

HPTLC METHOD FOR THE SEMI QUANTITATIVE ASSAY OF ALPRAZOLAM IN BIOLOGICAL SAMPLES

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Abstract

A simple high performance thin layer chromatography (HPTLC) method for the semi quantitative determination of alprazolam in plasma and urine has been developed. Pre-coated glass plates (silicagel 60 F254 as stationary phase) and chloroform : methanol (90:10 v:v) as mobile phase were used. The detection was performed by densitometry in the absorbance (reflectance) mode at 254 nm. A liquid-liquid extraction procedure (with tert butylmethyleter) at alkaline pH has been performed. A linear regression curve with correlation coefficients over 0.99 has been obtained in the range of 0.1 μg – 2 μg /spot; repeatability, recovery percent, detection limit (DL = 50 ng) and quantification limit (QL = 100 ng) have been demonstrated. The method can be used for alprazolam quantification at toxic/lethal levels in biological samples.

Rezumat

A fost elaborată o metodă HPTLC (cromatografie în strat subțire de înaltă performanță) simplă pentru determinarea semicantitativă a alprazolamului în plasmă și urină. Au fost utilizate plăci de sticlă pre-trasate (silicagel 60 F254 ca faza staționară) și cloroform: metanol (90:10 v:v) ca fază mobilă. Detecția a fost realizată densitometric prin înregistrarea absorbanței (modul reflexie) la 254 nm. Pentru izolarea din matrice biologică a fost aplicat un procedeu de extracție lichid-lichid (folosind terțbutilmetileter ca solvent), la pH alcalin. Curba de regresie liniară cu coeficienți de corelație peste 0,99 a fost obținută în domeniul 0,1 μg - 2 μg /spot; au fost demonstrate: repetabilitatea, acuratețea (ca procent de regăsire), limita de detecție (DL = 50 ng) și de cuantificare (QL = 100 ng). Metoda poate fi folosită pentru cuantificarea alprazolamului în probe biologice la nivele toxice/letale.

Keywords: alprazolam, HPTLC, semi quantitative analysis

Introduction

Benzodiazepines are a class of drugs with a variety of effects and different pharmacodynamic and pharmacokinetic properties; they are widely used in medical practice, especially for the anxiolytic action.

Benzodiazepines are relatively safe drugs, overdoses being fatal mainly in co-ingestion of other central nervous system (CNS) depressant drugs or alcohol [2]. The newly introduced triazolbenzodiazepine

compounds pose higher toxicity in overdoses. Because of the high consumption of benzodiazepines, they are involved in many forensic cases [3].

Alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4] triazole [4,3,-a]-[1,4] benzodiazepine) is a relatively new triazolobenzodiazepine compound with anxiolytic properties in humans, indicated in the treatment of depression and panic disorder. The main metabolite of alprazolam, α -hydroxyalprazolam possesses substantial biologic activity. However, as alprazolam metabolites are not present in significant concentrations in human plasma, the measurement of the initial compound is useful for both clinical and toxicological studies.

In recent years, the number of suicides or overdoses increased after ingestion of triazolobenzodiazepine. In addition, alprazolam is recognized as possessing a higher risk of abuse than any other triazolobenzodiazepine [4].

The objective of this study was to elaborate a HPTLC method for the semi quantitative determination of alprazolam in biological matrices (plasma and urine). An attempt was also made in order to assess the usefulness of these determinations in forensic and clinical toxicology.

Materials and methods

Reagents, Devices

- alprazolam (A) (a gift from LaborMed Pharma) – stock solution of 1 mg/mL in methanol; working solution 0.1 mg/mL in methanol;
- pre-coated TLC plates silicagel F₂₅₄ on glass, 20x20cm, from Merck;
- solvents for the mobile phases: chloroform (analytical grade reagent), methanol (HPLC, isocratic grade);
- normal human plasma (National Institute of Hematology “C.T. Nicolau”, Bucharest, Romania);
- stirrer system Vortex Genie 2 (Cole Parmer);
- evaporation system under nitrogen Techne Dry-Block (Bibby Scientific Inc.);
- cooling Centrifuge 2-15 K (Sigma);
- semiautomate spotting system Linomat 5 (Camag, Switzerland);
- TLC densitometer Scanner 3 (Camag, Switzerland);
- WinCATS software ver. 1.4.4.

Procedure

The solutions for analysis (alprazolam working solution and plasma or urine extracts) were spotted on the standard plates, under nitrogen flow,

in lines of 10 mm length using semiautomate Linomat 5 system equipped with a Hamilton micro syringe. The spotting scheme is described in Table I.

