Qualitative high performance thin layer chromatography (HPTLC) analysis of cannabinoids in urine samples of Cannabis abusers

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**Background & objectives:** Cannabis is one of the most commonly abused drugs worldwide. There is a distinct clinical correlation between cannabis abuse and mental disorders. However, it is essential to establish cannabis intake in the abusers in order to establish causality between cannabis and psychiatric illness. The limitations of current detection methods using commercial cassettes prompted us to standardize the method of extraction and detection of cannabinoids in the urine samples of cannabis abusers attending a de-addiction centre in south India.

**Methods:** In this study, diagnostic tests on 102 male patients suspected with cannabis abuse were done. Liquid-liquid extraction of cannabinoids from urine was done and screened by Duquenois-Levine, fast blue B salt and p-dimethylaminobenzaldehyde (p-DMAB) tests. All the results were confirmed by high performance thin layer chromatography (HPTLC). Samples were considered positive for cannabis based on the positive indication in colour test and by detection of 11-nor-Δ⁹ tetrahydrocannabinol-9-carboxylic acid (THC-COOH) on HPTLC.

**Results:** Based on the colour tests and HPTLC, cannabis abuse was detected in 64 of 102 patients tested. HPTLC method was found to be sensitive for detection and possible quantitation of THC-COOH.

**Interpretation & conclusion:** We report the standardization and utility of cannabinoid extraction, screening and detection by HPTLC in the urine samples of cannabis abusers. The HPTLC method was found to be high throughput, sensitive, reproducible and cost-effective compared to commercial kits.

**Key words** Cannabinoids - cannabis - deaddiction - drug abuse - thin layer chromatography

Many studies have demonstrated that psychosis, violence, aggression and crime are closely associated with drug abuse thereby making substance abuse a complicated psychosocial condition¹-⁴. Analysis of psychotic behaviour among substance abusers provides valuable clinical and biochemical information regarding the mechanism of action of the compounds and their role in psychosis⁵. However, it is necessary to demonstrate the presence of the implicated substance in order to make a more definitive diagnosis for a substance induced disorder. To establish such a positive correlation, it is necessary to develop and improve methods of drug detection in patient samples.

The abuse of cannabis, one of the most common illicit drugs and its association with psychosis has been observed in India⁶-⁸. Cannabis (Marijuana or charas/
ganja/bhang as it is known commonly in India) is predominantly obtained from *Cannabis sativa* L. It includes all parts of the plant except the seeds and woody material. Cannabis and cannabis resins (Hashish) are smoked with tobacco or ingested\(^9,10\). Once ingested, cannabis is metabolized to generate several metabolites in the human body. \(\Delta^9\text{-tetrahydrocannabinol} \) (THC) is considered as the primary psychoactive compound in cannabis. Once ingested, THC is metabolized to generate its hydroxylated and carboxylated metabolites of which, 11-nor- \(\Delta^9\)tetrahydrocannabinol-9-carboxylic acid (THC-COOH) is the major metabolite excreted in urine\(^11\). Although only a few cannabinoids are detectable in the urine, detection of THC-COOH is considered as a confirmatory test for cannabis\(^9\).

The presence of cannabinoids is usually detected using colour tests\(^12\), high performance liquid chromatography (HPLC)\(^9,13-15\), gas chromatography\(^16\) and commercially available immunoassay based cassettes\(^17-20\). Following screening tests for cannabinoid detection, it is necessary to perform confirmatory tests using advanced techniques such as fluorescence polarization immunoassay\(^21\), enzyme immunoassay\(^22\), high performance thin layer chromatography (HPTLC)\(^23\) and HPLC, which provide additional scope for quantitative monitoring of drugs during follow up of patients\(^24\). However, utilization of such confirmatory tests in the de-addiction centers of India is limited.

The de-addiction centre housed in the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore (www.nimhans.kar.nic.in / deaddiction), India, was started in 1994 as a nodal centre for south India. The current study describes a standardized method of extraction and detection of cannabinoids in the urine of 102 suspected cannabis abusers attending NIMHANS de-addiction centre of which, 40 samples were also tested for cannabis in parallel by commercial cassettes.

**Material & Methods**

All chemicals used were of analytical grade. Bulk solvents and routine chemicals were obtained from Sisco research laboratories (Mumbai, India) and Merck & Co. Inc (Whitehouse Station, NJ, USA). Ready-to-use silica gel coated plates were obtained from Merck. Commercial test kits for cannabis were obtained from Nano-Ditech corp. NJ, USA.

