DOI: https://doi.org/10.47391/JPMA.144

Diagnostic accuracy of cannabinoid testing by liquid chromatography-tandem mass spectrometry in human hair

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Abstract

Objective: To determine the diagnostic accuracy of Cannabinoids testing by LC-MS/MS in human hair and compare it with urine in civil heavy vehicle drivers.

Materials and Methods: Current study was a diagnostic accuracy study done in "Armed Forces Institute of Pathology Rawalpindi, Pakistan" from February to November 2017. Urine and hair samples were collected by non-probability convenient sampling technique from 151 heavy vehicle drivers from Punjab. Hair and urine samples were collected from each subject. Separation of compounds was performed on Agilent Poroshell and analyzed using 6460 Triple Quadrapole LC-MS along-with software Mass hunter ©.

Results: Study population (151 civil heavy vehicle drivers) was divided into three main divisions There were 69 (46%) truck drivers,43 (28.5%) twenty-wheeler drivers and 39 (26%) bus drivers. Mean age of study participants was 36±10.82 years. Paired t-test was applied to check mean difference between the two tests' concentration (i.e urine and hair analysis for cannabis) which showed significant difference at $p<0.001$. Among the different factors of diagnostic accuracy in hair and urine specimens were: Sensitivity (96% and 62%), Specificity (93% and 95%) Positive Predictive Value (88% and 87%), Negative Predictive Value (97% and 82%) respectively. Overall diagnostic accuracy of Cannabinoids detection in hair was 94% while in urine it was 83%. ROC curve showed area under curve of 0.79 and 0.96 for urine and hair samples respectively.

Conclusion: Current study signified hair as a substitute matrix owing to its non–invasive specimen collection, better diagnostic yield and wider detection period compared to urine.

Keywords: Cannabinoids testing in hair, liquid chromatography- tandem mass spectrometry (LC-MS/MS), diagnostic accuracy.

Introduction

Marijuana is extracted from a plant known as *Cannabis sativa*. The active compounds that are exclusive to the plant and are named as Cannabinoids. These include Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD) and tetrahydrocannabivarin (THCV). Cannabinoids act through two specific receptors located mainly in brain, immune system, lungs, kidneys etc.

Internationally cannabis (marijuana) is the most commonly used substances of abuse [1]. Currently it is being used by around 180 million people globally. According to WHO in EMRO region, the regional median annual prevalence of cannabis use in nine countries in population aged between 15-64 years is 3.6%. In Pakistan, about four million individuals (3.6%) were found to be under

influence of this evil [2]. This increasing prevalence also extended to automobile drivers. [3]

Δ9-tetrahydrocannabinol (THC) is the main psychotropic cannabinoid, causing elation, difficulties in concentration and cannabis withdrawal syndrome [4, 5]. Over the years, various studies have shown the adverse effects of cannabis use in drivers and its relationship with increasing risk of vehicle accidents. This is quite alarming as there is dose response relationship of usage of cannabis on coordination, which is essential to prudent driving [6]. So, it is the need of hour to ensure rapid and accurate detection of cannabis exposure of drivers in order to culminate this dangerous social evil.

Various biological matrices have been employed for detection and surveillance of cannabis addiction including urine, blood, oral fluids etc. LC-MS/MS has become benchmark in analysis of cannabinoids owing to low limit of detection, selectivity, but above all, due to its ability to determine both precursor and free ions and in a single analytical run [7]. In recent years, studies done on hair analysis have shown promising results. There is longer window period of detection in hair as compared to urine, which is about 30 days in chronic drug abusers [8]. Hair cannabinoid analysis mainly includes the psychoactive Δ9-tetrahydrocannabinol (THC) and its metabolite 11-nor-9-carboxy-Δ 9-tetrahydrocannabinol (THC-COOH). There is passive diffusion of drugs into hair from blood capillaries leading to drug deposition into basement membrane of hair follicle, thus providing a rough time related evidence of drug intake event[9]. On an average, 3months' time period is consistent with average hair growth of about 3.8-4cm [10]. Presence of THC-COOH, which is only metabolised in vivo, is considered a proof of consumption. However, there are some major difficulties for the detection of Cannabinoids in hair, mainly due to lower concentrations of THC-COOH, which is usually found in picogram per miligram range in hair. [11]