Table I.
Spotting scheme for the semi-quantitative assay of **A** in plasma and urine

Track No.	1-6						7-10				11-14			
Sample	Alprazolam						Plasma or urine extract 1 µg				Plasma or urine extract 2 µg			
µL	5*	1	5	10	15	20	10	10	10	10	20	20	20	20

*0.01 mg/mL spotted for verifying the detection limit

The developed technique was the ascending one, in a vertical chamber, previously saturated with the mobile phase vapors, in a dark place, using chloroform:methanol (90:10 v:v) as mobile phase. The chromatograms were acquired and processed with the TLC Scanner 3, by densitometry, using UV light at $\lambda=254$ nm, in the absorbance (reflectance) mode. The retardation factor (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 alprazolam.

Extraction procedure of urine and plasma

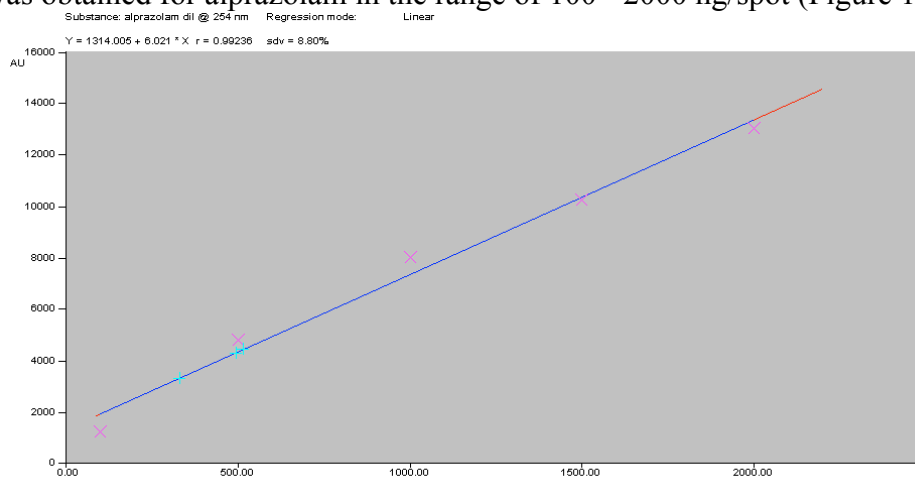
The samples of urine (5 mL) or plasma (1 mL) spiked with alprazolam were extracted by the liquid-liquid procedure, at alkaline pH (0.5 mL KOH 2M) using tert butylmethylether (5 mL). The samples were stirred on vortex 20 min., then centrifuged at 4000 rpm, 24°C, 10 min. After separating the organic phase, it was evaporated to dryness under nitrogen stream and the residues were dissolved with 1 mL methanol.

Results and Discussion

We performed a series of preliminary tests to define the linearity domain of the method. The developing system was also selected in the preliminary evaluations; the R_f of alprazolam in chloroform : methanol (90:10 v:v) system is about 0.55.

The preliminary experimental results indicated that the high quantities (1-20 µg) domain is not suitable for semi-quantitative evaluation, the calibration curve having a coefficient of 0.97 (as area peak) and 0.86 (as height peak). It may be noted that, at large amounts of substance (over 10 µg), the spot intensity tends to decrease due to adsorption of benzodiazepine in silica gel layer, leading to the difficulty of quantifying by absorption

(reflectance mode). Taking into account the peak area, a linear regression was obtained for alprazolam in the range of 100 - 2000 ng/spot (Figure 1).



Regression via area $Y = 6.021 \cdot x + 1314$ $r = 0.99326$

Regression via height $Y = 0.1709 \cdot x + 57.23$ $r = 0.97768$

Figure 1.

Calibration curve for HPTLC evaluation of alprazolam

The precision of the method was demonstrated as repeatability (Table II). At the same time, we have considered widening of the linearity range, testing the amount / spot below 100 ng.

Table II.

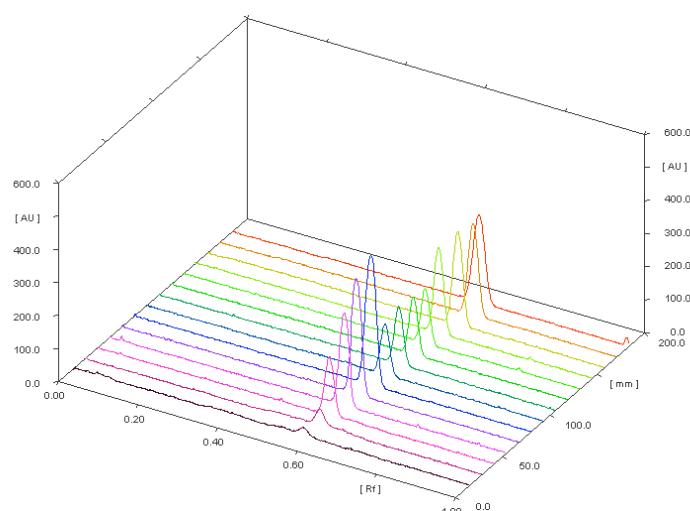
The repeatability of HPTLC method for the semi quantitative evaluation of alprazolam

