Assessment of malathion and its effects on leukocytes in human blood samples

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Abstract

In the present paper, we report a reproducible, cost effective, fast response method for detection of malathion and its effects on leukocytes in different human blood groups. Spectroscopic methods (UV-Vis spectrometry) and Fourier transform infrared coupled with solid phase extraction were applied for analyzing malathion content in human blood plasma. The spiking levels of malathion in the range of 0.1-1.7 μg/mL were extracted from blood plasma samples using SPE. The present active functional groups (C=O; P-O-C; -OH; P=S) were also characterized. The recovery rate of malathion was 80% ± 4.5%. The calculated correlation coefficient was 0.9799, indicating the linearity of the results. The limit of detection (LOD) and limit of quantification (LOQ) were (0.1-1.7) μg/mL and (0.3-1.5) μg/mL, respectively. Malathion <1.0 μg/mL showed no significant change while higher levels of malathion exposure (1.5 μg/mL and 3.0 μg/mL) reduced the number of white blood cells. In conclusion, the spectroscopic results may be useful to understand the mechanism of other pesticides such as methyl parathion and para-thion.

Keywords: leukocytes, malathion, solid phase extraction, UV-Vis spectroscopy, Fourier transform infrared, chromatography

Introduction

Malathion, an organophosphorus pesticide, may affect human blood cells through breathing or absorption through the skin. Leukocytes carry out specific effector functions related to homeostasis as well as to the host response to infection and inflammation. The efficient accomplishment of these functions requires a finely regulated cellular cytoskeleton to enable a marked reorganization of the leukocyte membrane, receptor localization, recruitment of signaling intermediates and changes in the morphology of the cell, as well as the induction and/or inhibition of cellular programs that lead to activation, proliferation, survival and differentiation. Some insecticidal organic compounds such as methyl parathion, dichlorodiphenyltrichloroethylene (DDT), ethyl parathion, malathion, and hexachlorocyclohexane cause toxicities to humans[1-6]. For example, malathion dose-dependently causes chromosomal aberrations in human peripheral leukocytes[7]. A significant increase in the number of micronucleated cells were also found in isolated lymphocytes exposed to high doses (75-100 μg/mL) of malathion[8].

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We previously reported a low-cost, sensitive colorimetric sensor instrument for quantitative analysis of methyl parathion \(^9\). Few spectroscopic techniques related to the determination of diazinon \(^{10-11}\) and fenthion \(^{12}\) have been reported. The different spectroscopic techniques are reported to determine insecticides, herbicides and chlorinated compounds \(^{12-14}\). The detection of pesticides in human blood is reported using sophisticated instruments such as high performance liquid chromatography and gas chromatography \(^{15-21}\). The extraction process and their conditions are reported in the literature \(^{22-23}\). The amount of a particular insecticide in various environments is considered to be the interest of the mode of detection by researchers using sophisticated techniques and developed sensors \(^{24-29}\). However, all these have several demerits such as wastage of excess solvents, time consuming, cost effectiveness, and the required expertise to operate these systems. The reported steps for analysis of the compound in urine/blood samples are well reported \(^{30-31}\). Previous studies have been shown the applicability of spectrometry, usually UV-Vis, FTIR and FT-Raman, to the analysis of insecticides in agrochemical formulations \(^{32-34}\).

The objective of the present work was to determine the concentration of malathion and analyze its effects on leukocytes in human blood using spectroscopic techniques via solid phase extraction (SPE) steps and to calibrate the quantitative results of malathion obtained by UV-Visible absorbance/refractive index. A comparative study was also done by gas chromatography-nitrogen phosphorus detector (GC-NPD) to support the validity of the spectroscopic method.

Materials and methods

Chemicals

Malathion was obtained from Dr. Ehrenstofer (Promochem, Wesel, Germany) and used without further purification. Three stock standard solution mixtures (50 μg/mL) were prepared and further diluted to make the required concentration samples of malathion (0.1 mg/mL-1.7 μg/mL) for analysis. The solvents and other added compounds such as methanol, n-hexane, methyl tert-butyl ether (MTBE), sodium hydroxide and hydroxyl ammonium hydrochloride used for extraction steps and colorimetric process were purchased from Merck, Germany.

