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TECHNICAL NOTE

TOXICOLOGY

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Development of a Simple and Low-Cost Enzymatic Methodology for Quantitative Analysis of Carbamates in Meat Samples of Forensic Interest

ABSTRACT: Foods contaminated with a granulated material similar to Temik (a commercial pesticide formulation containing the carbamate insecticide aldicarb) are often involved in accidental ingestion, suicides, and homicides in Brazil. We developed a simple technique to detect aldicarb. This technique is based on the inhibition of a stable preparation of the enzyme acetylcholinesterase, and it is specially adapted for forensic purposes. It comprises an initial extraction step with the solvent methylene chloride followed by a colorimetric acetylcholinesterase assay. We propose that results of testing contaminated forensic samples be expressed in aldicarb equivalents because, even though all other carbamates are also potent enzyme inhibitors, aldicarb is the contaminant most frequently found in forensic samples. This method is rapid (several samples can be run in a period of 2 h) and low cost. This method also proved to be precise and accurate, detecting concentrations as low as 40 μg/kg of aldicarb in meat samples.

KEYWORDS: forensic science, carbamates, acetylcholinesterase, forensic analysis, aldicarb, meat

Aldicarb (propanal, 2-methyl-2-(methylthio)-*O*-[(methyl-amino) carbonyl] oxime) is a systemic carbamate used in agriculture as an insecticide and nematocide. It has an oral LD_{50} of 0.9 mg/kg for rats, and it is poisonous to humans when they are exposed to it by dermal, oral, or subcutaneous routes. Aldicarb is currently sold as a dust-free, granulated material (Temik15—AVENTIS CROPSCIENCE). Carbamates, like organophosphates, are potent cholinesterase inhibitors. However, carbamates have a shorter half-life in organisms and a reversible toxic action (1).

Foods contaminated with a granulated substance similar to Temik are frequently involved in accidental ingestion, suicides, and homicides. Over the last 2 years, 157 cases involving aldicarb-contaminated commercial products, and liquid or solid foodstuffs (meat, coffee, milk, soup, juice, cookies, and chocolate) were reported in the State of Rio de Janeiro. Last year (2008), the majority of criminal events that involved materials contaminated with aldicarb were suicide and homicide events (Table 1). This event profile reflects the potential lethal effects of this praguicide.

The Forensic Laboratory in Rio de Janeiro (Criminalistic Institute, Carlos Éboli - RJ) is currently developing techniques to detect the presence of aldicarb in food samples. The methods being employed are liquid–liquid extraction followed by thin-layer chromatography as previously described (2). However, such foods as milk and meat are difficult to analyze because of their high fat content.

Several techniques are available for the detection of carbamates in foods. They usually include a liquid–liquid, solid phase, or supercritical fluid extraction (3,4) followed by gas or liquid chromatography (5–7). These instrumental techniques require sophisticated analytical structures and are expensive and time consuming. Enzymatic techniques based on the inhibition of the enzyme acetylcholinesterase, the biological target of carbamate pesticides, are also described. Some use amperometric sensors with immobilized cholinesterase (8–11) or an engineered enzyme to increase its sensibility (9). Most of these techniques were developed for the analysis of water samples.

The goal of this study is to detect the presence of aldicarb and other cholinesterase inhibitors in meat products for forensic purposes. The method proposed in this study involves a simple liquid–liquid extraction step followed by an enzymatic analysis using a freeze-dried preparation of the enzyme acetylcholinesterase obtained from rat brain. This method could also be adapted to detect thionophosphate insecticides (12,13). The proposed methodology can detect concentrations of aldicarb as low as 40 μg/kg with precision and accuracy, and it represents a quite inexpensive, simple, and rapid alternate approach for the forensic analysis of aldicarb and other cholinesterase inhibitors in meat products.

Materials and Methods

Reagents and Solvents

The enzyme substrate acetylthiocholine, color reagent sodium dithionitrobenzoate (DTNB) and Triton X-100 were purchased from

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TABLE 1—*Criminal events involving aldicarb intoxication registered at the Criminal Institute, Carlos Éboli during the year of 2008 in the city of Rio de Janeiro.*

Causes	Number of Cases
Suicides	26
Suicide attempts	2
Homicide attempts	12
Crimes against animals	7
Suspect death	1
Undetermined	3
Captured	4
Total	55

Sigma Chemicals Co. (St. Louis, MO). Anhydrous sodium sulfate P.A. and HPLC grade methylene chloride were obtained from Merch KGaA (Darmstadt, Germany). The analytical standard aldicarb (122.3 μg/mL in methanol) was purchased from Dr Ehrenstorfer GmbH, Germany.