*Receipt of urine samples:* This investigation is preliminary to a large study that involves analysis of substance abuse in first episode psychosis patients for which approval has been obtained from the institutional ethics committee.

Urine samples from 102 male patients (\(n=102; \text{age } 28.6 \pm 8.3 \text{ yr}\) exhibiting psychotic behaviour with suspected cannabis abuse admitted to outpatient department/ de-addiction centre or psychiatric wards of NIMHANS, Bangalore between September 2008 and March 2009 were collected for detection of cannabinoids. Each sample was accompanied with a requisition form containing the details of the patient including name, ward number, clinical profile, suspected drug used and last date of abuse. A detailed case history of individual patients was recorded during the OPD sessions. For 40 randomly selected patients, urine samples were analyzed by commercial test kits according to the specifications of the manufacturer. Approximately 200 \(\mu l\) of the urine sample was placed on the test window of the cannabis cassette through a dropper and was allowed to move along the cassette by capillary action. Cannabis positive sample were identified by the absence of a band against appearance of a distinct band in the positive control. The samples (10-20 ml per patient per evaluation) were collected in 20 ml labelled leak-proof sterile plastic containers. Temperature and \(pH\) of the sample was checked to monitor any adulteration. In most cases, the samples obtained were processed for drug test on the same day. If not, the samples were stored at 0-4\(^\circ\)C until extraction and analysis.

*Preparation of cannabis standard solution:* Cannabis resins were extracted from the dried parts of *Cannabis sativa* plants based on the method described earlier\(^24,26\). Briefly, the dried leaves and the flowering tops of cannabis plant were soaked in chloroform or petroleum ether (1 mg/ml w/v). The solvent enriched in cannabis resins was separated, evaporated to dryness and the residue was reconstituted in one ml methanol and used as a positive control (cannabis standard) throughout the study.

*Extraction of cannabinoids from urine samples:* Cannabinoids were extracted from urine by specific liquid-liquid extraction method that was slightly modified from the protocol described previously\(^24,27,28\). To 10 ml of urine, 1 ml of 6M NAOH was added, for alkaline hydrolysis, vortexed and heated at 60\(^\circ\)C for 30 min. The sample was cooled and \(pH\) adjusted to 4.0 with acetic acid to enrich for THC-COOH. For enrichment of cannabidiol (CBD) and cannabinol (CBN), \(pH\) was maintained in alkaline condition. In both cases, the cannabinoids were extracted with ethyl
acetate: iso-propanol (8.5:1.5 v/v). Total volume was about 22-24 ml per extraction. The aqueous phase was again extracted twice with ethyl acetate: isopropanol (8.5:1.5 v/v). The aqueous phase was discarded and the organic phase of all the three extractions was mixed and evaporated to dryness at 70-80°C in a water bath. The residue was reconstituted in 500 µl methanol and subjected to colour test and HPTLC analysis.

**Colour tests for cannabis:** The extracted samples were first screened by the following standard colour indicator based methods for the presence of cannabinoids and were confirmed by HPTLC followed by spray test.

(i) Duquenois-Levine test - This test was carried out based on the method previously published12. Briefly, to 100 µl extract, 200 µl of Duquenois reagent (2.5% v/v of acetaldehyde and 2% vanillin w/v in 95% ethanol) was added and mixed thoroughly for 1 min. To this mixture, 200 µl concentrated hydrochloric acid (HCl) was added and mixed gently followed by addition of 500 µl chloroform. Appearance of purple colour after a few minutes that moved into the chloroform layer was considered as a positive indicator of cannabinoids.

(ii) Fast blue B salt test - This was carried out based on the method described previously29. Briefly, to 10-20 µl of the extract, a pinch of fast blue B salt (fast blue B mixed with anhydrous sodium sulphate in the ratio 2.5 : 100) was added. To this mixture, 500 µl of chloroform was added and mixed thoroughly for 1 min. Later, 20 µl of 0.1 N aqueous NaOH was added and vortexed for 2 min. The presence of wine red colour in the chloroform layer was considered as a positive indicator of cannabis.

(iii) p-dimethylaminobenzaldehyde (p-DMAB) test - This test was carried out based on the method previously published12. Briefly, 100 µl fresh p-DMAB reagent (4% w/v of p-DMAB in 1:1 mixture of 95% ethanol and concentrated HCl) was added to 100 µl of the extracted sample and presence of red colour changing to violet on dilution was considered positive for cannabis.

