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Application of solid phase microextraction to the determination of strychnine in blood

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Abstract

A simple and rapid method based on solid phase microextraction (SPME) via direct immersion followed by gas chromatography coupled with electron impact ionization/mass spectrometry (GC/EI-MS) was developed for the determination of strychnine in blood. Papaverine was used as internal standard (I.S.). Two types of fibre coating were tested, 100 μm polydimethylsiloxane and 65 μm Carbowax™/Divinylbenzene, the latter giving higher recoveries of the compound. The main factors affecting the SPME process, such as sample dilution (1:10), adsorption and desorption times (20 and 10 min, respectively), carry-over effect (not observed), pH and salt addition (no modifications on pH or salt concentration) were optimized. The procedure was validated in terms of linearity (r^2 = 0.9992 for concentrations ranging from 0.10 to 5.00 μg/mL), intra and interday precision (0.93 and 4.62%, respectively at 0.50 μg/mL; 3.33 and 8.06%, respectively at 2.50 μg/mL), sensitivity (6.83 and 8.91 ng/mL for LOD and LOQ, respectively) and extraction recovery (0.54 and 0.39% at 0.50 and 2.50 μg/mL, respectively). The developed procedure was found suitable for forensic investigations and was considered a good alternative to the liquid–liquid extraction methods normally used for the determination of this compound in biological media.

Keywords: Solid-phase microextraction; Strychnine

1. Introduction

Strychnine is an alkaloid which was first isolated from St. Ignatius beans (*Strychnos ignatii*) in 1818. However, its commercial source is the ripe dried seed of *Strychnos nux-vomica*, a tree native in India [1]. This compound acts in the central nervous system [2], by selectively blocking the post synaptic inhibitory activity of glycine [3]. Strychnine is rapidly absorbed from the gastrointestinal tract. There is very little protein binding [4], and its distribution to tissues is rapid. The major route for removal and detoxification is hepatic metabolism [5], via enzymatic degradation involving the liver microsomal system, and up to 20% of the drug can be excreted unchanged in the urine [4]. Soon after ingestion, violent and generalized convulsions take place, and when death occurs, it is commonly due to asphyxia from respiratory arrest during convulsions [6]. Serum levels of 0.075–0.1 μg/mL are reported to produce toxic effects, whereas concentrations of 0.2–2 μg/mL have been associated to fatalities [7].

Several methods are described for the determination of strychnine, using paper chromatography after dissolution of the alkaloidal extract from Nux-vomica seed in chloroform [8], or liquid–liquid extraction followed by either thin-layer chromatography [9], high performance thin-layer chromatography [10], gas chromatography [2,6,11–15] or liquid chromatography [16–20].

Solid phase microextraction (SPME) was developed by Arthur and Pawliszyn in the early 1990 at the University of Waterloo (Ont., Canada) [21]. This technique displays

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some advantages when compared to other sample preparation methods, since it does not require the use of organic solvents, the handling of samples is easy and fast, and the equipment necessary to accomplish the analysis is simple [22]. This methodology has been used for the determination of several substances, such as pesticides [23–29], drugs of abuse [30–33], or medical substances [34,35], but its application to the determination of strychnine is not reported in the literature.

This paper describes a new method based on direct immersion SPME with gas chromatography/electron impact ionization mass spectrometry (GC/EI-MS) for the determination of strychnine in whole blood samples.

2. Experimental

2.1. Reagents and equipment

Analytical standards, crystallized strychnine and papaverine hydrochloride, were obtained from Merck Co. (Darmstadt, Germany) and Almirall laboratories (Barcelona, Spain), respectively.

Methanol (HPLC grade), phosphoric acid, sodium hydrogenphosphate and sodium chloride (analytical grade) were obtained from Merck Co.

Methanolic standard stock solutions at 1000 μg/mL were prepared and stored at 4 °C. Subsequently working solutions at 100 and 10 μg/mL for strychnine and at 10 μg/mL for papaverine [internal standard (I.S.)] were prepared in methanol.

