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SEPARATION AND IDENTIFICATION OF *AMFEPRAMONE* IN PLASMA AND URINE BY HPTLC

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Abstract: *Amfepramone, called also diethylpropion or diethylcathinone, is a stimulant drug of the amphetamine class that is used as an appetite suppressant. Regarding the abuse, amfepramone is considered to have a low potential. However, recently there have been reports of amfepramone abuse in teens and adults. We developed a simple HPTLC (High Performance Thin Layer Chromatography) method for determination of amfepramone in plasma and urine. Pre coated glass plates (silica gel 60 F254 as stationary phase) and methanol: ammonia as mobile phase were used, while the detection was performed by densitometry in the absorbance (reflectance) mode at 254 nm. A liquid-liquid extraction (with tertbuthylmethyleter) at alkaline pH has been applied. The obtained results indicate that amfepramone has been identified both in simulated samples and in real urine samples (collected from volunteer treated with amfepramone).*

Key words: *amfepramone, plasma, urine, HPTL.*

1.Introduction

In recent years the problem of extra weight is increasing and some people even turn into obsession that can evolve to depression of varying degrees. Another problem is the increasing number of child obesity cases even under the supervision of parents. Obesity is a medical condition of nutrition and metabolism characterized by the accumulation of excessive body fat, with the negative effects on health, leading to reduced life expectancy and /or health problems. People are considered obese when the body mass index (BMI), obtained by dividing the weight in kilograms by the square of that person's height in meters, is more than 30kg/m^2 or 27Kg/m^2, if there are comorbidities such as diabetes, hypertension, hyperlipidemia [7].

Amfepramone, also called diethylpropion or diethylcatinone, is an appetite suppressant belonging to the sympathomimetic amphetamine class. It works by affecting dopaminergic mechanism inhibiting the hunger center [1], [2], [3].

It is used in the treatment of obesity of various etiologies, in combination with a weight loss program based on a strict diet, exercise and behavior modification in an obese patient [9], [10], [11].

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An important aspect that argues this study is the presence of amfepramone on black market drugs that have an aggressive policy of promoting the general interest products in large quantities and prices. The "miraculous products" combined with diet and exercise guarantee visible results, but do not have a detailed prospectus, regarding the qualitative and quantitative chemical composition, and these effects in the body are not supported on clearly scientifically mechanisms.

2. Objectives

The study aimed the developing a new method for separation and identification of amfepramone in plasma and urine by HPTLC (High Performance Thin Layer Chromatography) [4], [5], [6], [7], [8].

3. Materials and methods

3.1. Reagents

- Solvents for the mobile phase:
  - methanol (*R)* – Sigma Aldrich, Germany;
  - ammonia (*R)* – Sigma Aldrich, Germany;
- Mobile phase: *Methanol (R) : ammonia (R) (100:1.5v /v)*
- Solvent for extraction from biological samples:
  - hexane*(R)* -Sigma Aldrich, Germany;
  - ethyl acetate (R) – Sigma Aldrich, Germany;
  - sodium carbonate (R) – Sigma Aldrich, Germany
  - a mixture of hexane(R): ethyl acetate (R) (7: 3)

3.2. Materials and equipment

- pre-coated TLC plates silica gel F254 on glass, 20x20cm, from Merck
- vertically developing tanks with cap
- semi-automatic pipettes with variable volume
- Vortex Genie 2 stirring system (Cole Parmer)
- cooling centrifuge Sigma 2-15 K
- system for evaporation under nitrogen (Dry-Block, Techne DB-3D Bibby Scientific Inc., England)
- semi-automatic spotting system Linomat 5 (Camag, Switzerland)
- TLC densitometer Scanner 3 (Camag, Switzerland)
- WinCATS software ver. 1.4.4.

3.3. Biological materials

- human plasma (National Institute of Hematology "C.T.Nicolau")
- urine from a female volunteers, nonsmoker, who ingested two Regenon capsules - Temmler® Pharma GmbH & CO.KG (amfepramone hydrochloride 25 mg/capsule)
- blank urine from healthy volunteer, non-smokers

The study was carried out in accordance with the Declaration of Helsinki [12].

The informed consent the participation in the study was obtained from the volunteer who was administered Regenon capsules -Temmler® Pharma GmbH & CO.KG.