Aldicarb Extraction from Meat

Beef samples were ground up in a high-speed grinder, and 5 g portions were weighed out. Some portions were fortified by vigorous mixing with adequate volumes of the aldicarb standard as described later. The extraction step was a modification of a previously described method (14). Ground meat aliquots (5.0 g) were blended at maximum speed in a Vortex-Genie II apparatus (Scientific Industries, NY) for 2 min with 20 mL of distilled water and centrifuged at 5 × *g* at 8°C for 20 min. Supernatant layers were collected and filtered through Whatman No.1 filter paper under vacuum to remove fat and fiber. A 12.5-mL aliquot from each filtrate was transferred to a separatory funnel and extracted by vigorous mixing with 10 mL of methylene chloride. After phase separation, most of the methylene chloride bottom layer was collected in a 25-mL Erlenmeyer flask with a Teflon cap and mixed with 3 g of anhydrous sodium sulfate. Following sodium sulfate decantation, a 0.5-mL aliquot of the dried methylene chloride extract was transferred to a glass test tube (1 × 10 cm) and completely evaporated in a 46°C water bath under a stream of pure nitrogen.

Acetylcholinesterase Preparation

The acetylcholinesterase enriched fraction was prepared by detergent extraction from a rat brain particulate fraction, in a method previously described (15). In this case, the Triton X-100 concentration was increased to 4 g% instead of 0.5 g%, which substantially increased the stability of the enzyme preparation. The protein concentration was determined according to Peterson's method (16), and the preparation was diluted about five times with 0.2 M sodium phosphate buffer (pH 7.5), which contained 4% Triton X-100 to give a final protein concentration of 2 mg/mL. Aliquots (10 mL) of the final acetylcholinesterase preparation were freeze-dried and kept in sealed flasks under nitrogen atmosphere. Each dried aliquot was suspended in 10 mL of distilled water when used. Under these conditions, dried preparations could be stored for more than 6 months, and refrigerated (8°C) suspended preparations for about 3–4 weeks without losing their properties.

Acetylcholinesterase Assay

In total, 20 μL of the suspended enzyme preparation and 105 μL of 0.2 M sodium phosphate buffer (without detergent) were added to each test tube containing the extracted residue. Tubes were sealed with Teflon films, vigorously mixed in the Vortex apparatus and incubated at 37°C for 30 min. Measurement of the acetylcholinesterase activity was based on the Elmann's colorimetric method (17). After incubation, 250 μL of the color reagent (1.0 mM sodium DTNB in 0.2 M sodium phosphate buffer, pH 7.5) was added and, after mixing, the tube contents were poured into a 1-cm light pass spectrophotometer cuvette with a reading capacity of 600 μL. Then, 250 μL of the substrate solution (1.25 mM acetylthiocholine in the phosphate buffer) was added to the cuvette and mixed. Cuvettes were immediately read at a wavelength of 412 nm for 3 min using the kinetic mode of a Spectronic Genesis 2 spectrophotometer (Spectronic Industries Inc., USA). Increases in the absorbance per min (ΔA/min), which expresses the acetylcholinesterase activity, were recorded.

Calibration Curves and Recovery of Aldicarb after Extraction

The direct inhibition curve of aldicarb (reference curve) was made by adding, to 1 × 10 cm test tubes, appropriate amounts of the carbamate standard conveniently diluted with the phosphate buffer to 20 μL of the enzyme preparation, and 0.2 M phosphate buffer to reach a volume of 125 μL. In these incubation mixtures, 0.05, 0.10, 0.20, and 0.30 μg/mL aldicarb concentrations were tested. Controls (100% enzyme activity) contained only the enzyme and phosphate buffer.

