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ANALYTICAL STUDY OF AMPHETAMINE AND METHAMPHETAMINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD

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Abstract. Amphetamine and methamphetamine were tested compounds using RP-HPLC method. On the basis of analytical studies, a new procedure for the chromatographic separation and determination of analyzed drugs was completed in about 5 minutes. The samples were eluted isocratically using a mobile phase consisting of acetonitrile and ortho-phosphoric acid (pH 2.1) [15: 85, *v/v*] at a 1.0 mL/min flow rate, with UV (205 nm) detection and temperature at 40°C. The described procedure allows the quantification of amphetamine and methamphetamine with adequate linearity, reproducibility and accuracy in the concentration interval 5.0 – 500.0 μg·mL^{-1}. For both compounds, the limits of detection were 0.5 μg·mL^{-1}. The utility of the described assay was tested by determining the analyzed compounds in seized tablets.

Keywords: analytical study, amphetamine, methamphetamine, high-performance liquid chromatography, diode array detector, seized tablets

Introduction

Amphetamine-type stimulants (ATS) are a group of substances, mostly synthetic in origin, that are structurally derived from *β*-phenethylamine (*β*-PEA, Fig. 1a). ATS generally simulate the central nervous system (CNS). Therefore, to varying degrees,

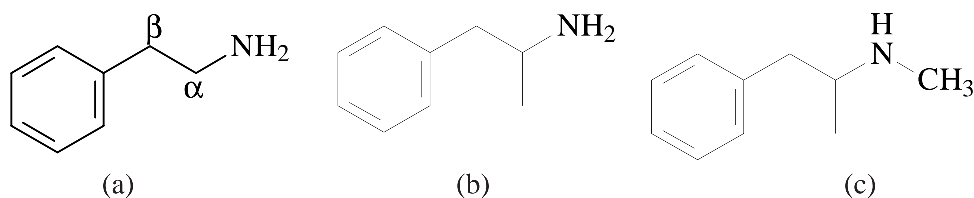


Figure 1. Chemical structure of: (a) *β*-phenethylamine, (b) amphetamine, and (c) methamphetamine.

they are considered as prototypes of central nervous system stimulants with a potential of psychotic toxicity when overdosed or abused for long periods.

ATS may produce one or more dose-related symptoms, including increased alertness and euphoria, increased heart rate, blood pressure, respiration and body temperature [1]. The original drug is called amphetamine ($C_9H_{13}N$) (Fig. 1b), but in terms of structural characteristics, the major sub-group without substitution on aromatic ring includes and methamphetamine ($C_{10}H_{15}N$), smokable methamphetamine (ice) (Fig. 1c), and dextroamphetamine (dexies) [2].

Illicit amphetamine frequently encountered as the sulphate salt in powder form, and rarely as tablet. Amphetamine base may seized in clandestine laboratories, typically as a dark brown oily liquid with a characteristic unpleasant smell of 1-phenyl-2-propanone (P-2-P) and/or solvent residues.

Methamphetamine is an amphetamine derivative that has a history as a periodically popular drug of abuse [1,2]. Ogata first synthesized the drug in Japan in 1919 [3], patented in 1920, and later licensed to Burroughs Wellcome, who marketed it as the anorectic Methedrine®. There are a variety of popular terms including meth, crystal, crystal meth, ice, speed, whiz, and crank, for the name of methamphetamine. No term is specific for particular grade or chemical product, although these terms generally reserved for illicit preparations, as opposed to diverted pharmaceuticals. Frequently, drugs sold as methamphetamine may in fact contain no methamphetamine at all, and are actually substitutes such as caffeine, ephedrine, pseudoephedrine, or even cocaine, depending on local drug availability.

Unfortunately, supply of these drugs has increased dramatically on the European illegal market [4,5], including Macedonia. Consequently, the analysis of amphetamines has become of increased interest from a point of view of toxicology, occupational medicine and law enforcement.

A variety of analytical techniques, for example titrimetry [6], spectroscopy [7,8], capillary electrophoresis [9-11], liquid chromatography [12-14] and gas chromatography [15,16] have been used for quantisation of amphetamine and methamphetamine in different real samples. Undoubtedly, GC coupled on-line to a MS detection system is the most powerful technique for identification and confirmation of amphetamines. However, there are laboratories around the world, especially in developing countries,

which cannot afford such an expensive instrument. In these laboratories, identification of amphetamines, especially in forensic cases, has been performed by High-performance liquid chromatography – Diode array detector (HPLC-DAD) as the most rational and universal separation and identification technique.

