Experimental central composite design-based dispersive liquid-liquid microextraction for HPLC-DAD determination of diazinon in human urine samples: method development and validation

Reza Mohammadzaheri1,2, Mehdi Ansari Dogaheh3, Maryam Kazemipour1,2, and Kambiz Soltaninejad4

1 Department of Chemistry, Science and Research Branch, Islamic Azad University, Kerman, Iran
2 Department of Chemistry, Kerman Branch, Islamic Azad University, Kerman, Iran
3 Department of Pharmaceutics, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran
4 Department of Forensic Toxicology, Legal Medicine Research Center, Legal Medicine Organization, Tehran, Iran

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Diazinon poisoning is an important issue in occupational, clinical, and forensic toxicology. While sensitive and specific enough to analyse diazinon in biological samples, current methods are time-consuming and too expensive for routine analysis. The aim of this study was therefore to design and validate a simple dispersive liquid-liquid microextraction (DLLME) for the preparation of urine samples to be analysed for diazinon with high performance liquid chromatography with diode-array detector (HPLC-DAD) to establish diazinon exposure and poisoning. To do that, we first identified critical parameters (type and volume of extraction and disperser solvents, pH, surfactant, and salt concentrations) in preliminary experiments and then used central composite design to determine the best experimental conditions for DLLME-HPLC-DAD. For DLLME they were 800 µL of methanol (disperser solvent) and 310 µL of toluene (extraction solvent) injected to the urine sample rapidly via a syringe. The sample was injected into a HPLC-DAD (C18 column, 250×4.6 mm, 5 µm), and the mobile phase was a mixture of acetonitrile and buffer (63:37 v/v, pH 3.2; flow rate: 1 mL/min). Standard calibration curves for diazinon were linear with the concentration range of 0.5–4 µg/mL, yielding a regression equation \( Y = 0.254X + 0.006 \) with a correlation coefficient of 0.993. The limit of detection and limit of quantification for diazinon were 0.15 µg/mL and 0.45 µg/mL, respectively. The proposed method was accurate, precise, sensitive, and linear over a wide range of diazinon concentrations in urine samples. This method can be employed for diazinon analysis in routine clinical and forensic toxicology settings.

KEY WORDS: disperser solvent; extraction solvent; high performance liquid chromatography; liquid phase microextraction; Taguchi orthogonal array

Diazinon (O,O-diethyl O-[4-methyl-6-(propan-2-yl) pyrimidin-2-yl] phosphorothioate) is one of the most common causes of occupational, clinical, and forensic organophosphate (OP) poisoning in the world (1–14). In biological samples it can be determined with several analytical methods for experimental, clinical, and forensic purposes (15–18), such as solid-phase extraction (SPE) followed by gas chromatography/mass spectrometry (GC/MS) in postmortem blood samples (15), high performance liquid chromatography with diode-array detector (HPLC-DAD) in serum and urine of patients with acute poisoning (16), or liquid chromatography with tandem MS in gastric content and blood for forensic toxicology (18).

Although these methods are sensitive, and specific enough to analyse diazinon in biological samples, they are too time-consuming and expensive for routine analysis. This issue has called for the development of simple, fast, low-cost, user- and environment-friendly sample preparation methods such as liquid phase microextraction (LPME), which requires a small volume of a water-immiscible solvent (19).

One of the LPME methods is the so called dispersive liquid-liquid microextraction (DLLME). It is rapid, simple, inexpensive, efficient, and requires minimal (microlitre) volumes of low- and high-density solvents for the extraction of many water-based samples (20, 21). However, to obtain optimal efficiency (20–22), this method has to be fine-tuned through trial and error, which is time-consuming, or through statistical models and experimental designs, such as the Taguchi orthogonal array design and central composite design (CCD) (23, 24).
The aim of this study was to make use of experimental design and develop a fast, simple, inexpensive and specific DLLME-HPLC-DAD method for the determination of diazinon in human urine samples for routine analysis in clinical and forensic toxicology laboratories.

MATERIALS AND METHODS

Chemicals
HPLC-grade methanol, acetonitrile, water, toluene and dichloromethane were purchased from Merck Chemical Co. (Darmusdat, Germany). HPLC-grade standards for diazinon, pirimiphos-methyl, azinphos-ethyl, and chlorpyrifos were purchased from Dr. Ehrenstofer GmbH (Augsburg, Germany). All other chemicals and reagents were of analytical grade, purchased from Merck Chemical Co.

