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Hair as a Medicolegal Evidence in Detection of Cannabis and Pregabalin residues

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Abstract

Hair samples are one of the most important resources in the forensic analysis of crime scenes, often providing valuable information that can lead to the identification of a suspect or a victim. This study was conducted to HPTLC quantitative evaluation of Cannabis and Pregabalin residues in hair and serum. Thirty Wistar male rats were grouped into three groups; first group kept as negative control, second group (Cannabis group) administered Cannabis by gastric tube at a dose of 3 mg/kg b.wt.) and third group (Pregabalin group) administered Pregabalin by gastric tube at a dose of 30 mg/kg b.wt. daily for eight weeks. Results revealed that Cannabis and Pregabalin were both deposited in hair and serum compared with control groups. Using of hair samples is strongly recommended as routine test for pregabalin and cannabis abuse beside blood and urine tests.

KEYWORDS
Cannabis, HPTLC, Hair, Pregabalin, Serum

INTRODUCTION

Hair samples are one of the most important resources in the forensic analysis of crime scenes, often providing valuable information that can help to lead to the identification of a suspect or victim. Worldwide, drug problems are as old as drugs themselves. Addictive drugs whether licit/legal eg cannabis and Pregabalin because they are used in ways that are not medically approved have the unique ability to perpetuate their use by compromising rationality and wreaking havoc on the faculty of self preservation (Cami and Fame, 2003). The cannabis plant has been used as a drug for both recreational and entheogenic purposes and in various traditional medicines for centuries as it is very rich in phytochemicals (Bonini *et al*., 2018).

Although at therapeutic dosages Pregabalin can be regarded as a drug with low addictive liability, misusing of Pregabalin at high doses producing the entactogenic, euophoric, and dissociative feelings in several studies and case reports (Yargic and Ozdemiroglu, 2011; Carrus and Schifano 2012; Gahr *et al*. 2013).

Kintz *et al*. (2017) mentioned that for analysis of new psychoactive substances, hair testing is a good complement to urine testing. Cirimele *et al*. (1996b) reported that Chromatographic techniques are best to identify and quantify drugs due to high capacity for the separation of components. If some chromatographic technique is coupled with mass spectrometry then the detection window becomes broader in terms of specificity and sensitivity.

High Performance Thin Layer Chromatography (HPTLC) is a sophisticated and automated form of the thin-layer chromatography (TLC) with better and advanced separation efficiency and detection limits. It is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks (Sharma *et al*., 2008; Kadam *et al*., 2012).

Therefore, the objective of the current study was to investigate cannabis and Pregabalin addictions or abuse and their deposition in hair using high performance thin layer chromatography (HPTLC)

MATERIALS AND METHODS

Experiments were conducted on Wistar male rats received Cannabis and Pregabalin in their drinking water for a period of 8 weeks to detect the deposition of Pregabalin in serum and hair.

Drugs

Pregabalin was used in this study under the trade name Pregavalex® (capsule containing water soluble powder), each capsule contains 150 mg pregabalin base. It is produced by Eva Pharma Company, Egypt.

Cannabis was purchased from Alibaba online chemical store, (100ml cannabis extract), with a dose of 3mg/kg, catalogue number : 5059808006627.

Experimental animals

Thirty clinically Healthy Wistar male rats, weighting about 120 to 130 g. Rats were obtained from the animal house laboratory, Faculty of Veterinary Medicine, Suez Canal University, Ismailia. The animals were kept three weeks for adaption prior to study.

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Animals were clinically examined specially for their skin and hair and all were apparently healthy and had normal skin and hair. Animals were housed and maintained on standard ration (72% corn, 27% soya bean and 1% fish meal) they kept with feed and water ad libitum.

Experimental design

Animals were weighted and randomly allocated one of the treatment groups to have almost the same initial body weights. the experimental animals were classified into 3 groups (n=10 / group); Cannabis group: received a daily dose of ½ ml of containing 3mg extract diluted by DMSO 1% /kg body weight (by stomach tube) (Gabriel, 1972). Pregabalin group: received ½ mg of the water contains pregabalin every day by stomach tube, with a dose of 30 mg/kg. control group: received blank tap water without medications (Sarah *et al*., 2020).