In the current work, we presented a detailed analytical study of amphetamine and methamphetamine by HPLC–DAD using reverse phase column Lichrospher® 60 RP Select B. The dependence of column back pressure (*P*) and column's plate height (*H*) on flow rate of mobile phase (*F*) using high-performance liquid chromatography is studied. The optimal flow rate for simultaneous separation of amphetamine and methamphetamine, from the minimum of Van Deemter plot that have a hyperbolic form, was been selected. The developed method for direct separation and determination of these two drugs was been validated for linearity, precision, and accuracy. The utility of the described assay was tested by determine the analyzed compounds in seized tablets.

EXPERIMENTAL

Solvents and reagents

The reagents used were of highest purity (>99.95 % purity), methanol and acetonitrile HPLC grade (Merck, Darmstadt, Germany), *ortho*-phosphoric acid (Alkaloid, Skopje, R. Macedonia). Authentic samples of amphetamine ($C_9H_{13}N \cdot SO_4$) and methamphetamine ($C_{10}H_{15}N \cdot HCl$), were supplied from the United Nations Drug Control Program (Vienna, Austria).

Sample preparation

Stock solutions of amphetamine and methamphetamine were prepared in methanol at concentrations of 1.00 mg/mL. The solutions were stored at 4°C until analysis. Series of standards for each of the substances have been prepared by progressive dilution of the stock solution. All the samples analyzed (tablets, powders), have been seized by Macedonian police in the period from 2005 to 2006, mainly in the area of Skopje. Ten milligrams of ground tablets, were weighed and dissolved in 7 mL of methanol. The solution is sonicated for 5 min, filtered, made up to 10 mL with methanol and 20 μL was injected into the chromatographic system.

Instrumentation and materials

A Varian HPLC system equipped with a ternary pump Model 9012 and UV-Diode Array detector Model 9065 is used. The chromatographic system was under control by the software package Varian Star 4.50. Separations were performed on the reverse phase column LiChrospher® 60 RP Select B (250 x 4.6 mm, 5 μm particle diameter), protected by a guard column LiChrospher® 60 RP Select B (4 mm x 4.6 mm, 5 μm) (Merck). A mixture of acidified water with H_3PO_4 (pH 2.1) and acetonitrile was selected as an optimal mobile phase. An isocratic elution 85 % (H_3PO_4, pH 2.1):

15 % CH_3CN was performed at temperature of 40°C and mobile phase flow rate of 1.5 mL/min. Samples were injected through injector valve Rheodyne Model 7125 with a 20 μL sample loop. The identity of each compound was established by comparing the retention times and UV spectra in real samples with those obtained for standards. The wavelength of 205 nm for quantifying of both, amphetamine and methamphetamine was used. To obtain reproducible results, the column was thermostat with column heater (CH-30) and Eppendorf controller of temperature (TC-45).

Results and discussion

The determination of basic compounds requires special RP chromatographic sorbents. Retention, selectivity and peak symmetry of basic compounds are strongly been influenced by the silica matrix. Strongly distorted peaks of the basic compounds are often been observed when unsuitable RP sorbents are used, due to the interaction of the basic compounds with unreacted SiOH groups on the silica matrix [17]. LiChrospher® 60 RP-select B is a spherical porous silica carrier, in which the starting silica material optimized in order to prevent any secondary interactions with basic compounds. The usage of this type of column allows separation of basic compounds (such as amphetamine and methamphetamine with dissociation constants values (p*K*a) of 9.9 and 10.1, respectively [1, 18] without the need of ion pair reagents.

Development and optimization of HPLC method

In order to achieve a satisfactory separation of amphetamine and methamphetamine on LiChrospher® 60 RP Select B column some of the chromatographic parameters, including composition and pH of mobile phase, detection wavelength, mobile phase flow rate and temperature were been varied. Series of mobile phases containing acetonitrile/water with different volume fractions and acetonitrile/buffer solutions with different pH values (from 2.1 to 4.4) and different volume fractions were been investigated.

The most satisfactory results were obtained when the mobile phase consisted of a mixture of acidified water with H_3PO_4 (pH 2.1) and acetonitrile (ACN) in volume fraction ratio of 85:15 (Fig. 2), and UV detection at 205 nm (Fig. 3).