3.4. Preparation of working solution and spiked samples

Stock solution of diethylpropion hydrochloride 1.0 mg/mL in methanol (Sigma Aldrich) was used as standard. The working standard solution was prepared by 1:2 dilution of stock solution.

Spiked plasma and urine samples were prepared as follows: 500 µL stock solution and 500 µL plasma, respectively 500 µL stock solution and 500 µL urine.

3.5. The extraction of samples

- **Liquid-liquid extraction**

The 1 mL sample was treated with 5 mL of extraction solvent, a mixture of hexane (R): ethyl acetate (R)(7:3). Then 200 µL of saturated sodium carbonate solution were added. The samples were extracted on vortex for 10 minutes and then were centrifuged for 10 minutes at 3000 rpm at a temperature of 15°C. The upper organic phase was separated and evaporated under nitrogen stream, at a temperature of 40 °C. After evaporation of the solvent, the residue was taken up in 500 µL methanol (R).

4. Results and discussion

4.1. Interpretation of the results obtained for the liquid - liquid extraction of spiked urine and plasma samples

We performed a series of preliminary tests to define the detection limit of the method. The chromatograms were acquired and processed with the TLC Scanner 3, by densitometry, using UV light at λ=254 nm, in the absorbance (reflectance) mode. The R_f value was automatically computed for each track on the basis of the maximum peak of the spot. The quantitative analysis facility of the software WinCATS ver. 1.4.4 was used for the semi quantitative evaluation of amfepramone. The solutions for analysis were spotted on 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 scheme is described in Table 1. Samples obtained and treated according to the method described in liquid - liquid extraction were used. All samples (biological and blank), were made in parallel and in the same conditions. We developed a plan to spot the analysis solutions, including the sequence of spotting and the volumes to be spotted for each migration track (Table 1).

Table 1

The spotting plan for amfepramone analysis

Track no.	1 – 5					6 – 7		8 – 11			
Sample	Amfepramone					Blank plasma or urine extract		Plasma or urine extract			
Volume (µL)		10	15	20	25	20	10	10	10	20	20

According to the plan for obtaining chromatographic plate, the solutions were spotted in band with semi-automatic system Linom 5 (Camag) using Hamilton microsyringe in a stream of nitrogen. The plates were developed in the developing vertically chamber (ascending technique was applied), previously saturated with the vapor of the mobile phase. After development, the plates were evaluated using densitometer TLC Scanner 3 (Camag). Determination was performed at a wavelength of 254 nm, which is the maximum absorption for amfepramone. In the solvent system used as a mobile phase, amfepramone is characterized by a R_f value of 0.85 ± 0.01, according to the software generated report. R_f value falls within the acceptance condition deviation of ± 5%, as it is shown in 3D chromatograms. In the 3D chromatograms obtained the peak of amfepramone is observed at approximately the same R_f, both in the standard solution and in the extracts from the biological samples (Fig. 1).

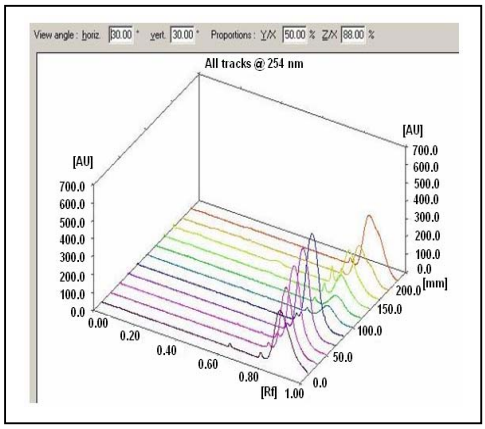

Fig. 1. *The chromatogram obtained at the evaluation of spiked biological samples of amfepramone by HPTLC method (3D representation) for track no 1 to 11 in order mentioned in table 1*

4.2. Interpretation of the results obtained for the liquid-liquid extraction of urine samples collected from volunteer

The study included collecting the urine samples from a young female volunteer, healthy, non-smoker.

The first sample was collected 4 hours after amfepramone administration (sample 1), considering the amfepramone half-life between 4-6 hours. The second sample was collected at 7 hours to observe the metabolic rate (sample 2).