Samples collection

Blood and hair samples

At the end of the 14th day of repeated oral administration of cannabis and Pregabalin, 5 rats were lightly anesthetized (6% diethyl ether) for blood and hair collection from experimental and control groups. At the end of the experiment, 5 rats were also lightly anesthetized (6% diethylether) for blood and hair collection from experimental and control groups for assaying of residues of Cannabis and Pregabalin after the last dose in falcon tube then kept in deep freezer at -20 till transported in ice box to assay the level of Cannabis and Pregabalin residues at Central Lab of Faculty of Veterinary Medicine, Suez Canal University, for HPTLC examination serum samples were obtained from collecting blood from the medial canthus of rats eyes and keeping it without using anticoagulant, allow blood to clot for 30 min. at 25ºC, centrifuge the blood at 4,000 rpm for 15 min. at 4ºC. pipette off the top yellow serum layer without disturbing the white buffy layer, the serum stored on ice freeze at -80ºC.

Chemicals and Standard drugs

All used chemicals, reagent and standards were HPLC grade with purity of at least 99.9%.

The standard for cannabis and Pregabalin were obtained from Sigma Chemical Co. (St. Louis, MO, USA), for Pregabalin, the standard solution was stored in-20°C till be used.

Hair digestion

Hair was digested according to the procedures recommended by Joseph and Fred (2019) using organic solvent incubation method. The hairs were digested in an ultrasound bath for several hours at 45°C using methanol as solvent. After evaporating the organic solvent, the samples were analyzed directly.

Extraction of Cannabis and pregabalin residues from hair and serum

Cannabis and pregabalin residues were extracted from serum and hair as follow:

Cannabis residues were extracted from serum and hair according to the methods described by Taufik *et al*. (2016) with slight modifications. Briefly, 1 ml of serum was extracted with 2 ml of n-hexane:toluene mixture (1:1, v:v) and 1 gm of hair was extracted with a mixture of n-hexane:toluene:formic acid (50:50:0.3, v:v:v). The hair samples were incubated at room temperature overnight, sonicated for 30 minutes and filtrated using Whitman filter paper. Both serum and hair tubes were centrifuged at 1000G for 5 minutes, then the supernatants were collected in other clean caped tubes. The supernatant was evaporated to dryness under reduced pressure using rotatory evaporator at 40ºC, the residue was reconstituted in 100 µL of the mobile phase and kept in -20ºC deep freezer till HPTLC assay.

Pregabalin was extracted from serum or clean hair by incubating exactly 1ml of serum or hair with 2 ml of extraction solution (toluene: methanol: formic acid, 7.5:2:0.5, v:v:v) for 30 minutes in clean Falcon tubes. Only the hair tubes were placed in an ultrasound bath for 2 h at room temperature to allow hair digestion. Then all the tubes were centrifuged at 1000G for 5 minutes, the supernatant was collected in another clean caped tubes and evaporation to dryness using rotatory evaporator at 40ºC, the residue was reconstituted in 100 µl of the mobile phase and kept in deep freezing till analysis with HPTLC (More *et al*., 2019).

Analytical procedure

Cannabis and Pregabalin residue were determined in rat's hair and serum samples by using High Performance Thin Layer Chromatography (HPTLC).

HPTLC analysis

The principle of HPTLC analysis

It works on the same principle as TLC, the principle of separation is adsorption. Different components in the sample have varying affinities to the adsorbent material. The higher affinity components travel slower toward stationary phase, allow affinity component travel rapidly toward the stationary phase on chromatographic plate, then components are separated.

Standard preparation

Stock solutions of Cannabis and Pregabalin (1,000 µg ml −1) were separately prepared using methanol and kept in the refrigerator at -20ºC before analysis. Aliquots equivalent to 0.05–2 mg of Cannabis (50-2000 ng) and 0.9–1.7 mg of Pregabalin (900-1700ng) were withdrawn separately from their related stock solutions into a set of 10 ml volumetric flasks and methanol was used to make up to the mark. A 10 µl aliquot of each calibration standard was applied to HPTLC plates.

HPTLC technique

HPTLC technique was performed on 20cm×10cm HPTLC silica gel 60F254 plates (Merck) with mobile phase consisting of Diethylamine: Toluene (% v/v): 6:94 ratios Cannabis (Yifan *et al*., 2020) and Toluene: Methanol: Formic acid (%v/v/v): 7.5:2:0.5 ratios (More *et al*., 2019) for Pregabalin.