Since the very beginning of the modern chromatographic separation era, it was been recognized that a plot of analysis time versus plate number provides the most direct and unbiased comparison of the performance of chromatographic systems with different physicochemical properties or with different support morphologies or using different flow driving methods.

The key to understanding the importance of using smaller column packing materials (5 μm, in this case) follows from the Van Deemter equation:

$$H = A + B/u + Cu,$$

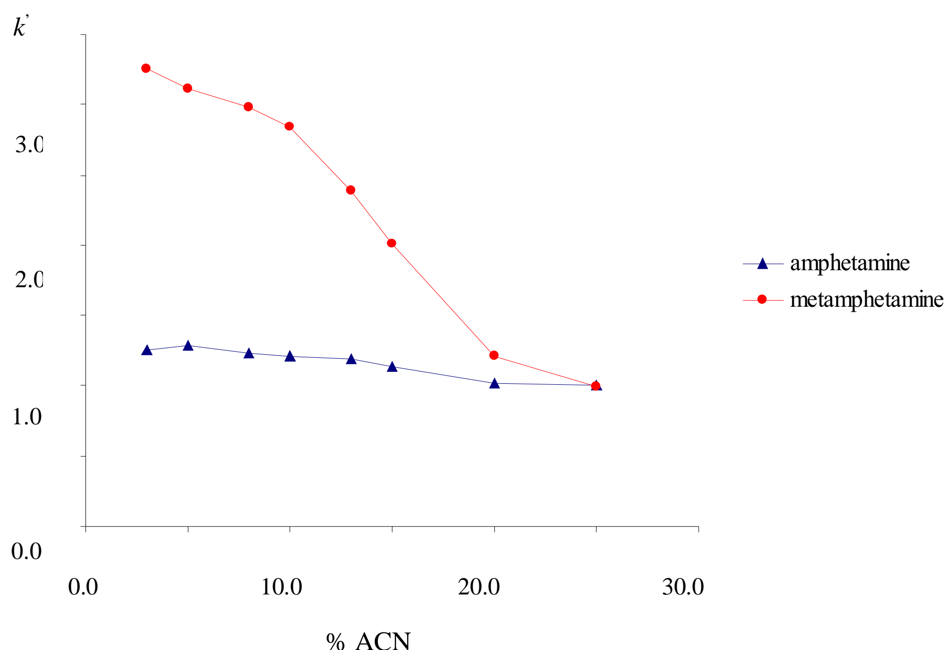


Figure 2. Effect of percent acetonitrile in the mobile phase on capacity factor of amphetamine (▲), and methamphetamine (●).

where A – eddy diffusion term, that results from multiple flow paths in the column; directly proportional to the packing particle diameter and is independent of mobile phase flow rate; B – longitudinal diffusion coefficient, related to the diffusion coefficient of the molecule in the mobile phase – if the analyte is eluted very quickly, there will be little time for diffusion to occur; C – analyte mass – transfer coefficient, related to the time needed for the analyte molecules to equilibrate between the mobile and stationary phases; u – linear flow rate of the mobile phase; H – plate height.

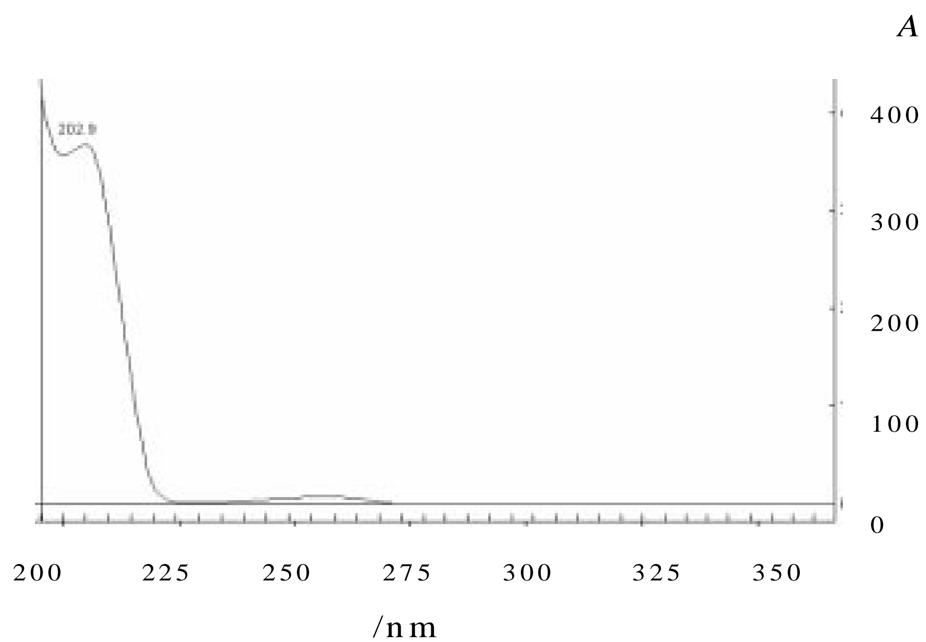
In our studies was used the flow rate F in place of the linear flow rate u of the mobile phase, and the amphetamine was used as a void marker to measure and the retention void of the column.

