DETECTION OF PREGABALIN IN URINE USING THIN LAYER CHROMATOGRAPHY: AN EGYPTIAN STUDY

BY

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ABSTRACT

Pregabalin is an anticonvulsant drug that is used for the treatment of partial seizures, generalized anxiety disorder, and neuropathic pain. It has been demonstrated to have potential abuse. This study aimed to detect pregabalin using thin-layer chromatography (TLC). This study recruited 120 patients from the Emergency-Toxicology Unit with disturbed consciousness level and no history of abusive drug intake. Urine samples were collected from patients and tested for pregabalin and other drugs of abuse (opiates, cannabis, benzodiazepines, barbiturates, amphetamines, codeine and tramadol) using EMIT and TLC. Different mobile phase solvents were adopted for the development of pregabalin in TLC plates. The present results showed that 60% of cases were positive for pregabalin using two mobile phases (toluene: glacial acetic acid) and (chloroform: methanol, while other mobile phase (Ethyl acetate: methanol: concentrated ammonia) did not allow for the development of pregabalin. From the present results it can be concluded that TLC can be used as a method for detection of pregabalin in urine.

Keywords: Pregabalin, TLC, mobile phase.

INTRODUCTION

Pregabalin is a structural analogue for the inhibitory neurotransmitter gammaaminobutyric acid (GABA) which exhibits anticonvulsant and analgesic activities via binding to either GABAA or GABAB receptors or alters GABA uptake or degradation (Arnold et al., 2010).

In 2004, pregabalin was approved by the Food and Drug Administration (FDA) for the treatment of peripheral neuropathic pain and post-therapeutic neuralgia and has been approved as an adjunctive therapy for partial seizures (Plested et al, 2010). By the year 2006, it was prescribed by the European Commission for the treatment of generalized anxiety disorder (Gajraj, 2007).

Pregabalin is widely prescribed by pain management specialists (O'Connor and Dworkin, 2009); in addition it has been

suggested to have potential abuse, which placed pregabalin into Schedule V of the Controlled Substances Act by the Drug Enforcement Administration in 2006 (Cada et al., 2006). However, prescribing pregabalin is still not under legislative control in Egypt unlike its legislation in other different countries including Turkey (Yargic and Ozdemiroglu, 2011) and the United States (USA) (Heltsley et al., 2011).

In many areas of the world, there is an urgent need to qualitatively and quantitatively estimate drugs using minimal laboratory resources with a rapid, inexpensive and acceptable screening technique; that can specifically determine whether more sophisticated testing is or is not warranted (El-Bagary et al., 2012).

The thin-layer chromatography (TLC) method is dependent on the use of comparative testing to reference products. It is an inexpensive and rapid screening test that can determine whether an agent can be detected via a number of tests that distinctly attribute, or confirm, using appropriate legally prescribed methods, which is required prior to take regulatory action (Flinn et al., 1989).

Although several analytical methods have been adopted for the measurement of pregabalin in biological samples, including liquid chromatography-tandem mass spectrometry, gas chromatography, high performance liquid chromatography and capillary electrophoresis (Heltsley et al., 2011), in Egypt, the application of such high cost methods is not widely available. In addition, the detection of pregabalin using the enzyme immune assay has not been applied until recently. Thus, the objective of this study was to develop a simple and cost-effective method for detecting pregabalin in urine using thin-layer chromatography as a tool for diagnosing its abuse among patients referred to the Mansoura Toxicology Unit.

SUBJECTS, MATERIAL AND METHODS

Subjects :

One hundred and twenty patients (112 males and 8 females) were recruited from the Toxicology Unit-Mansoura Emergency Hospital from January 2009 to December 2009. Patients attended the hospital complaining of a disturbed consciousness level, with no history of drug abuse or a past history of medical disorders. Socio-demographic data regarding age, sex, residence, occupation, marital status and educational level were recorded.

Urine samples were collected from patients (10 ml each); at the time of admission and prior receiving any treatment, in clean dry labeled test tubes. The samples were preserved at -18°C until the time of analysis. All data were encoded with an identifier for confidentiality.

Legal consent was obtained from each patient according to the Declaration of Helsinki, as a requirement for approval by the Ethical Committee in the Faculty of Medicine – Mansoura University.

Material and Methods :

(I) Preliminary test (EMIT[®] d.a.u. TM : *drug of abuse in urine*) : the samples were analyzed by the EMIT qualitative detection of opiates, cannabis, benzodiazepines, barbiturates, amphetamines, codeine and tramadol in urine using a Viva-E® System.