The calibration curve of aldicarb fortified and extracted samples was constructed as follows: 5 g portions of uncontaminated ground beef were mixed with small volumes of diluted aldicarb standards to give final aldicarb concentrations of 0.40, 0.80, 0.16, and 0.25 μg/g. These samples corresponded to aldicarb concentrations of 0.04, 0.08, 0.16, and 0.25 μg/mL, respectively, in the enzyme incubation mixture because of dilution and concentration steps during the extraction process. Percent recovery of aldicarb was calculated by plugging in the percent inhibition obtained for fortified meat samples into the linear regression equation of the reference curve, and using the percent enzyme inhibition obtained for the same concentration in the reference curve as the 100% marker.

Intraday and Interday Precision and Accuracy

To evaluate intraday precision and accuracy, concentrations used in the extracted calibration curve were analyzed in quadruplicate. Precision was determined as the %CV (coefficient of variation) of the enzyme inhibition calculated for each of the quadruplicates. Accuracy was evaluated by calculating the concentration of each extracted sample using a calibration curve that was run on the same day and determining, for each quadruplicate, the percent deviation from the expected concentration. To determine interday precision and accuracy, extracted samples were prepared during four separate weeks. Precision was determined as the %CV of the concentration measured for each sample from these 4 weeks. Accuracy was evaluated by calculating the percent difference between the nominal concentration and the mean concentration calculated for similar samples extracted during these 4 weeks. Limits of acceptable intraday and interday precision and accuracy were set at <15% CV and ±15% deviation from expected, respectively.

Limit of Quantification

The limit of quantification (LOQ) was set as the minimum compound concentration that could be quantified with an acceptable level of precision of ≤15% and accuracy of ±15%. LOQ samples were analyzed as unknown samples on nine different days.

Stability of Samples During Storage

For stability studies, 100 g of uncontaminated ground beef was fortified with the aldicarb standard, to give a concentration of 0.20 μg/g. After homogenization, aliquots containing 5 g of meat were taken. Some of them were analyzed immediately, some were stored frozen (−20°C), some were kept refrigerated (8°C), and others were maintained at room temperature (*c.* 24–28°C). Percent enzyme inhibitions of stored samples were reevaluated 30 days later. Controls of uncontaminated meat were also stored under the same conditions stated above.

Quantification of Real Samples

The analyte concentration in real forensic samples was calculated by inverse regression using the aldicarb calibration curve for fortified samples. We expressed the results of testing contaminated forensic samples in μg/g of aldicarb equivalents as all carbamates are also potent inhibitors of the cholinesterase enzyme. The enzymatic method described previously was not able to identify the chemical nature of the organophosphate in forensic samples.

Results and Discussion

Calibration Curves

The linearity of the curves was confirmed over the aldicarb concentration ranges used in both fortified meat (0.04–0.25 μg/mL) and reference samples (0.05–0.30 μg/mL) as shown in Figs. 1 and 2, respectively. Each time, assays were performed in triplicate, using four different concentrations on four different days. Typical regression equations for fortified samples and reference calibration curves were $y = 96.06 + 23.24x$ ($r^2 = 0.9925$) and $y = 90.62 + 22.13x$ ($r^2 = 0.9987$), respectively.

Positive forensic samples will most likely be contaminated with much higher enzyme inhibitor levels than the maximum concentration shown in calibration curves. For this reason, the rest of the methylene chloride extracts of unknown samples must be saved until the 0.5-mL aliquots initially taken for analysis are completely processed. This will allow for repetition of assays using convenient dilutions of extracts.

Extraction Efficiency—Aldicarb is often extracted from vegetables and other low-fat foods using acetone or ethyl acetate. Methylene chloride was selected because of its properties of lower polarity and small water carryover from the organic matrix as this method focuses on fat soluble pesticides. Homogenization and sampling strategies have been thoroughly discussed in relation to preparation of food samples for chromatographic analysis (14). Such studies have shown that 5 g or more is required for testing portions using conventional noncryogenic homogenization, therefore, a test portion of 5 g was chosen for this study. Table 2 shows recovery data obtained after the extraction of aldicarb from meat at different fortification levels. As shown in this table, recoveries of aldicarb were close to 100% with this extraction method.