The plate height was calculated as $H=L/N$, where L is the length of the column (in this case, $L = 250$ mm), and N is the effective plate number.

The effective plate number for the other analyte was calculated using the formula

$$N = 5,54\left(t_R / w_{1/2}\right)^2,$$

where t_R is the retention time substracted by the retention void (as determined by the amphetamine peak), and $w_{1/2}$ is the full-width half-maximum of each of the other analyte peaks [19].



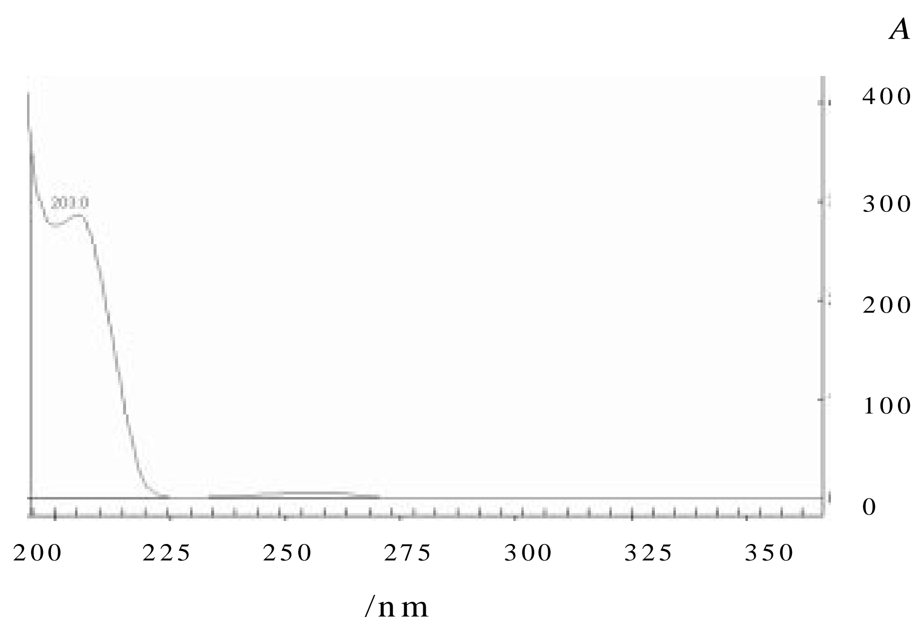


Figure 3. UV spectra of: (a) amphetamine and (b) methamphetamine.

The van Deemter equation for the column is plotted in Fig. 4 that included the fitted data points.

For the LiChrospher® 60 RP Select B column, the curve has a minimum (i.e. highest chromatographic resolution) near a mobile phase flow rate of 1.0 mL/min. Therefore, the flow rate, from the minimum of Van Deemter plot (that have a hyperbolic form), was selected as optimal for simultaneous separation of amphetamine and methamphetamine using HPLC method.

During each run, the syringe pump pressure was recorded in order to estimate the column back pressure for the different flow rates. These pressure data have been plotted in Fig. 5.

Linear response for the column's back pressure to flow rate of mobile phase was observed, and equation

$$y = 98{,}857 \cdot x + 7{,}286$$

with correlation coefficient of 0.9979 was obtained.

With respect to the location and shape of the peaks of amphetamine and methamphetamine, temperature of 40°C, was selected for recording all chromatograms.

Under optimal chromatographic conditions: column LiChrospher® 60 RP-select B, isocratic elution of mobile phase: 0.01 mol/L H_3PO_4 (pH 2.1) and acetonitrile 85:15 (*v/v*), flow rate of 1.0 mL/min, the column temperature 40°C, UV detection performed at 205 nm, the obtained retention times were approximately 3.68 min for amphetamine and 4.50 min for methamphetamine (Fig. 6b).