Apparatus

A Hitachi U-2800 double beam spectrophotometer (Tokyo, Japan) with UV solutions 2.1 software was used for recording UV-Vis spectra and absorption measurements for quantitative analysis. A hemocytometer with a cover-slip (improved Neubauer) was used to count white blood cells (WBCs) in different blood samples. A Perkin-Elmer autosystem model RS-1 spectrometer (USIC, Delhi University, Delhi, India) was used for recording all IR spectra before and after the reaction process that shows functional group characterization. All IR spectra were obtained by the KBr technique. A Gas Chromatography-Nitrogen Phosphorus detector (GC-NPD) Perkin Elmer Clarus 500, PE Auto-System GC with built-in Autosampler with TURBOMASS Program was used for correlation of UV-Vis data.

Sample collection and preparation

We collected blood samples from subjects who did not take drugs that may affect on the counting of WBCs in the present study. Malathion was prepared in methanol at 10 μg/mL. Malathion samples of 0.1 mg/mL-1.7 μg/mL were prepared using serial dilution method with the help of micro-pipettes (Eppendorf-AG, Hamburg Germany). Human blood plasma samples were collected from healthy subjects aged 25 to 40 years in a 5 mL glass lithium heparin vacutainer. After centrifugation at 3500 rpm for 15 minutes, the supernatant was saved in a 10 mL glass vial at -20°C. WBCs were isolated through centrifugation as described by Balaji et al. \(^7\).

Solid phase extraction

A malathion containing 10 mL sample (2 mL serum diluted with 8 mL water) was passed through a 500 mg C\(_{18}\) Cartridge previously conditioned by passage of 5 mL methanol, 5 mL methyl tert-butyl ether (MTBE) in (1:1), and 3 mL deionized water, avoiding dryness. After loading the sample, cartridges were washed with 3 mL deionized water. The sample flow-rate was adjusted to approximately 6-8 mL/min. The cartridge was dried by passage of air by use of a vacuum for 10-15 min and compounds were then eluted with 5 mL MTBE \(^{21-22}\). The extract was evaporated to dryness under a gentle nitrogen stream at 40°C and the residue was dissolved in 1 mL n-hexane for GC-NPD and UV-Vis analysis.

Refractive index

Refractive indices for malathion are often quoted as single number which is independent of wavelength. The refractive index n at different wavelength was calculated using the envelope curve method for T\(_{\text{max}}\) (T\(_{\text{M}}\)) and T\(_{\text{min}}\) (T\(_{\text{m}}\)) in the transmission spectra of extracting
samples. The mathematical equation for calculation of the refractive index was given by

\[ n = \left[ N + (N^2 - n_s^2)^{1/2} \right]^{1/2} \]  

(1)

Where,

\[ N = 2n_s(T_M - T_m)/T_M T_m + (n_s^2 + 1)/2 \]  

(2)

where \( n_s \) the refractive index of substrate taken 1.52 (glass holder) and \( N \) was a variable quantity that depended on \( T_m \) and \( T_M \).

The spectrum of malathion was taken as a standard level at wavelength 319 nm to determine the absorbance of malathion. The Beer’s law requires monochromatic radiation because of absorptivity is constant at a single wavelength. When light passes through a colored sample, the emerging light intensity (I) is reduced relative to the incident light intensity (I₀) due to the observance by the analyst at certain wavelengths. The sample absorbance (A) is related to these intensities and the sample concentration by the Beer-Lambert law:

\[ A = \ln(I_0/I) = \varepsilon lc \]  

(3)

where \( l \) is the optical path length through the solution, \( \varepsilon \) is the molar extinction coefficient (mol/l/cm) at a particular wavelength and \( c \) is the concentration of the absorbing species [9].

**Results**

**Effect of leukocytes with spiking level of malathion**

The UV-Vis absorbance spectra were taken for primary analysis of plasma sample. The maximum absorbance of blood plasma was found at 225 nm, 440 nm, -541 nm and 576 nm ([Fig. 1](#)) in which the peak at 225 nm is the intrinsic UV-Vis absorbance of hemoglobin (Hb) caused by the tryptophan and tyrosine in the Hb peptide. The peaks at 576 nm, -541 nm and 440 nm correspond to the \( \alpha \), \( \beta \) and Soret \( \gamma \) bands in the visible region, respectively.

