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Deltamethrin Binding to *Triatoma infestans* (Hemiptera: Reduviidae) Lipoproteins. Analysis by Solvent Bar Microextraction Coupled to Gas Chromatography

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ABSTRACT The binding of deltamethrin (DLM) to the hemipteran *Triatoma infestans* (Klug) hemolymph lipoproteins was evaluated in vitro. After DLM incubation with the insect hemolymph, lipoproteins were fractioned by ultracentrifugation. DLM binding was analyzed by a microextractive technique—solvent bar microextraction—a solventless methodology to extract DLM from each lipoprotein fraction. This is a novel use of the technique applied to extract an insecticide from an insect fluid. Capillary gas chromatography with microelectron capture detection was used to detect DLM bound by the *T. infestans* hemolymph lipoproteins and to identify the preferred DLM carrier. We show that Lp and VHDLp I lipoproteins are mainly responsible for DLM transport in *T. infestans*, both in DLM-resistant and DLM-susceptible bugs. Our results also indicate that DLM amounts transported are not related to DLM susceptibility.

KEY WORDS pest control, deltamethrin, insecticide transport, insect lipoprotein

Current pest control strategies heavily rely on pyrethroid insecticides. A large number of studies have been addressed to understand their mechanism of action and toxicokinetics (Soderlund 1995, Davies et al. 2007). Regarding contact insecticides, a large piece of knowledge has been gained in toxicological effects, although the early stages of insecticide–insect contact has not been fully examined. The mode of entry and transport of the toxic molecules to the target site has been rather unexplored after pioneer studies by Gerolt (1983) and, although the carrier role of lipoproteins was early proposed, whether this mechanism of insecticide transport decreases or enhances their toxicity has not been addressed in insects. The extensive use of chemical insecticides paved the way to growing level of insecticide resistance in most insect pests (Hemingway and Ranson 2000, Soderlund 2008, Hardstone et al. 2009). A great deal of research has focused on detoxification mechanism, although much less efforts have been devoted to pharmacokinetic studies, both in mammals and insects (Kim et al. 2008). Understanding absorption and distribution mechanisms might help model the contribution of putative differential insecticide uptake, binding to lipoproteins, insecticide bioavailability, and distribution among internal tissues in insecticide-susceptible compared to insecticide-resistant insects. Intoxication and death of the insect target will depend not only on the applied dose and formulation, but on product stability and insect physiology, and among others, on its penetration through the insect cuticle. The primary vehicle of most contact insecticides through the insect body has been proposed to be the insect hemolymph. Earlier studies attempting to understand the penetration of insecticides through the insect cuticle and its transport to target organs showed that ^{14}C-DDT incubated with cockroach hemolymph was mostly bound to hemolymph proteins (Winter et al. 1975). Helling et al. (1986) showed that the binding of insecticides to the hemolymph proteins in *Manduca sexta* (L.) was related to polarity. Bioconcentration and water solubility of organic molecules are correlated to the octanol: water partition coefficient (K_{ow}). The log K_{ow} value is useful to represent the lipophilicity of a molecule. Insecticides of relatively low polarity and water solubility (DDT and parathion, log $K_{ow} \sim \geq 6$) were detected bound to high molecular weight protein fractions whereas those of higher polarity (carbaryl and propoxur, log $K_{ow} \leq 2$) were not bound to the proteins (Helling et al. 1986). Haunerland and Bowers (1986) reported that *Heliothis zea* lipoproteins showed rather unspecific binding of xenobiotics of different hydrophobicities.

Triatoma infestans (Klug) is the major Chagas disease vector in Argentina. Pyrethroids were shown to be effective against this insect, until growing reports of insecticide resistance and tolerance were reported (Mougabure-Cueto and Picollo 2015). Among mechanisms of deltamethrin (DLM) resistance studied in this bug, increased esterase detoxification and alterations of the target site have been reported (Fabro et al. 2012,

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Germano et al. 2012), together with higher amounts of both cuticle hydrocarbons and cuticle thickness, associated with reduced DLM penetration through the cuticle (Pedrini et al. 2009, Juárez et al. 2010).

