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A selective method for quantification of diazinon in human plasma by GC-MS

Méthode sélective pour la quantification du diazinon dans le plasma humain par GC-MS

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Abstract. Diazinon is a synthetic molecule known as an organophosphorus insecticide. It is used for gardens and for agriculture. This molecule represents a toxicological concern for humans. For this reason, the detection and the quantification of the diazinon in human samples are important in order to monitor an exposure and to diagnose intoxications. The aim of this work is to develop a selective method for the quantification of diazinon in human plasma by means of a gas chromatography (GC) with a mass spectroscopy (MS) detector. The method presented in this article includes a liquid-liquid extraction (LLE) using dichloromethane/propanol-2/heptane. The correlation coefficient for the calibration curve is 0.992. The limit of detection (LOD) and the limit of quantification (LOQ) are respectively 5 µg/L and 2 µg/L. The average recovery of the three different concentrations (15, 250 and 375 µg/L) varies from 22.8% to 31%. A test of stability at ambient temperature for 24 hours and at 4 °C for 48 hours was conducted. The relative error factor was found to be inferior to 15%. The results obtained show that the method developed gives satisfying validation parameters in human plasma. Therefore, the compound could be detected at very low concentration with a good linearity. This method can be used with other matrices, such as blood or urine, for a partial validation.

Key words: *diazinon, LLE, GC-MS, plasma, limit of detection, limit of quantification*

Résumé. Le diazinon est une molécule synthétique utilisée comme insecticide organophosphoré pour contrôler les insectes dans les jardins et pour l'agriculture. Cette molécule a des effets toxicologiques qui menacent les êtres humains. La détection et la quantification du diazinon chez l'homme sont importantes en cas d'exposition à cette molécule. Par conséquent, il est particulièrement important de développer une technique pour faire ces mesures. L'objectif de cette étude est d'élaborer une méthode sélective pour la quantification du diazinon dans le plasma humain par la chromatographie gazeuse (GC) couplée à la spectrométrie de masse (MS). Cette méthode utilise une extraction liquide liquide avec dichlorométhane/propanol-2/heptane. Le coefficient de corrélation de la courbe de calibration est 0,992. La limite de détection (LOD) et la limite de quantification (LOQ) sont respectivement 5 g/L et 2 g/L. Le pourcentage de recouvrement moyen est entre 22, 8 % et 31 %. Un test de stabilité à température ambiante après 24 heures et à 4 °C après 48 heures a été fait avec une erreur relative inférieure à 15 %. Les résultats de validation montrent des paramètres acceptables dans le plasma humain, cette molécule peut être détectée à des concentrations minimes avec une bonne linéarité. Cette méthode peut être utilisée pour d'autres matrices comme le sang et les urines avec une revalidation partielle.

Mots clés : *diazinon, LLE, GC-MS, plasma, limite de détection, limite de quantification*

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Background

Diazinon ($C_{12}H_{21}N_2O_3PS$) is a synthetic molecule used as an organophosphorus insecticide to control insects at home and gardens. Moreover, these compounds are used for agriculture and veterinary purposes [1]. For humans, diazinon is defined as a moderately hazardous compound (class II) according to the World Health Organization's (WHO) classification [2]. The toxic effects of this molecule are due to the inhibition of the acetylcholinesterase, an important enzyme for the nervous system function. The absorption of this pesticide in the body, its distribution and its extraction has been reported for rats [3] and human [4]. Diazinon is responsible for fatal [5] or non-fatal [6] cases of poisoning around the world, especially in developing countries [7]. The number of cases worldwide is estimated at one to five million [8].

The extensive use of this compound, especially in agricultural areas, suggests that the best means to monitor exposure and the intoxication is through human samples [9-13]. For the bio-monitoring of pesticides, metabolites are used as exposure biomarkers', mainly in the urine. Organophosphorus are rapidly metabolized in the body [13] resulting in common metabolites known as dialkyl phosphates [9, 13, 14]. The detection of the parent compound proves recent exposure [15-17].

