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Exposure of Chlorpyrifos Coated Long-Lasting Insecticidal Bed Net into the Artificial Saliva and Determination of Residual Concentration Using LC-MS/MS

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Abstract Malaria is one among the major public health problems with an estimated million cases worldwide every year. WHO recommends the use of insecticide-treated nets, particularly long-lasting insecticidal nets (LLIN), to reduce malarial deaths [1]. The treated bed nets have been factory incorporated and coated with long-lasting insecticidal Nets (LLINs) to protect people from malaria and also reduce incidence of malaria in a population [2]. Pyrethroid insecticides have been extensively used for the treatment of nets to protect against malaria and other vector-borne diseases. The new LLIN products are under the development and its requiring assessment of risks to humans. Which have been shown to be cost-effective to reduce malaria for children (< 5 years of age). Striving plans for their extensive use in worldwide for self-protection from mosquitoes [3], prevention from malaria and another severe disease. The health risks from the LLINs have not been sufficiently investigated and reported in the peer-reviewed scientific literature [4,5]. Here, we use a probabilistic risk assessment approach to estimate the risks when a child chewing the pesticide treated nets while sleeping under the bed nets [6]. The experiment was conducted in artificial biological fluid to determine the amount of potential active to be leaching into the biological fluid which is artificial saliva during overnight contact. The concentration of active ingredient content release proportion from the long-lasting insecticidal net into the artificial saliva was determined by using LC-MS/MS [7,8]. During the leaching experiment at predetermined intervals at 0.5, 2, 4, 8, 12, 16, and 24 hours after immersing in artificial saliva at 36±2℃.

Keywords: *LC-MS/MS, leaching, LLIN, pesticide, residue, household, malaria, MRM, risk of LLIN*

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1. Introduction

Insecticide coated long-lasting Nets (LLIN) is the usage of protection from a mosquito that has been revealed to reduce malaria, sickness, and death due to malaria in rife regions [1]. Especially Insecticide-treated bed nets have been shown to reduce the malaria of children and fragment women [2]. Bed nets are protecting the people from a mosquito while sleeping under them. However, bed nets treated with a pesticide are significantly more defensive than untreated nets [3,4]. The insecticides that are utilized for treating bed nets that protect and killed the insects. The mosquitoes enter the house and attempt to feed on the child also reduced. In addition, the number of mosquitoes and their length of life will be decreased [5,6]. Striving plans for their extensive use in worldwide for self-protection from mosquitoes and prevention from malaria and another severe disease. The health risks from the LLINs have not been sufficiently investigated and reported in the peer-reviewed scientific literature. We use a probabilistic risk assessment approach to estimate the residues when a child chewing the nets while sleeping under the bed nets. The laboratory experiment was conducted in artificial biological fluid to determine the amount of potential active to be leaching into the artificial saliva during overnight. The concentration of active ingredient content release proportion from the long-lasting insecticidal net into the artificial saliva and sweat were determined by using LC-MS/MS [7,8]. During the leaching experiment at predetermined intervals at 2, 4, 8, 12, 16, and 24 hours after immersing in artificial saliva and sweating at 36±2℃.

2. Materials and Methods

2.1. Materials and Reagents

Mass Spectrometry grade reagents were used in the experiments. Optima LCMS grade acetonitrile (purity 99.9%) and Formic acid (purity 99.5%) were procured from Fisher chemical (United States), Heptane (Fraction from petroleum) (Purity 99%) was purchased by S.D. Fine Chemicals, Mumbai and Chlorpyrifos standards was purchased from Sigma-Aldrich (Buchs, Switzerland). The water was produced Milli-Q purification system. Artificial saliva purchased from Pickering solutions (California, USA). The rectangular insecticide (chlorpyrifos) coated long lasting insecticidal nets samples were procured from Local market.

2.2. Calibration Standards

A stock solution of Chlorpyrifos reference standard was prepared by weighing 50.15 mg of 99.3% pure reference standard into a 50 mL volumetric flask and bringing it to volume with acetonitrile. A series of calibration solutions were then prepared by diluting the appropriate volume of various stock solutions into different 10 mL flasks and bringing to volume with acetonitrile. The prepared calibration solutions were 0.1 ng/mL, 1.0 ng/mL, 5.0 ng/mL, 10 ng/mL, 20 ng/mL and 50 ng/mL and analyzed by LC-MS/MS chromatography. A linear curve was plotted for the concentration of standard versus observed peak area and the correlation coefficient was determined. The calibration shown in Figure 1. The LOD of the method was determined based on the minimum signal to noise ratio 3:1. The LOQ was determined based on the recovery study and minimum signal to noise ratio 10:1.