Globally many studies have been done to assess cannabinoid exposure by hair analysis because of its advantages over classical matrices. However, local data is sparse. Present study followed the method development and validation study, done at our institute, for cannabis detection by LC-MS/MS [12]. The main objective of this study was to assess the diagnostic accuracy of Cannabinoids testing by LC-MS/MS in human hair and to compare it with urine for cannabis detection in civil heavy vehicle drivers. This alternative biological matrix testing would prove useful in scenarios of cannabis addicts monitoring, easy road side specimen collection for surveillance of drivers, post-mortem forensic testing [13] and situations where urine samples are not available e-g road traffic accidents, drug facilitated crimes etc. [14]

Methodology

It was a diagnostic accuracy (validation) study done in "Department of Forensic Medical Sciences Laboratory, Forensic Toxicology Section, Armed Forces Institute of Pathology, Rawalpindi, Pakistan" from February to November2017, using non-probability convenience sampling method. Self-declaration or denial of cannabis use / addiction was taken as reference standard (gold standard). A total of 151 civil heavy vehicle drivers were included in study (95% confidence interval, level of significance 0.05%). Adult male civil heavy vehicles (including truck, twenty-wheeler and bus) drivers, with an average travelling time ranging from 12 to 15 hours per day, between ages of 20-65 years, who were active smokers were included in this study. Passive smokers were excluded by detailed interview. Current research study was approved from the Institutional Ethical Review Board (IERB) of Armed Forces Institute of Pathology, Pakistan. Informed Consent was taken from the participants.

These drivers were interviewed thoroughly to record their present or past history of cannabis usage. This self-reported presence or absence of active cannabis usage was taken as reference standard (gold standard); true and false positives,

true and false negatives were labeled on the basis of this self-report. Active/current smoker was considered as an individual who had smoked hundred cigarettes in his life and who was at present smoking cigarettes (joint, marijuana or tobacco) [15]. The participants were inquired about their consumption of marijuana within the preceding 3 months.

Ten milliliter of urine was collected in urine container and was kept at -20 degrees centigrade till further analysis. Hair strands were collected from the posterior apex of scalp and cut as near to the root as possible. Samples were placed in zip lock bags and placed at room temperature till these were analyzed.

Chemicals that were used for extraction and sample preparation included10N NaOH (Merck–Germany), Acetoacetate buffer, Glacial acetic acid, Internal Standard of THC-d3 and THC-COOH-d3 (Cerilliant Corporation-USA) and Acetonitrile + Ultra-pure water from Millipore apparatus (Merck-Germany).

Limit of detection (LOD) in urine samples was 0.1ng/ml, whereas in hair it was 0.025 ng/mg. Limit of quantification (LOQ) was 5ngm/ml and 100pgm/mg in urine and hair respectively. For hair samples, a cut-off of 0.05ng/mg and for urine samples a thresh hold of 15ng/ml was taken for positive results. Both for Urine and hair positive and negative controls were analyzed with each batch of samples.

Two ml of urine sample was taken and mixed with NaOH. After incubation, acetoacetate buffer and glacial acetic acid were added to mixture. Then one ml of sample was taken and internal standards added and vortexed. Extraction solution of THC was made by combining ethyl acetate with N-hexane. Post centrifugation, the supernatant containing THC was transferred to another tube and placed in evaporator at 60°C. The residues of THC were then reconstituted with Acetonitrile + Ultra-pure water and vortexed. With the help of syringe, 200µl of solution was filtered and transferred to Gas Chromatography vial and assessed on LC-MS/MS System.

About 20mg of hair strands were taken and decontaminated. The dried hair specimens were then carefully cut into sections of 1mm size and added to labeled tubes. Samples were then incubated with NaOH, and internal standard at 60°C overnight, then vortexed. Formic acid was added and vortexed. Extraction was done by addition of N-hexane + Ethylacetate. Supernatant was taken post centrifugation and dried in Bio base fume hood. The dried samples were reconstituted with methanol. Sample (10 µl) was injected into GC vials and run on LC-MS/MS System.

