC-with tandem mass spectral detection for the determination of THC in hair.

Many MS/MS methods monitor only one transition in the multiple reaction-monitoring mode (MRM), which is inadequate for forensic defensibility of the result. Recently, the need to monitor a second transition, allowing the ratio between the abundance of the primary and secondary transitions to be calculated and establishing more confidence in the final result, has been a focus of the literature.[8] Maralikova and Weinmann noted that guidelines for confirmatory analysis using LC-MS/MS have not yet been established and suggest that the monitoring of at least two transitions is required to provide sufficient identification of drugs.[9]

Several authors have reported on the disposition of THC in hair following cannabis use. Skopp *et al*. presented the determination of THC in hair using gas chromatography-mass spectrometry (GC/MS) with a detection limit of 25 pg/mg using 50 mg of hair as a sample volume. The concentration of THC detected in hair following long-term marijuana use ranged from 20–90 pg/mg.[10] Huestis *et al*. reported that concentrations detected in hair from 38 cannabis users ranged from 3.4 to greater than 100 pg/mg of THC. The median concentration for African American subjects was 34.3 pg/mg and for Caucasian users 18.8 pg/mg.[11] In order to assess the utility of the methods as applied to specimens from

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authentic drug users both the immunochemical screening method and the confirmatory procedure were applied to proficiency specimens provided by the Society of Hair Testing from August 2008.

Materials

Standards and reagents

The enzyme-linked immunosorbent assay kit, S-THC ELISA, hair extraction buffer and neutralizing buffer were obtained from Immunalysis Corporation (Pomona, CA, USA). Triply-deuterated internal standard (d_3-THC), as well as unlabelled drug standards were obtained from Cerilliant (Round Rock, TX, USA). Solid-phase extraction columns (Cerex® Polychrom ™THC 682-0353) were purchased from SPEWare (Baldwin Park, CA, USA). All solvents were HPLC grade or better and were obtained from Spectrum Chemicals (Gardena, CA, USA). All chemicals were ACS grade.

Calibrators: immunoassay

The enzyme-linked immunosorbent assay (ELISA) was operated in semi-quantitative mode (Log Absorbance. v. Log concentration). Calibrators were prepared using THC (Figure 1) in drug-free hair matrix at concentrations of 25, 50, 100, 500 and 1000 pg/mg using 10 mg aliquots of hair.

Calibrators: LC-MS/MS

For the chromatographic calibration standards, a working solution for the deuterated internal standard was prepared in methanol at a concentration of 1000 ng/mL. Unlabelled drug standard was prepared in methanol at the same concentration. All the working solutions were stored at −20 °C when not in use. For each batch, seven calibration standards excluding the drug free negative specimen were prepared in authentic drug-free human hair (20 mg). Drug concentrations of 10, 25, 50, 150, 250, 500 and 1000 pg/mg of hair were prepared (internal standard concentration: 500 pg/mg) for the calibration. Quality-control (QC) materials were prepared at 20 pg/mg and 100 pg/mg using separate lot numbers from the calibration standards.

Methods

Sample preparation for enzyme linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) technology is based upon the competitive binding to antibody of enzyme-labelled antigen and unlabelled antigen in proportion to their concentration in the reaction well. Hair was cut into small pieces and then an aliquot was weighed (10 mg). Extraction buffer (pH 4.8, 0.5 mL) was added and the tubes were capped and incubated for 2 hours at 75 °C. After cooling, the neutralization buffer was added (50 µL) and 0.1 mL of the total extract was removed. Phosphate buffered saline (100 mM) containing 0.1% w/v of bovine serum albumin (0.4 mL) was added to give a 1 : 5 dilution of the extract. An aliquot of 25 µL was used for ELISA and the assay was run according to the manufacturer's instructions. Each calibrator was analysed in duplicate and the process was repeated five times with different Lot numbers.

Sample preparation for chromatographic analysis

Hair specimens were washed with methanol (2 mL), rinsed, and allowed to dry before being cut into small pieces. An aliquot (20 mg) was used for analysis. The internal standard and 1 M sodium hydroxide (1 mL) were added to the hair in a glass tube. The tube was capped and incubated at 75 °C for 15 min. The specimens were allowed to cool, then deionized water (3 mL) was added and the tubes were centrifuged (2144 g; 20 min). Solid-phase extraction columns (Cerex®) were placed into a positive pressure manifold. Ethyl acetate (3 mL) and methanol (1 mL) were passed through. The samples were allowed to flow through the columns and then the columns were washed with a fresh solution of deionized water: acetonitrile: ammonium hydroxide (84 : 15:1, v,v; 3 mL). The columns were dried under pressure (30 psi for 10 min), then hexane (1 mL) was passed through. Δ^9-tetrahydrocannabinol was eluted with hexane : ethyl acetate (50 : 50 v,v 3 mL) and evaporated to dryness under nitrogen at 40 °C. The extracts were reconstituted in 20 mM ammonium formate pH 8.6: methanol (50 : 50 v,v; 50 µL), transferred into auto sampler vials, capped and analysed using LC-MS/MS.