In order to explore DLM transport to the target tissues via hemolymph, our aim was to discriminate the actual DLM carrier and to perform a preliminary comparison of the transport mechanism between DLM-resistant (R) and DLM-susceptible (S) bugs. To this end, a first step was to find and develop a suitable simple, fast, and reliable method to estimate DLM amounts in hemolymph. Insect hemolymph lipoproteins are named depending on their buoyant density as high density lipoprotein, namely, lipophorin (Lp), and very high density lipoproteins (VHDLps). Lp has multiple functions as reusable shuttle in the transport of diacylglycerols, hydrocarbons, and lipid-related compounds throughout the insect body by loading dietary and newly synthesized lipids to be utilized as metabolic fuel, and delivering them to other tissues (Gilbert and Chino 1974, Gu et al. 1995, Canavoso et al. 2001, Marinotti et al. 2006).

Fichera and Brenner (1982a) reported Lp (density 1.11–1.15 g/ml) and two VHDLps (density 1.19–1.23 and 1.25–1.26 g/ml, named VHDLp I and VHDLp II, respectively) in the hemolymph of adult males of the hemipteran *T. infestans*. Before ultracentrifugation techniques were applied to insect lipoprotein fractionation, in vivo and in vitro experiments showed the role of lipoproteins in xenobiotic binding, modulating their transport, metabolism, and elimination (Olson 1970, Winter et al. 1975, Helling et al. 1986).

It has been reported that binding depends on the insecticide lipophobicity, among other factors. Haunerland and Bowers (1986) showed that *H. zea* lipophorin binds preferentially compounds of high or low hydrophobicity, whereas arylphorin, a storage lipoprotein, binds more strongly insecticides of intermediate polarity. Other studies reported that lindane was mostly associated with the male Lp, although DLM was also additionally recovered in female vitellogenin (Breton et al. 1992).

Among most popular methods, assays for DLM and other pyrethroid chemical detection include gas chromatography (Meneghini et al. 2014) and high-performance liquid chromatography (HPLC) in a variety of substrates—foods, environmental samples, and various tissues, as revised by Kim et al. (2006). The relatively small size of insects compared to other organisms studied requires new or more sensitive methodologies to help detect minute amounts of insecticide at picogram levels. Traditional extraction methods usually require solvent extraction procedures with relatively high amounts of high purity organic solvents (Liu and Dasgupta 1996). To reduce solvent use, liquid–liquid microextraction techniques are being used increasingly, among them, hollow fiber liquid phase microextraction (HF-LPME; Shen and Lee 2002, Lin et al. 2011, Liu et al. 2012). Basically, all these techniques use some water-immiscible organic solvent in suspension within a larger aqueous volume. The major advantage of microextractive techniques is the use of barely microliter amounts of solvents; in addition, it does not require purification steps to extract DLM. Solvent bar microextraction (SBME) is a modification of the HF-LPME; the organic solvent is placed within a hollow fiber that is sealed in both ends. This "solvent bar" containing the extraction solvent is placed in a flask with aqueous solution and stirred (Jiang and Lee 2004).

In this study, we used SBME coupled to capillary gas chromatography (CGC) with microelectron capture detector (μECD) to measure the amount of DLM bound in vitro by hemolymph lipoproteins in *T. infestans*. We are not aware of previous reports of the application of this microextraction technique in insects. We show that Lp and VHDLp I are mainly responsible for DLM transport, and compared DLM transport between R and S bugs.