Instrumentation and chromatographic conditions
Separation, identification, and quantification were carried out on a Knauer HPLC system (Smartline Series 1200, Berlin, Germany). Chromatography was run isocratically on a Nucleosil® C18 analytical column (250×4.6 mm, 5 μm particle size, Perfectsil® Target). An RP-18 guard column was fitted upstream of the analytical column. The mobile phase was a mixture of acetonitrile and buffer, optimised (63:37 v/v, pH 3.2) and delivered by a Knauer 1050 HPLC pump at a flow rate of 1 mL/min. A diode array detector (K-2800, Knauer) with a wavelength range of 190–740 nm was used for detection. The system was equipped with ChromGate® software (version 3.3.2., Knauer).

Sample preparation
We used diazinon-free urine samples provided by healthy volunteers in our laboratory. They were kept frozen at -20 °C until analysis and then thawed to room temperature. Each sample was added 10 µL of pirimiphos-methyl (internal standard, IS) (2.5 µg/mL) and vortexed at 1250xg for 10 min. Then we added 100 µL of sodium lauryl sulphate (SLS) (3 % w/v) and 100 µL of sodium chloride (NaCl) (1 %,w/v) to the glass tubes containing 1000 µL of urine. The final solution was then prepared following the DLLME procedure.

DLLME procedure
A mixture of 800 µL of methanol (disperser solvent) plus 310 µL of toluene (extraction solvent) was quickly injected to the samples with a syringe (Hamilton, NV, USA), which dispersed fine droplets of toluene to form a cloudy solution. Over just a few seconds, the analytes were extracted on toluene droplets and after centrifugation at 1250xg for 15 min, these droplets became a supernatant on the surface of the conical test tube. The supernatant phase was then completely transferred into another conical test tube and the residue dried by evaporation with nitrogen in a water bath, dissolved to a mobile phase, and then 20 µL of the sample injected into the HPLC.

DLLME optimisation with experimental design
To achieve maximum recovery, the selection of extraction efficiency variables was based on preliminary experiments that yielded distinct responses of eight variables on three levels of experimental designs (Table 1). Optimisation was performed on spiked samples.

Preparation of standard solutions
Standard solutions were prepared by serially diluting the diazinon stock solution (100 µg/mL) with HPLC-grade water to 0.5, 1.0, 1.5, 2, 2.5, 3, and 4 µg/mL. The stock solution (2.5 µg/mL) of pirimiphos-methyl (IS) in methanol was prepared and stored at -20 °C. The stock and standard solutions were prepared on a daily basis and stored in the dark at 4 °C. All solutions were used on the day they were prepared.

Experimental design
To obtain optimal conditions, we relied on the four-factor-two-level central composite design (CCD), which is used in response surface methodology. Briefly, each numeric factor is varied over five levels: plus and minus alpha (axial points), plus and minus 1 (factorial points), and the centre point. If categorical factors are added, CCD will be duplicated for every combination of the categorical factor

| Table 1 Variables and their levels for experimental design |
|-----------------|-----------------|-----------------|-----------------|
| Symbol          | Level 3          | Level 2          | Level 1          | Factor                      |
| A               | methanol         | acetonitril      | type of disperser solvent |
| B               | 10               | 0               | sonication duration (minute) |
| C               | dichloromethane  | chloroform       | toluene          | type of extraction solvent  |
| D               | 600              | 300             | 100             | volume of extraction solvent (µL) |
| E               | 1000             | 500             | 0               | volume of disperser solvent (µL) |
| F               | 5                | 3               | 1               | surfactant concentration (% w/v) |
| G               | 5                | 3               | 1               | salt concentration (% w/v)   |
| H               | 10               | 7               | 4               | pH                           |
levels. It was also used to investigate parabolic interactions between the following parameters: volume of extraction solvent (toluene), salt percentage (NaCl), surfactant percentage (SLS), and the volume of disperser solvent (methanol). This CCD design allowed modelling the response surface by fitting a second-order polynomial with the number of experiments equal to 21 for four factorial designs at five levels and five replicated points. Table 2 shows the range of independent variables used in this study in terms of actual and coded values.