2.3. Precision and Assay Accuracy

The system precision was determined initially by injecting six times a typical standard solution prepared at 0.005 µg/mL concentrations. The precision of the method was determined by analyzing the relative standard deviation in the observed area. The analytical method was validated for the recovery of Chlorpyrifos at two concentration levels (n=5) one at LOQ (0.001 µg/mL) and the other at a higher concentration (0.01 µg/mL) level in artificial saliva. A 0.1 mL aliquots of the 0.1 µg/mL (stock solution 1) solution were transferred to the 5 different 10 mL flasks and volume was made up to mark with artificial saliva, resulting in the final concentration of 0.001 µg/mL. A second concentration was prepared by transferring 0.1 mL aliquots of the 1.0 µg/mL to the 5 different 10 mL of flasks and volume was made up to mark with artificial saliva resulting in a final concentration of 0.01 µg/mL.

2.4. Methods

Two replicate net samples were used for the experiment and the sampling of long lasting net sample was done by cutting five pieces of 50x50 cm at five different places in duplicate sampling (Side-1, Side-2, Roof-3, Side-4 and Side-5) as per the WHOPES [World Health Organization Pesticide Evaluation Scheme- Use of FAO and WHO specifications for pesticides - 8.21]. The samples were coded as S1, S2, S3, S4 and S5. Five pieces representing one from each side of net were exposed to the artificial saliva. The five cut pieces (50 x 50 cm) of Chlorpyrifos 13g/kg coated onto polyester filaments of Long Lasting Insecticidal Net was subjected to leaching test by immersing the five pieces of LLIN material into five separate 250 mL glass containers containing 200 mL of artificial saliva. Ensured that the net samples were fully in contact with biological fluid. The artificial saliva samples were coded as S1, S2, S3, S4 and S5. The flasks were kept shaking continuously for 18 hours to mimic the action of chewing and sucking. During the tests, the biological fluid in the beaker was maintained at a specific temperature of 36 ± 2℃. While the solutions in the flasks were in contact with the net sample a 10 mL volume of sample solution was withdrawn from each flask for analysis at different pre-determined intervals 0.5, 2, 4, 6, 12 and 18 hours. The samples were coded as S1, S2, S3, S4 and S5 with a prefix of time interval and with saliva. The collected saliva samples were partitioned three times with 10 mL portions of heptane. The heptane layer was combined and transferred into a round bottomed flask and dried near to dryness under nitrogen stream.

The sample solutions were reconstituted with 10.0 mL of acetonitrile solvent. A small quantity of sample solution was filtered through the 0.45 µm PTFE syringe filters and injected to the LC-MS/MS. The leaching concentrations of Chlorpyrifos 13g/kg coated onto polyester filaments of Long Lasting Insecticidal Net in to the artificial saliva were determined by analysing the duplicate injections of each sample solution along with a standard solution using LC-MS/MS.

The extract was analyzed using triple-quad Mass spectrometer in Multi reaction monitoring (MRM) mode. The analysis of Chlorpyrifos content in artificial saliva was performed using a Agilent 6490 triple-quad Mass spectrometer interfaced with Agilent 1290 Infinity series UHPLC with MassHunter software. The mass spectra operated in the positive Agilent jet stream Electro spray Ionization (AJS-ESI) mode. The Separation was achieved on a Zorbax Eclipse Plus C18 reversed phase column with 50 mm length x 3.0 mm i.d., x 1.8µ Particle size. The mobile phase compositions of 0.1% formic acid in water 40% and 0.1% formic acid in Acetonitrile (60%) at a flow rate of 0.8 mL·min–1 were used. The column oven temperature was operated at 45℃. The total running time was 6 min. The MS operating conditions were Gas Temperature 270℃, Gas Flow 11 L·min–1, Nebulizer gas flow 35 Psi, Sheath gas temperature 350℃, Sheath gas flow 11 L·min–1 and Capillary voltage 3500 V.

The MS/MS instrument was operated in the Multiple Reaction Monitoring mode (MRM). The pseudomolecular ions of the analytes $[M+H]^+$ were selected by the first quadrupole. These precursor ions were impulsed with Nitrogen gas in the collision cell (second quadrupole) and the resulting fragment ions (product ions) were separated according to their m/z ratio in the third quadrupole. Two of these product ions were selected: one product ion (MRM-transition) serving for quantitation ion m/z 350→198.0 with 24v of collision energy and the second for confirmation ion m/z 350→125.0 with 16v of collision energy in a positive mode of analysis.