Materials and Methods

All animal care and laboratory experimental protocols were approved by the Directive Board of the INIBIOLP (Instituto de Investigaciones Bioquímicas de La Plata's Animal Welfare Assurance No. A5647–01) and carried out following the AVMA Animal Welfare Policies and AVMA Guidelines on Euthanasia: https://www.avma.org/kb/policies/pages/default.aspx, https://www.avma.org/KB/Policies/Documents/euthanasia.pdf, accessed 9 September 2015.

Insects. Male *T. infestans* of S and R colonies were reared at the INIBIOLP and fed weekly on rats at 30°C and 50–60% relative humidity under a photoperiod of 12:12 (L:D) h. Colonies are periodically renewed by incorporating first-generation insects, usually from Formosa province, provided by the Servicio Nacional de Chagas, Cordoba (DLM-susceptible) and from Salta province, provided by Dr Ruben Cardozo, National University Salta (DLM-resistant), both in Argentina (Pedrini et al. 2009).

Reagents and Materials. DLM (98%, Pestanal, analytical standard) and phenylmethylsulfonyl fluoride (≥98.5%, GC) were purchased from Sigma-Aldrich (St. Louis, MO). Acetone (ACS) was from Tedia (Fairfield, OH). Octanol (RPE) and other solvents came from Carlo Erba (Milan, Italy). Ultrapure water was obtained from Milli-Q reference system (Millipore, Bedford, MA). The Q3/2 Accurel polypropylene HF membrane (wall thickness 200 μm, wall pore size 0.2 μm, and inner diameter 600 μm) came from Membrana GmbH (Wuppertal, Germany).

Hemolymph Collection and DLM Incorporation. Three days after being fed, males from both colonies were ice-chilled and individually placed head down in 1.5-ml eppendorf tubes with the bottom cut-off enough to enable head and front legs come out the tube. The eppendorf was placed pending within a glass tube (12 by 100 mm^2) prior cutting off legs and head in order to collect hemolymph (25 μl/insect) after 5 min of centrifugation at 300 rpm at room temperature. Then, hemolymph samples of 20 insects each were placed in a refrigerated tube added with phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2 mM to inhibit hemolymph proteases. A solution of DLM in acetone (560 ng/μl) was incorporated to 600 μl of hemolymph and vigorously vortexed every 10 min for a