Quantity/spot	50 ng	100 ng	500 ng	1000 ng	1500 ng	2000 ng
Average*	703.53	1222.72	4826.16	8045.67	10522.41	12830.43
Standard error	47.43	8.61	123.29	67.052	160.20	171.06
CV**	6.74%	0.70%	2.5%	0.83%	1.52%	1.33%

* $n = 3$ ** variation coefficient

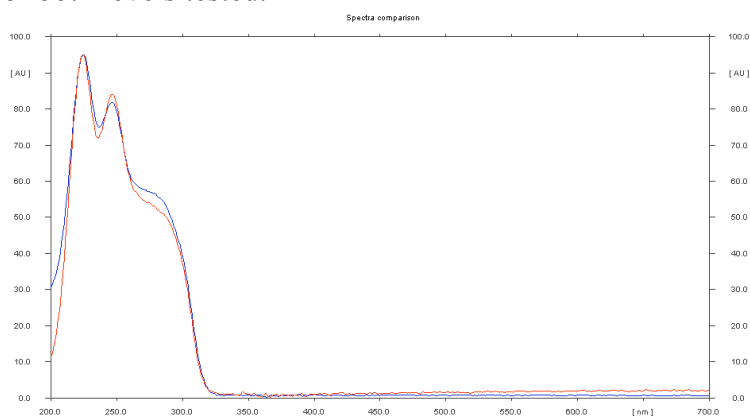
Based on the experimental data and the regression curves [1], the detection (DL) and quantification limits (QL) of alprazolam by the method described above were as following DL=50 ng, and QL=100 ng.

For the evaluation of alprazolam in biological samples, two levels included in the calibration curve were tested: 1 μg (1000 ng) and 2 μg (2000 ng); for each level four replicated samples have been performed. The representative 3-D densitogram obtained for the determination of alprazolam in plasma is presented in Figure 2 (the spotting scheme is described in Table I).

**Figure 2.**

3-D representation of the densitogram for the semi quantitative evaluation of alprazolam in plasma

The UV spectra of the corresponding spots of alprazolam confirmed its presence in standard solution and plasma extract (Figure 3). The repeatability of the method for the semi quantitative evaluation of alprazolam in plasma and urine has been demonstrated (Table III). The coefficient of variation is appropriate, according to the bio-analytical method for both levels tested.

**Figure 3.**

UV absorption spectra (reflectance mode) of the corresponding spots for alprazolam in standard solution and plasma extract

The accuracy of the method as recovery after extraction from plasma and urine has been evaluated. Satisfactory extraction yields for both urine and plasma samples have been obtained (Table III).

Table III.

The repeatability and recovery of HPTLC method for the semi quantitative evaluation of alprazolam in plasma and urine

Sample	Plasma		Urine	
Amount (ng/spot)	1000	2 000	1000	2000
Average*	942.56	1.55	944.71	1.77
Standard Error	9.190	0.124	18.482	0.065
CV**	0.97%	7%	1.96%	4.17%
Recovery (%)*	94.47	88.82	94.25	78.26

* n = 4 ** variation coefficient

The reproducibility of the method was assessed by performing a standard curve using plasma extracts. The experimental data regarding the standard curve of plasma extracts fit a linear model, the correlation coefficient of the regression line being greater than 0.99 (data not shown). HPTLC analysis of plasma samples from patients who received a therapeutic dose of alprazolam 1 mg indicated that the sensitivity of the method is insufficient to assess alprazolam at therapeutic concentrations.

Conclusions

We developed a high performance thin-layer chromatographic method for the semi quantitative assay of alprazolam in biological samples. The method allows the evaluation of alprazolam both in plasma and urine samples, after liquid-liquid extraction (with tert butylmethyleter) at alkaline pH. The linearity (in the range 0.1µg – 2µg/spot), repeatability, accuracy (as recovery percent), detection limit (DL=50ng) and quantification limit (QL=100ng) have been demonstrated. The method can be applied for the quantification of alprazolam at toxic/lethal levels in biological samples.

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