Sample application

All samples and standards were be applied to the plates by means of CAMAG Linomat 5 with dosing syringe 100 µl as 7 mm bands with 10.5 mm Distance between tracks, application X 15 mm and 10 mm application Y edges of plate and the application volume was 5 µL for both samples and standards in Cannabis and 5-15 µL for both samples and standard in Pregabalin.

Chromatogram Development

According to Yifan *et al.* (2020), the HPTLC plates with Cannabis were developed to 70 mm in the ADC 2 with chamber saturation for 20 min with mobile phase and after conditioning at 33% relative humidity for 10 min using a saturated solution of magnesium chloride. Then 5 min of drying after development.

The HPTLC plates with Pregabalin were developed to a distance of 80 mm in Camag Automatic Developing Chamber ADC 2 at room temperature. The development occurring in a two-steps (preconditioning with 10 ml mobile phase for 5 minutes and development with 25 ml mobile phase for 20 minutes). Then plates dried for 5 min by a stream of warm air (More *et al*, 2019).

Detection

For cannabis densitometric analyses were performed with the CAMAG TLC Scanner 4 at 209 and 285 nm for quantification (multi-wavelength scan), slit dimension 5.0 x 0.2 mm, scanning speed 20 mm/s, spectra recording 190 to 450 nm. For post derivatization, the immersed into freshly-prepared Fast Blue Salt B (FBS) reagent (1 g of FBS (o-dianisidine bis(diazotized) zinc double salt dissolve it in 200 mL of water)using the Chromatogram Immersion Device, immersion time 5 s and immersion speed 3 cm/s and then dried in flow of cold air in the fume hood for 5 minutes. Subsequently, the plates were scanned at 450 nm.

For PGB the plates were scanned and examined densitometry at l=5 nm by means of CAMAG TLC Scanner 4 with slit dimension of 6×0.30 mm at wavelength of 205 nm (More *et al*., 2019). For post derivatization, the plates were immersed with 3% (w/v) ethanolic Ninhydrine solution, then heated for 5 min at 100ºC using a thermoblock heater. Subsequently, the plates were scanned at 550 nm.

RESULTS

HPTLC results

Cannabis and pregabalin concentrations in hair and serum, following 2 and 8 weeks of oral administration of a dose of ½ ml of water containing 3mg extract /kg body weight for cannabis and for Pregabalin received ½ mg of the water in a dose of 30 mg/kg daily by stomach tube in Wistar male rats were studied using high performance thin layer chromatography (HPTLC).

The following tables and charts show the different concentrations of Cannabis & Pregabalin in hair & serum then studied after method calibration using standard curves in hair and in serum.

Standard curves

Cannabis detection and standard curve

The best absorbance wavelength and RF (Retention factor) time were confirmed from all tracks absorbance scanning as shown in Figure 1. In addition, several AUC peaks are pointed up and compared after samples were spiked with several concentrations of cannabis (Figure 2).

For preparing standard curves, sequential concentrations of Cannabis (50, 500, 1000 and 2000 ng) Cannabis per milliliter were assayed and their corresponding absorbance AUC are illustrated in Table 1 and shown in Figure 3. Cannabis concentrations in rat's serum hair after 2 and 8 weeks of oral administration are shown in Table 2 and Figure 4.

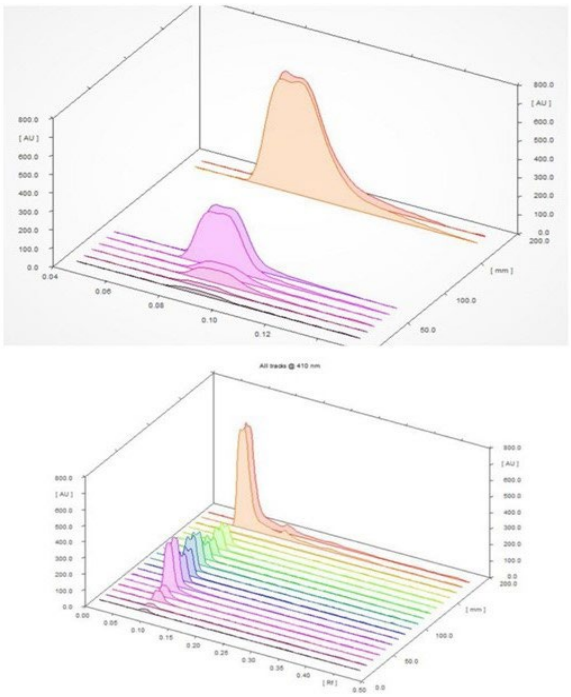

Fig. 1. Scanning of Cannabis standards and samples using the high performance thin layer chromatography (HPTLC), the scan viewing the best absorbance wavelength and RF time.