Phosphate buffer solutions were prepared by mixing solutions of phosphoric acid and sodium hydrogenphosphate, in variable proportions, according to the desired pH [36].

The biological sample used was fresh human blood, obtained from the exceeding of the Portuguese Institute of Blood, preserved with citrate phosphate dextrose (1:7).

The SPME fibre holder for manual use, 100 μm polydimethylsiloxane (PDMS) and 65 μm Carbowax^{TM}/Divinylbenzene (CW/DVB) coated fibres were obtained from Supelco (Bellefonte, PA, USA).

Chromatographic analysis was performed using a 6890 Series gas chromatograph (Hewlett-Packard, Wilmington, DE, USA), equipped with a model 5973 mass selective detector (Hewlett-Packard, Palo Alto, CA, USA). A capillary column (12 m × 0.25 mm i.d., 0.25 μm film thickness) packed with 5% phenylmethylsiloxane (Ultra 2), supplied by J&W Scientific (Folsom, CA, USA), was used. The carrier gas was helium at a constant flow rate of 1 mL/min.

The GC oven temperature program started at 150 °C for 1 min, then raised by 35 °C/min to 200 °C, held for 1 min and finally elevated by 40 °C/min to 270 °C, where it was kept constant for 7 min. The injector port (splitless) and the detector temperatures were set to 240 and 280 °C, respectively. The mass spectrometer was operated with a filament current of 300 μA and an electron energy of 70 eV in the electron impact (EI) mode. Quantification was done in the selected ion monitoring (SIM) mode, and the monitored ions were 338, 324 and 308 for papaverine, and 334, 120 and 162 for strychnine.

The retention times were 7.69 and 10.73 min for papaverine and strychnine respectively, obtaining a good separation of both compounds.

2.2. Extraction procedure

New fibres were conditioned in the injector of the GC system as follows: PDMS fibres were heated at 250 °C for 30 min and CW/DVB at 220 °C for 30 min, according to the suppliers' specifications.

The parameters that could influence the extraction of strychnine, such as adsorption and desorption times, pH, ionic strength and agitation during the adsorption process were optimized preliminarily, as described in Section 3.1, and the final conditions were as follows.

After addition of 250 ng of papaverine (I.S.) to 100 μL of blood, a final volume of 1 mL was obtained with water. The sample was then agitated for 30 s, and a 65 μm CW/DVB coated fibre was directly immersed in it for 20 min. After extraction the fibre was thermally desorpted in the injection port of the GC system for a total period of 10 min (5 min before the run was started and for another 5 min after the run was started).

2.3. Validation procedure

The procedure was validated in terms of selectivity, linearity, precision, accuracy, sensitivity and absolute recovery. Selectivity was evaluated by analyzing a set of ten blank samples, and it was checked for interferences at the retention times and monitored ions. Calibration data were generated using spiked blood samples, and the calibration curve was established between 0.10 and 5.00 μg/mL. Precision was characterized in terms of relative standard deviation (R.S.D.%) by analyzing sets of six spiked blood samples at both low and high concentrations in the same day (intraday precision) and on 4 different days (interday precision). Accuracy was evaluated in terms of percentage error (RE%) between the measured and the spiked concentrations for all calibrators, and also for the intra and interday precision assays; the limits of acceptable variability were set at 15% for all concentrations. The limits of detection and quantification (LOD and LOQ, respectively) were determined using blank samples. LOD was defined as the mean calculated analyte concentration in 10 blank samples (the background noise was interpolated in the calibration curve) plus three times the standard deviation, and LOQ was defined as the mean value plus 10 times the standard deviation.

Absolute recovery was calculated at both low and high concentrations by comparing peak areas of spiked samples with those obtained from splitless injections of methanolic solutions of strychnine.