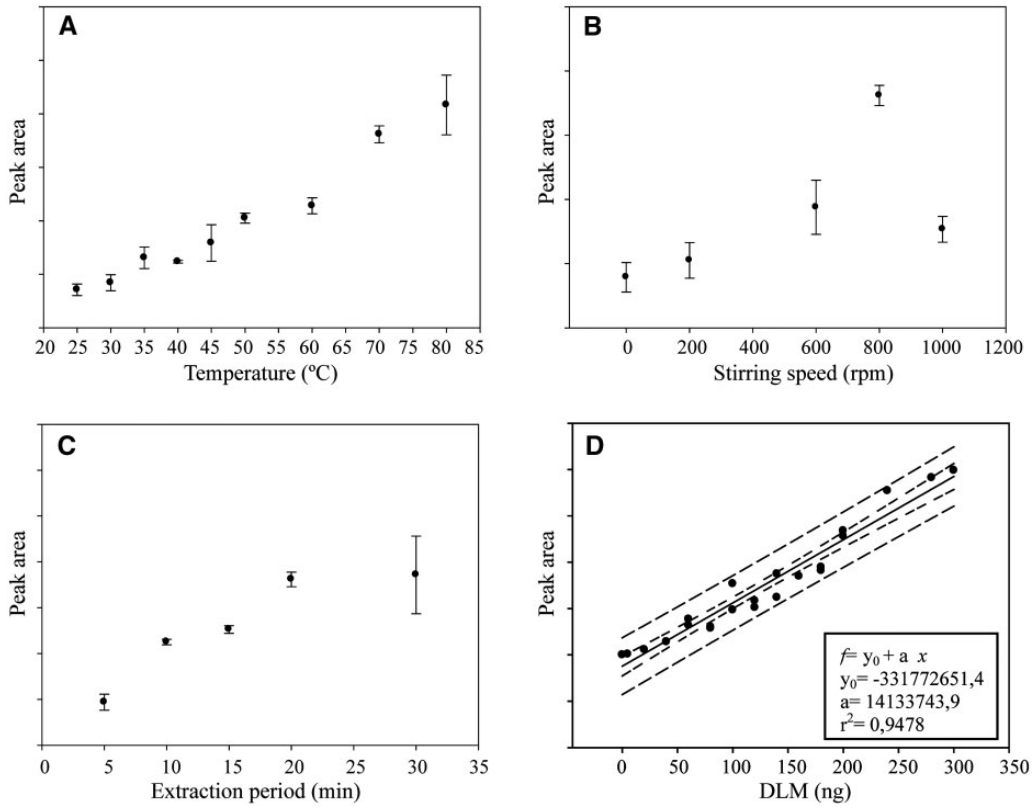

Fig 1. SBME optimization and validation. Evaluation of DLM extraction efficacy with temperature (A), stirring speed (B), and extraction time (C). Each point corresponds to the mean value of three replicates ($n = 3$). Linearity of DLM detection response (D) was investigated under optimized SBME conditions (65°C, 800 rpm, 20 min). Figure 1D shows 95% confidence band (–) and 95% prediction band (-).

period of 1 h at 30°C of temperature. Then, the tube was centrifuged for 10 min at 6,500 rpm to pellet the hemocytes. This procedure was performed separately both for R and S hemolymph samples.

Lipoprotein Fractionation. Hemolymph lipoproteins were isolated following the NaBr density-gradient ultracentrifugation method described by Rimoldi et al. (1999), and routinely used in this institute. The hemolymph supernatant was layered over 3 ml of NaBr solution (density 1.28 g/ml, 0.196 M NaCl, 0.01% EDTA) and was placed into a 4.4-ml polyallomer thin-walled tube (11 by 60 mm^2, Beckman). Centrifugation was performed at 45,000 rpm for 22 h at 4°C in a Beckman L8 70 M ultracentrifuge using a SW60Ti rotor (Beckman Coulter, Inc., Fullerton, CA). Fractions (100 µl per fraction) were collected from the top of the tube. Ten microliters of each fraction were used to monitor the presence of proteins by light absorption at 280 nm in an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences UK Ltd, Buckinghamshire, United Kingdom) and the remaining 90 µl were used for DLM estimation by solvent bar microextraction followed by gas chromatography analysis.

Solvent Bar Microextraction (SBME). The hollow fiber (HF) was washed in acetone, air dried, and finally impregnated by immersion in 1-octanol. The solvent bars were obtained by sealing each 12 mm of the HF membrane with a FS-200 heat sealing machine (Dasa, Argentina), and then cut (Wang et al. 2012). 1-Octanol was selected as solvent based on fiber compatibility, low water solubility, low vapor pressure, and gas chromatographic behavior (Shen and Lee 2002). Ninety microliters of each ultracentrifugation fraction were placed in a head-space vial (6 ml), and mixed with 150 µl of acetone and 2,760 µl of milliQ water to obtain a final volume of 3 ml. The extraction temperature was set at 65°C. Each solvent bar was placed into a vial and stirred (800 rpm) for 20 min, then removed from the solution and a 2-µl aliquot of the DLM-enriched solvent was taken out with a syringe (10 µl, Model 1701 N SYR Cemented, Hamilton, NV) and injected in the chromatograph.

Capillary Gas Chromatography (CGC)–Microelectron Capture Detector (µECD). CGC was performed in a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard, Wilmington, DE) operated in the splitless mode at 280°C. Samples were injected into a 4.0-mm-ID recessed double taper inlet liner (Restek Bellefonte, PA) and fitted with a nonpolar fused silica DB-5ht capillary column (length 30 m, inner diameter 0.32 mm, film thickness 0.10 µm; Agilent J&W, Santa Clara, CA), with helium as carrier gas.