The number of white blood cells (WBCs) in human blood was reduced by 40% within 42 hours after a spiked quantity of malathion (0.5, 1.5 and 3.0 \( \mu g/\) mL) in a sample of different blood groups. Furthermore, malathion did not show any effect on WBCs, if the quantity was less or equal to 1.0 \( \mu g/\) mL. The biological reaction rate with malathion was initially slow, but a major change was found after one day ([Fig. 2 A–C](#)).

**UV-Vis analysis**

The present study involved the coupling reaction of malathion with hydrolyzed product under alkaline conditions (at pH 9.2, 27 \( \pm 2 \) °C) and gave light lime yellow color to dark colored product having a maximum absorption at 319 nm. UV-Vis absorption results of malathion showed a blue shift (hypsochromic shift) due to substitution reaction effect or chromophore and auxochrome effect (presence of covalent unsaturated groups like C=O, P=S and -OH) and another property of spectra, i.e. hyperchromic effect, showed an increase in absorption as well as concentration of solute. The slight blue shift in spectra was used as a measure of the strength or energy of the band ([Fig. 3A](#)).

**Quantification**

We used optical parameters (i.e. refractive index and absorbance) for determination of malathion in human blood samples. The dry residues were reacted with (0.95 mol/L) sodium hydroxide and (0.045 mol/L) hydroxyl ammonium hydrochloride to quantify malathion by UV-Vis spectrometry and its functional groups characterized by IR transmission spectra before and after the reactants. The sampling time of 15 min was recommended to determine the concentration of malathion for this process. The quantity of reactants showed the maximum linearity (i.e. sodium hydroxide and hydroxyl ammonium hydrochloride) with 100 \( \mu L \) and 50 \( \mu L \), respectively [10]. The quantity of hydrolyzing agents has been adjusted through UV-Vis spectra for different concentrations of malathion. The optimization of the method was validated by comparison of the obtained reference results in the same environmental conditions. The reflective index showed linear behavior with
concentration of malathion (Fig. 3B). The absorbance concentration is shown in Fig. 3C. A correlation in both techniques was produced for the validation of quantitative results with gas chromatography-nitrogen phosphorus detector (GC-NPD) Table 1 and 2).

**Colorimetric assay analysis**

The IR transmission spectrum of pure malathion in methanol solvent is shown in Fig. 4A. Here, it has been observed that the active wave number region lies between 1800 to 650 per cm and above this region (2900 cm$^{-1}$) it may conflict due to a hydroxyl group, because the selected region is mainly used to analyze malathion. In the region 1800 to 650/cm, the untreated groups located at 1737.77, 1602.26, 1174.82, 1016.75, 795.20, and 655.76 cm$^{-1}$ are the most intense ones in the residue (Fig. 4B). Therefore, the IR spectra were verified for the presence of hydroxyl groups and other groups in malathion before and after the addition of reactants. The bands are frequently intense and may be used for the qualitative and quantitative determination of relative concentrations of malathion residue (Fig. 4B).

The observed band at 1737.77/cm is due to the carbonyl stretching of the ester group. The band located at 1016.75/cm due to P-OCH$_3$ group stretching. This band may be the stretching of the O-CH$_2$ group of an aliphatic ester. Absorption around 600-800/cm is the indicator of alkanes group. The band present in a
reaction sample of malathion at 1633.27/cm is due to a strong C-O stretching highly absorption band at 1750-1600/cm in the region of untreated IR spectra of the malathion residue and the direction of shifting depends on the resonance and predominant effects while the inductive effect reduces the length of the C=O band. Due to this effect, the force constant and absorption frequency increases.

The resonance effect increases the C=O band length and reduces the frequency of absorption due to conjugation with phenyl group. The two closer bands at 795.20/cm and 655.76/cm are due to alkanes

**Fig. 3** Analytical information from UV–Vis. A: UV-Vis energy spectra of different extracted samples of malathion from blood sample. B: Refractive index analysis of extracting samples of malathion. C: Linear representation of absorbance and concentration of malathion.
group and another band located at 1015.68/cm is due to an aliphatic this or tone stretching in the P-OCH₃ group. The band obtained at 1173.42/cm lies in the region 1260-1160/cm is due to P-O-C group. The positions of highly absorbed functional groups are mainly in the regions 2950-1190/cm that shows the characteristic of the number of adjacent hydrogen atoms in the molecular structure of malathion. 

