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ORIGINAL ARTICLE

SCREENING METHOD FOR THE DETECTION OF DEXTROMETHORPHAN ABUSE BY HPTLC

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Abstract

Dextromethorphan (DXM), the methylated dextrorotatory analogue of a μ-opioid receptor agonist, levorphanol, is a widely available antitussive medicine. At higher levels compared to therapeutic doses, DXM acts as a dissociative hallucinogen and has been shown to be often abused by individuals seeking this particular type of effects. Some reports indicate that DXM causes false positives results in the initial immunoassay urine screening for drugs of abuse. The paper presents a new high performance thin layer chromatographic method (HPTLC) for the determination of DXM in urine samples collected from DXM abusers. The separation has been achieved on chromatographic glass plates precoated with silicagel 60 F254 using as solvent system the mixture methanol: ammonia (100:1.5, v/v). The detection was performed by densitometry in the absorbance mode at 278 nm. An unknown spot, presumably a metabolite of DXM was detected in all urine samples, suggesting that the subjects could be extensive DXM metabolizers. The results indicate that the proposed HPTLC method is a simple and rapid procedure that can be applied as a screening tool for an analytical diagnostic in DXM abuse or intoxication.

Rezumat

Dextrometorfanul (DXM), analogul dextrogir metilat al levorfanolului, unui agonist al receptorului μ-opioid, este un medicament antitusiv disponibil pe scară largă. La doze mai mari față de dozele terapeutice, DXM acționează ca un halucinogen disociativ și s-a dovedit că este adesea cunsumat abuziv de către persoanele care caută acest tip special de efecte. Unele rapoarte indică faptul că screening-ul inițial al drogurilor de abuz în urină prin imunoanalize poate conduce rezultate fals pozitive în cazul DXM. Lucrarea de față prezintă o nouă metodă cromatografică în strat subțire de înaltă performanță (HPTLC) pentru determinarea DXM în probele de urină colectate de la subiecți care au consumat DXM în mod abuziv. Separarea a fost realizată pe plăci cromatografice de sticlă preacoperite cu silicagel 60 F254, utilizând ca sistem de solvenți amestecul metanol:amoniac (100:1,5, v/v). Detecția s-a făcut densitometric, în modul de absorbanță, la 278 nm. Un spot neidentificat, datorat probabil unui metabolit al DXM, a fost detectat în toate probele de urină analizate, sugerând că subiecții ar putea fi metabolizatori extensivi ai DXM. Rezultatele indică faptul că metoda HPTLC propusă este un procedeu simplu și rapid care poate fi aplicat ca instrument de screening pentru un diagnostic analitic în abuzul sau intoxicația cu DXM.

Keywords: dextromethorphan abuse, DXM, HPTLC, urine screening

Introduction

Dextromethorphan (DXM, (+) 3-methoxy-17-methyl-morphinan) is the methylated dextrorotatory analogue of levorphanol. Although DXM has opioid "roots" DXM itself is not an opioid agent, acting by different mechanisms of action. Unlike levorphanol (a μ-opioid receptor agonist), DXM binds with high affinity to sigma receptors, which accounts for its suppression of the cough reflex. At high dosages, DXM and its main active metabolite dextrorphan (DXO) were shown to act as N-methyl-D-aspartate (NMDA) antagonists [5, 22, 41].

DXM has been used in clinics as an antitussive agent (15 - 30 mg, 3 to 4 times *per* day in adult) for more than 50 years. It is available in more than 140 over-the-counter cough and cold preparations [23]. At doses significantly exceeding the maximum prescribed dosages (*i.e.* at 5 to 10 times the recommended dose), DXM acts as a dissociative hallucinogen, producing effects similar to those of ketamine and phencyclidine (PCP) [2, 29, 41]. Thus, at very high doses, over 1500 mg/day, DXM can induce a state of psychosis, including symptoms such as delusions, dissociative states, paranoia, and visual hallucinations [23]. Therefore, DXM can be subject of drug abuse. In addition to medical use, the abuse of psychotropic or different drug groups is reported and has increased in many countries [4, 7]. In fact, an increase in the recreational abuse of DXM-containing formulations is reported, mainly in adolescents and young adults [25, 29, 39], who are seeking the dissociative effects of DXM. A