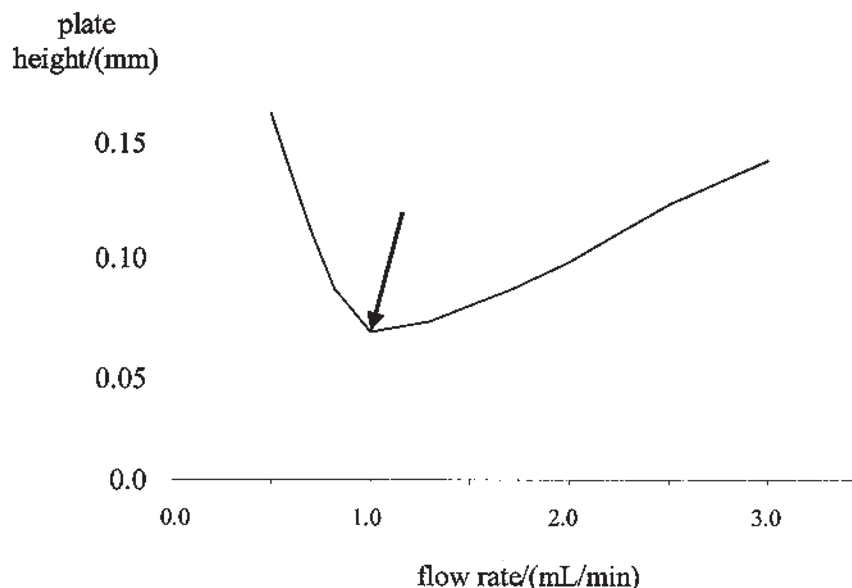


Figure 4. The dependence of column's plate height (*H*) on flow rate of mobile phase (*F*) – van Deemter curve.

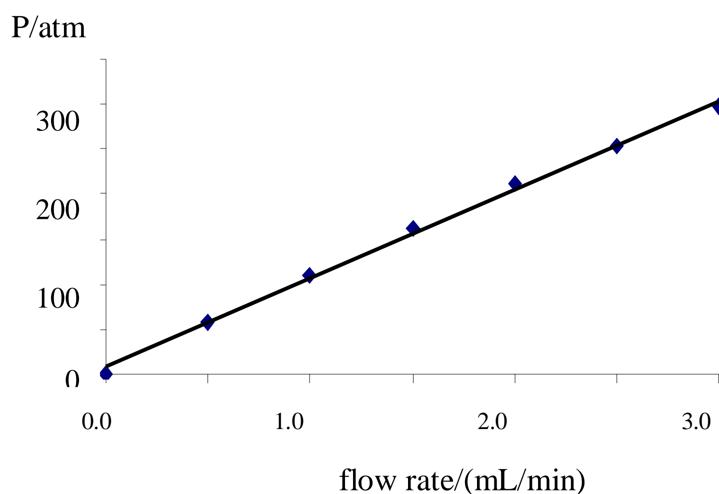


Figure 5. The dependence of pressure (*P*) on flow rate of mobile phase (*F*).