Evaluation of Matrix Interference

Enzyme activities (expressed as ΔA/min) of control assays (containing only enzyme and buffer) and assays of uncontaminated meat extracts were compared. Results (mean ± SD of five different experiments) were 0.140 ± 0.0036 and 0.137 ± 0.0057, respectively. Therefore, under the conditions proposed for this method,

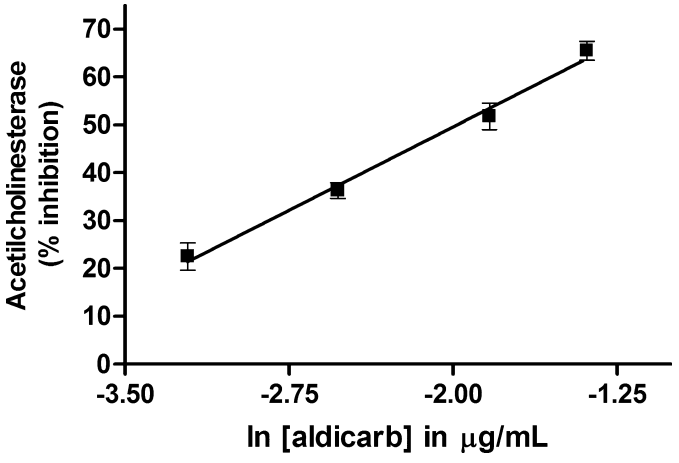

FIG. 1—*Linear regression analysis of the calibration curve for extracted meat samples. Natural logarithm values (ln) of nominal concentrations were used as the independent variable and the % enzyme inhibition as the dependent variable. Each point is the mean ± SD of four experiments performed on different days.*

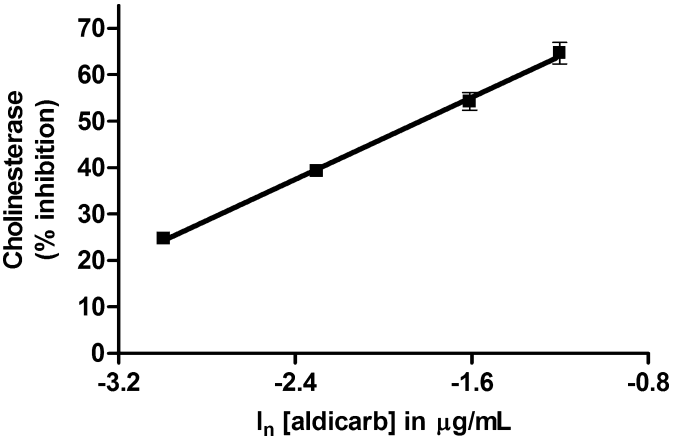

FIG. 2—*Linear regression analysis of the direct inhibition curve for aldicarb (reference calibration curve). Natural logarithm values (ln) of the nominal concentrations were used as the independent variable and the % enzyme inhibition as the dependent variable. Each point is the mean ± SD of four experiments performed on different days.*

TABLE 2—*Recovery index for aldicarb in raw meat samples.*

Fortified Concentration (μg/mL)	Recovered Concentration* (μg/mL)	Recovery Index (%)†
0.04	0.039 ± 0.06	97.5
0.08	0.086 ± 0.013	107.5
0.16	0.156 ± 0.024	97.5
0.25	0.275 ± 0.020	110.0

*Recovered concentration was determined by calculating the concentration of each extracted sample using the linear regression equation of a direct reference calibration curve run on the same day. Each value is the mean of four experiments performed on different days ± SD.

†Mean percent recovery from the expected concentration.

TABLE 3—*Characterization of the limit of quantification (LOQ)*.*

Day of Analysis	Enzyme Inhibition (%)* Mean ± SD (*n*=5)	Relative Standard Deviation (%)
1	21.76 ± 3.23	14.84
2	21.02 ± 2.61	12.41
3	20.13 ± 2.22	11.02
4	21.01 ± 2.74	13.04
5	21.01 ± 1.97	9.37
6	21.78 ± 2.21	10.14
7	23.02 ± 2.42	10.51
8	20.04 ± 4.67	23.30
9	20.41 ± 1.97	9.65
Mean	21.13 ± 0.94	4.44

*Meat portions fortified with 40 μg/kg of aldicarb were analyzed on nine different days.

Control values for uncontaminated meat samples (100% enzyme activity) were recorded each day.