The detection and the quantitative assessment of these compounds is a challenging task for laboratories in the clinical and forensic toxicology field. There are different separating techniques used for this purpose. The chromatographic ones are the most commonly used and the most efficient. A prior preprocessing extraction step is required. The most common extraction methods are related to the solid (SPE or SMPE) or the liquid (LLE or LMPE) extraction [18-23]. Both of them provide reliable results for analyzing pesticides in human samples.

The majority of previous studies [9-23] confirm the effectiveness of the measurement of diazinon and its metabolites for toxicological researches. These measurements contribute greatly in the elaboration of scientific data. Therefore, the aim of this study is to develop a simple and selective method for the identification and the quantification of diazinon in human plasma by the use of liquid-liquid extraction (LLE) using GC-MS chromatography.

Methods and materials

This study describes a method using the GC-MS for the determination and the quantification of the insecticide diazinon ($m/z = 304$) (*figure 1*). Diazinon was prepared in the isooctane as a solvent. The extraction was conducted with a sodium carbonate buffer and a dichloromethane/promanol-2/heptane solvent mixture.

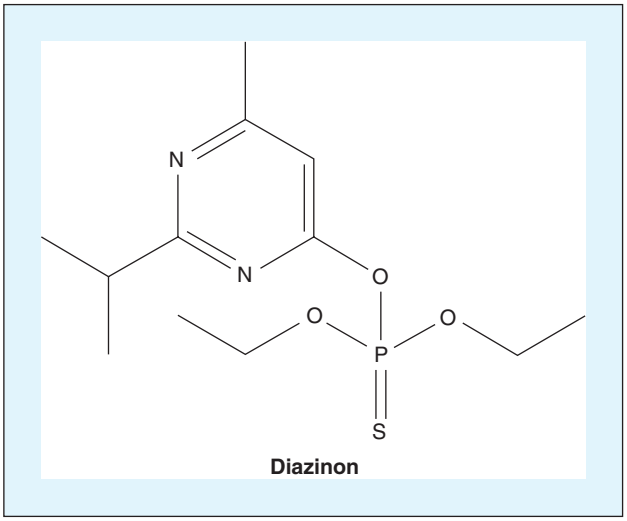

Figure 1. Chemical structure of diazinon (Chemdraw16).

Reagent

We used standard diazinon (250 mg, 98.3%) purchased from Fluka Analytical (Germany) and the isooctane solvent (2, 2, 4 trimethylpentane, 99%) from Sigma Aldrich (United States). The human plasma was obtained from the National blood transfusion center of Morocco in Rabat. All other chemicals and solvents had organic residue analytical grade.

Instruments

A PerkinElmer MS, Clarus® SQ 8C coupled to GC Clarus® 680 was used to identify and quantify the compound. The capillary column used was Rxi R- 5 ms (30 m × 0.25 mm ID 0.25 µm film thickness). The injector temperature was 280 °C. The splitless mode is used. The carrier gas was helium (1 mL/min).

Calibration standards

Individual stock standard solutions (10 and 1 mg/mL) were prepared in isooctane and stored in the dark at -20 °C. In the same solvent, spiked calibration standards were set at six different levels (5, 10, 20, 100, 200 and 500 ng/mL). These concentrations were prepared daily for three days and injected five times a day for each preparation.

Sample preparation

Three working standards with diazinon (15, 250 and 375 µg/L in isooctane) and the internal standard (praezpam, 200 ng/mL) were prepared. We spiked three aliquots

of 1 mL of plasma sample with 15, 250 and 375 µg/L of diazinon. The sample was vortexed for five seconds. We added one milliliter of the sodium carbonate buffer, 5 mL of dichloromethane/promanol-2/heptane and 100 µL of prazepam.