Liquid chromatography – tandem mass spectrometry (LC-MS/MS)

An Agilent Technologies 1200 Series liquid chromatograph pump coupled to a 6410 triple quadrupole mass spectrometer, operating in positive electrospray mode (ESI) was used for analysis. The liquid chromatographic column was a narrow-bore Zorbax Extend C18 threaded system (2.1 × 50 mm ×1.8 µm), designed for high-pressure operation. The double end-capping of the stationary phase protected the silica support from dissolution at high pH extending the allowable pH range of operation from 2 to 11.5. The column temperature was held at 40 °C and the injection volume was 5 µL. The mobile phase consisted of 20 mM ammonium formate (pH 8.6, Solvent A) and methanol (Solvent B). At the beginning of the run, the mobile phase composition was 30% A:70% B at a constant flow rate of 0.3 mL/min. After 2 min, the percentage of solvent B was 100%, and at 3 min the percentage of B returned to 70%. The equilibration time was 3 min. The gas temperature was 350 °C, the gas flow was 13 L/min and the nebulizer pressure was 50 psi. Nitrogen was used as the collision gas and the capillary voltage was 3500 V.

Two transitions were selected and optimized for THC using flow injection analysis. For tri-deuterated THC, the precursor ion 318.4 was fragmented to 196.3 at optimized fragmentor voltage of 150 V and collision energy of 35 V. For THC, the precursor ion of 315.4 was fragmented to 193.3 (quantitative transition) and 123.3 (qualitative transition) at fragmentor voltages of 125 and 150 V respectively; collision energy of 20 eV. Each subsequent analysis required the ratio between the quantifier transition and the qualifier transition to be within +−20% of that established by calibration standards, in order to meet the criterion for a positive result. The transition ratio was determined at the concentration of 20 pg/mg and was a mean of values taken over five days.

Validation

Data Analysis

Calibrations of the assay, slope, intercept and correlation coefficients were calculated using linear regression analysis over the concentration range from the limit of quantitation to 1000 pg/mg.

Peak area ratios of the target analyte and the internal standard were calculated using Mass Hunter software (Agilent). The data were fitted to a linear least-squares regression curve with a 1/x weighting and was not forced through the origin.

Selectivity

Commonly encountered drugs were added to hair specimens obtained from drug-free volunteers (n = 6). The specimens were subjected to the same extraction and analysis procedures described. The following drugs were included at a concentration of 10 000 ng/mL: cannabinol, cannabidiol, cocaine, benzoylecgonine, cocaethylene, norcocaine, morphine, 6-acetylmorphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, tramadol, fentanyl, gamma-hydroxybutyrate (GHB), phencyclidine (PCP), amphetamine, methamphetamine, methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), carisoprodol, methadone, diazepam, nordiazepam, oxazepam, alprazolam, chlordiazepoxide, bromazepam, temazepam, lorazepam, flurazepam, nitrazepam, triazolam, amitryptiline, nortriptyline, imipramine, protriptyline, doxepin, nordoxepin, trimipramine, secobarbital, pentobarbital, butalbital and phenobarbital.

Linearity and sensitivity

The linearity of the assays was established with seven calibration points, excluding the drug-free matrix. The sensitivity of the method was determined by establishing the limit of quantitation (LOQ) defined as the lowest concentration detectable with a signal-to-noise (S : N) ratio of at least 10 for the quantifying ion and retention time within 0.2 minutes of the calibration standard. The limit of detection (LOD) was defined as the lowest concentration detectable with a signal-to-noise (S : N) ratio of at least 3 for the quantifying ion. In both cases, the qualifying ion was required to be present within the range established (+−20%), with acceptable chromatography. The limits were determined by the method of serial dilution, whereby hair samples fortified with decreasing THC concentration were analysed, until the specified criteria were no longer met.