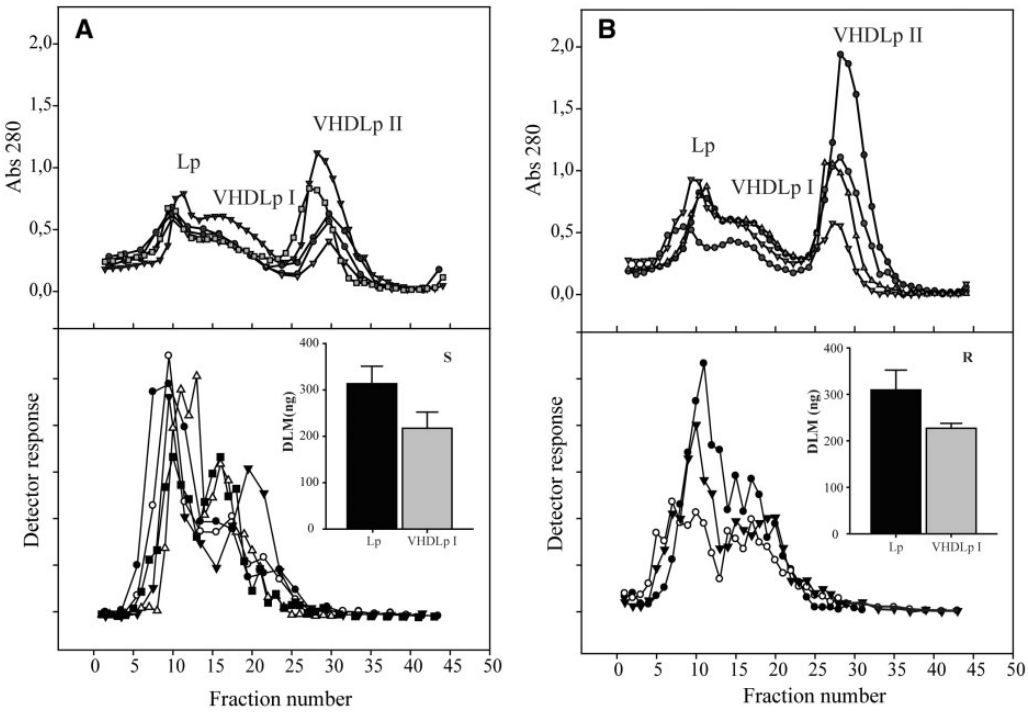

Fig. 2. Density-gradient ultracentrifugation profiles of S (A) and R (B) adult male *T. infestans* hemolymph lipoproteins after incubation with deltamethrin. One hundred-microliter ultracentrifugation fractions were separately collected for protein (absorbance 280 nm) and DLM (SBME-GC-μECD) measurement. (Top) Absorbance profile of S (A) and R (B) lipoprotein fractions. (Bottom) DLM distribution in S (A) and R (B) lipoprotein fractions. The inset shows DLM amounts associated to Lp (S: 313.42 ± 84.12 ng; R: 309.52 ± 70.14 ng) and VHDLp I (S: 217.27 ± 95.18 ng; R: 227.26 ± 20.80 ng); no significant differences were detected between S and R samples. Data corresponds to $n = 5$ (S) and $n = 4$ (R) hemolymph samples prepared as described in Materials and Methods. Symbols correspond to different hemolymph samples.

Oven temperature was programmed from 150°C to 300°C at 15°C/min. The μECD was held at 320°C. DLM peak areas were calculated for each chromatogram (HP ChemStation, Hewlett-Packard) and expressed as normalized area. The peak identity was confirmed by comparison with a DLM standard.