The prazepam is used as an internal standard because of its availability in our laboratory. This molecule is used for its stability under our analytical conditions and it is not interfering with our compound (all compounds had different retention times).

The preparation was mixed for 10 minutes and centrifuged at 4.000 rpm for another 10 minutes. The supernatant is transferred to a 20 mL tube and evaporated under nitrogen gas at 40 °C. The residue was reconstructed with 100 µL of isooctane. After shaking for 1 minute, only 10 µL is injected into the GC-MS.

GC/MS analysis

We choose an optimum temperature program and a selected ion recording (SIR) mode. The oven temperature was initially at 80 °C for 1 minute and raised to 290 °C for 10 minutes 20 °C/min ramp rate. The temperatures of the injector, the ion source and the transfer line were respectively 250 °C, 230 °C and 270 °C. The mass spectrometer was operated in the electron impact (70 eV), with a total scan time of one second. The analysis took 18 minutes.

GC-MS data analysis was done with version 6.0 of TurboMass software and the 2008 version of the NIST library. For a perfect matching, the R.Match must be between 900 and 1.000; the software indicates the level of similarity between the mass spectrum found and the data in the NIST library. After acquisition of the total ion chromatogram in scan mode, peaks were identified by retention time and mass spectra. The confirmation step used the SIR mode, by choosing the parent ion and characteristic mass fragment for diazinon (m/z = 304, 179, 137,152 and 29) and for prazepam (269, 295 and 91). The parent ion was the most abundant for each compound.

Results

Gas chromatographic determination

The analysis was executed with the SIR mode using diazinon and prazepam's ions. The identification is based on the retention time and the mass spectra. Diazinon was detected at 6.93 min and prazepam at 17.30 min (*figure 2*).

Five blank samples were injected respectively. There is no detection at 6.93 and 17.30 min. Therefore, this study is selective for diazinon and the matrix effect is assessed.

A new molecule, O,O-diethyl phosphonothioate (m/z 137) was found (*figure 3*). It is a transformed product of diazinon metabolism [9]. Seifert *et al.* [24] named this molecule as the major hydrolytic diazinon product. The authors identified it by controlling the hydrolysis at pH 2.4 and 50 C° for 48 hours.

For three days, three concentrations (15, 250, 375 µg/L) of standard mixture were repeated and injected five times. The error coefficient is under 20% for the calculated concentrations. The detection by GC-MS provides selective results and the background noise is removed. This analysis determines the specificity of the method.

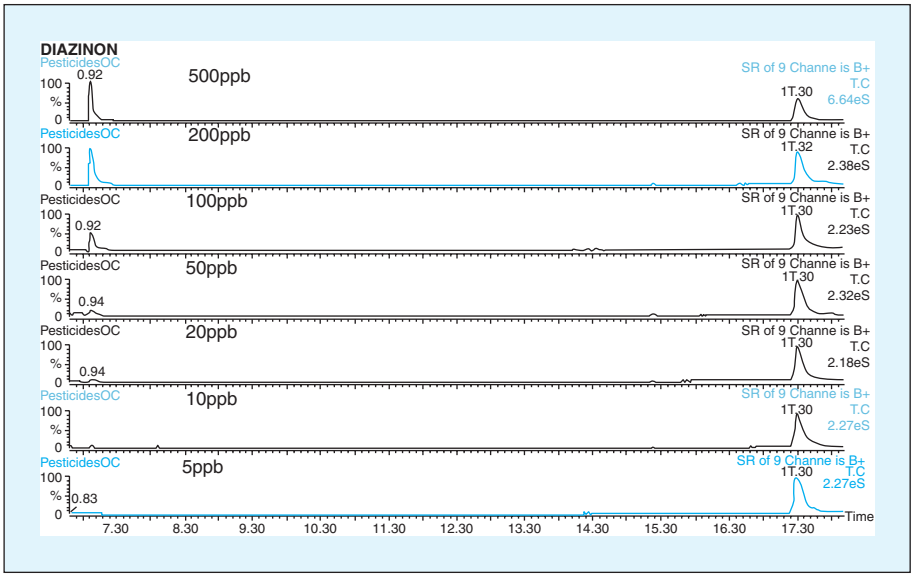

Figure 2. Chromatograms of different concentrations of calibration standards.