Accuracy, precision and extract stability

The accuracy of the procedure was determined using six replicates at two concentrations (20 and 100 pg/mg). Accuracy was calculated as

$$\frac{\textit{mean measured concentration} - \textit{fortified concentration}}{\textit{fortified concentration}} \times 100\%$$

Inter-day and intra-day precision were determined at the calibration points of 20 and 100 pg/mg. Intra-day data were obtained from six analyses performed on one day; inter-day data were obtained by analysing six specimens on each of five days (n = 30).

In the event that specimens needed to be reinjected, the stability of the drug extracts at concentrations of 20 and 100 pg/mg were determined by allowing the auto-sampler vials to remain in the liquid chromatographic chamber for 24 and 48 hours after which time they were reanalysed. The unit was maintained at 7 °C. The responses were compared to those achieved on the first day of analysis; quantification was performed using the freshly prepared calibration curve each day.

Extraction efficiency and ion suppression

In 2007, Chambers *et al.*[12] discussed a systematic and comprehensive strategy for reducing matrix effects in LC-MS/MS. The relative efficiency of the extraction method for THC in the hair matrix was determined by first assessing the response of extracted samples (n = 3) at concentrations of 20 and 100 pg/mg {R_{ES}}. Next, hair was extracted and drug was added post-extraction at concentrations of 20 and 100 pg/mg (n = 3) {R_{PES}}. The efficiency was then calculated from the equation (R_{ES}/R_{PES}) × 100.

The percentage reduction/improvement in response due to matrix effects (ion suppression/ion enhancement) was determined by assessing the peak area response of a non-extracted neat drug standard (n = 3) at concentrations of 20 and 100 pg/mg {R_{NES}}. The non-extracted solution was analysed in the same reconstitution solvent as the extracted specimens. The percentage matrix effect was then calculated using the equation (R_{PES}/R_{NES}) − 1) × 100. Chambers *et al*. modified the original equation by applying the subtraction of 1 to the quotient so that a negative result indicates ion suppression and a positive result indicates ion enhancement of the signal.

Application to proficiency specimens

The Society of Hair Testing (SoHT) provides proficiency testing samples on an annual basis. In August 2008, five samples were received and since specimen was remaining, the described procedures were applied. The samples were from authentic drug users, with the exception of one drug-free specimen.

Results and Discussion

The development of a rapid aqueous extraction for the immunometric screening of THC in hair was described, followed by a simple LC-MS/MS assay for THC confirmation with an improved limit of quantitation to 10 pg/mg. The method provides excellent sensitivity without the need for derivatization, and a much shorter run-time than related GC/MS procedures. The procedure is useful in routine testing for the determination of THC in hair, providing forensic defensibility by the inclusion of two monitored transitions.

ELISA screening

When applied to hair calibrators at concentrations of 25, 50, 100, 500 and 1000 pg/mg, in semi-quantitative mode (Log Absorbance. v. Log concentration), the correlation coefficient of the slope was r^2 = 0.98776 (Figure 2). The average separation (B/Bo) from the drug-free negative hair over the tested range was 78.1% (25 pg/mg); 68.9% (50 pg/mg); 48.5% (100 pg/mg); 26.4% (500 pg/mg) and 15.3% at 1000 pg/mg, where B = absorbance

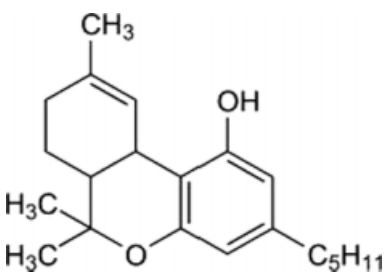

Figure 1. Molecular structure of tetrahydrocannabinol (THC).

value of the specimen and Bo = absorbance value of the negative calibrator.

LC-MS/MS confirmation

While THC has been detected in hair, the increasing utility of LC-MS/MS in laboratories makes development of confirmatory procedures necessary and timely. The monitoring of a second qualifying transition is included for THC in hair and is necessary for confidence in the identification of the analyte. The pH of the mobile phase (8.6) is slightly above the recommended limit for the usual LC columns (pH 8), so the Extend C18 column was chosen, allowing a pH range of 2 to 11.5 to be employed.

The confirmatory procedure developed for THC was validated by determining accuracy, precision, linearity, limits of detection and quantitation, selectivity and extract stability. The limit of quantitation and detection was 10 pg/mg for THC, which surpasses the recommended concentration for the analysis of THC in hair for various European organizations.