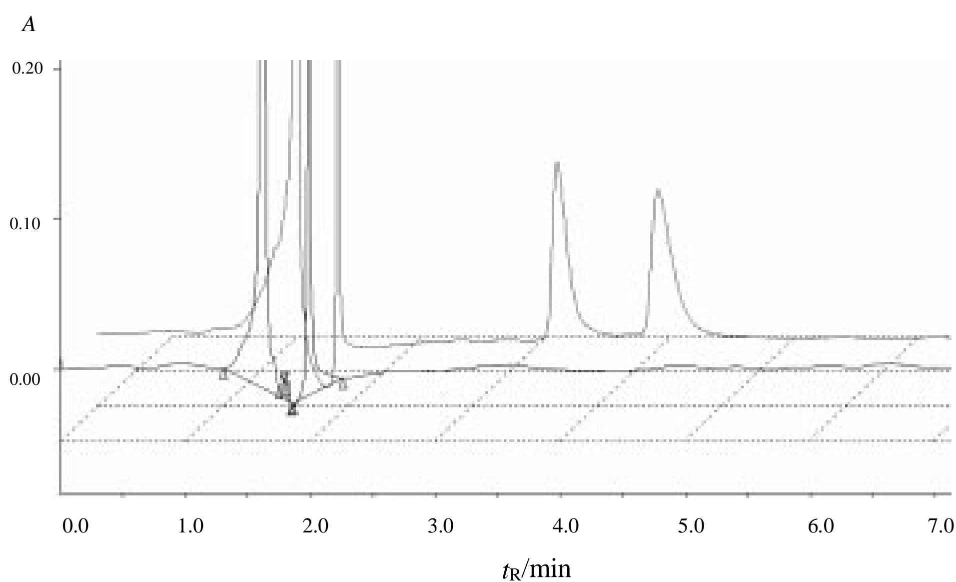


Figure 6. Chromatograms obtained from: (a) methanol and (b) standards of amphetamine 50 μg/mL and methamphetamine 50 μg/mL. Chromatographic conditions: column LiChrospher® 60 RP-select B, isocratic elution of mobile phase: 0.01 mol/L H_3PO_4 (pH 2.1) and acetonitrile 85:15 (*v/v*), flow rate of 1.0 mL/min, the column temperature 40 °C, UV detection was performed at 205 nm.(A-amphetamine, M-metamphetamine)

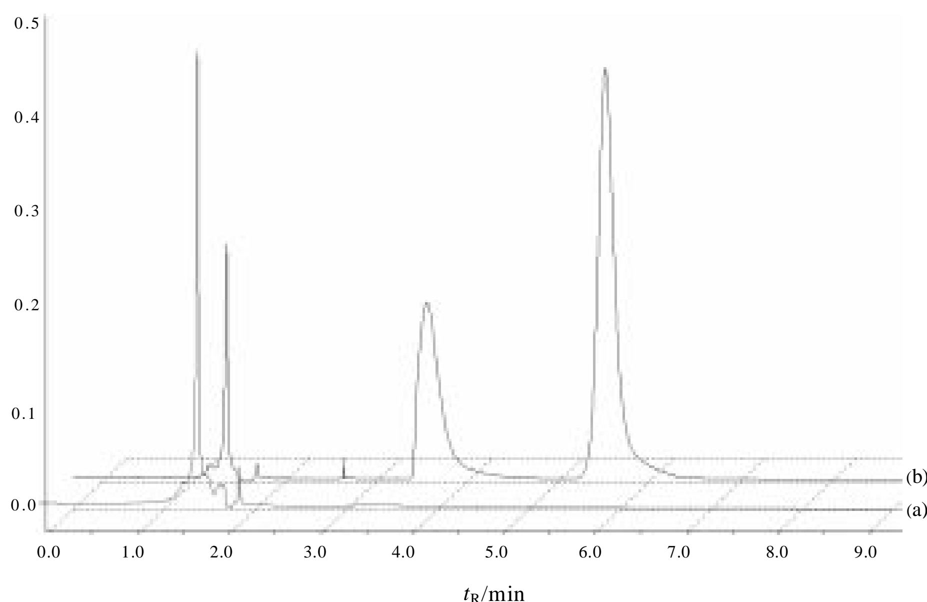


Figure 7. Chromatograms obtained from: (a) methanol, and (b) extract of seized tablet containing amphetamine and caffeine. Chromatographic conditions are identical as Fig. 6. (A-amphetamine, C-caffeine)

Using the retention times for amphetamine and methamphetamine, the capacity factor – k', selectivity factor – α (for amphetamine/ methamphetamine) and resolution factor – R_s (for amphetamine/methamphetamine) were calculated. The obtained values of these three parameters ($1 < k' < 10$, $\alpha > 1$, $R_S > 2$) show that the proposed chromatographic conditions are suitable for separation and quantification of the analyzed components. The column efficiency was determined by the number of plates whose value shows a good separation efficiency of the applied column. The values for the repeatability of the system (RSD % ≤ 2.0, $n = 7$) show that the system is precise (Table 1).

Linearity and method validation

The linear range of the method was studied by analyzing in duplicate seven concentrations of each compound ranging from 5.0 to 500.0 μg/mL. The obtained linear ranges for each of the drugs with corresponding correlation coefficients (R^2) are given in Table 2.