**HPTLC based detection of cannabinoids:** Following extraction and colour tests, cannabis standard and extracted samples (up to 20 samples/batch) were processed on the automated HPTLC system (CAMAG, Muttenz, Switzerland) according to the instructions of the manufacturer. Ready-to-use silica coated plates (Cat. No. 1.05547.0001 by Merck) were activated by blowing hot air for 5-10 min and placed in the automatic sample applicator. The HPTLC was programmed to automatically spray 5-10 µl of each sample in band form using specialized Hamilton syringe on one-side of the TLC plate in individual tracks. The TLC plate was developed in ethyl acetate: methanol: ammonia (8.5:1:0.5) or petroleum ether: diethyl ether (ratio 9:1 v/v) solvent system as described earlier26,27. The plate was developed in the automated developing chamber (CAMAG) until the solvent front reached the maximum distance (80 mm distance in a typical 20 x 10 cm plate). The developed plate was dried with a plate drier and subjected to UV analysis (wavelength: 200-600 nm) in the dedicated UV detector. All tracks in the plate were scanned at user-defined wavelength (278 and 350 nm for cannabis) and individual Rf values of peaks were obtained. These data were matched with the cannabis standard and compared against the in-built CAMAG drug/chemical library to identify the cannabinoids in the urine sample.

**Spray test for cannabinoids:** Following HPTLC, visualization of cannabinoids was also carried out by spraying fast blue B solution onto the developed plate. Appearance of red, orange and purple spots was considered as positive evidence for the presence of THC-COOH, CBD and CBN respectively29.

**Results**

This study was done to generate a standardized protocol for detection of cannabinoids in the urine samples of patients with cannabis abuse and associated psychosis. A standard cannabinoid mixture (positive control) utilizing the street sample of cannabis plant was prepared and the presence of cannabinoids in this mixture was confirmed by all the color indicator based tests. The presence of cannabinoids only in organic phase and not in aqueous fraction confirmed the enrichment of cannabinoids by the extraction procedure (data not shown). It was observed that the colour tests were specific only to cannabis patients and were not reliable in multiple drug-abusers.

To further characterize the components of cannabis, the cannabis standard was subjected to HPTLC analysis in different solvent systems. Based on preliminary experiments, it was found that the cannabinoids exhibited different Rf values in different solvent systems (Table I). We selected THC-COOH, CBN and CBD as major cannabis metabolites for detection in the standard and urine samples24,30.

HPTLC based separation of cannabinoids was done using ethylacetate: methanol: ammonia (8.5: 1 : 0.5) solvent system. Fig. 1A and 1B show the chromatogram and spectral scanning curve (200-700 nm) for THC-COOH.
Following extraction of cannabinoids from urine samples of all 102 patients, all the samples were screened by Duquenois-Levine, fast blue BB salt and p-DMAB tests. Table II shows findings of ten representative samples from among the 102 samples analyzed by the three screening tests. The samples were further confirmed by HPTLC analysis.

For HPTLC, 5-10 µl of each sample was spotted on pre-activated silica gel (11 samples per plate including one cannabis standard as positive control) and developed in ethyl acetate-methanol-ammonia solvent system in the automatic developing chamber (Fig. 2). The dried plates were scanned in the TLC scanner at wavelength 278 nm. The peaks obtained in all the tracks were analyzed and the Rf value was compared to the standard. The presence of a specific peak for THC-COOH at Rf around 0.32 ± 0.06 was recorded and considered as a positive result for cannabinoids (Fig. 3 and Table II). We observed that two of the samples not confirmed by the colour tests were found to contain the THC-COOH peak indicating the level of sensitivity of the HPTLC and the necessity for confirmation following colour based screening tests. Further, the absorbance (OD) value of THC-COOH varied differently in different samples indicating the

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**Table I.** Rf values of cannabinoids in solvent systems: (a) ethyl acetate: methanol: ammonia (8.5:1:0.5) (b) petroleum ether: diethyl ether (4:1) (c) cyclohexane: di-isopropyl ether: diethylamine (5.2:4:0.8) (d) heptane: diethyl ether: glacial acetic acid (80:10:4) (e) plate sprayed with di-ethylamine; solvent system: xylene: hexane: diethylamine (2.5:1:0.1)