Linearity, precision, accuracy and stability

The accuracy and precision of the assay were determined as described. The procedure was very accurate, with a variation of +10% for THC at 20 pg/mg; −3.3% at 100 pg/mg. Inter-day (between day) and intra-day (same day) precision of the assay was determined using replicate analyses. The inter-day and intra-day precisions were 9.8% and 4.0% at a concentration of 20 pg/mg respectively and 6.7% and 2.2% at 100 pg/mg respectively.

Linearity was obtained with an average correlation coefficient for THC of >0.99 over the range 10–1000 pg/mg of hair. The mean correlation coefficient and equation of the calibration curve for the THC based on five replicate analyses on five separate days was THC: $r^2 = 0.9978$; y = 0.002x (SD 0.0001) +0.0042 (SD 0.0008) (Table 1). The allowable qualifying ratio for the intensity of the second transition for THC was 35.8–53.7% (n = 5). The ranges were established at a concentration of 20 pg/mg over five days.

The extracted specimens were stable for at least two days when kept in the instrument rack inside the auto-sampler, which was maintained at 7 °C. There was less than a 15% difference in the quantitation of the extracts after 24 and 48 hours.

Selectivity

Hair specimens collected from drug-free individuals (n = 3) showed no interference with any of the assays. For exogenous interference, commonly encountered drugs of abuse, as well as cannabinol and cannabidiol, were studied as described in the experimental section. The absence of interference was assessed by the observation that no chromatographic interference was observed in the channels of the monitored transitions. The quantitation of the THC was not affected by the presence of the other drugs. Matrix effects were reduced, but not eliminated, through the use of sample dilution and a specific solid-phase extraction.

Extraction recovery, ion suppression/matrix effects process efficiency

The solid-phase extraction of drugs from complex matrices reduces ion suppression effects. Accordingly our specimens were subjected to extraction using Cerex® columns specifically intended for the extraction of THC from hair. The efficiency of the extraction procedure and matrix effect was established. Hair, being a

Table 1. Linearity, intra and inter-day precision of the assay

Linearity (established over 5 days)

Day	Slope (1/x)	Intercept	r^2 on the Linearity part
1	0.0019	0.0048	0.998
2	0.002	0.0038	0.999
3	0.0021	0.003	0.998
4	0.0019	0.005	0.996
5	0.002	0.0043	0.998
Mean	0.002	0.0042	0.9978
Standard deviation	0.0001	0.0008	0.001

Intra-day precision (n = 6) determined at two concentrations

Replicate	20 pg/mg	100 pg/mg
1	23	99
2	21	95
3	23	96
4	22	97
5	22	101
6	21	98
Mean	22	97.6
Standard deviation (SD)	0.89	2.16
Coefficient of variation (CV %)	4.07	2.21

Inter-day precision (n = 30) determined at two concentrations

Day	20 pg/mg	100 pg/mg
Day 1	21	96
2	20	95
3	19	96
4	17	96
5	20	95
6	17	96
Day 2 1	23	99
2	21	95
3	23	96
4	22	97
5	22	101
6	21	98
Day 3 1	25	90
2	23	93
3	20	94
4	22	85
5	22	92
6	20	89
Day 4 1	24	112
2	23	95
3	23	111
4	23	106
5	23	115
6	24	101
Day 5 1	25	100
2	18	100
3	21	95
4	24	103
5	24	100
6	22	105
Mean	21.73	98.2
Standard deviation (SD)	2.15	6.64
Coefficient of variation (CV, %)	9.89	6.76

Table 2. Matrix effects for THC in hair at a concentration of 20 and 100 pg/mg

THC	Average peak area response (n = 3)		
	Extracted hair (R_{ES})	*Post-extracted hair (R_{PES})*	*Non-extracted standard (R_{NES})*
20 pg/mg	284	505	1035
100 pg/mg	1274	2227	4975
THC	% SPE recovery ($R_{ES}/R_{PES} \times 100$)		% Matrix effect ($R_{PES}/R_{NES} - 1 \times 100$)
20 pg/mg	56.2		−51.2
100 pg/mg	57.2		−55.2

Table 3. Screening and confirmation results from SoHT 2008 hair proficiency specimens compared to mean and median reported concentrations from laboratories enrolled in the proficiency scheme

SoHT specimen	Mean (pg/mg)	Median (pg/mg)	ELISA semi-quantitative result (pg/mg)	LC/MS-MS quantitative result (pg/mg)
A	220	160	212	68
B	Negative	Negative	Negative	Negative
C	290	200	204	82
D	230	150	174	87
E	250	140	129	59

solid matrix, has unique disadvantages when determining drug recovered from the matrix, because there is often insufficient authentic material to extract multiple times. In this assay we assessed the efficiency of the extraction procedure, rather than attempting to extract an authentic hair several times, in order to preserve specimens.