recent systematic review on Over-the-Counter anti-histamines, cough medicines and decongestants' misuse revealed that DXM was the most reported misused drug [30]. DXM abuse is maintained by wide availability of DXM-containing medicines, reduced price, legislative and extensive information on abusive consumption, available on various websites [40]. However, concentrated pill formulations are also available, so abusers of DXM can ingest large doses without having to drink large volumes of the less palatable cough syrup formulation. Particularly popular in the adolescent community, DXM is known as DXM, robo, skittles, triple C, and red hots [16] and the practice of using large amounts of DXM to achieve psychoactive effects is known as "robotrippin" [20, 27, 30]. In addition, some fatal cases, especially in teenagers and young adults, were reported as a result of the ingestion of large doses of DXM, alone or in combination with other drugs, for recreational purposes [13, 21].

In overdose cases, monitoring the serum levels of DXM does not seem to be useful as the correlation between serum DXM levels and clinical effect has not been established. However, plasma levels can be evaluated in order to assess the phenotype. DXM undergoes extensive first-pass hepatic metabolism via the cytochrome P450 enzyme CYP2D6 to its N-demethylated metabolite, DXO. A small percentage (5 - 10%) of white Europeans are poor metabolizers of DXM and are consequently exposed to toxic risks if they abuse DXM in large doses.

In the abuse circumstances, the presence of DXM should be confirmed by qualitative determinations in urine or serum. Several analytical interferences have been reported for DXM testing in biological fluids. For instance, DXM can produce a false positive test for PCP in the urine. The explanation would be the high molecular similarity of DXM to PCP shown by computational analysis. In contrast, although DXM is anecdotally reported to cause a false positive opioid assay, it has been demonstrated that the ingestion of a single normal (or even twice normal) dose of DXM is not likely to produce a false positive six hours urine opioid Enzyme multiplied immunoassay (EMIT) screening [36]. Other studies report DXM to cause false-positive results in immuno-assay testing of opioids [35].

Urine drug screening is commonly used to monitor the patients' compliance to treatment, and to detect the drug abuse. Standard immunoassay tests are the favourite initial test for urine drug screening. However, immunoassay tests have several limitations regarding the cross reactivity leading to false positive or false negative test results. Therefore, more efficient techniques, implying the separation of analytes (such as the chromatographic methods) are required to overcome the disadvantages of screening immunoassay.

Different chromatographic methods are reported to determine DXM in biological fluids. The methods used in literature for the analysis of DXM and its metabolites in biological samples are mostly chromato-graphic methods such as gas chromatography with mass spectrometry detection (GC-MS), high-performance liquid chromatography (HPLC) with direct fluorescence detection (HPLC-FL) or liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Thin-layer chromatography (TLC) techniques were reported to evaluate the urinary metabolic profile of DXM [9, 11, 14]. TLC has been also used as screening test to identify DXM in drug abuser's urine samples [18]. DXM and its active metabolite DXO were measured in human urine by an isocratic liquid chromatography-mass spectrometry (LC-MS) method [8]. A sensitive LC-MS/MS method has been reported to assay DXM and three metabolites (DXO, 3-methoxymorphinan and 3-hydroxymorphinan) in human urine [38]. DXM and its metabolite, DXO were determined in human urine also by using a sensitive and selective reversed-phase high performance liquid chromatography with UV-spectrophotometric detection [10]. A gas chromatography-mass spectrometry confirmation method for the detection of DXM and DXO in urine and oral fluid was reported [28].

A summary of some bioanalytical methods for the identification and quantification of DXM and its metabolites (DXO, 3-methoxymorphinan and 3-hydroxymorphinan) in biological samples is presented in Table I.

Table I

Bioanalytical methods for determination of DXM and its metabolites

Matrix	Analyte	Method of extraction	Method of analysis	Application	Reference
Human urine	DXM, DXO	LLE Ethylacetate, under basic conditions (pH = 11)	TLC, aluminium sheet 20 x 20, silica gel 60 F254, solvent mixture methylene chloride: ethanol (9: 1), detection of DXM with Dragendorff reagent	Samples from drug abusers	[13]
Human urine	DXM, DXO	LLE Ethylacetate, under basic conditions (pH = 11) Lidocaine as internal	GC-MS, full scan mode Linearity in the range of 100 - 2000 ng/mL	Samples from drug abusers	[13]