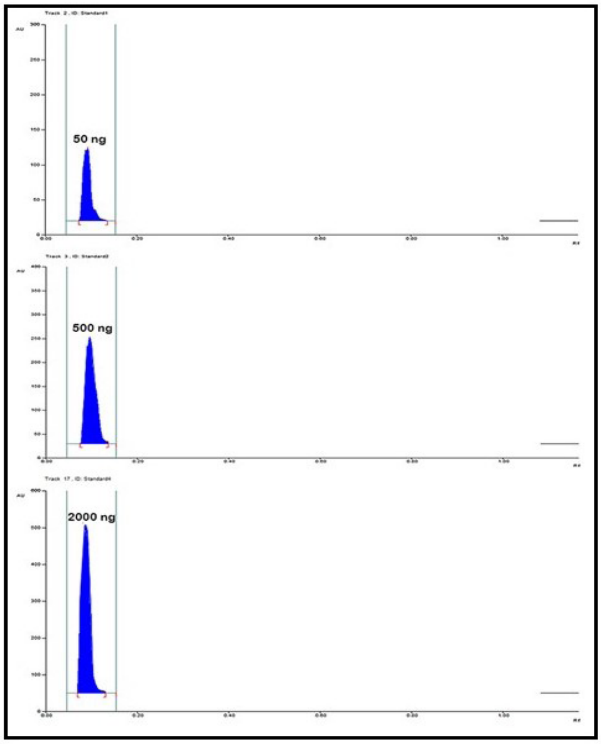

Fig. 2. Comparison between 3 concentrations (50, 500 and 2000 ng/ml) of cannabis showing the reliability of RF time and the sharpness of peaks with no interferences.

Table 1. Standard curves of Cannabis in samples after being spiked with known concentrations.

Cannabis concentrations (ng/ml)	Corresponding absorbance AUC values Spiked samples
50	1294.8
500	3835.1
1000	6375.4
2000	10748.7

Table 2. Cannabis Concentrations in rat's serum& hair after 2 and 8 weeks of oral administration.

Week	Serum (µg/ml)	Hair (µg/g)
2	3.29±0.20**	67.99±4.22***
8	3.11±0.14**	85.41±3.44***

Mean ± SE. T. test analysis of serum and hair significancy was applied,*significant at p≤0.05 **significant at p≤0.01; ***: p≤0.001.

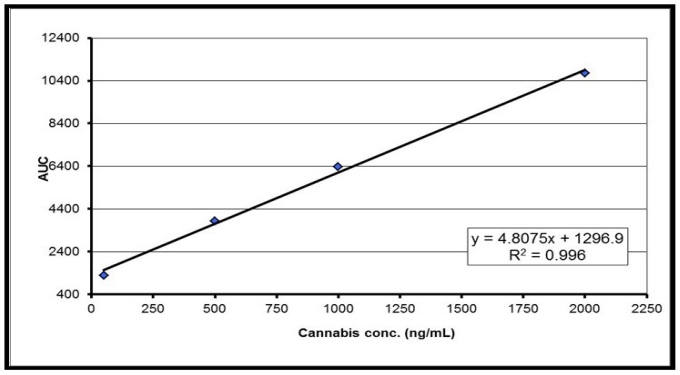

Fig. 3. Standard curve of Cannabis in samples using the first 4 concentrations to obtain coefficient of determination (R2) equal to 0.996.