The chromatograms were acquired and processed with the TLC Scanner 3, by densitometry, using UV light at λ=254 nm, in the absorbance (reflectance) mode. The Rf value was automatically computed for each track on the basis of the maximum peak of the spot. The quantitative analysis facility of the software WinCATS ver. 1.4.4 was used for the semi quantitative evaluation of amfepramone. The solutions for analysis were spotted on standard plates, under nitrogen flow, in lines of 10 mm length using semi-automate Linomat5 system equipped with a Hamilton micro syringe. The spotting scheme is described in Table 2. Samples obtained and treated according to the method described in liquid - liquid extraction were used. All samples were made in parallel and in the same conditions. We developed a plan to spot the analysis solutions, including the sequence of spotting and the volumes to be spotted for each migration track (Table 2).

Table 2

The spotting plan for amfepramone analysis from real (collected from volunteer) biological samples

Track No.	1-5					6 -7	
Sample	Amfepramone					urine extract	
						real Sample 1	Real Sample 2
Volume (µL)	5	10	15	20	25	20	20

According to the plan for obtaining chromatographic plate, the solutions were spotted in band with semi-automatic system Linom 5 (Camag) using Hamilton microsyringe in a stream of nitrogen. The plates were developed in the developing vertically chamber (ascending technique was applied), previously saturated with the vapor of the mobile phase. After development, the plates were evaluated using densitometer TLC Scanner 3 (Camag). Determination was performed at a wavelength of 254 nm, which is the maximum absorption for amfepramone. With the solvent system used as a mobile phase, amfepramone is characterized by an

R_f value of 0.88 for standards, but variation within the samples is very high, according to the report generated by the software. The R_f values are not within the acceptance condition deviation of ± 5%, but supports the hypothesis of the presence of active metabolites.

In the 3D chromatogram representation (Fig. 2), for the tracks of the real samples, it has been shown two supplementary peaks at R_f values lower than the R_f of standards.

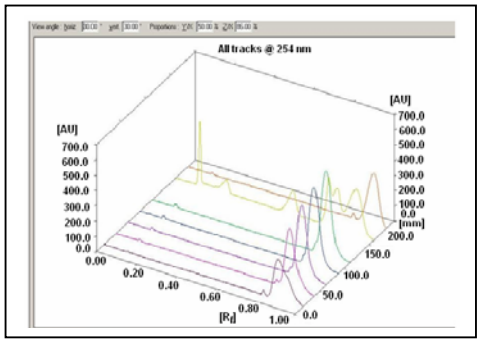

Fig. 2. *The chromatogram obtained at the evaluation of real (collected from volunteer) biological samples amfepramone by HPTLC method (3D representation) for track no 1 to 7 in order mentioned in table 2*

Having in view the extensive biotransformation of amfepramone, these spots can be attributed to amfepramone metabolites, such as ethylaminopropio - methoxyisobutyrophenone or 2-N-diethylamino-1-phenylpropanol.
According to the literature data, these metabolites are at the same time the main amfepramone impurities [5].

The UV spectra were recorded at the wavelength at which amfepramone has maximum absorption (254 nm). Comparing the spectra of simulated plasma and urine samples with spectra of the reference solution, the exact correspondence is shown, or otherwise, in simulated samples, real samples and reference solutions amfepramone has been identified, indicating that the applied method is sensitive and reliable.

As can be shown, there is a clear superposition between spectra of simulated samples and standard, the proposed method are suited to this type of determination, taking into account any improvements that may be made to. The spectrum of real urine samples collected from a volunteer at different times, is very similar in shape, as it is shown in the following figure (Fig. 3).

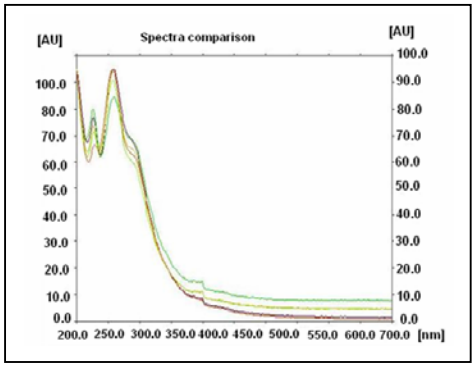

Fig. 3. *The spectra of spiked, real sample, and standard spectrum*

5. Conclusions

Considering the experimental data presented in this study, as well as the information reported in the literature, it is suggested that a large study is needed, involving more volunteers, as amfepramone shows a great variability among people. The extraction method should be also improved to provide quantitative satisfactory results.

We developed a new method, reproducible and with accurate results in terms of identifying amfepramone in both urine and plasma samples.

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