Matrix	Analyte	Method of extraction	Method of analysis	Application	Reference
		standard (IS)			
Human plasma, urine and *in vitro* incubation matrix	DXM, DXO, 3-methoxymorphinan and 3-hydroxymorphinan	IS levallorphan	GC-MS, Selected ion monitoring (SIM) mode, without derivatization	*In vitro* CYP2D6 and CYP3A4 inhibition study	[32]
Urine, oral fluid	DXM, DXO	SPE trideuterated DXO as IS Hexane:2-propanol 97:3, v/v, pH = 9	GC-MS, full scan mode Derivatization of DXO with N,O-bis(trimethylsilyl)tri-fluoroacetamide (BSTFA) containing 1% trimethyl-chlorosilane (TMCS) Linearity in the range 10 - 100 ng/mL	Authentic urine and oral fluid specimens obtained from volunteers following therapeutic ingestion of DXM	[21]
Human hair	DXM, DXO	Washing with distilled water and acetone and extraction with methanol Codeine-d3 as deuterated IS	GC-MS, SIM mode Derivatization with BSTFA containing 1% TMCS Linearity in the range of 2.13 - 75 ng/mg	Hair samples from drug abusers	[19]
Human urine Liver microsomes	DXM, DXO, 3-methoxymorphinan and 3-hydroxymorphinan	SPE Silica cartridges Dichloromethane/hexane (95:05, v/v) as eluent	HPLC- FL Linearity over the range of 0.2 - 8.0 µM	Evaluation of the activity of CYP2D6 and CYP3A	[3]
Human urine	DXM, DXO	LLE Hexane-butanol (95:5 m/m) Verapamil hydrochloride as IS	HPLC-FL Linearity in the range: 0.015 - 10 microg/mL for DXM and 1 - 10 µg/mL for DXO	Pharmacogenetic and pharmacokinetic studies	[12]
Human urine	DXM, DXO, 3-methoxymorphinan and 3-hydroxymorphinan	LLE Chloroform, under basic conditions 3-ethylmorphine as IS	LC-MS/MS Linearity in the range of 5 - 500 ng/mL for DXM	Drug interaction studies assessing potential CYP3A and CYP2D6 inhibition	[27]
Human urine	DXM, DXO	SPE from acidified hydrolysed biological matrix using Waters Oasis HLB 3 cc cartridge Levallorphan as IS	LC–MS/MS with atmospheric pressure chemical ionization (APCI) Linearity in the range of 2 - 200 ng/mL for DXM and 250 - 20,000 ng/mL for DXO	Drug interaction studies in a clinical trial	[8]
Oral fluid	DXM, DXO	SPE DXM-d3 as IS	LC–MS/MS electrospray ionization (ESI) Linearity in the range of 2 - 100 ng/mL	Authentic samples tested to evaluate the applicability of the method	[1]

Human urine is the preferred biological sample used. At the same time, *in vitro* incubation matrix such as liver microsomes is used as the methods were applied for the CYP2D6 and CYP3A4 inhibition study or for the evaluation of the activity of CYP2D6 and CYP3A.

For analysis of DXM in drug abusers, oral fluid samples have been used, as this type of sample becomes a popular matrix for drug testing having in view its ease of collection. One report indicates the use of hair as a sample for DXM analysis in drug users. However, few methods are applicable for the determination of DXM and its metabolites in drug

users, most of the methods are applicable in pharmacogenetic and pharmacokinetics studies as well as in drug interaction studies. As DXM undergoes O-demethylation to generate the metabolite DXO, it is useful as a probe to study metabolism. Thus, the urine molar ratio of DXM and DXO can be used to define the type of metabolizer (extensive and poor) phenotypes for CYP2D6 polymorphism [12].

Regardless of the analysis method used, sample preparation is a very important step. As extraction methods, both SPE (solid phase extraction) and LLE (liquid-liquid extraction) were applied. In most methods, an internal standard is used, usually an opioid compound, deuterated or not, but also compounds with other structures (*e.g.* verapamil, lidocaine).

In addition to a GC-MS method, only one report presents a TLC method applied for urine sample screening collected from DXM drug users. In contrast to the high performance thin layer chromatography (HPTLC) method with densitometric detection presented in our paper, this method is a simple TLC, without semi-automatic application of the samples and with the detection made by using the colour reaction with Dragendorff reagent.

In this context, for clinical and forensic purposes, there is a need for some sensible and accurate methods for DXM assay in biological specimens and material evidence.