The extraction itself was relatively efficient with an average recovery of over 56% at 20 pg/mg, 57% at 100 pg/mg) but ion suppression even incorporating deuterated internal standard and solid-phase extraction was significant (Table 2). The ion signal was suppressed for THC at both concentrations but the use of deuterated internal standards allowed accurate quantitation. The sensitivity of the instrument was adequate for the determination of THC in hair even when significant suppression of the signal was occurring and two transitions were monitored for each drug.

Proficiency specimens

Of the five proficiency samples received from the Society of Hair Testing in 2008, four were from authentic drug-users and one was a drug free hair specimen. All samples were analysed as described and results are shown in Table 3.

ELISA screening

The screening aqueous extraction method used no organic solvents, so evaporation and reconstitution steps were avoided; further, the process was fast and simple, requiring only 10 mg of hair. The ELISA results correlated well with reports from several laboratories (n = 13) participating in the SOHT testing program. As the proficiency samples had been stored for at least 8 months

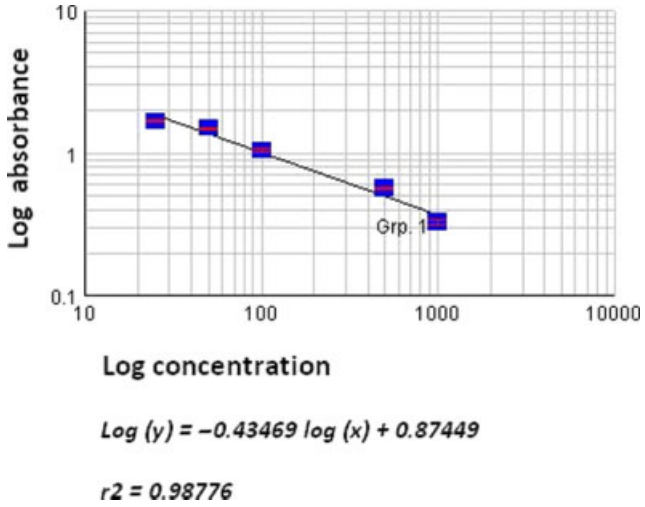

Figure 2. Semi-quantitative analysis of hair calibrators fortified with THC and analysed using ELISA.

(provided to laboratories in August 2008), the results were well within reported ranges.

LC-MS/MS confirmation

Figure 3 shows transitions for an authentic hair specimen (Sample C) taken from a drug user as part of the proficiency program. The THC concentration overall was significantly lower than that predicted by ELISA screening.

It has been reported that both hashish and marijuana lose potency during storage due to the decrease in concentration of THC.[13] In 2000, Skopp *et al.*[14] reported such a phenomenon in hair specimens, particularly when exposed to light. Interestingly they also noted that such degradation was unrelated to hair colour. It has been further suggested that cannabinol (CBN) is produced from THC by oxidation and is a potential marker for the degradation of THC. During their experiments, the group showed that while CBN was initially formed from the THC degradation, CBN was further oxidized to other cannabinoids. In another study,[15] experiments with marijuana components in hair showed that THC was the most unstable cannabinoid compared to CBN and cannabidiol (CBD) under various storage conditions.

Considering the proficiency specimens were at least 8 months old and had been stored at room temperature, the difference in quantitation can be explained by oxidation of THC in the hair specimen to other cannabinoids, which were recognized by the immunoassay but not the LC-MS/MS, as the MS/MS mode of detection was multiple reaction monitoring (MRM) as opposed to full scan.

Characterization of the ELISA showed only 4% cross-reactivity towards CBN at an equivalent concentration of 4 ng/mL THC, but 800% cross-reactivity with 11-nor-9-carboxy-Δ^9-THC and 133% with Δ^8-THC. Obviously cross-reactivity percentages vary across concentration range but the specificity of the antibody towards cannabinoids is demonstrated by the results. The ability of an immunoassay to detect other products becomes a significant advantage for analysis, as even aged or degraded specimens may be found to be positive for cannabinoids due to ELISA recognition of other structurally related compounds.

