

HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF THE COMPONENTS OF AN AQUEOUS ANTIDOTE SOLUTION

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Abstract

The objectives of this study were to develop and validate a method for the simultaneous HPLC-UV determination of obidoxime (OBI), scopolamine (SCA), Atropine (ATR) and imipramine (I) in an aqueous antidote solution. An ion-pair mechanism with DAD (diode array detector) detection was used to quantitatively determine the four drugs. The proposed HPLC method uses a Kromasil 100-5C8 (250x4.6mm) column, gradient elution, at 25°C column temperature. The mobile phase consisted of 25% acetonitrile and 75% aqueous phase (5mM sodium 1-heptanesulfonate monohydrate, pH=3.5). The analyte detection was an UV detection at $\lambda=230\pm4$ nm, with reference $\lambda=360\pm 8$ nm. The calibration curve was linear between 1.3-152.5 $\mu\text{g/mL}$ ($r=0.9959$, $n=6$) for OBI, between 0.13-13.75 $\mu\text{g/mL}$ ($r=0.9951$, $n=6$) for SCA, between 0.25-25.5 $\mu\text{g/mL}$ ($r=0.9999$, $n=6$) for ATR and between 0.58-50.80 $\mu\text{g/mL}$ ($r=0.9996$, $n=6$) for I. In conclusion, this chromatographic assay fulfilled all the requirements for being a reliable and feasible method, including linearity accuracy, precision and stability.

Rezumat

Obiectivul acestui studiu a fost dezvoltarea și validarea unei metode HPLC-UV pentru determinarea simultană a obidoximei (OBI), scopolaminei (SCA), atropinei (ATR) și imipraminei (I) dintr-un amestec-antidot, sub formă de soluție apoasă. Pentru determinarea cantitativă a celor patru substanțe s-a utilizat procedura perechilor ionice și detectorul cu șir de diode (DAD). Metoda HPLC propusă utilizează o coloană Kromasil 100-5C8 (250x4.6mm) cu eluție în gradient și o temperatură de lucru în coloană de 25°C. Faza mobilă a fost constituită din acetonitril 25% și soluție apoasă 75% (5mM 1-heptansulfonat de sodiu monohidrat, pH = 3,5). Detecția analiților s-a efectuat în UV la $\lambda = 230\pm4$ nm cu referința $\lambda = 360\pm8$ nm. Curba de calibrare este liniară în intervalul 1,3-152,5 $\mu\text{g/mL}$ ($r=0,9959$, $n=6$) pentru OBI, 0,13-13,75 $\mu\text{g/mL}$ ($r=0,9951$, $n=6$) pentru SCA, 0,25-25,5 $\mu\text{g/mL}$ ($r=0,9999$, $n=6$) pentru ATR și respectiv 0,58-50,80 $\mu\text{g/mL}$ ($r=0,9996$, $n=6$) pentru I. În concluzie, această metodă cromatografică a îndeplinit toate cerințele de fezabilitate și robustețe incluzând liniaritatea, acuratețea, precizia și stabilitatea.

Keywords: HPLC-UV; organophosphate compounds; antidote.

Introduction

Organophosphorous compounds (OPC) are well known as substances developed in the past for military purpose (nerve agents - tabun, sarin, soman, and VX) [1]. Though the Chemical Weapons Convention (CWC) has been signed and ratified by more than 180 countries, the threat persists.

The absorption of these agents into the organism is performed through inhalation, dermal absorption and through mucous membranes. As soon as OPC enter the system, symptoms of poisoning appear and affect the muscarinic and nicotinic receptors. Ultimately death results due to the respiratory arrest [2]. These compounds irreversibly inhibit the enzyme acetyl cholinesterase (AChE) [1, 5].

Some of the OPC with low toxicity are used as insecticides in agriculture. Since they have also a toxic effect on human beings, the staff working in agriculture is exposed [4-6].

The effective antidotes used to defeat the effects of OPC intoxications are oxime reactivators. Pralidoxime, trimedoxime, obidoxime (Toxogonin®), methoxime and HI-6-oxime {1-[[[4-(aminocarbonyl) pyridinio] methoxy] methyl]-2- [(hydroxyimino) methyl] pyridinium dichloride} are frequently used AChE reactivators [1-5]. The reactivators are usually combined with atropine sulphate to suppress the cholinergic symptoms [1-6].