Our study aimed to develop a fast and less expensive HPTLC method used for DXM screening in the abuser's urine samples. DXM was evaluated in urine samples from 3 male volunteers with a history of DXM abuse.

Materials and Methods

Chemicals

Methanol (HPLC, isocratic grade), ammonia 25% (analytical grade reagent), ethyl acetate (analytical grade reagent), sodium bicarbonate (analytical grade) and Dextromethorphan hydrobromide (DXM) (USP specifications, Sigma Aldrich) were used.

Instrumentation: Stirrer system Vortex Genie 2 (Cole Parmer), Evaporation system under nitrogen Techne Dry-Block (Bibby Scientific Inc.) and Cooling Centrifuge 2-15 K (Sigma) were used for sample preparation. Pre-coated TLC plates silicagel F_{254} on glass, 20 x 20 cm (Merck), Semiautomate spotting system Linomat 5 (Camag, Switzerland), TLC densitometer Scanner 3 (Camag, Switzerland) with a WinCATS software ver. 1.4.4 were used for the chromatographic analyses.

Urine samples

The urine samples were collected from three male DXM users who volunteered for the study and ingested DXM at doses within the range of 800 - 1080 mg. The samples were collected at 60, 90 and 120 minutes after the ingestion of DXM doses. The group of subjects was represented by a homogenous group of male volunteers around the same biological age, which voluntarily ingested various quantities of DXM. All the study participants sign an informed consent for the inclusion in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee. Also, a blank urine sample was obtained from a healthy volunteer, non-user, after his Informed Consent had been collected.

Procedure

DXM stock solution of 5 mg/mL was prepared in methanol. The DXM work solution of 1 mg/mL was prepared by appropriate dilution in methanol. The positive control urine sample was prepared at the concentration of 1 mg/mL by spiking the blank urine with the DXM stock solution. A blank urine sample was used as negative control.

Extraction of urine samples

The samples of urine (1 mL) were extracted by liquid-liquid procedure, at alkaline pH (0.2 mL $NaHCO_3$ 8%) using the mixture hexane: ethyl acetate 1:1 (v/v). The samples were stirred on vortex for 20 minutes and subsequently centrifuged for 10 minutes at 3000 rpm, at a temperature of 15ºC. The organic layer was separated and then evaporated to dryness under a nitrogen stream; the residue was collected with 1 mL methanol.

The solutions for analysis were spotted on the standard plates, under nitrogen flow, in lines of 10 mm length using semi-automate Linomat 5 system equipped with a Hamilton micro syringe. The spotting sequence was as follows: the standard, the blank urine, the control urine samples and the subjects' urine samples, previously prepared through liquid-liquid extraction. Table II resume the spotting scheme.

Table II

Spotting scheme for TLC evaluation of **DXM** in urine

Track No.	**1 - 5**					**6**	**7 - 9**			**10 - 12**		
Sample	**DXM**					**Blank urine**	**Control urine sample**			**Subject urine sample**		
Volume (µL)	5	10	15	20	25	5	5	15	25	25	25	25

A vertical plate development was performed in a chromatographic chamber previously saturated with the mobile phase vapours represented by the solvent system methanol:ammonia (100:1.5 v/v). The obtained chromatograms were processed with the TLC Scanner 3, by densitometry, using UV light at λ = 278 nm. The automatically computing of the R_f values and

the semi quantitative evaluation of DXM have been performed by using the software WinCATS ver. 1.4.4.

Results and Discussion

Preliminary experiments were performed using DXM standard solutions and spiked urine samples in order to select the solvent systems and to establish the conditions for the extraction procedure. Different solvent systems such as cyclohexane:toluene: diethylamine (75:15:10, v/v), ethyl acetate: methanol: ammonia (85:10:5, v/v) and methanol:ammonia (100:1.5, v/v) were tested. Best results were obtained by using the mixture methanol:ammonia (100:1.5, v/v) as developing system. The R_f of DXM in the selected developing system is about 0.22 (Figure 1).