(II) Confirmatory Thin Layer Chromatography (TLC):

Thin layer chromatography plates were used (25 DC-Alufolien 20x20 cm kieselegal 60, MERK, Germany).

Chemicals :

All chemicals were purchased from Sigma Aldrich, Egypt. The following standards were used: phenobarbital, butabarbital, morphine, 6-monoacetylmorphine (6-MAM), delta-9-tetra-hydrocannabinol and amphetamine, which were obtained from the Vienna International Toxicology Center. Diazepam (Valinil) and Clonazepam (Apetryl) were purchased from La Roche Company. Tramadol hydrochloride (Tramal) was purchased from October Pharma S.A.E. and Codeine

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was obtained from Macferlane, England. A standard solution of pregabalin was prepared as a stock solution (0.1 mg mL⁻¹) in water according to Shaalan, (2010). All TLC standards were stored in dark bottles at 4°C (dissolved in chloroform as 1 mg/ ml).

Solvents :

- 1. Ethyl acetate: methanol: concentrated ammonia solution (85:10:5) for the development of barbiturates, opiates, codeine, tramadol, amphetamines and pregabalin.
- 2. Toluene: glacial acetic acid (97:3) for the development of benzodiazepines and pregabalin.
- 3. Toluene for the development of cannabis.
- 4. Chloroform: methanol was used at two different concentrations, (4:1) and (3:2), for the development of pregabalin.

Spraying reagents (all reagents were freshly prepared) :

- Acidified iodoplatinate solution for opiates, tramadol and amphetamines.
- 2. Spraying reagents for benzodiazepines:
 - (a) Sulfuric acid (18 N): prepared assulfuric acid: extra pure water (1: 2).
 - (b) Sodium nitrite (1%).
 - (c) Ammonium sulfamate (5 gm in

100 ml pure water).

- (d) Naphthyl-ethylene-diamine dihydrochloride (benzo reagent): 1% naphthylethlenediamine in 80% acetone.
- 3. Zwikker's reagent for barbiturates: Copper sulfate (40 ml of a 10% solution) was mixed with 10 ml of pyridine and water was added for a total volume of 100 ml.
- 4. Fast Blue BB solution for cannabis:0.1 % solution of fast blue BB salt in water and methanol (1:3) .
- 5. Ninhydrin reagent (20 mg of Ninhydrin 0.4% in 100 ml of acetone (0.2% in n-butanol), stored in a brown bottle in a refrigerator for 20 minutes. After spraying, all plates were exposed to a 254- and 366-nm UV viewing lamp (P.W, Allen & Co.) (Kaye, 1980).

Digestion of the samples and TLC development were performed according to George and Braithwaite (1995) for cannabis, barbiturates, benzodiazepines and pregabalin detection. Detection of opiates, codeine and amphetamines was performed according to Meadway et al. (1998).

To capture images of the TLC plates, a Minolta MAXUM DYNAX 7XI camera equipped with a NINOLTA AF lens 100 MMF (2- 8 Macro equipped with Konica film for color prints VX100 – 36/135); was used for 3-5 minutes .

RESULTS

Figure (1) shows that 60% of the cases were positive for pregabalin by TLC, while 40% were negative.

The patients' ages ranged from 19 to 45 year-old (mean age: 29.81 ± 5.5). As shown in table 1, the highest incidence of positive pregabalin cases were males (91.7%), urban residents (79.2%) and those who had private work (40.3%), those who were married (56.9%), and those with only a secondary school education (50%).

Estimation of drugs by EMIT: all cases were proven to be negative for opiates, cannabis, benzodiazepines, barbiturates, amphetamines, codeine and tramadol.



Fig. (1) : Percentages of positive and negative cases for pregabalin using TLC in the studied group (n=120).

	Positive cases for pregabalin (n=72)	Negative cases for pregabalin (n=48)
Sex Male Female	66 (91.7%) 6 (8.3%)	46 (95.8%) 2 (4.2%)
Residence Urban Rural	57 (79.2 %) 15 (20.8%)	38 (79.2%) 10 (20.8%)
Occupation No employment Governmental Private	19 (26.4%) 24(33.3%) 29 (40.3%)	17 (35.4%) 11 (22.9%) 20 (41.7%)
Marital status Single Married	31 (43.1%) 41(56.9%)	24 (50%) 24(50%)
Educational level Illiterate Secondary school High graduate	2 (2.8%) 36 (50.0%) 34 (47.2 %)	5 (10.4%) 25 (52.1%) 18 (37.5%)

Table (1) : Socio-demographic characteristics of the studied patients (n=120).