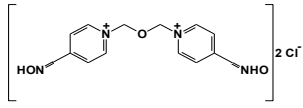
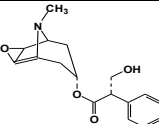
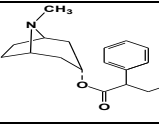
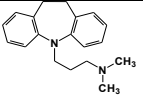
Most techniques reported in the literature are quantifying only one or two components. There are few methods that can be applied directly to both atropine and obidoxime derivatives in parenteral injection devices. HPLC techniques were used to determine both of these drugs in one run at a single wavelength using UV detection [1].

The present study was aimed to develop and validate the simultaneous HPLC-UV determination of obidoxime, scopolamine, atropine and imipramine in an aqueous antidote solution, named ATOX, designed for OPC poisoning treatment [7].

Materials and methods

Analytes and reagents used for the quantitative determination were: acetonitrile HPLC grade (Fluka); sulphuric acid 98% (Merck); ultra pure deionized water locally produced by NANOpure Diamond ultra pure water system (conductivity < 2.3 μ S/cm). Sodium 1- heptansulfonate monohydrate $\geq$ 96% and ortho phosphoric acid 85% (Fluka); atropine sulphate, scopolamine hydrochloride, imipramine bromide, obidoxime hydrochloride, standards provided by Sigma. The analytes properties are shown in table I.

Table I
Analyte properties

Analyte	Properties
 Obidoxime	- <i>dissociation constant</i>: $pK_a=7.6, 8.3$ (25°C); - <i>partition coefficient</i>: $\log P$ (octanol/water) = - 2.0; <i>solubility</i>: slightly in ethanol and methanol, insoluble in chloroform and ether, freely soluble in water.
 Scopolamine	- $pK_a=7.6$ (23°C); $\log P$ (octanol/buffer, pH 7.4) = 1.2, - <i>solubility</i>: 1 in 1.5 of water and 1 in 20 of ethanol.
 Atropine	- $pK_a=9.9$ (20°C); $\log P$ (octanol/water) = 1.8, - <i>solubility</i>: 1 in 1.455 water, 1 in 2 of ethanol, 1 in 1 of chloroform.
 Imipramine	- $pK_a=9.5$ (24°C); $\log P$ (octanol/buffer, pH 7.4) = 2.5; <i>solubility</i>: in ethanol, chloroform and ether, insoluble in water.

Instrumentation and chromatographic conditions

The HPLC system was a HP 1100 with diode array detector (DAD), binary pump, vacuum degasser and thermostatted auto sampler. Chromatographic separations were carried out by a 5 μ m particle size Kromasil 100-5C8 (AKZO NOBEL), 250x4.6mm; 100Å. The column temperature was maintained at 25°C. The mobile phase was acetonitrile (25%) and 5mM sodium 1-heptansulfonate pH=3.5 (with ortho phosphoric acid 85%) and gradient elution delivered at a flow rate of 1.0 mL/min. The eluent was monitored at $\lambda=203\pm 2$ nm; $\lambda_{ref}=360\pm 4$ nm and $\lambda=230\pm 4$ nm; $\lambda_{ref}=360\pm 8$ nm. The injection volume was 10 μ L.

Results and discussion

The UV absorption spectra for the analytes are presented in figure 1. The specific absorbance (A^1_1) for each compound at maximum lambda: obidoxime = 947 (285nm), imipramine = 298 (251nm), atropine = 6.3 (258nm) and scopolamine = 6.3 (257nm) [8]. We have chosen to monitor the chromatograms in UV at $\lambda = 203$ and 230 nm because of the large amount of obidoxime in the ATOX composition. With these values for UV wavelengths the monitoring during the chromatographic elution reached the lowest detection limit for scopolamine and atropine at 203 nm and in the same time reduced the absorption for obidoxime at 230 nm.