10 µl of sample was injected from GC vial and chromatographic separation was done. Passage through Electrospray ionization (ESI)-source caused ionization, resulting in formation of parent ion which then passed to MS1 (Quadrupole 1), (Qaudrapole 2) and MS 2(Qaudrapole3). High energy dynode detector detected the daughter ions and transmitted the signals to computer software in the form of chromatograms, which were then assessed and results were compiled.

Data analysis was done on SPSS Version 16. Descriptive statistics mean and ±SD were calculated for continuous variables like age, Urine for cannabis and Hair for cannabis, while frequencies with percentages were computed for qualitative variables (age, age in groups, smoking status, occupation, geographical area). Paired t-test was applied to check mean difference between the two tests' concentration (i.e. urine and hair analysis for cannabis) that was considered significant at $p<0.05$. Among different parameters of diagnostic accuracy in hair and urine samples including Sensitivity, Specificity, Positive and Negative Predictive Value were assessed. Receiving Operating Characteristics (ROC) curve was plotted both for hair and urine keeping self-declaration or denial of cannabis use / addiction as gold standard.

Results

All 151 included subjects were male civil heavy vehicle drivers, which were stratified into three groups. Truck drivers were 69(45.7%), 20-wheeler drivers

were 43(28.5 %) while 39(25. 8%) individuals were bus drivers. Mean age was 36±10.82 years. Subjects were divided according to the age into four main strata.: a) 20-25 y: 28(18.5%), b) 26-40 y:73(48.3%), c) 41-60 y:47(31.1%) and d)>60 y:3(2%). Participants who belonged to rural area were 59(39.1%), and 92(60.9%) were from urban population. Among the total subjects, 63(41.2%) were smokers and 87(58.3%) were non-smokers. While among the subjects who were active smokers, 53 (35.1%) were also cannabis smokers.

Among the total 151 subjects whose urine and hair samples were analyzed for cannabis detection, 36 (23.8%) had both positive urine and hair samples, about 22(14.6%) had only hair positive, while in 90(59.6%) both the analyzed matrices were negative, and in only 3(2%) subjects, urine was positive. Hair samples were negative for THC.

ROC curve (Fig: 1) showed area under curve of 0.96 and 0.79 for hair and urine respectively. This highlighted the significance diagnostic accuracy of hair when compared to urine for detection of cannabinoids.

Several parameters of diagnostic accuracy in hair and urine samples including Sensitivity, Specificity, Positive and Negative Predictive Value were assessed (Table I). Paired t test was applied to check mean difference between the t two tests' concentration which was significant at $p<0.001$. Hair analysis have shown promising results. Its advantages included not only an easy method of sample collection and storage but also a very high index of analyte stability in hair. There is wider window period of detection up-to three months as compared to urine, which is about a month in chronic abusers. When compared to hair sampling, urine samples have the disadvantage of less stable matrix, lower window of detection, dependency on type of container used, adulteration and risk of infection transmission. Thus, making hair a better and sensitive matrix for detection of cannabinoids abuse.

Discussion

Illicit usage of marijuana has been on the rise in recent past and has become a major social issue [16]. Li et al (2011) (reported a pooled odds ratio of 2.66 (95% CI:2.07–3.41) in a meta-analysis of about 20 years research papers, in which vehicles' accidents association with cannabis usage was addressed[17]. In order to curtail this grave situation various biological matrices have been developed for detection and monitoring of cannabis use. In previous years, urine was considered to be a gold standard in detection of cannabis, but now hair is being considered as substitute matrix due to its several additional advantages.

In a Swedish pilot study, hair analysis of drivers was done for 20 drugs (including cannabis), in order to assess their abstinence and re granting of license [18]. Hair specimens were screened by Liquid Chromatography Mass Spectrometry and positives results were confirmed by analysis on Gas Chromatography-Mass Spectrometry or Liquid Chromatography Mass Tandem Spectrometry. Cut-off of 0.05ng/mg was kept in hair samples, which is the same as used for hair analysis in present study. Results of study revealed more positive hair samples than urine, 8.3% hair samples were positive, of which 4.7% were positive for THC.