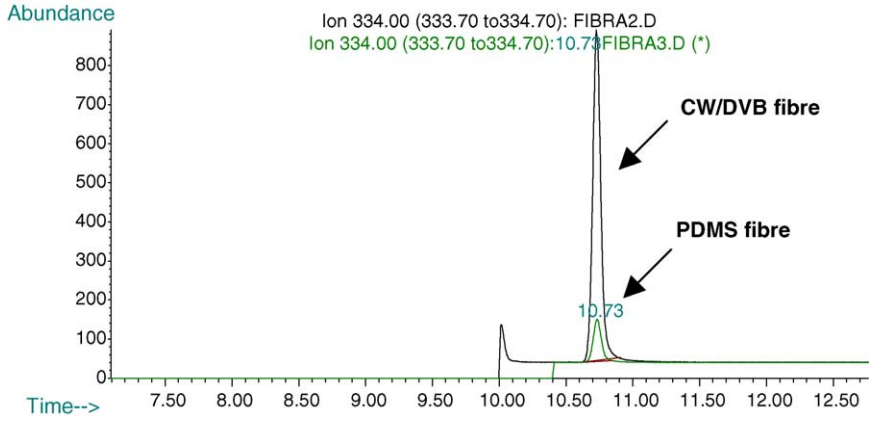

Fig. 1. Merged fragmentograms (ion 334) obtained with both coated fibres in the extraction of strychnine at 1 μg/mL.

3. Results and discussion

3.1. Optimization of SPME

In the present work, sample dilution, adsorption and desorption times, pH, ionic strength and sample agitation during adsorption were optimized ($n = 3$).

Two types of fibre coating were tested: the 100 μm PDMS and the 65 μm CW/DVB. PDMS fibre was excluded because of very low recovery, when compared with CW/DVB (Fig. 1).

3.1.1. Dilution of the sample

In order to study the influence of matrix constituents on the extraction yield, blood was diluted with water prior to the extraction. Dilution factors of 2, 5 and 10 were tested (500, 200 and 100 μL of blood were spiked with 1 μg of strychnine, and a final volume of 1 mL was achieved by addition of water).

The best extraction yield was obtained using a sample volume of 100 μL, e.g., applying to the sample a dilution factor of 10. This dilution factor also originated better resolved chromatograms, and therefore a sample volume of 100 μL was chosen to carry out this work.

3.1.2. Adsorption and desorption times

Solid phase microextraction is an equilibrium process that involves partitioning of analytes between two phases: an aqueous or gaseous phase (the sample) and a solid phase (the fibre coating). Therefore, the optimization of the contact time between these two phases is crucial. The influence of the adsorption time on the extraction yield was evaluated using aqueous solutions of strychnine at 1 μg/mL. To find the best adsorption time, the fibre was directly immersed in the sample for 1, 5, 10, 15, 20, 30 and 45 min, and peak areas obtained for each extraction time were compared. Equilibrium was reached after 20 min of contact. Longer adsorption times did not significantly improve the extraction yield, and therefore the selected time for adsorption was 20 min.

The optimization of desorption time aims at achieving the complete desorption of the adsorbed analyte, to improve sensitivity, and simultaneously avoid carry-over effects.

After extraction, the fibre was desorpted in the injection port of the GC for 1, 2, 3, 5 and 6 min before the run was started. On the basis of peak areas evaluation the best desorption time was 5 min. To test for carry-over effects, further desorption times were tested. After 5 min of desorption, the run was started, and the fibre was left in the injector for additional time before it was retracted (1, 3, 5 and 6 min). The largest peak areas were obtained for a total desorption time of 10 min (5 min before and 5 min after the run was started). Carry-over effects were not observed after adopting this procedure, even at concentrations twice superior to the highest calibrator of the calibration curve.