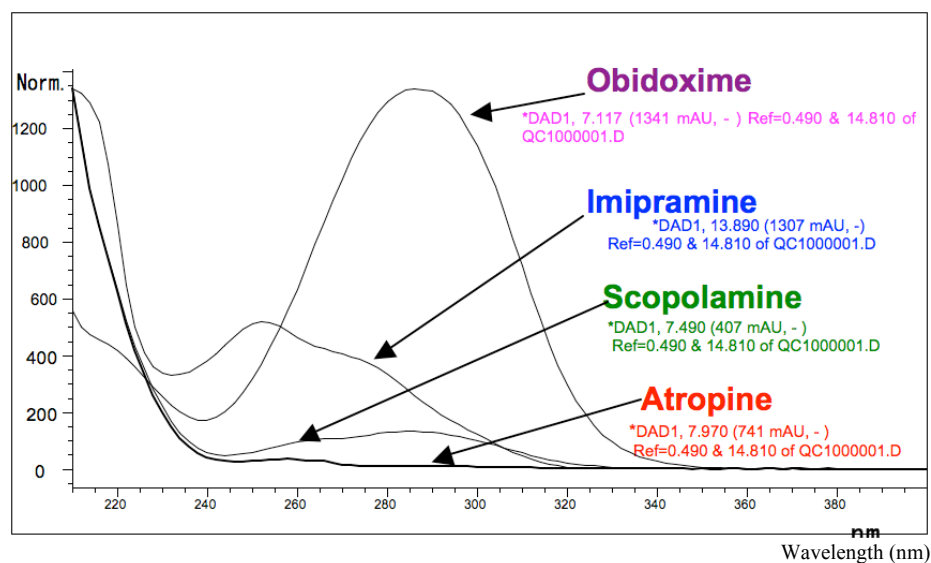


Figure 1

Overlay of UV spectra of atropine sulphate, scopolamine hydrochloride, imipramine bromide and obidoxime hydrochloride

In order to develop a suitable and robust HPLC method for the determination of all four compounds, different mobile phases, at different pH values, and columns (C18 or C8) were employed to achieve the best separation and resolution. It has also been tried a reversed phase chromatographic mechanism. The retention time for obidoxime using the above-mentioned conditions was not a satisfactory one and it has not a stable value (2-3 minutes).

Due to the chemical structure of obidoxime, a cationic one, having two quaternary nitrogen atoms, the mechanism of choice was a ion pair one [9]. The ion-pair reagent sodium 1- heptanesulfonate was found to be appropriate allowing a good separation and quantification for all compounds, in one run, without preliminary separation procedures. For the lower limit of quantification (LLOQ) UV absorption at 203nm was used.

Method validation

Linearity

Calibration curves were performed in the following concentration ranges: 1.3 – 152.5 µg/mL ($r=0.9959$, $n=6$) for obidoxime –table II, between 0.13 – 13.75 µg/mL ($r=0.9951$, $n=6$) for scopolamine – table III, 0.25 – 25.5 µg/mL ($r=0.9999$, $n=6$) for atropine – table IV- and 0.58 – 50.8 µg/mL ($r=0.9996$, $n=6$) for imipramine –table V.

Table II
Calibration curve parameters for the standard concentrations of obidoxime

ANALYTE	Concentration (µg/mL)	Area (mAU•s)	Mean	SD	CV (%)
Obidoxime (OBI)	1.325	101.0	99.58	0.96	0.966
		99.0			
		98.5			
		99.4			
		100.5			
		99.1			
	7.625	616.0	615.60	1.44	0.234
		616.8			
		614.0			
	15.25	1271.1	1269.80	1.30	0.102
		1268.5			
		1269.8			
	30.5	3354.0	3359.00	4.58	0.136
		3360.0			
		3363.0			
	61	4911.0	4910.61	1.93	0.039
		4908.5			
		4912.3			
	152.5	11718.0	11718.00	1.50	0.013
		11716.5			
		11719.5			

where CV – coefficient of variation; SD – standard variation.