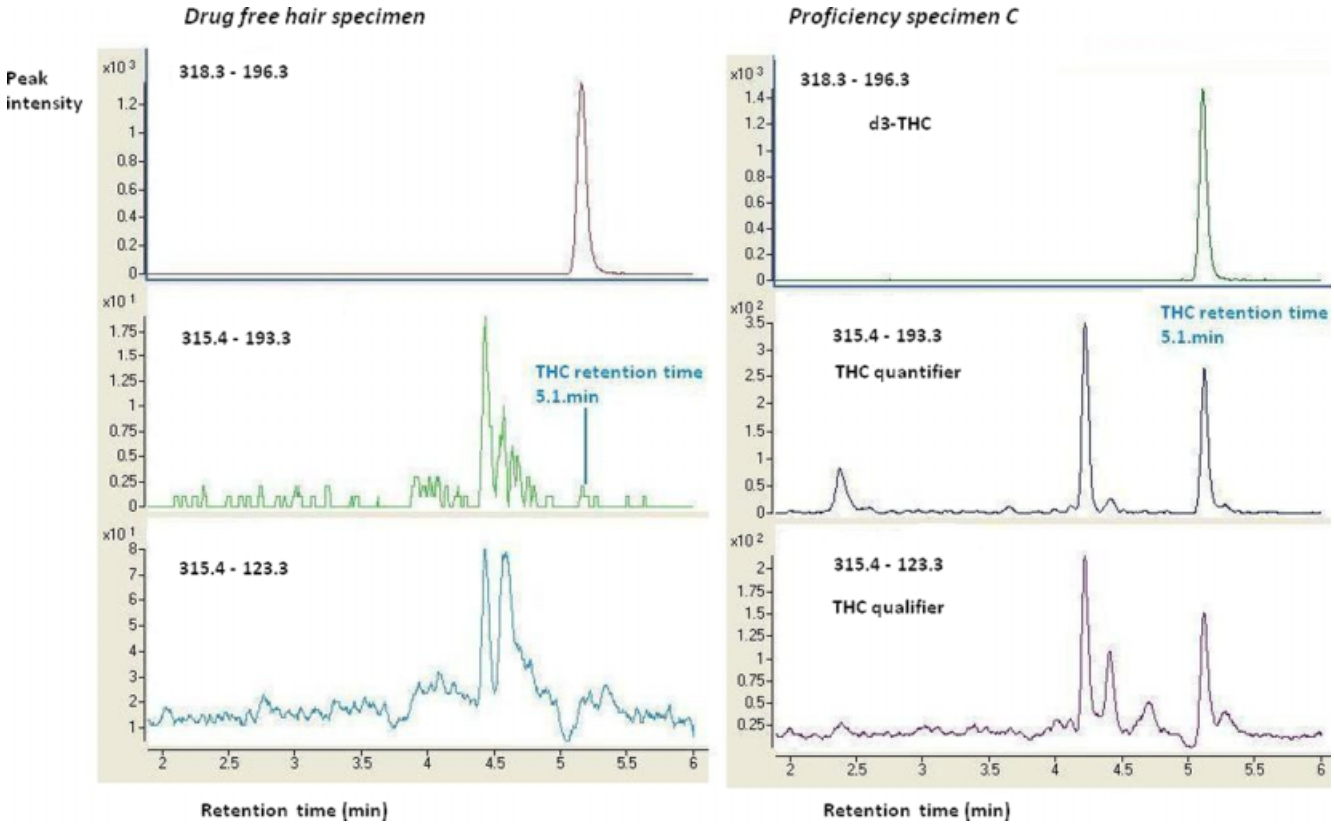

Figure 3. Extracted transitions for (a) negative hair sample, (b) authentic proficiency specimen C (quantified at 82 pg/mg).

Summary

The determination of THC in hair by both immunochemical and LC-MS/MS was described for the first time. The ELISA assay made use of a rapid aqueous extraction, thereby eliminating evaporation and reconstitution steps. The semi-quantitative screening mode resulted in concentrations in good agreement with proficiency tests.

Further, a validated LC-MS/MS confirmatory method with a limit of quantitation of 10 pg/mg was described. The assay included monitoring a qualifying transition and calculation of a ratio, required to be within 20% of that of a known calibration standard in order for a definitive identification to be made. The quantitative results for THC were significantly lower than that predicted by the immunoassay, indicating that THC had oxidized over a period of 8 months to other cannabinoids, which were recognizable by the ELISA assay. It is recommended that hair specimens considered to contain marijuana be analysed as soon as possible after collection to avoid extensive degradation of THC.

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