According to a research conducted by Han E et al, samples were analyzed on GC/MS/MS-NCI system. Of total subjects, 37%had both positive urine and hair samples, 18.9% participants had positive hair and negative urine, 41.2% had both matrices negative, while 2.6% had urine positive and hair negative [19]. A similar trend has been seen in our study, keeping self reporting of cannabis abuse as gold standard. Receiver operating characteristic curve has been made of urine and hair samples from same individuals. About a quarter subjects (23.8%) had both positive urine and hair samples, about 14.6% had only hair positive, in 59.6% both urine and hair were negative and only 2% had urine positive and hair negative for cannabis. Although urine is used in routine for cannabinoid testing, but now researchers are focusing more towards hair as

being more sensitive and specific with long detection period as compared to urine. Moreover, it's easier to collect hair samples as compared to urine specially in forensics. It is emphasized in settings of strong clinical suspicion of cannabis abuse with negative urine test. False negative results should always be ruled by hair analysis.

Agius et al found sensitivity of 95% and specificity of 97% for THC detection in hair, when authentic hair samples, with sufficient concentration of cannabis were screened according to medical and physiological assessment guide lines by ELISA techniques and further confirmation was done by GC-MS or LC-MS/MS [20]. Musshoff et al conducted preliminary analysis of hair samples of drivers on LUCIO-Direct ELISA kit with further quantitation on GC-MS or LC-MS. When a cut-off of 0.1ng/mg was kept, which is according to guidelines of Society of Hair Testing (SoHT), sensitivity of 92% and specificity of 87% was found [21]. These results are in concordance to sensitivity (96%) and specificity (93%) of hair found in our study.

Taylor et al reported a sensitivity of 77%, when hair samples of heavy cannabis smokers were analyzed on GC-MS/MS, keeping a cut-off of 0.05ng/mg for THC, which is similar to that used in our research [22]. The difference in results might be due to complimentary advantages including better quantitation and detection ability of LC-MS/MS technology used in our study.

An observational study published in 2015, that revealed the sensitivity of 79% and a specificity of 95% for THC-COOH detection in urine by GC-MS [23]. These findings are in agreement with our results in urine, and had showed sensitivity of 62% and specificity of 95 %. (Table I)

Area Under curve (AUC) of 0.75 has been reported by Gryczynski et al when ROC curve was plotted for hair testing vs self-report [24]. While results of our research reveal AUC OF 0.96. (Fig 3).

Although this study has revealed hair as an appropriate matrix for cannabinoid analysis, yet it has limitations in terms of very low concentration of THC-

COOH in hair, which might not always be detected by our instrument due to its manufacturer specifications. Also, it requires state of the art technology and lab expertise, which is present in our institute, yet not commonly available in other setups of our country.

Conclusion

This study indicated that hair as an alternative biological matrix has a better diagnostic yield as compared to urine. Its noninvasive and easy specimen collection, better analyte constancy, as well as broader detection period give hair sampling a distinctive potential as compared to urine.

Competing interests: None to declare

Funding disclosure: None to declare.

Disclaimer: The abstract of this article has been published in conference proceedings of following international conferences.

a) Alveena Younas, J Chromatogr Sep Tech 2018, Volume 9 DOI: 10.4172/2157-7064-C1-040 Date: June 20-22, 2018, Location: Rome, Italy Page number: 53(volume 9), (conferenceseries.com Chromatography & Separation Technique Volume 9 ISSN: 2157-7064 Euro Mass Spectrometry 2018, 7th World Congress on Mass Spectrometry)

b) Clin Psychiatry 2018, Volume 4 DOI: 10.21767/2471-9854-C1-003, Date: July 16-18, 2018, Location: London, UK, Page number: 60(volume 4), (Joint Event on 7th World Congress on Addictive Disorders & Addiction Therapy & 29th International Conference on Sleep Disorders and Psychiatry, ISSN:2471-9854)

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patients with moderate-risk illicit drug use. Drug Alcohol Depend. 2014;141:44-50.