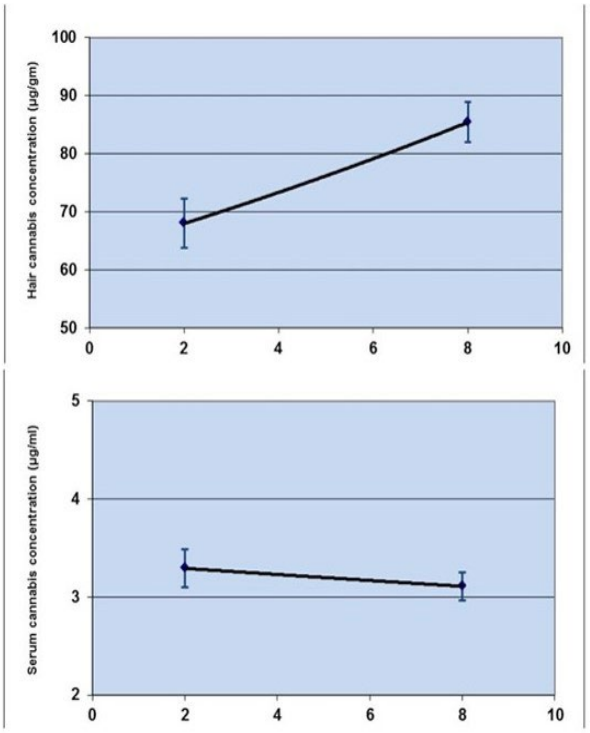

Fig. 4. Cannabis Concentrations in rat's hair and serum after 2 & 8 weeks of oral administration.

Pregabalin detection and standard curve

The best absorbance wavelength and RF (Retention factor) time were confirmed from all tracks absorbance scanning as shown in Figure 5. In addition, several AUC peaks are pointed up and compared after samples were spiked with several concentrations of Pregabalin Figure 6.

For preparing standard curves, sequential concentrations of Pregabalin (900, 1100, 1300, 1500 and 1700 ng) Pregabalin per milliliter were assayed and their corresponding absorbance AUC are illustrated in Table 3 and shown in Figure 7. Pregabalin concentrations in rat's serum and hair after 2 and 8 weeks of oral administration are shown in Table 4 and Figure 8.

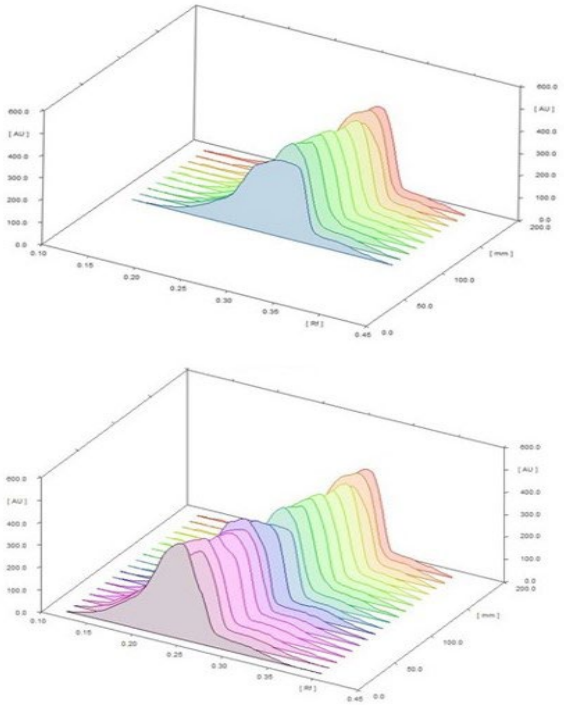

Fig. 5. A multi-track scanning of Pregabalin using the high performance thin layer chromatography (HPTLC), the scan viewing the best absorbance wavelength and RF time.