3. Results and Discussion

3.1. Calibration Curve

A plot of the peak area and the concentration of Chlorpyrifos showed acceptable linearity for the quantifier ion (350.0→198.0), as indicated by the correlation coefficient of 0.9999. A representative calibration curve is presented in Figure 1 and representative overlaid chromatograms have been presented in Figure 2. The limit of detection and limit of quantification for Chlorpyrifos were established at 0.0001 µg/mL and 0.001 µg/mL based on the minimum signal to noise ratio (S/N) 3:1 and 10: 1. The ion ration between quantifier and qualifier ion are well within the limit (<30%).

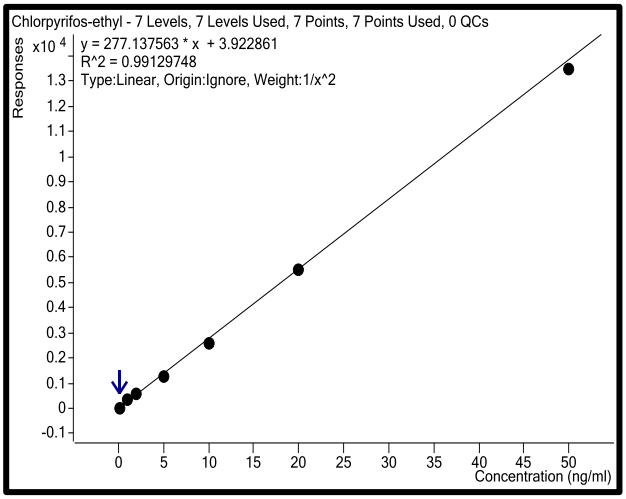

Figure 1. Calibration curve

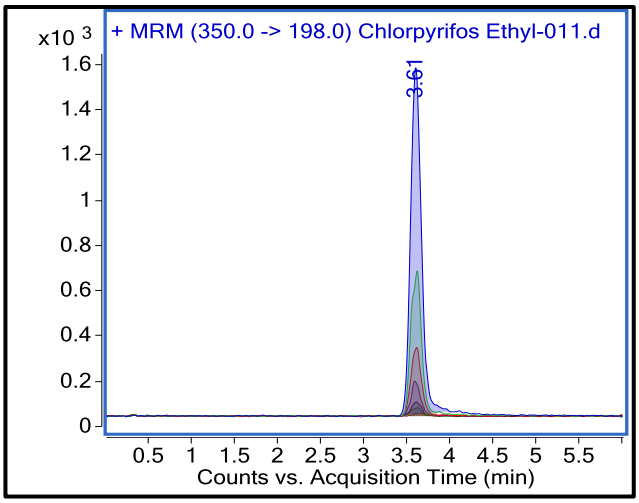

Figure 2. Overlaid chromatograms of calibration

3.2. Precision and Assay accuracy

The relative standard deviation of peak area for the 6 measurements of a mid-range standard was 1.90%. This indicated an acceptable system precision. The results of multiple recoveries in control artificial saliva samples were studied at two fortification levels equivalent to 0.001 µg/mL and 0.01 µg/mL of Chlorpyrifos standard. The results are presented in Table 1. The overall assay accuracy of standard at LOQ level and at higher level was 97.96 and 95.63% respectively. The standard deviation at LOQ level and higher level were 0.69 and 1.66 an acceptable method of analysis. The data presented in the Table 2 and the representative Chromatogram of blank artificial saliva and LOQ level is presented in Figure 3 and Figure 4.

Table 1. System Precision - Chlorpyrifos

Sample Code	Peak Area (Counts*Sec)	Slope	Intercept	Concentration of injected sample (µg/mL)	Average Recovery (%)	Standard Deviation	Relative Standard Deviation (%)
S1R1	2245	441544.84	-55.16	0.0052	0.0051	0.0001	1.90
S1R2	2176	441544.84	-55.16	0.0051			
S1R3	2187	441544.84	-55.16	0.0051			
S1R4	2182	441544.84	-55.16	0.0051			
S1R5	2116	441544.84	-55.16	0.0049			
S1R6	2154	441544.84	-55.16	0.0050			