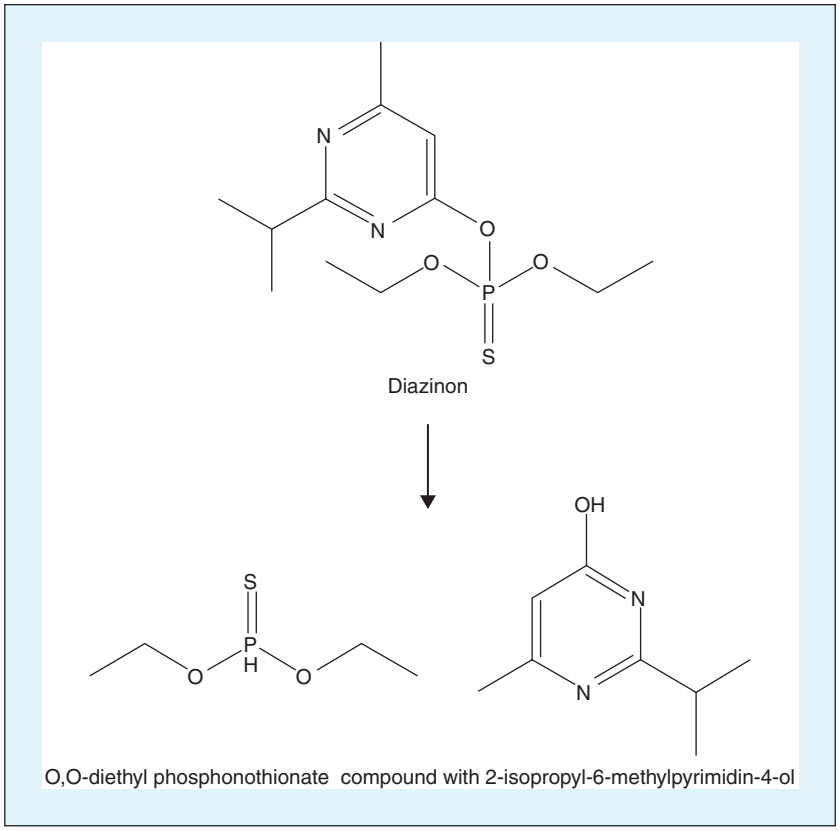

Figure 3. Dissociation of diazinon (O,O-diethyl phosphonothioate; m/z and 2-isopropyl-6-methylpyrimidin-4-ol) (Chemdraw16).

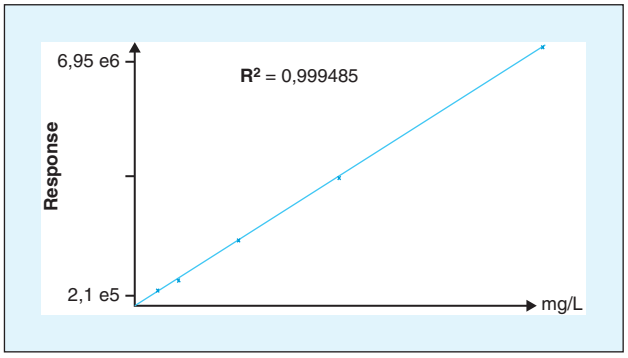

Figure 4. Diazinon linear calibration curve.

Validation method

Linearity of the calibration curves

The calibration curve showed an excellent linearity. The calibration curve was obtained by plotting the peak areas of each concentration detected compared to the concentration prepared using the TturboMass software. The linearity is provided by calculating the regression line expressed by the correlation coefficient R^2 (0.999). Our linearity results are illustrated in *figure 4*. *Table 1* shows that the coefficient errors found are acceptable.

Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) are determined as the lowest concentration detected or quantitated, taking into consideration a 1:3 and 1:10 ratio of the baseline noise and calibration point, respectively. The LOQ and LOD were repeated five times for confirmation. The LOD and LOQ are 2 µg/L and 5 µg/L.

Accuracy and precision

The analysis is repeated five times for 3 days in the same conditions. The average recovery percentage of the three different concentrations of the spiked plasma samples (15, 250 and 375 µg/L) varies from 22.8% to 31%. The repeatability is evaluated by the coefficient of variation (CVr). All the results show a CVr between 2.24% and 15.8%.

Discussion

Our goal was to develop a selective method for the quantification of diazinon in human plasma by GC-MS. Because of the nature of the use of this compound and its harmful

Table 1. Calculated concentrations for the calibration standards and the relative error ER

Standards (µg/L)	First assay	ER*
5	4.55	9
10	11.49	14.9
20	19.37	3.15
50	52.04	4.08
100	91.70	8.3
200	212.30	6.15
500	526.51	5.3
R^2	0.9995	

*ER< 20%.

effect, our method is optimized and adapted for application on real samples. The fundamental source of inaccuracy in detection of pesticides, especially in biological samples, is associated with the presence of interfering elements, also called the matrix effect.

We were able to specify and select our target by means of GC-MS. The optimization by the SIR method provides a good performance and accuracy. In addition, different oven programs were tested; we used an 18 minutes program. The injection of a free diazinon plasma and a spiked one at 100 µg/L confirmed that there are no interferences in the preparation. Therefore, the specificity and the selectivity of the method were verified.

Six concentrations of spiked plasma varying linearly from 5 to 500 µg/L, demonstrate an excellent linearity expressed by a correlation coefficient exceeding 0.99.

The LOD and LOQ found, at 2 µg/L and 5 µg/L respectively, enable the detection and quantitative evaluation of diazinon in a spiked plasma. This concentration is lower than other limits found by other authors [18-21] with the exception of Naksen, Pérez and Lacassie [22, 23, 25, 26] who found limits close to ours.

For the three tested concentrations, the average recovery percentages were found to be varying between 22.8% and 31%. Our study shows higher values compared to those of Naksen *et al.* [22] who used a solid phase extraction (SPE). Gallardo *et al.* [27] found a recovery percentage of 6.7% for blood samples, using a solid phase microextraction (SMPE) method. Moreover, the recovery obtained by Guan *et al.* [28] was between 3.2% to 7.2 % for the whole blood samples.

The low values found for our "recoveries" can be caused by different parameters. First, the use of LLE can cause the loss of an interesting part of the compound. In addition, the over shaking or reduced time of contact between the compound and the solvent can enhance the chances to have lower recoveries. Besides, pH influences the extraction amount [27]. In our work, we did not use any acids because it can degrade compounds [29]. Furthermore, the evaporation step, which concentrates the molecule, may eliminate it in the case it is volatile. Also, some handling mistakes, as pouring the sample or over measuring the solvents' volume, may interfere with the yielding of the extraction.

Although the recoveries of diazinon extraction were low, we managed to have an excellent linearity expressed by R^2, a perfect precision and relevant LOD and LOQ. Our method is then validated, verified and suitable for use for other biological samples.

Conclusion

Chromatographic methods became more efficient to separate compounds from different complex samples. Most of analytical researchers recommend those methods for precision. Furthermore, an analytical method must be validated to ensure good results for laboratory's measurements.

In this study, a selective method of quantification and detection of diazinon in human plasma was validated. The results show satisfying validation parameters in human plasma because diazinon could be detected at very low concentration with a good linearity showed with the calibration curve. This selective method for diazinon can be considered as a good addition and contribution to the clinical and forensic toxicology field.

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Conflict of interest: none of the authors have any conflicts of interest to disclose concerning this article.

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