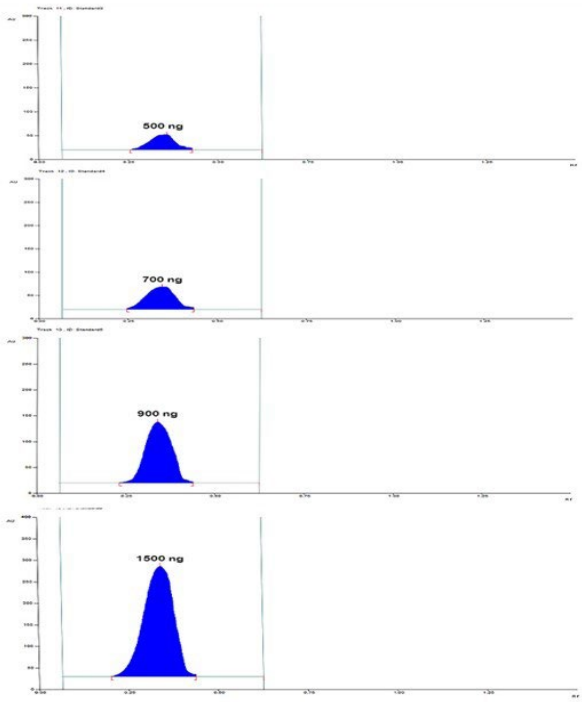

Fig. 6. Comparison between 3 concentrations (900, 1100, 1300, 1500 and 1700 ng) of pregabalin showing the reliability of RF time and the sharpness of peaks with no interferences.

Table 3. Standard curves of Pregabalin in samples after being spiked with known concentrations.

Pregabalin concentrations (ng/ml)	Corresponding absorbance AUC values Spiked samples
900	3657.1
1100	5380
1300	8012.5
1500	10851.06
1700	13318.87

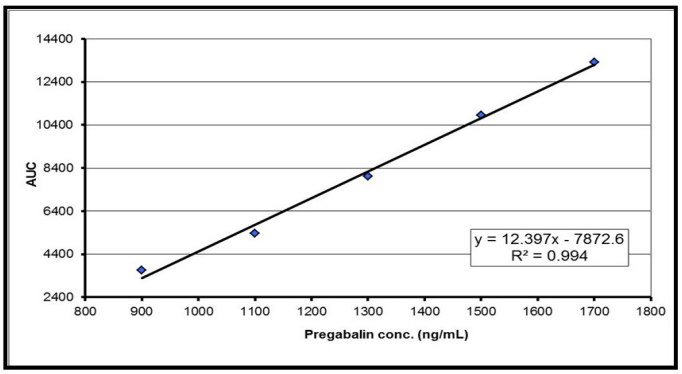

Fig. 7. Standard curve of Pregabalin in samples using the first 5 concentrations to obtain coefficient of determination (R2) equal to 0.994.

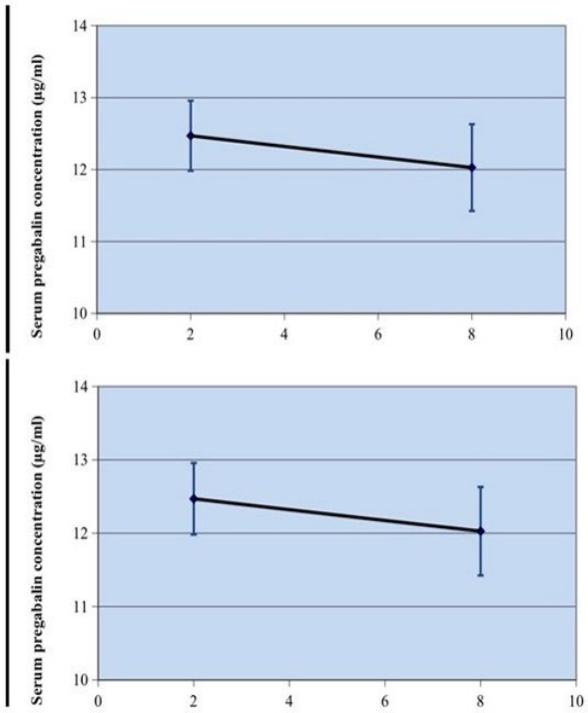

Fig. 8. Pregabalin concentrations in rat's serum and hair after 2 and 8 weeks of oral administration.

Table 4. Pregabalin Concentrations in rat's serum& hair after 2 and 8 weeks of oral administration.

Time (weeks)	Serum (µg/ml)	Hair (µg/g)
2	12.47±0.49**	3.75±0.19***
8	12.03±0.60**	5.51±0.66***

Mean±SE. T. test analysis of serum and hair significancy was applied. *: significant at p≤0.05; **high significant at p≤0.01 very high significant at***: p≤0,001.

DISCUSSION

In the scientific community, until recently, hair analysis was considered a quite esoteric method typical of forensic analysis for investigating suspected poisoning (Tagliaro *et al*., 1997).