Table 2. Assay Accuracy - Chlorpyrifos

Sample Code	Peak Area (Counts*Sec)	Slope	Intercept	Concentration of Injected Sample (µg/mL)	Fortified Concentration (µg/mL)	Recovery (%)	Average Recovery (%)	Standard Deviation
S0R1	-	441544.84	-55.16	-	-	-	-	-
S0R2	-	441544.84	-55.16	-	-	-	-	-
S1R1	375	441544.84	-55.16	0.0011	0.001	97.42	97.96	0.69
S1R2	379	441544.84	-55.16	0.0011	0.001	98.33		
S1R3	382	441544.84	-55.16	0.0011	0.001	99.01		
S1R4	376	441544.84	-55.16	0.0011	0.001	97.65		
S1R5	375	441544.84	-55.16	0.0011	0.001	97.42		
S2R1	4201	441544.84	-55.16	0.0096	0.01	96.39	95.63	1.66
S2R2	4200	441544.84	-55.16	0.0096	0.01	96.37		
S2R3	4125	441544.84	-55.16	0.0095	0.01	94.67		
S2R4	4248	441544.84	-55.16	0.0097	0.01	97.46		
S2R5	4062	441544.84	-55.16	0.0093	0.01	93.24		

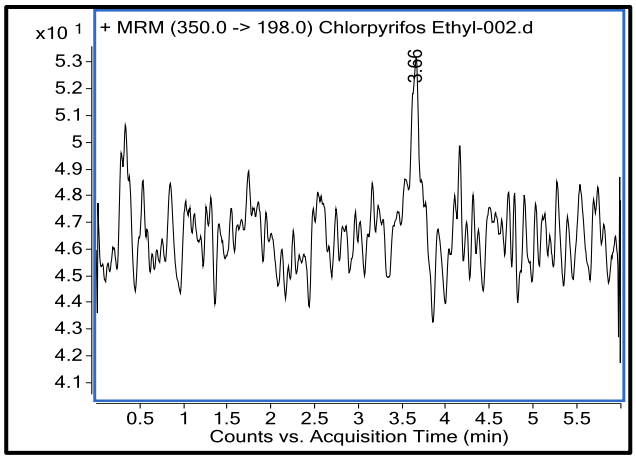

Figure 3. Control Saliva extracts chromatogram (m/z 350->198.0)

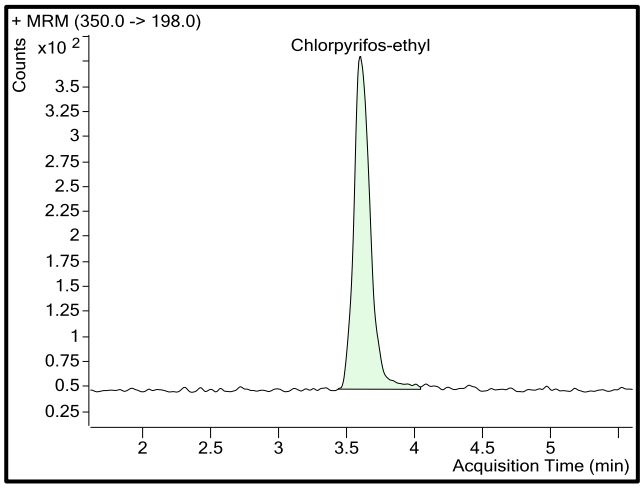

Figure 4. LOQ Level chromatogram (m/z 350->198.0)

3.3. Determination of Chlorpyrifos Residue in Artificial Saliva-Leaching Test

The leaching of residues in Artificial saliva from long lasting insecticidal net (LLIN) coated with Chlorpyrifos was studied using the representative net samples cut from different positions. The study was conducted at the predetermined intervals of 0.5, 2, 4, 6, 12 and 24 hours at 36 ± 2℃ of five pieces from each panel determinations were conducted. The average leaching residues of five replications of Chlorpyrifos in Artificial saliva from the side panel position 1, side panel position 2, roof panel position 3, side panel position 4 and side panel position 5 were determined. Each concentration was assayed by LC-MS/MS and the standard deviation was calculated. From the observed area, Y (the recovery Leaching potential) was calculated using the linear regression equation y = mx + b, the slopes and intercept (b) were derived from the linear curve. The individual results are presented in Table 3, the representative results and chromatograms are presented in Figure 5 to Figure 7.