TABLE 4—*Influence of sample storage conditions on aldicarb decay.*

Sample Storage Condition	Enzyme Inhibition (%) Mean ± SD (*n*=4)	% Recovery After Storage
Control (1st day)	60.98 ± 5.16	–
Room temperature	23.49 ± 1.75	38.5
Refrigerated (8°C)	48.32 ± 2.1	79.2
Frozen (−20°C)	60.66 ± 1.24	99.4

A meat portion was fortified with 200 μg/kg of aldicarb. Some aliquots were assayed immediately for cholinesterase inhibition (controls), while others were stored under different conditions and assayed 30 days later.

matrix components and solvent impurity interferences are negligible.

Intraday and Interday Precision and Accuracy

Intraday precision were 2.34% (lower limit of detection [LOD]), 2.16%, 2.84%, 2.26% based on % R.S.D. (Relative Standard Deviation) values for extracted samples containing 0.04, 0.08, 0.16, and 0.25 μg/mL of aldicarb, respectively. Interday precision were 4.58% lower limit of detection [LOD], 9.76%, 12.98%, and 7.05% based on % R.S.D. values for extracted samples also containing 0.04, 0.08, 0.16, and 0.25 μg/mL, respectively.

Limit of Quantification

The LOQ, defined as the lowest quantifiable concentration on the calibration curve at which both accuracy and precision should be within the maximum tolerable CV of 15% expressed as the % R.S.D., was deemed to be 0.04 μg/mL (40 ppb). Table 3 shows the mean enzymatic percent inhibition and the % R.S.D. obtained when the LOQ concentration was assayed in quintuplicate on nine different days. This LOQ is adequate for the analysis of forensic samples, as this value falls within the concentration range of maximum allowable levels of aldicarb residues in many foods.

Stability of Stored Meat Samples During Storage—If samples cannot be analyzed just after collection, they must be stored frozen (−20°C). As shown in Table 4, this is the only condition under which the inhibitory capacity of aldicarb remains intact after 1 month.

Reagents can be prepared as a kit containing three sealed flasks: freeze-dried acetylcholinesterase preparation, powdered substrate (both flasks must be vacuum sealed or kept in nitrogen atmosphere), and color reagent solution (highly stable). Under these storage conditions, reagents remain stable for at least 6 months at room temperature. After solubilization with the adequate volume of water, the enzyme preparation must be stored refrigerated and the substrate frozen. Soluble preparations can be reused within the next 3–4 weeks.

Quantification of Real Samples

Two real forensic samples of sausage supposedly contaminated with aldicarb were used to test the performance of the aforementioned method. Methylene chloride extracts of both samples had to be diluted 10^5 times with the solvent to fall within the calibration curve for fortified samples. Results showed that the two samples of sausage were contaminated with 5.8 and 22.1 μg/g aldicarb equivalents, respectively, which could cause the death of an adult human in a few minutes even if only small portions are ingested.

The method is evidently not specific for aldicarb. All other carbamates are potent acetylcholinesterase inhibitors and would also be efficiently detected by this method. Unpublished data from our laboratory showed that the inhibitory potencies of some important carbamates, as judged by carbamate concentrations in aqueous solutions (extracted with methylene chloride and processed by our methodology) that inhibit 20% of the enzyme activity, are in parts per billion. Inhibitory potencies are approximately: 3 for carbofuran, 9 for propoxur, 11 for carbaril, 36 for metomil, 40 for pirimicarb, and 40 for aldicarb. Therefore, taking into account that aldicarb is the cholinesterase inhibitor most frequently found in forensic samples, we propose that the results analysis of such unknown samples be expressed in aldicarb equivalents. If considered necessary, chromatographic techniques can be used to identify the specific contaminating inhibitor.

Conclusions

The proposed method is sufficiently precise and accurate. It is also a low-cost technique and a rapid technique (several samples can be processed in a 2 h period) demanding only basic laboratory equipments and glassware. Furthermore, it can be easily adapted for other food materials. The method was proved to be effective for detecting the presence of aldicarb and other potent cholinesterase inhibitors in forensic samples of fatty foods. The novelty of the approach lies in the clean-up achieved through the combined use of gravimetrical fat removal and liquid–liquid extraction of a fatty matrix. Under the proposed conditions, it is quite improbable that compounds other than the well-known cholinesterase inhibitors would interfere with enzymatic activity.

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