It is possible to estimate when specific drug intake occurred, over a time period as long as the length of the hair allows (weeks, months, or even years) (Wilco *et al*., 2014).

Furthermore, compared to the other bio-indicators such as blood ,nail ,urine and saliva , hair has attractive advantages, such as finer stability, easy collection and transportation, convenient storage, and reflects long-time exposure to drugs and higher drugs concentrations than in other bio-indicators, so in our study we used hair samples is our indicator for Cannabis an Pregabalin deposition in hair as other studies used hair as an indicator for drug deposition (Doğan-Sağlamtimur and Kumbur, 2010; Cooper *et al*., 2012 and Kintz, *et al*. 2017).

Cutting a hair specimen at a certain point in time as performed in our present study will result in a sample that contains hair in the growing, transition and resting stages, other studies curtained that such as Pragst and Balikova (1998), who collected the hair samples at 2 and 8 weeks of oral administration of cannabis and pregabalin.

Hair analysis has shown great potential in the detection and control of drug use and may provide information on the severity of an individual's drug exposure In fact; the disposition of drugs in the body includes its incorporation into growing hair. Drugs became entrapped during hair fiber formation and stabilized in the keratin matrix such as cannabis (Po¨tsch *et al*., 2004), that make clear interpretation for our study that showed great presence of cannabis in hair rather than blood it was up to 18 times its presence in blood serum.

HPTLC may be helpful in screening for high Cannabis content. A HPTLC densitometric method for the analysis of cannabinoids in Cannabis sativa L. has been previously reported. (Fischedick *et al*., 2009).

It also has the potential to analyze large numbers of samples in much less time than conventional HPLC and GC–MS methods since it is possible to spot up to 15 samples on one plate with an automated sampler. HPTLC has been applied in the pharmaceutical field for years in conventional botanical and chemical characterization of natural herbal products, yet it is not widely used in the forensic science field, An additional benefit of TLC is that it is considered a green technique because it uses much less solvent then many HPLC methods (Vadivel *et al*., 2018).

In the present study liquid chromatography methods (HPTLC) were used to detect the deposition of the Cannabis and Pregabalin in hair samples of the intakers, these results proved that they are positively deposited in hair.

Cannabis and Pregabalin concentrations in hair and serum, following 2 and 8 weeks of oral administration of a dose of ½ ml of water containing 3mg extract /kg body weight for cannabis and for Pregabalin received ½ ml of the water in a dose of 30 mg/kg daily by stomach tube in Wistar male rats were studied using high performance thin layer chromatography (HPTLC).

In the present study, the detection of cannabis concentrations in the hair by using HPTLC showed that the hair samples had detectable concentrations of cannabis 8 weeks after the last dose (Figures 1 and 2). This long lasting hair concentration came in agreement with other researchers data such as Wilco *et al*. (2014) who have detected levels of cannabis presence in hair after it was confirmed by quantitative liquid chromatography (HPTLC) after 7 weeks and it was spiked with definite of blank and standard concentrations.

HPTLC analysis Using the proper mobile phase system with HPTLC is a superior method for qualitative identification of the common cannabinoids in cannabis products, This method offers a good alternative for the measurement of CBN samples in hair that mobile phase was dimethylamine and toluene (Yifan *et al*., 2020), this came in matching with our results as the hair samples showed very high significant results for the presence of cannabis in hair using HPTLC with the appropriate mobile phase of Diethyl amine : Toluene (% v/v) 6:94 ratios.

The results of Fischedick *et al*. (2009) CBN experiments indicate that the HPTLC method allows for the simultaneous quantification CBN at 206 nm without a loss in accuracy and T-test values were very high significant, that was matching to our HPTLC results in cannabis treated group that showed high significant result at 205 nm, The best absorbance wavelength and RF (Retention factor) time were confirmed from all tracks absorbance scanning as shown in Figure 1. In addition, several AUC peaks are pointed up and compared after samples were spiked with several concentrations of cannabis (Figure 2).