Table III
Calibration curve parameters for the standard concentrations of scopolamine

ANALYTE	Concentration (µg/mL)	Area (mAU•s)	Mean	SD	CV (%)
Scopolamine (SCA)	0.1375	20.5	20.50	1.037	5.06
		19.2			
		20.5			
		19.5			
		21.5			
		21.8			
	0.6875	109.0	107.50	1.323	1.23
		107.0			
		106.5			
	1.375	238.5	239.10	1.307	0.55
		240.6			
		238.2			
	2.75	432.9	431.10	1.709	0.39
		430.9			
		429.5			
	5.5	904.1	902.90	1.153	0.13
		901.8			
		902.8			
	13.75	1815.8	1813.00	2.476	0.14
		1812.1			
		1811.1			

Table IV

Calibration curve parameters for the standard concentrations of atropine

ANALYTE	Concentration (µg/mL)	Area (mAU•s)	Mean	SD	CV (%)
Atropine (ATR)	0.255	38.10	37.80	0.876	2.32
		37.90			
		36.60			
		38.50			
		38.80			
		36.90			
	1.275	162.5	161.22	2.713	1.68
		163.05			
		158.1			
	2.57	354.26	354.19	1.950	0.55
		352.2			
		356.1			
	5.1	675.5	674.65	0.769	0.11
		674.0			
		674.45			
	10.2	1313.0	1311.95	1.408	0.11
		1312.5			
		1310.35			
	25.5	3265.0	3264.90	1.153	0.04
		3266.0			
		3263.7			

Table V

Calibration curve parameters for the standard concentrations of Imipramine

ANALYTE	Concentration (µg/mL)	Area (mAU•s)	Mean	SD	CV (%)
Imipramine (I)	0.58	223.0	222.1	1.543	0.69
		222.0			
		224.0			
		220.0			
		223.0			
		220.6			
	2.9	1255.3	1254.47	2.079	0.16
		1252.1			
		1256.0			
	5.8	2631.0	2630.85	1.779	0.07
		2632.55			
		2629.0			
	11.6	5175.5	5174.00	1.323	0.03
		5173.0			
		5173.5			
	23.2	9681.0	9680.30	0.818	0.01
		9680.5			
		9679.4			
	50.8	22257.0	22256.00	1.000	0.004
		22256.0			
		22255.0			

Table VI
Statistic parameters of the calibration curves

Statistic parameter	Obidoxime	Scopolamine	Atropine	Imipramine
B	75.93	131.50	127.69	435.55
A	267.86	55.2959	11.24	-13.44
r_{xy}	0.9959	0.9951	0.9999	0.9997
S_{xy}	246200.20	3462.43	11558.53	135055.70
S_x	56.94	5.13	9.51	17.61
S_y	4341.49	678.09	1214.93	7671.75
S_o^2	191970.00	5617.90	159.67	37065.77
S_o	438.14	74.95	12.64	192.52
S_b^2	11.84	42.67	0.35	17.07
S_b	3.44	6.53	0.59	4.13
t^*S_b	6.69	12.69	1.15	8.03
S_a^2	55655.69	1630.51	46.37	7035.22
S_a	235.91	40.38	6.81	83.88
t^*S_a	458.38	78.46	13.23	162.97

where:

B = slope; A = origin ordinate; r_{xy} = correlation coefficient; S_{xy} = dispersal xy values; S_x = dispersal x values; S_y = dispersal y values; S_o^2 = the all population dispersal for y values; S_o = standard deviation for the all population y values; S_b^2 = dispersal slope of the regression line; S_b = slope's standard deviation of the regression line; t^*S_b = B variation range; S_a^2 = origin ordinate dispersal; S_a = origin ordinate standard deviation; t^*S_a = A variation range.

For LLOQ the best results were obtained at 203 nm as it can be seen in figure 2.