Discussion

A spectroscopic methodology is developed for detection of malathion in blood plasma samples and the reproducible results are calibrated with GC-NPD and optical measurement. In this method, the regression equation of the observed calibration curve and their corresponding correlation coefficient, RSD, limits of detection and quantification (LOD and LOQ) are also calculated. The quantitative results are reproducible with ±2.2% error. The recovery data from UV-Vis spectrometry (in both techniques; absorbance and refractive index) were compared with GC-NPD, which showed that the results have 5-10% higher recovery than UV-Vis analysis data. However, the proposed techniques are economical and suitable for routine analysis. In quantification methods, absorbance and refractive index provide unique information in analyzing malathion residue in human blood samples.

The characteristics of the observed bands in the spectrum of malathion produces a hydroxyl (-OH; above 2900/cm) and ester structure, which may be the outcome of 'OH stretching and C-O is stretching that is responsible for high absorbance property in malathion residue due to the 'OH group. The 'OH group is responsible for higher absorbance in pure malathion and extracted malathion residue.

The spectroscopic data are standardized for the determination of malathion residue in human blood samples. It has been concluded from the present results that the coupling of spectroscopic analysis with extraction technique is quite adequate for quantitative analysis. It has several merits like high sensitivity, specificity, simple determinations and reduced cost. The presence of malathion in human blood affects the WBC count. It was studied with more precautions and taken reference to interference drugs. It has also been observed that the number of WBCs in human blood is reduced up to 40% (average for all blood groups) within 42 h after a spiked quantity of malathion (2μg/mL) in samples of different blood group. Initially, the effect of malathion was found very slow but, after one day a significant change has been observed as a result of counting WBCs in order of blood group O⁺, A⁺, B⁺ and AB⁺. A standardization of UV-Vis data with both quantification of optical parameters (like absorbance and refractive index) were correlated with GC-NPD data that will approve the quantification methodology for malathion in human blood plasma. To find the role of functional groups of malathion with the colorimetric reagents, the IR studied were done. The IR spectrum of malathion is interpreted in terms of vibrations, namely the stretching and bending of having various functional groups of malathion that can be further used for qualitative analysis. The IR spectrum of malathion is interpreted in terms of vibrations, namely the stretching and bending

| Analytical parameters                  | Optimum value |
|---------------------------------------|---------------|
| LOQ μg/mL                             | 0.1 -1.7      |
| LOD μg/mL                             | 0.3 -1.5      |
| Maximum wavelength                    | 319 nm        |
| Correlation coefficient (R²)          | 0.989         |
| Y=a+b (X); a. Slope                   | Y=0.6941+0.18425X |
| b. Intercept                         | 1.1145        |
| Chisquare                             | 0.00557       |
| Average                               | X=1           |
| Variance                              | X=1.361       |
| LOD: limit of detection; LOQ: limit of quantification |

Table 1 A comparative analysis of UV-Vis and GC-NPD results.

| (Concentration of malathion in μg/mL) | *Absorbance method | *Refractive index method | *GC-NPD method |
|---------------------------------------|--------------------|-------------------------|---------------|
|                                       | Recovery (%) ± R.S.D. (%) | Recovery (%) ± R.S.D. (%) | Recovery (%) ± R.S.D. (%) |
| 0.1                                   | 78.2 ± 4.5         | 79.4 ± 2               | 88.1 ± 5     |
| 0.5                                   | 80.8 ± 6           | 80.2 ± 4               | 87.4 ± 3     |
| 0.9                                   | 83.0 ± 7           | 84.2 ± 3               | 89.8 ± 6     |
| 1.3                                   | 77.8 ± 4           | 78.5 ± 2               | 87.0 ± 4     |
| 1.7                                   | 76.7 ± 6           | 77.4 ± 4               | 85.7 ± 3     |

*Three determinations
of having various functional groups of malathion and can be used for qualitative analysis.

As to future aspects, it may be applied for routine analysis in quality control laboratories by using an absorbent/refractive index based UV-Vis methodology for quantitative analysis and color variation with concentrations of malathion shows the qualitative analysis in blood plasma samples. The methodology is suitable for pathological/forensic laboratory to study poisoning or exposure in a person by malathion, and human health resources to attain present effects on leukocytes. These results are encouraging and useful to apply this method for other pesticides and to develop a cost effective detection method.

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