The products of the colorimetric test reaction using fast blue salt to detect the presence of cannabis in samples was used to ensure the appearance of cannabis on the silica gel plate (Joseph and Fred, 2019), that obtain and agree with the aim of using FBS

in our study we used for post derivatization, the immersed into freshly-prepared Fast Blue Salt B (FBS) reagent (1 g of FBS (o-dianisidine bis(diazotized) zinc double salt dissolve it in 200 mL of water) using the Chromatogram Immersion Device, immersion time 5 s and immersion speed 3 cm/s and then dried in flow of cold air in the fume hood for 5 minutes. Subsequently, the plates were scanned at 450 nm.

Using fast blue salt in detecting presence of cannabis was also confirmed by The United Nations suggested identifying cannabinoids fast and easily with color tests-e.g. the Fast Corinth V salt test, the Fast Blue B salt test, and the Rapid Duquenois test. Thin layer chromatography (TLC) in both normal and reverse phase systems, besides visualizing with Fast Blue B salt as a reagent, may be used to detect cannabinoids generally. TLC is typically used for fast screening of cannabinoids and does not provide further information; in addition, it has low specificity and sensitivity and false-positive results may be seen (Leghissa *et al*., 2018; Citti *et al*., 2018).

On our study Pregabalin was detected in hair samples after oral administration in 2 and 8 weeks using HPTLC, the best absorbance wavelength and RF (Retention factor) time were confirmed from all tracks absorbance scanning as shown in Figure 5. In addition, several AUC peaks are pointed up and compared after samples were spiked with several concentrations of Pregabalin (Figure 6), this came with an agreement with (Pauly *et al*., 2013) who reported one real case of Pregabalin determination has been performed using HPTLC in hair sample. The analysis has been carried out the method validation has been successfully completed using HPTLC; the analysis method has been proved fast and simple with a run time for an analysis of hair samples.

This study of Sunil *et al*. (2019) aimed to development of sensitive, economical and less time consuming HPTLC technique for the determination of Pregabalin in pharmaceutical dosage form in samples. Well resolved by the relevant ICH guidelines and other current regulatory guidelines. The chromatographic conditions were optimized to achieve the best resolution and peak shape for Pregabalin. UV scanning at 200-400 nm for Pregabalin show that 205 nm is the suitable wavelength for detection of drug. Different mobile phase in different proportion were tried and the mobile phase containing Toluene: Methanol: Formic acid (7: 2.5: 0.5 v/v/v) was selected as optimal for obtaining well resolved peaks of Pregabalin, this also support our study as we used the mobile phase that consists of Methanol: Formic acid (%v/v/v) : 7.5:2:0.5 ratios and a multi-track scanning of Pregabalin using the high performance thin layer chromatography (HPTLC), the scan viewing the best absorbance wavelength and RF time (Figure 5).

Fast and simple HPLC methods for the determination of Pregabalin, in whole blood sample were developed. The analytical performance this method was documented. Satisfactory linearity and acceptable limits of detection and limits of quantification were obtained. It performed very well in routine analysis, showing within- and between-assay precisions, extraction recoveries of 100% and no major matrix effects (Sandra *et al*., 2011), this result came in a matching with our study of the Pregabalin treated groups at both 2 and 8 weeks of oral administration, HPTLC showed perfect linearity and very high significant results in Pregabalin deposition in serum, as very long linearity indicate high significancy.

PGB does not bind to plasma proteins and is excreted predominantly unchanged by the kidneys, it does not undergo extensive metabolism in the body, with about 92% excreted unchanged in urine (Brockbrader *et al*., 2010), that clear and support the result in our study that is Pregabalin concentration in hair was more less than cannabis concentration in hair.

The colored complex that appeared in drevatization step in PGB treated group was first formed as a Schiff base owing to the condensation of the amino group of PGB with the carbonyl function of NIN, followed by decarboxylation (Armenta *et al*., 2008). In our study, for post drivatization, the plates were immersed with 3% (w/v) ethanolic Ninhydrine solution, then heated for 5 min at 100ºC using a thermoblock heater. Subsequently, the plates were scanned at 550 nm, this came in matching with (Naguib *et al*., 2021) who used for Ninhydrin as a derivatizing reagent which produced a purple-colored complex called Ruhemann's purple.

CONCLUSION

The intake of Cannabis and Pregabalin orally leads to their deposition in hair and serum. Cannabis and Pregabalin deposition in hair makes hair samples very important and accurate samples for drug addict testing. HPTLC showed a great quantitative method for residues of Cannabis and Pregabalin in hair and serum.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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