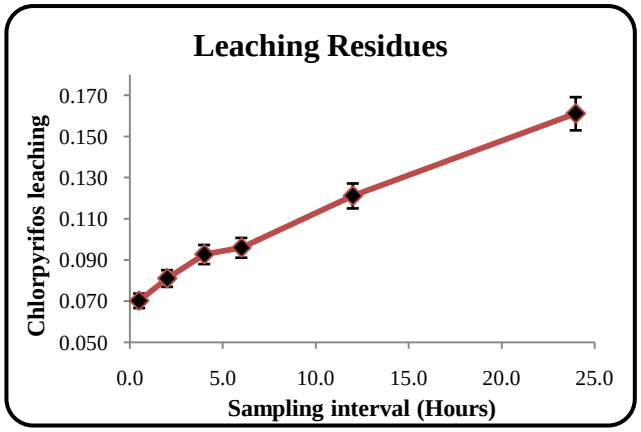

Figure 5. Trent of the leaching potential in artificial saliva

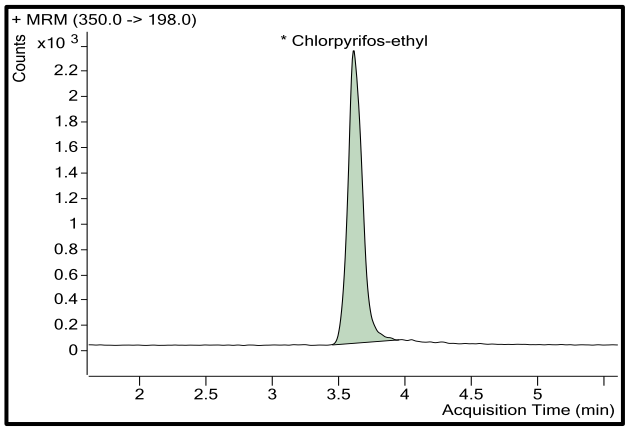

Figure 6. Representative Chromatogram for the leaching sample (m/z 350->198.0)

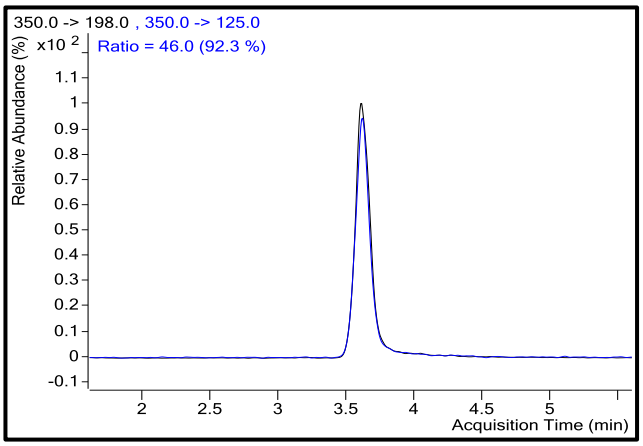

Figure 7. Representative Chromatogram for the leaching sample (m/z 350->198.0 and m/z 350->125.0)

Table 3. Chlorpyrifos Residues in Biological fluid (Artificial)

Sample Occasions (Hours)	Side panel position 1	Side panel position 2	Roof panel position 1	Side panel position 4	Side panel position 5	Average Residue in %
0.5	0.0687	0.0700	0.0725	0.0703	0.0699	0.0703
2.0	0.0835	0.0833	0.0830	0.0774	0.0783	0.0811
4.0	0.0917	0.0925	0.0958	0.0925	0.0915	0.0928
6.0	0.0975	0.0952	0.0961	0.0975	0.0938	0.0960
12.0	0.1210	0.1244	0.1157	0.1197	0.1254	0.1212
24.0	0.1602	0.1606	0.1642	0.1626	0.1581	0.1611

The leaching residues of chlorpyrifos in artificial saliva was determined. The chlorpyrifos coated long lasting insecticidal net (LLIN) samples representing the sides and roof panel positions were exposed to the artificial saliva at 36±2℃ and collected the samples at predetermined intervals were analyzed for the leaching residual concentrations of chlorpyrifos by the LC-MS/MS method.

4. Conclusion

The leaching residues of chlorpyrifos in artificial saliva was determined. The chlorpyrifos coated long lasting insecticidal net (LLIN) samples representing the sides and roof panel positions were exposed to the artificial saliva at 36±2℃ and collected the samples at predetermined intervals of 0.5, 2, 4, 8, 12, 16, and 24 hours and analyzed for the leaching residual concentrations of chlorpyrifos by the LC-MS/MS method. The concentration of the content release proportion range between 0.0703 % to 0.1611 % with respect to the 24 Hours immerses in the artificial saliva. The residual concentrations of the pesticides were used for the WHO generic risk assessment model for insecticide treated nets.

Acknowledgements

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