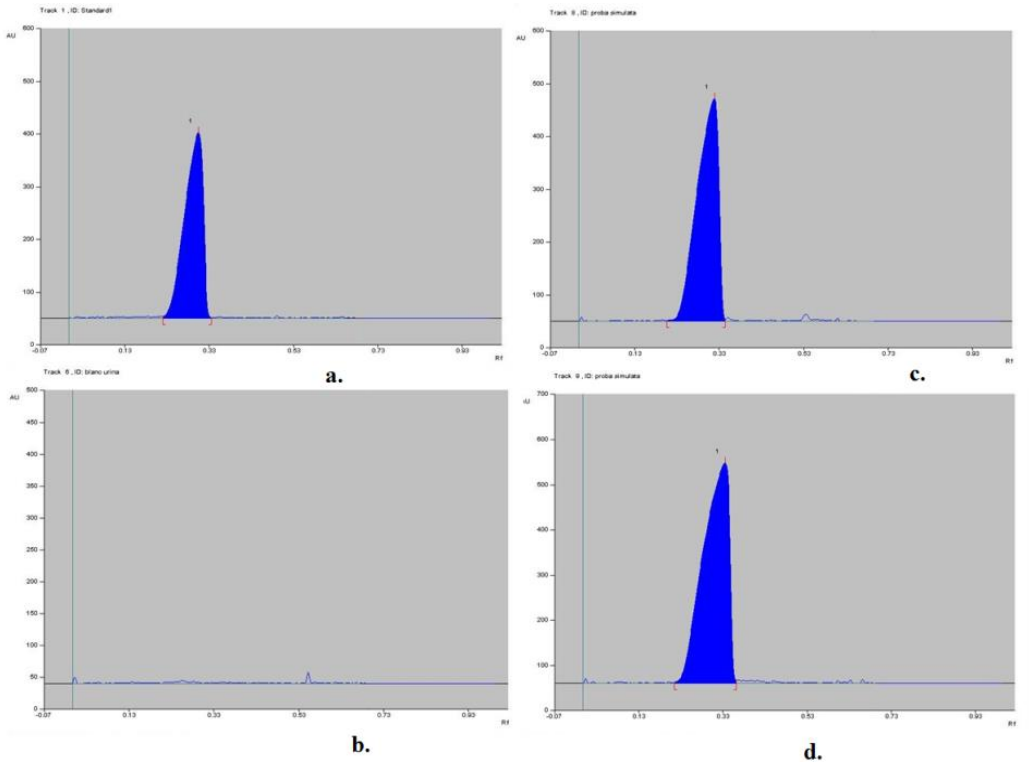

Figure 1.
The chromatographic peaks for a. Standard DXM; b. Blank; c. Spiked sample 1; d. Spiked sample 2

The identification of DXM is confirmed by the UV spectra of the corresponding spots in standard solution and spiked urine extract. An absorption maximum at 278 nm was observed in the UV spectrum.

Based on the peak area, a linear regression was obtained for DXM in the range of 5 µg - 25 µg *per* spot. The semi-quantitative analysis indicates an extraction yields around 75%, requiring the optimization of the extraction procedure.

The subjects selected for this study were recreational DXM users with a history of poly-drug use (Table III). They experienced different drugs from hallucinogenic class (hallucinogenic mushrooms, Cannabis, Ecstasy, solvents), stimulants (amphetamines, mephedrone, cocaine) or medicines such as barbiturates, benzodiazepines (including alprazolam), tramadol, codeine, selegiline. The subjects also declared the preference for the dissociative drugs, such as PCP, ketamine or gamma-hydroxybutyrate (GHB). The DXM users in this study were young male around 25 years old and two of them are common alcohol users.

The TLC analysis of the urine samples collected from the three DXM male users (Table III) indicates that DXM was found in amounts below the limit of detection (Figure 2). As it can be noticed in the Figure 5, a new compound with a R_f of 0.77 has been identified in the urine sample collected from the subject 2. Similar results have been obtained after HPTLC analyses of the urine samples collected from the other two volunteers (data not shown).

Table III
Characterization of the study group of DXM users for HPTLC evaluation of DXM in urine

Subject	1	2	3
Age (years)	24	23	26
Ingested dose	880 mg	800 mg	1080 mg
Observations	Declared alcohol consumption	No alcohol consumption	Declared alcohol consumption