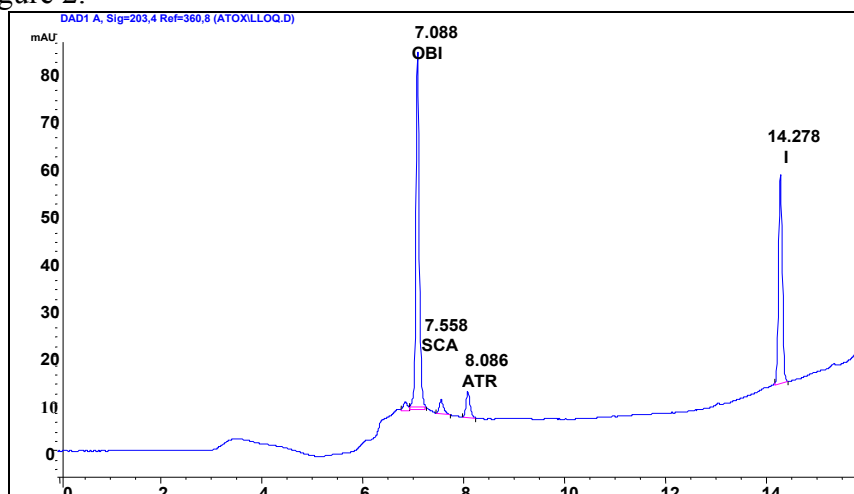


Figure 2
Chromatogram for LLOQ: OBI, SCA, ATR and I

Accuracy and Precision

In this work the precision was calculated for three concentration levels for each compound. Precision was measured using five spiked samples for each concentration level. Accuracy was calculated as deviation of the mean from the nominal concentration. Precision was expressed as the relative standard deviation (RSD) of each calculated concentration.

$$\text{Accuracy \%} = C_{\text{exp}}/C_{\text{th}} \times 100$$

where C_{exp} - experimental concentration; C_{th} – theoretical concentration.

C_{exp} was calculated from the calibration curve $y=mx+b$; C_{th} was the concentration for each analyte in the quality control (QC).

Table VII
Statistic parameters

Analyte	QC level	Theoretical Concentration (C_{th} µg/mL)	Statistic parameters
Obidoxime	QC1	305.000	Mean: 286.40 µg/mL; s= 1.292; RSD= 0.45 %; Recovery: 93.90%
	QC2	61.000	Mean: 73.98 µg/mL; s=0.062; RSD= 0.084 %; Recovery: 121.27%
	QC3	7.625	Mean: 8.02 µg/mL; s=0.157; RSD=1.96%; Recovery: 105.23%
	LLOQ	1.325	Mean: 1.47 µg/mL; s=0.084; RSD=5.74%; Recovery: 110.9%
Scopolamine	QC1	27.50	Mean: 25.51 µg/mL; s = 0.608; RSD= 2.38 %; Recovery: 92.75 %
	QC2	5.50	Mean: 5.99 µg/mL; s = 0.092; RSD= 1.54 %; Recovery: 108.95 %
	QC3	0.687	Mean: 0.62 µg/mL; s= 0.009; RSD= 1.52%; Recovery: 90.59 %
	LLOQ	0.1375	Mean: 0.12 µg/mL; s= 0.0053; RSD= 4.54 %; Recovery: 87.3 %
Atropine	QC1	51.00	Mean: 50.78 µg/mL; s= 0.3123; RSD= 0.62 %; Recovery: 99.57 %
	QC2	10.20	Mean: 10.076 µg/mL; s=0.056; RSD=0.56 %; Recovery: 98.78 %
	QC3	1.275	Mean: 1.266 µg/mL; s=0.005; RSD=0.43%; Recovery: 99.29%
	LLOQ	0.255	Mean: 0.208 µg/mL; s= 0.0068; RSD= 3.29 %; Recovery: 81.57 %
Imipramine	QC1	116.00	Mean: 114.792 µg/mL; s= 0.1134; RSD= 0.099%; Recovery: 98.96 %
	QC2	23.20	Mean: 22.502 µg/mL; s=0.027; RSD=0.12 %; Recovery: 96.99 %
	QC3	2.90	Mean: 22.502 µg/mL; s=0.027; RSD=0.12 %; Recovery: 96.99 %
	LLOQ	0.58	Mean: 0.56 µg/mL; s= 0.01193; RSD= 2.135 %; Recovery: 96.5 %

Stability

The results obtained demonstrated that the stock solutions prepared are stable for 24 h at 20°C. The stock solutions and the samples had to be kept into dark vials to avoid light degradation. The standard solutions were stable when stored at a temperature between 2-8°C for 6 months.

Conclusions

A HPLC method was developed and validated for the simultaneous determination and quantification of the compounds in the ATOX antidote. This chromatographic assay fulfilled all the requirements for being a reliable and feasible method, including linearity, accuracy, precision and stability applied to aqueous samples [10]. Therefore, this HPLC method can be used as a routine sample analysis.

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