Limit of detection (LOD) and limit of quantification (LOQ) by an empirical method that consisted of analyzing a series of standard solutions containing decreasing amounts of drugs were determined. This method, although not applicable for complex matrices, is useful for simple samples. The LOD was the lowest concentration that presented a CV that did not exceed 20% and the LOQ the lowest concentration that presented a

Table 1. Characteristic parameters for system suitability of the method

Parameters	amphetamine	methamphetamine
t_R / min.	3.68	4.50
k'	1.13	2.13
α		1.88
N	4676	3807
R_S		5.50
Repeatability of the system (RSD %)	0.95	1.03

t_0 (migration time, nonretained species) = 1.73 min

CV that did not exceed 10%. The results are given in Table 2.

Precision was expressed as relative standard deviation (%RSD). For *intra-day*, 10 replicates of 50, 100 and 250 μg/mL were analyzed on the same day. These standards were analyzed in five replicates over 5 days to establish *inter-day* precision (Table 2).

With no reference materials, the accuracy of the method was determined by analyzing spiked samples at six concentration levels over the range of 25 and 500 μg/mL. Results were calculated as experimental values compared to theoretical values and were expressed as percent recovery (Table 2).

The limit of detection was calculated by LOQ=10σ/a where σ is the standard deviation of the response of the blank and a is the slope of the calibration curve. The limit of quantification was calculated by LOQ=10σ/a under the ICH guidelines. The limits of detection for amphetamine and methamphetamine 0.5 μg/mL, and the limits of quantification 1.5 μg/mL were obtained. The limits were validated by analysing standards prepared at the concentrations of the LOQs for each standard and their precision and accuracy were assessed (Table 2).

Application of HPLC procedure to seized tablets and powders

A number of seized tablets, powders (145 samples) were analyzed to demonstrate that the column Lichrospher® 60 RP Select B could be used in everyday analysis in the laboratory. The chromatographic conditions selected were a mobile phase consisting of acidified water with H_3PO_4 (pH 2.1)/acetonitrile (85/15, *v/v*) maintained in an isocratic mode, flow rate at 1.0 mL/min, column temperature at 40 °C with

Table 2. Linearity and validation studies for the Lichrospher® 60, RP Select B, 250 x 4.6 mm, 5 µm particle diameter

Parameter	Drugs standards	
	Amphetamine	Methamphetamine
Correlation coefficient (R^2)	0.9993	0.9996
y-intercept - *a*	4816.1	4588.7
Slope - *b*	3241	2890
Intra-day precision (% RSD)		
50 µg/mL	2.3	1.7
100 µg/mL	1.4	0.9
250 µg/mL	2.5	2.1
Inter-day precision (% RSD)		
50 µg/mL	2.2	1.9
100 µg/mL	1.7	2.3
250 µg/mL	1.9	1.5
LOD µg/mL	0.5	0.5
LOQ µg/mL	1.5	1.5

Amphetamine and methamphetamine standards were tested for intra-day precision, inter-day precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) under the guidelines of ICH (International Conference of Harmonisation).

Lichrospher® 60, RP Select B, 250 x 4.6 mm, column. Sample detection was at 205 nm.

Fig 7b, is a sample chromatogram from a seized tablet that contained 17.4 % amphetamine and caffeine, that is identified from its UV spectra. Amphetamine and caffeine were the main components present of samples tested. Quantification of the components was by the regression equation.

Conclusion

The objective of this study was to demonstrate the ability of the Lichrospher® 60, RP Select B column to detect and quantify active ingredients, amphetamine and methamphetamine in seized tablets, powder. The method uses a simple procedure for sample preparation and allows separation of analyzed analytes on HPLC column in about 5 min. The application of the method to a large number of seized tablets demonstrated the suitability of the method for the laboratories with a heavy workload.

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АНАЛИТИЧНО ОПРЕДЕЛЯНЕ НА АМФЕТАМИН И МЕТАМФЕТАМИН ПО МЕТОДА НА ВИСОКОСКОРОСТНАТА ТЕЧНА ХРОМАТОГРАФИЯ (HPCL)

Резюме. Амфетамин и метаамфетамин са определяни по метода на обратно-фазовата високоскоростна течна хроматография (RP-HPCL). Предложена е нова процедура за разделяне и определяне на анализираните дроги за около 5 мин. Подвижната фаза е съставена от ацетонитрил и ортофосфорна киселина и скорост на потока 1,0 mL/min, с UV детекция (205 nm) при температура 40 °C. Процедурата позволява охарактеризиране на определяемите вещества с добра възпроизводимост и точност в концентрационния интервал 5,0 – 500,0 $\mu g.mL^{-1}$. За двете вещества границата на определяне е 0,5 $\mu g.mL^{-1}$.

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