Fig. (2) : Thin layer chromatograms of drugs of abuse showing negative results for: (A) amphetamines and barbiturates, (B) cannabis, (C) benzodiazepines and (D) opiates. A positive spot was detected on plate C after spraying with ninhydrin.

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Fig. (3) : Thin layer chromatograms of the pregabalin standard and urine samples extract revealing positive or negative cases of pregabalin: (A) and (B) with solvent mobile phase (toluene: glacial acetic acid 97:3).



Fig. (4) : Thin layer chromatograms of the pregabalin standard and urine samples extracts revealing positive and negative cases of pregabalin: (A) with the solvent mobile phase (chloroform: methanol 4:1) and (B) solvent mobile phase (chloroform: methanol 3:2).



Fig. (5) : Thin layer chromatograms of the pregabalin standard and urine samples extracts revealing negative cases of pregabalin: (A) with the solvent mobile phase (chloroform: methanol 4:1) and (B) solvent mobile phase (chloroform: methanol 3:2).

As shown in figure (2), all of the TLC plates showed negative results for different drugs of abuse (amphetamines, cannabis, benzodiazepines, barbiturates, opiates, codeine, tramadol). However, a spot was found in plate C after spraying with ninhydrin reagent, which indicated a positive result for pregabalin as shown in the following figures.

In figures (3-5), different mobile phases were used for the development of pregabalin in the TLC plates. These results indicated that using (toluene: glacial acetic acid) and (chloroform : methanol) enabled the development of pregabalin in urine samples [Figs. 3A-B, 4 A-B]. While in fig. (5), the mobile phase (Ethyl acetate: methanol: concentrated ammonia) did not enable the development of pregabalin in urine samples.

DISCUSSION

Pregabalin has been identified as a novel class of analgesic drugs that are broadly prescribed for the treatment of epilepsy and chronic pain and has potential abuse. Thus, it was placed into Schedule V of the Controlled Substances Act by the Drug Enforcement Administration in 2006 (Yargic and Ozdemiroglu, 2011).

Although, several analytical methods

have been adopted for the measurement of pregabalin in biological samples, including liquid chromatography-tandem mass spectrometry, gas chromatography, high performance liquid chromatography and capillary electrophoresis (Heltsley et al., 2011), these adopted methods were found to be very complicated and could not be used for routine analysis in emergency hospitals (Navneet et al., 2010).

In Egypt, pregabalin has not yet been listed by the national authorities as a drug of abuse. Investigations are still underway to determine the prevalence of its use in an addictive manner to recommend for its abandonment. Application of the previously described tools is not widely available due to the high cost needed to perform screening for pregabalin detection. This research study aimed to establish a simple and cost-effective screening tool for the detection of pregabalin using thin-layer chromatography. It was performed on 120 patients, they were selected from patients attending the Emergency Toxicology Unit with disturbed consciousness state and no history of drug abuse.

To the best of our knowledge, this study was the first to be performed using TLC plates for the detection of pregabalin in urine samples. Urine samples from those patients were examined by Emit and TLC to detect different drugs of abuse (opiates, cannabis, benzodiazepines, barbiturates, amphetamines, codeine and tramadol) which may explain the clinical conditions of these patients. The results of this study revealed that samples were negative for all tested agents, with a positive result for pregabalin as detected by TLC. It was found that TLC positively detects pregabalin in urine samples in 60% of cases, while 40% of the cases were negative. This finding could explain the disturbed consciousness of those patients, who may have been administered other drugs rather than those tested in this study.

The positive cases for pregabalin were tested using three different mobile phases. The developing mobile phase (chloroform: methanol) proved to be efficient for developing pregabalin at two different concentrations (4:1 and 3:2). However, using (ethyl acetate: methanol: concentrated ammonia) as a mobile phase did not enable the development of the drug. This finding indicated the usefulness of the mobile phase (chloroform: methanol) instead of toluene: glacial acetic acid.