Table 1: Accuracy of hair and urine testing for Cannabinoids detection by LC-MS/MS

Parameter	Hair (%)	Urine (%)
Sensitivity	96.30	62.26
Specificity	92.78	94.90
Positive Likelihood ratio	13.34	12.20
Negative Likelihood ratio	0.04	0.40
Positive Predictive value	88.14	86.84
Negative Predictive Value	97.83	82.30
Diagnostic Accuracy	94.04	83.44

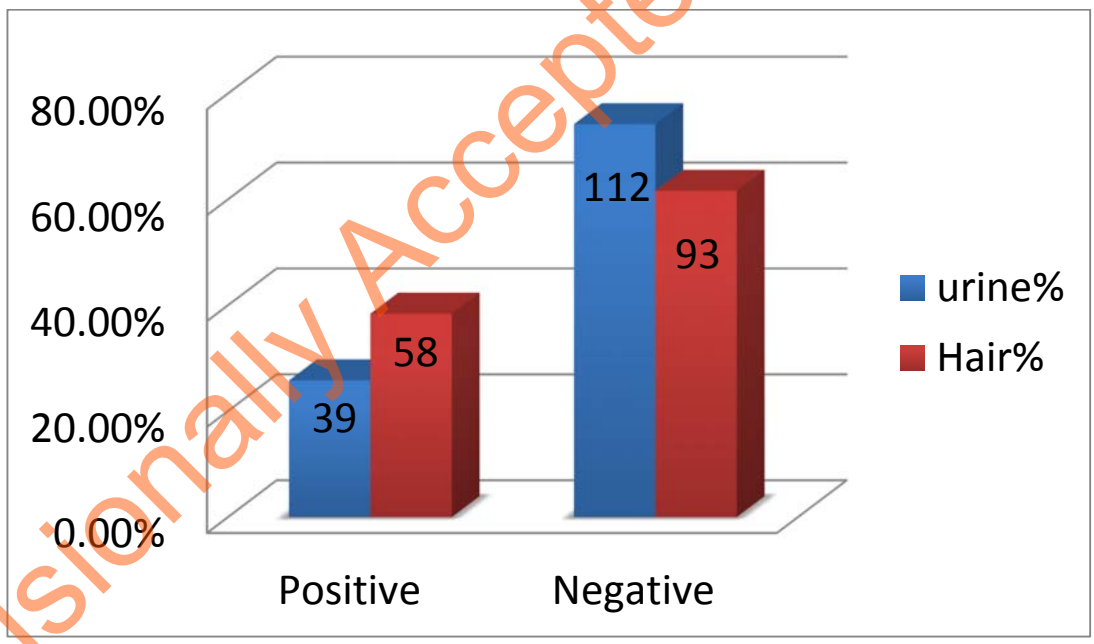

Figure 1: Qualitative results for cannabis in urine and hair samples of 151 subjects