History of drug use			
Chemical solvents	Yes	No	Yes
Cannabis	Yes	Yes	Yes
Psilocybe mushrooms	Yes	Yes	Yes
GHB	Yes	No	Yes
Ketamine orally	No	Yes	Yes
PCP, orally/sniffing	No/Yes	Yes/Yes	No/Yes
Cocaine sniffing	No	No	Yes
Amphetamines, sniffing/orally/ intravenously	Yes/Yes/Yes	Yes/Yes/No	Yes/Yes/Yes
Ecstasy, orally/intravenously/sniffing	Yes/Yes/Yes	Yes/No/Yes	Yes/Yes/Yes
Mephedrone, sniffing/orally	Yes/Yes	Yes/Yes	Yes/Yes
Codeine	Yes	Yes	Yes
Tramadol orally	Yes	Yes	Yes
Barbiturates	Yes	Yes	Yes
Benzodiazepines, including alprazolam	Yes	Yes	Yes
Trihexyphenidyl	Yes	Yes	Yes
Selegine	Yes	No	Yes

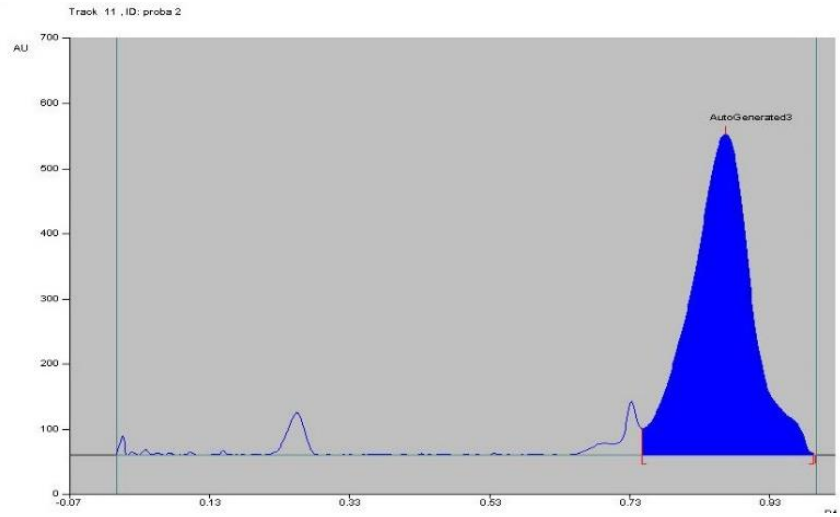

Figure 2.
Chromatographic peak for the urine sample collected from the subject 2

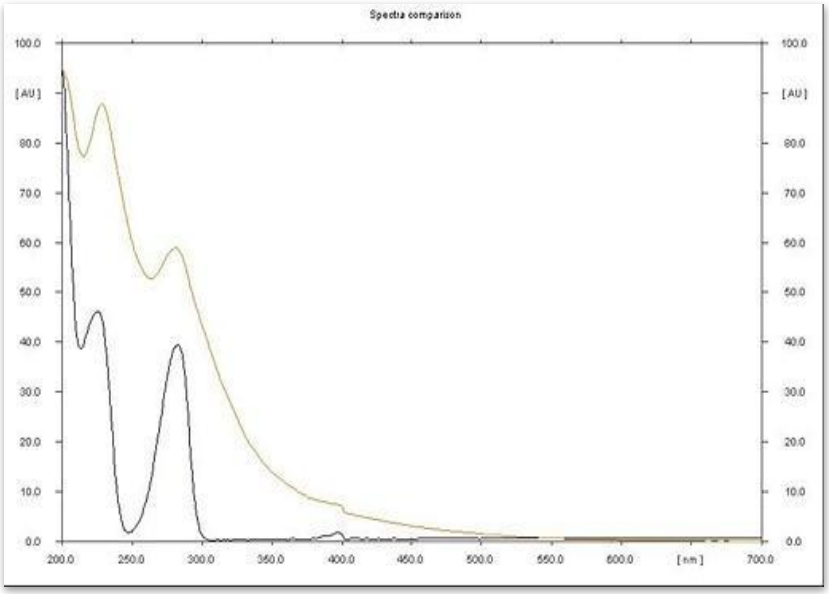

Figure 3.
Comparison between the spectrum of DXM reference solution and of the second subject's sample

The UV spectrum corresponding to the spot with Rf = 0.77 is very similar to DXM UV spectrum (Figure 3). Based on the obtained spectra, it is suggested that the unknown compound with Rf = 0.77 could be DXO, the main metabolite of DXM.