SBME Optimization and Validation. A DLM standard solution was prepared with 0.8 ng DLM in 200 μl of acetone and then added to a saline solution (3,800 μl); salt concentration was similar to that used in hemolymph protein fractionation. Experiments were carried out at different temperatures (25–80°C), stirring speeds (up to 1,000 rpm), and extraction periods (5–40 min), to evaluate their effect on extraction efficiency. The performance and reliability of the SBME-CGC-μECD method was studied by determining the limit of detection (LOD), limit of quantification (LOQ), and the linearity range for DLM at the same conditions as described above. The linearity of the detection response was investigated within a DLM concentration range between 5 to 300 ng under the optimized SBME conditions. To establish the LOD, the amounts of DLM used ranged from 0.05 to 0.4 ng, diluted in similar conditions as described above.

Statistical Analysis. The amount of DLM bound to R and S lipoproteins was compared using the nonparametric Mann–Whitney U test (Instat 3.10, GraphPad Software Inc., La Jolla, CA, 2009).

Results and Discussion

SBME Optimization and Validation. Figure 1 shows the effect of temperature, stirring speed, and extraction time for SBME optimization. Temperature plays a major role in the extraction process because it influences mass transfer rates and the partition coefficients of DLM. Linear response and low dispersion were observed below 70°C; thus, a temperature of 65°C was selected for DLM extraction (Fig. 1A). Stirring facilitates DLM extraction from the aqueous phase; a stirring speed of 800 rpm was chosen because extraction efficiency diminished at higher speed probably due to tumbling of the solvent bar in the aqueous solution leading to solvent loss (Fig. 1B). An extraction period of 20 min was selected because at longer extraction periods (30 min) we observed organic phase loss into the aqueous phase affecting DLM measurement (Fig. 1C). Linearity of the response was measured up to 300 ng and the detection limit was 0.05 ng, with a signal/noise ratio = 3 (Fig. 1D). A simple and sensitive microextraction method (SBME) coupled to CGC-μECD is here shown to be useful to measure

small amounts of insecticide in insect hemolymph avoiding solvent consumption and purification steps. This solventless technique can be applied to other xenobiotics, after appropriate response calibration, and given the high sensitivity it could be also used in small insects. Among other applications, it can be also used to follow insecticide penetration in target tissues.

Deltamethrin Binding by *T. infestans* Hemolymph. Ultracentrifugation of adult male *T. infestans* hemolymph showed the characteristic lipoprotein profile (Fichera and Brenner 1982a, 1982b; Rimoldi et al. 1997) with Lp, VHDLp I, and VHDLp II components, both in S and R insects (Fig. 2). DLM amounts in each lipoprotein fraction were estimated using the linearity curve of Fig. 1D by adding the DLM amounts of all fractions corresponding to each peak. DLM was detected bound to the Lp- and VHDLp I-enriched fractions in an approximate 60/40 ratio, with similar distribution profile between R and S bugs. No DLM was associated to VHDL II (Fig. 2). Similar amounts of DLM were bound by R and S insects ($P = 0.90$). DLM is a lipophilic compound of high molecular weight with a $K_{ow} = 4$. (Tomlin 1994). The differential lipoprotein binding can be related to DLM lipophilicity. In addition to the internal core of phospholipids common to the three lipoproteins, both Lp and VHDL I have a similar lipid composition transporting large amounts of diacylglycerols; differently, VHDLp II mostly transports nonpolar components (i.e., hydrocarbons; Fichera and Brenner 1982b). Thus, although DLM resistance in *T. infestans* was shown to be associated to cuticle width enlargement (Pedrini et al. 2009), and DLM penetration in S bugs was >2-fold than that detected in R bugs (Juárez et al. 2010), DLM binding to lipoproteins does not seem to contribute to this mechanism. However, we are far from fully understanding insecticide penetration and traffic through insect body; further studies will be addressed both in vivo to follow DLM catabolism by cuticle enzymes and at the molecular level in order to identify the cuticle proteins participating in the penetration resistance mechanism.

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