<table>
<thead>
<tr>
<th>No.</th>
<th>Metabolite</th>
<th>Rf in (a)</th>
<th>Rf in (b)</th>
<th>Rf in (c)</th>
<th>Rf in (d)</th>
<th>Rf in (e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>THC-COOH</td>
<td>0.32±0.06 (n=57)</td>
<td>0.32</td>
<td>0.39</td>
<td>0.32</td>
<td>0.29</td>
</tr>
<tr>
<td>2</td>
<td>CBN</td>
<td>0.95</td>
<td>0.27</td>
<td>0.28</td>
<td>-</td>
<td>0.20</td>
</tr>
<tr>
<td>3</td>
<td>CBD</td>
<td>0.95</td>
<td>0.36</td>
<td>0.44</td>
<td>-</td>
<td>0.36</td>
</tr>
</tbody>
</table>

In all the solvent systems, the Rf values are subject to minor variation depending on the laboratory conditions such as temperature, humidity and other parameters (e.g., age and quality of the patient material)

CBD, cannabidiol; CBN, cannabinol; THC-COOH, 11-nor-Δ9 tetrahydrocannabinol-9-carboxylic acid

**Table II.** Summary of the diagnostic tests on ten samples of cannabis abusers

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Rf for THC-COOH</th>
<th>Duquenois-Levine</th>
<th>Fast blue B</th>
<th>p-DMAB</th>
<th>Final result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/- (colour)</td>
<td>+/- (colour)</td>
<td>+/- (colour)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.36</td>
<td>+ (purple)</td>
<td>+ (red)</td>
<td>+ (red)</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>0.36</td>
<td>+ (purple)</td>
<td>+ (red)</td>
<td>+ (red)</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>0.36</td>
<td>+ (purple)</td>
<td>+ (red)</td>
<td>+ (red)</td>
<td>+</td>
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<td>4</td>
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<td>6</td>
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<td>7</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>0.35</td>
<td>+ (purple)</td>
<td>+ (red)</td>
<td>+ (red)</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cannabis standard</td>
<td>0.35</td>
<td>+ (purple)</td>
<td>+ (red)</td>
<td>+ (red)</td>
<td>+</td>
</tr>
</tbody>
</table>

Following extraction of cannabinoids from urine samples of all 102 patients, all the samples were screened by Duquenois-Levine, fast blue BB salt and p-DMAB tests. Table II shows findings of ten representative samples from among the 102 samples analyzed by the three screening tests. The samples were further confirmed by HPTLC analysis.

**Fig. 1.** HPTLC profile of cannabis standard run on silica plates and developed in solvent system A (ethyl acetate: ammonia 8.5:1 : 0.5). (A) shows absorbance at 278 nm (A.U= arbitrary units) of THC-COOH in the cannabis standard plotted against Rf value. (B) shows the spectral scanning of THC-COOH in the cannabis standard with absorbance (AU) plotted against wavelength.
Fig. 2. HPTLC profile of extracted cannabinoids from patient samples developed in solvent system A (ethyl acetate: ammonia 8.5:1 : 0.5). + corresponds to positive control (Cannabis standard). Rest of the lanes correspond to the profile of cannabinoids extracted from patient samples (patient nos. 1 to 10 as shown in Table II). Reference scale for Rf value is also shown.

Fig. 3. 3-dimensional representation of the scanning data of the HPTLC plate shown in Fig. 2. The HPTLC profile of cannabinoids extracted from urine samples patients and plotted as absorbance (OD) vs. Rf value. “+” indicates the profile of cannabis standard. The numbers 1 to 10 correspond to the HPTLC profile of samples 1-10 (shown in Table II). Arrows indicate the position of the THC-COOH peak (Rf ~0.32) in cannabis positive samples.
level of abuse among the patients. Specific quantitation of THC-COOH levels would be more useful and could be correlated with psychiatric symptoms. In this study, of the 102 suspected cases of cannabis abuse, based on positive result in the colour test and HPTLC analysis, 64 samples were found to be positive (Table III).