DXO has a very similar UV spectrum to DXM, with an absorption peak at 280 nm [37]. Without DXO standard analysis, the precise identification of the unknown peak could not be made. Based on the results, it can be assumed that the spot with Rf = 0.77 belongs to a metabolite of DXM, DXO most probably, as it is the main metabolite of DXM. In this context, the results suggest that subjects included in the study are fast metabolisers of DXM. Several reports indicate that TLC can be used as rapid and less expensive procedure to make a clear distinction between extensive and poor DXM metabolizers [9, 14, 15]. DXM is rapidly metabolized by the liver and is O-demethylated by CYP2D6 to its pharmacologically active metabolite DXO, a potent NMDA antagonist with a half-life of approximately 3.5 to 6 h [22, 26, 40]. Current data indicate that about 37.3% of women and 57.3% of men are fast DXM metabolizers. Preliminary current data suggests that extensive DXM metabolizers may report a greater DXM abuse potential due to the increased rate of metabolism to the active metabolite DXO [23].

The cough preparations containing DXM have been adopted by many recreational drug users for the psychoactive effects of DXM [33]. DXM use at mega doses (5 to 10 times the dose recommended for control of nonproductive coughs), a practice called "robotripping", "robo-ing" or "robo-copping" (after the popular cough syrup Robitussin) has profound psychological and physiological effects and may result in a neurobehavioral toxidrome with significant psychiatric effects consisting of psychomotor agitation, hostility, grandiose behaviour, hallucinations, paranoia and panic [34, 14, 31]. The standard doses of DXM for adults in cough formulations range from 15 mg to 30 mg up to four times daily, with a maximum dose of 120 mg for 24-hour period. At doses between 100 mg and 300 mg, symptoms such as mild stimulation, euphoria and hallucinations are described. Doses of 300 mg to 600 mg cause loss of motor coordination, visual distortions and dissociation. At doses of 600 mg or higher, complete dissociation, out-of-body sensations and coma can occur [17].

The symptomatology described by the subjects of this study is in line with the plateaus of DXM toxicity reported in the literature, as well as in accordance with the ingested dose. Therefore, the subjects experienced visual and auditory disturbances, altered consciousness, delayed reaction times, hallucinations, delusions, ataxia, mania, panic, partial dissociation. Thus, the subjects showed all the DXM plateau specific features, even though the fourth was not pithy. The delimitation between the phases is rather difficult to ascertain. Still, there are certain classic dissociative reflection patterns, such as the feeling of complete understanding of abstract concepts on various abstracting levels, observed through the use of hierarchical logic elements and self – referential statements. Consistent with the particular features of every plateau, DXM has the ability to allow the mind to create and to maintain the association between distant non related concepts.

The prolonged use of DXM for recreational purposes implies a series of severe hazards. The most critical side effect of using hallucinogens and/or dissociative substances is brain damage. This is a well-documented risk, as they are able to produce the impairment of cognitive functions, and temporary or irreversible mental deterioration.

An abstinence syndrome may be associated with cessation of DXM abuse that is characterized by dysphoria and intense cravings. In addition, chronic use of the drug was related with toxic psychosis and cognitive deterioration.

The use of high DXM doses may result in more profound and potentially life-threatening effects at even higher doses [24]. In the last decades, a guideline for the management of patients with a suspected ingestion of DXM has been proposed [6]. Patients who have ingested more than 7.5 mg/kg should be referred to an emergency department for evaluation. The diagnostic of DXM intoxication is based mainly on the clinical evaluation, as DXM is not detected by basic drug screens [2].

DXM was reported to be abused in very high doses, sometimes exceeding 12 to 15 times the maximum recommended daily dose, with urine DXM levels exceeding 2000 ng/mL [20]. In these cases, TLC technique can be useful as a screening procedure for the analysis of the urine samples, as it can assure a good separation of the analytes and has no risk for false positive results due to cross-reactivity.

Conclusions

A new HPTLC method used to identify DXM in abusers' urine is presented. As a modern version, HPTLC is one of the complex chromatographic techniques based on the full capabilities of thin layer chromatography and offering several advantages such as automation, scanning, selective detection and quantification by using densitometry. The proposed HPTLC method is a simple and rapid procedure that can be applied as a screening tool for an analytical diagnostic in DXM intoxication. At the same time, this HPTLC method has the major advantage of being cost-efficient and flexible, requires minimal amounts of solvents and presents a low risk of environmental pollution.

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Conflict of interest

The authors declare no conflict of interest.

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