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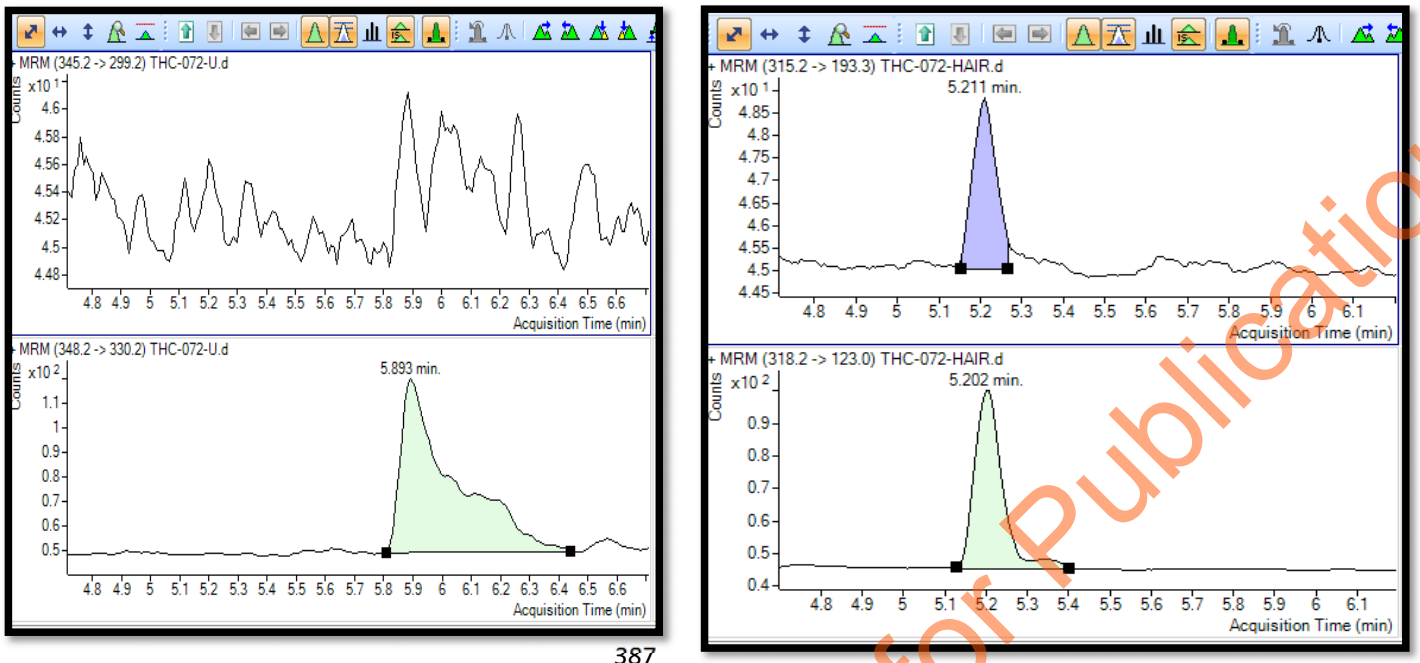

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388 **Figure 2: Chromatograms (THC)of negative urine and positive hair**
389 **samples of same subject**

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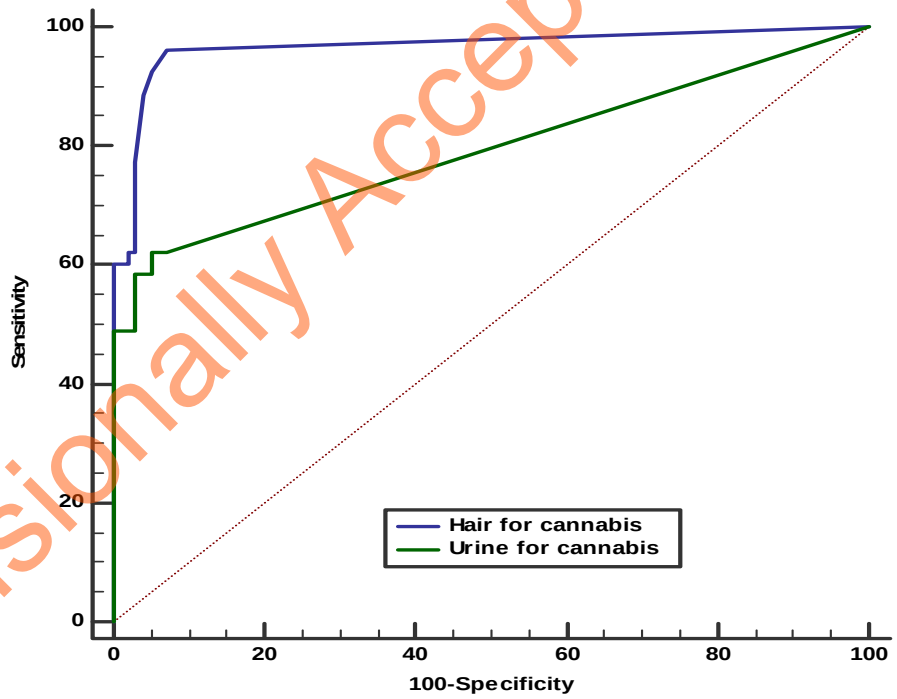

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394 **Figure 3: ROC Curve showing area under curve for hair and urine**

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