**Discussion**

Toxicology of substance abuse poses a great challenge to biochemists in terms of chemical analysis and characterization of the effects of these drugs on the human system. There seems to be convincing evidence that cannabis abuse is linked with subsequent occurrence of schizophrenia and psychosis\(^2,5\). For reliable psychiatric correlation, there needs to be well-standardized methods for cannabinoids. Although cannabis test based on commercially available ready-to-use cassettes is available, it has limited utility. Because it is relatively expensive and has limited sensitivity threshold, and gives only qualitative and not absolute quantification. Most of the commercial kits clearly state that the test provides only a preliminary result and more specific alternative testing method should be used to confirm the immunoassay result\(^9\). This could be by either HPTLC or GC/MS or HPLC\(^11,18\). In routine TLC testing, the detection is only by spray method and the Rf value is not accurately recorded\(^9\). However, UV based scanning after developing HPTLC plate not only provides opportunity for scanning at specific wavelengths but could also be useful for quantitation. As mentioned by Meatherall and Garriott\(^23\), the limit of detection for THC-COOH by HPTLC is 5 ng/ml when 2 ml of urine is used indicating the sensitivity of the technique. The limit of detection of THC-COOH was in the similar range in the current study. There have been some reports on the utilization of TLC based qualitative and semi-quantitative analysis of cannabis and cannabinoids such as THC-COOH\(^30-32\). However, utilization of HPTLC based detection is very limited in Indian centers. It was observed that the HPTLC based detection of cannabinoids is ideal for a hospital setting involving several patients. It is a cost-effective (operational costs approximately INR 20 per sample), highly sensitive, accurate and less time consuming (following extraction, for 20 samples, starting from sample application up to the final data analysis and reporting only one hour is required) method. Further, with specific standards, quantification is possible which could help correlate the progress in rehabilitation/detoxification with the levels of cannabis in the urine.

Urine analysis for drug testing is advantageous because it is non invasive, easy to collect with no risk to patient. However, urine analysis has its own limitations. For most compounds, drug abuse screening yields qualitative data indicating either presence or absence of a drug. Nevertheless, it is difficult to predict the route of administration, quantity, frequency and the date of last abuse. In addition, a positive drug test does not reflect drug dependence but only indicates recent consumption. However, if a patient is found repeatedly positive for drug abuse and exhibits clinical history of behavioural abnormality, then the tests might imply drug dependence. This kind of monitoring during the follow up of such patients could give better qualitative correlation. Therefore, based on a single analysis, not all the information related to drug abuse of a patient can be obtained\(^24,33,34\). Therefore, in this study, clinical history and last date of abuse were recorded to get better association and distinction between acute and chronic users.

It has been established that cannabis metabolites including THC-COOH are lipid soluble and tend to accumulate in the fat tissues of the human body.

### Table III. Summary of the drug analysis carried out on urine samples of all cannabis abusers included in this study

<table>
<thead>
<tr>
<th>Result</th>
<th>Colour test</th>
<th>Spray test</th>
<th>HPTLC analysis</th>
<th>Immunoassay*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duquenois-Levine test</td>
<td>Fast blue B Test</td>
<td>p-DMAB</td>
<td>Fast blue –B Spray</td>
</tr>
<tr>
<td>Positive</td>
<td>62</td>
<td>62</td>
<td>62</td>
<td>64</td>
</tr>
<tr>
<td>Negative</td>
<td>38</td>
<td>38</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Not determined</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>102</td>
<td>102</td>
<td>102</td>
<td>40</td>
</tr>
</tbody>
</table>

*Immunoassay based commercial cassette screening was initially carried out on 40 randomly selected from among the 102 samples. Further confirmation by HPTLC showed that the cassette-test results matched with the outcome of the HPTLC analysis (i.e., 26 positive and 14 negative among 40 samples) indicating the consistency of analysis.
Consequently, its availability in the serum and urine is limited, making its levels decline rapidly in these body fluids after consumption. If very high levels of cannabis metabolites are detected in the patient urine for a long time that indicates chronic cannabis abuse. However, a quantitative correlation between THC-COOH and severity of psychosis is not clear. In the future, quantitative comparison with known quantity of purified THC-COOH as a standard could give a better picture regarding its reliability in clinical correlation with the severity of psychosis.

In the current study, cannabis consumption was suspected based on colour test and HPTLC data and visualization by spray test. In the samples studied, Rf value of THC-COOH in some of the samples varied between 0.32 and 0.39. This minor discrepancy might be either due to chronic abuse or due to the difference in the quality of the abused substance. The change in Rf might also depend on the source and species of the cannabis obtained by the user.

In conclusion, colour based tests for cannabinoids were standardized and screened for urine samples from patients of drug abuse. HPTLC was found to be a powerful technique for detection and potential quantitation of drugs and compounds in clinical samples.

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References


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