3.1.3. pH and ionic strength

To enhance the extraction of organic analytes from aqueous matrices it is common to use pH adjustment and salting. The pH of the sample has an important role in SPME, and a basic pH is expected to improve the extraction of a basic analyte [37]. The effect of pH on the extraction yield was evaluated by diluting spiked blood samples at 1 μg/mL in phosphate buffer 0.07 M (pH 5–8) instead of water. The peak areas obtained for each of the pH values were compared. The largest amount of strychnine was extracted at pH 8, as it would be expected, since strychnine is a basic analyte. However, the dilution of the sample with water yielded larger recovery of the compound, which may have been due to a possible effect of an increase in salt concentration derived from the phosphate buffer. In fact, if the analyte is in dissociated form, a decrease in the amount extracted may be observed when the salt concentration is increased, since the activity coefficient of the ionic species in the aqueous matrix increases with the increase of the sample's ionic strength [37]. In the case of strychnine, the compound would be mainly in dissociated form, since its pK_a values are 2.3 and 8.0 [38], being negatively affected by the increase in salt concentration. It was not possible to convert strychnine into its neutral form

because, as the maximum pH value allowed for this type of coated fibre is 9, higher pH values could not be tested, since the lifetime of the fibre would be dramatically decreased. Therefore, this study was carried out diluting the sample with water.

The effect of ionic strength on extraction efficiency was evaluated by analyzing the amount of strychnine extracted in sample solutions containing 0, 0.375, 0.75, 1.5 and 3% of sodium chloride. The extracted amount decayed with the increase of salt concentration in the sample, which corroborates the results obtained in the pH experiments.

3.1.4. Sample agitation during the adsorption process

To evaluate the influence of agitation on the extracted amount of strychnine, spiked blood samples at 1 μg/mL were analyzed with, and without agitation during the adsorption process, and both peak areas were compared. Sample agitation did not improve significantly the extraction yield.

3.2. Validation of the analytical method

Spiked blood samples were analyzed by direct immersion of the 65 μm CW/DVB coated fibre for 20 min without agitation. After this period of time, the fibre was retracted and desorpted in the injector of the GC during 5 min before the run was started and for further 5 min after the run was started.

3.2.1. Selectivity

Selectivity, as the ability of the method to distinguish between the analyte and matrix constituents, was checked analysing blank samples ($n = 10$). No interferences by matrix constituents at the retention times and selected ions of strychnine and papaverine were observed (Fig. 2).

3.2.2. Calibration curve and linearity

The calibration curve (peak area ratio between analyte and I.S. versus analyte concentration) was established in spiked blood samples, prepared and analyzed using the above mentioned procedure, between 0.10 and 5.00 μg/mL. The correlation coefficient of the calibration curve was 0.9992. The data related to the calibration curve and linearity are listed in Table 1.

Table 1
Validation data of the calibration curve

Concentration	R.S.D. (%)	Regression line	r^2	RE (%)
0.10	7.43	$y = 0.3976x - 0.0018$	0.9992	10.44
0.25	7.22			−12.97
0.50	4.60			−2.93
1.00	6.65			−2.21
1.50	7.36			−2.43
2.50	8.80			3.80
4.00	7.07			−1.93
5.00	8.31			0.13

Concentrations expressed in μg/mL ($n = 6$); R.S.D.: relative standard deviation; RE: relative error.

3.2.3. Limits of detection and quantification

Theoretical limits of detection and quantification (LOD and LOQ, respectively) were determined using blank samples ($n = 10$), as described in Section 2.3.

The obtained values were 6.83 and 8.91 ng/mL for LOD and LOQ, respectively.

3.2.4. Intra- and interday precision

To evaluate the intraday precision a set of six spiked blood samples at both low and high concentrations (0.50 and 2.50 μg/mL) was analyzed in the same day using the above mentioned procedure. The samples were quantified and the coefficient of variation (CV) was calculated. The CVs were 0.93 and 3.33% at 0.50 and 2.50 μg/mL, respectively.

Interday precision was calculated by analyzing sets of six spiked blood samples at 0.50 and 2.50 μg/mL on 4 consecutive days. The procedure was the same used above. The calculated CVs were 4.62 and 8.06% for 0.50 and 2.50 μg/mL, respectively.