Several previous studies have detected pregabalin using different techniques. Gujral et al., (2009) reported that UV spectrophotometry can be used as a simple, precise, accurate, reproducible and low cost method for the determination of pregabalin in bulk, in formulations and in human urine. Shaalan (2010) successfully developed three new, simple, sensitive and selective spectrofluorimetric and spectrophotometric methods for the determination of pregabalin in capsules and spiked urine. He concluded that the proposed methods were simple, accurate and less tedious compared to chromatographic procedures.

In 2011, Dahl et al. studied the effectiveness of using ultra-high pressure liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS) in the detection of pregabalin in whole blood and urine. They demonstrated that both methods were simple high-throughput methods that are suitable for screening.

Mudiam et al. (2012) proved that the use of solid-phase microextraction (SPME) and dispersive liquid-liquid microextraction (DLLME) to extract pregabalin from urine and pharmaceutical formulations were simple, fast, efficient and inexpensive techniques followed by GC-MS analysis. They found wide application for the routine determination of pregabalin in biological samples as well as in quality control samples of pharmaceutical formulations.

Rodríguez et al. (2013) studied the determination of pregabalin in human urine using nonaqueous CE-TOF-MS, which showed a great improvement in its qualitative ability. This may find wide application for the routine determination of pregabalin in biological samples. Moreover, Priez-Barallon et al. (2014) studied the detection of pregabalin overdose in postmortem human samples (central and peripheral blood, urine, and bile) via hydrophilic interaction HPLC-high-resolution MS.

CONCLUSION AND RECOMMENDATIONS

Thus, it is recommended to test the performance characteristics of TLC to detect pregabalin in large-scale clinical studies, particularly in the high risk group, to indicate the effectiveness of using this tool as a screening method and to determine whether it can be compared to newly developed sensitive tools as previously described.

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الكشف عن البريجابالين في البول باستخدام الكرو ماتوغرافيا ذو الطبقة الرقيقة: دراسة مصرية

المشتركون في البحث

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 i.c. سهــام جـاد الحــق

قسم الطب الشرعي والسموم الإكلينيكية - كلية الطب - جامعة المنصورة

يعد البريجابالين دوا ، مضاد للصرع حيث يستخدم لعلاج نوبات الصرع الجزئى، القلق العام ، وآلام الأعصاب. وقد ثبت أنه قد يسبب الإدمان. وقد هدفت هذه الدراسة إلى الكشف عن البريجابالين باستخدام الكروماتوغرافيا ذو الطبقة الرقيقة. وأجريت هذه الدراسة على عدد ١٢٠ مريضا من وحدة السموم بمستشفى الطوارئ الجامعى، وقد كان جميعهم مضطربى الوعى وليس لديهم تاريخ مرضى دال على إدمان العقاقير. وقد تم جمع عينات البول من المرضى وتم اختبارها لوجود المخدرات (الأفيون والقنب، البنزوديازيبينات، الباربيتورات والأمفيتامينات، الكودايين والترامادول) باستخدام الكروماتوغرافيا فو الطبقة الرقيقة وجهاز الفحص الإنزيمي المناعى، وقد تم استخدام العديد من مختلف الذيبات ذات الطور المتحرك لإجراء الدراسة. وأظهرت نتائج الدراسة أن ٢٠٪ من الحالات كانت إيجابية للبريجابالين باستخدام الذيبات ذات الطور المتحرك لإجراء الدراسة. وأظهرت نتائج الدراسة أن ٢٠٪ من الحالات كانت إيجابية للبريجابالين باستخدام الذيبات ذات الطور المتحرك لإجراء الدراسة. وأظهرت نتائج الدراسة أن ٢٠٪ من الحالات كانت إيجابية للبريجابالين باستخدام الذيبات ذات الطور المتحرك (التولوين: حامض الخليك الجليدي) و (الكلوروفورم: الميثانول)، في حين أن استخدام الذيبات (الأثيل اسيتات: الميثانول: الأمونيا المركزة) لم تسمح بظهور البريجابالين. ونستنتج من هذه الدراسة أن الكروماتوغرافيا ذات الطبقة الرقيقة يمن مندام المديرك من المركزة عامض الخليك الجليدي) و الكلوروفورم: الميثانول)، في حين أن استخدام المديرك المني اسيتات: الميثانول: الأمونيا المركزة) لم تسمح بظهور البريجابالين. ونستنتج من هذه الدراسة أن الكروماتوغرافيا ذات الطبقة الرقيقة يمكن استخدامها كوسيلة للكشف عن البريجاليان في البول.

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