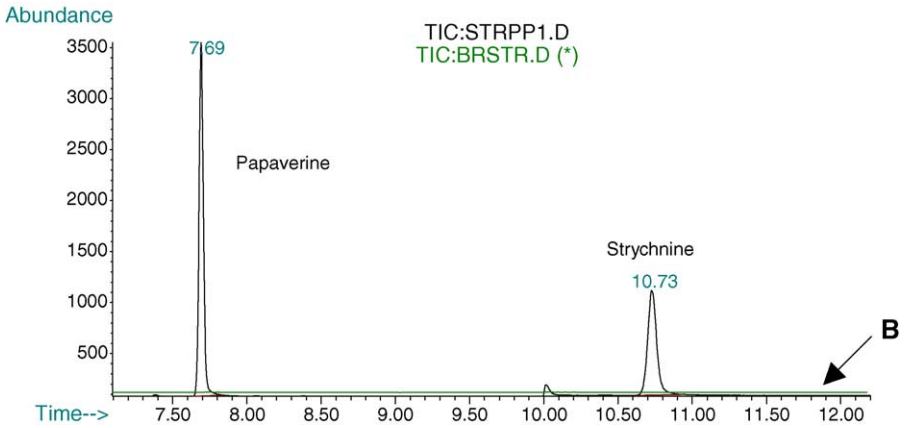

Fig. 2. Merged chromatograms obtained from a spiked blood sample at 1 μg/mL of strychnine and from a blank blood sample (B).

Table 2
Validation data

Concentration (μg/mL)	Intraday precision ($n = 6$)				Interday precision ($n = 6$)				Recovery (%)	LOD (ng/mL)	LOQ (ng/mL)
	Spiked	Found	CV	RE	Spiked	Found	CV	RE			
0.50	0.50	0.48	0.93	−3.34	0.50	0.47	4.62	−5.11	0.54	6.83	8.91
2.50	2.50	2.38	3.33	−4.80	2.50	2.49	8.06	−0.40	0.39		

CV: coefficient of variation (%); RE: relative error (%).

Table 3
Results obtained by applying the proposed method to three authentic samples

	Strychnine (μg/mL)
Case 1	2.39
Case 2	1.27
Case 3	1.03

3.2.5. Absolute recovery

To calculate the absolute recovery of the described method, sets of six spiked samples at 0.50 and 2.50 μg/mL were analyzed, and peak areas were compared with those obtained from a splitless injection of 1 μL of methanolic solutions of strychnine at 10 and 50 ng/1 μL, respectively. Because strychnine amount in the methanolic solutions was 50 times lower than the theoretically extracted by the fibre (if analyte recovery was 100%), it was necessary to apply a factor of 50 to peak areas obtained with the methanolic solutions.

Absolute recovery was calculated for both concentrations according to the formula:

$$\text{Absolute recovery} = \frac{\text{SPME peak area}}{\text{Methanolic solution peak area} \times 50} \times 100$$

The calculated values were 0.54 and 0.39% at the concentrations of 0.50 and 2.50 μg/mL, respectively. These recovery values are low when compared with other described in the literature for the determination of strychnine in blood [2,10]. However, this finding is quite normal, since recoveries reported for SPME are in general considerably lower than those observed for liquid–liquid extractions [19].

Table 2 resumes, LOD, LOQ, precision and accuracy, and absolute recovery at both low and high concentrations.

3.2.6. Application to authentic samples

After being optimized and validated, the described method was then applied to three real blood samples belonging to persons intoxicated with strychnine, which were obtained from the Laboratories of Forensic Toxicology from the Delegations of Coimbra and Lisbon of the National Institute of Legal Medicine in Portugal (Table 3).

4. Conclusions

Direct immersion SPME coupled with GC/MS-EI has proved to be a fast, simple, solvent-free method for the determination of strychnine in blood samples. Since the methodology is selective, linear within the studied range, precise, accurate and sensitive, we may conclude that it can be successfully applied to forensic cases, in spite of the low extraction recovery presented. Also, its fastness when compared to other techniques, and the fact that no organic solvents are required to accomplish the analysis, make this method a good alternative to the conventional extraction procedures normally used for the determination of this compound in biological media.

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