**HPLC method validation**

Validation included the following parameters: linearity, precision, accuracy, limits of detection and quantification, and selectivity (25). For calibration we used seven concentrations ranging from 0.5 to 4 µg/mL of diazinon. Each concentration was prepared in triplicate and analysed three times. Calibration curves were constructed by plotting the concentration of compounds versus peak area response. The linearity was evaluated with the least square regression method.

The limit of quantification (LOQ) was determined during the evaluation of the linear range of calibration curve.

**RESULTS AND DISCUSSION**

**Results of DLLME optimisation**

Sonication and pH had negative effects on maximum recovery (p>0.05). Other parameters had a positive effect (p<0.05) and were selected for further optimisation.

**Data analysis**

For regression analysis and diagram plotting for the experimental results we used the Design Expert v. 7.01 software (Stat-Ease Inc., Minneapolis, MN, USA).

**Ethical approval**

This project was approved by the Ethics Committee of the Legal Medicine Research Centre.

**Response surfacing based on CCD**

The actual and statistically predicted diazinon recoveries for experiments are shown in Table 4, while Figure 1 shows (Table 3). Methanol (as disperser solvent) and toluene (as extraction solvent) had positive effects on all variables and were used in the experiments.

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the relationship between the two. The mathematical model was as follows:

\[(\text{Recovery})^{0.81} = +22.87 - 1.01 \times A - 3.76 \times B + 2.98 \times C + 2.97 \times D - 2.64 \times AB + 2.58 \times AC - 3.35 \times AD - 1.56 \times BC + 1.09 \times A^2 - 1.79 \times C^2 + 1.01 \times D^2\]

This equation represents the relationship that diazinon recovery (R) has with the volume of the disperser solvent (A), salt concentration (B), surfactant concentration (C), the volume of extraction solvent (D), and their combinations (AB, AC, AD, and BC). Table 5 shows the results of the analysis of variance (ANOVA) for the CCD model and the significance of each coefficient determined by F-values (variation of data about mean value) and P-values (probability). The model turned out to be highly predictive of the experimental results. Extraction solvent volume had a high linear and quadratic effect on response. In addition, the interaction effects of combined variables were significant. The correctness of the model was also ensured by multiple correlation coefficient ($R^2$), which was 0.9872 and showed high prediction of the actual value and excellent response, with 0.85% of the total variation. The predicted $R^2$ (0.8624) was in reasonable agreement with the adjusted $R^2$ (0.9716). Furthermore, the coefficient of variance (CV=4.76%) was low, which indicates significant precision and reliability of the experimental data.

For precision to be adequate, the signal-to-noise ratio should be >4. With our model it was 30.73, indicating that it could be used to evaluate experiments.

Figure 2 shows a 3D response surface diagram of the effects of two factors on diazinon recovery. Figure 2A shows significant interactions of extraction and disperser solvent volume with diazinon recovery (p<0.0002). Diazinon recovery increased with the increase in extraction solvent volume (from 300 to 450 µL) and disperser solvent volume (from 400 to 800 µL). Significant increase in diazinon recovery

**Table 4** Experimental conditions according to the central composite design and observed response values

| Experiment No. | Methanol volume (µL) | NaCl conc. (%w/v) | SLS conc. (%w/v) | Toluene volume (µL) | Actual recovery | Predicted recovery |
|----------------|----------------------|-------------------|------------------|---------------------|----------------|------------------|
| 1              | 800                  | 3.00              | 3.00             | 300.00              | 43.70          | 40.72            |
| 2              | 800                  | 3.00              | 1.00             | 300.00              | 20.10          | 21.55            |
| 3              | 800                  | 1.00              | 3.00             | 450.00              | 82.00          | 80.49            |
| 4              | 400                  | 3.00              | 1.00             | 450.00              | 69.27          | 68.99            |
| 5              | 800                  | 1.00              | 1.00             | 450.00              | 43.52          | 43.50            |
| 6              | 400                  | 1.00              | 3.00             | 300.00              | 46.3           | 43.06            |
| 7              | 400                  | 3.00              | 3.00             | 450.00              | 63.00          | 61.23            |
| 8              | 400                  | 1.00              | 1.00             | 300.00              | 31.80          | 32.99            |
| 9              | 200                  | 2.00              | 2.00             | 375.00              | 63.00          | 64.14            |
| 10             | 1000                 | 2.00              | 2.00             | 375.00              | 53.50          | 54.13            |
| 11             | 600                  | 0.00              | 2.00             | 375.00              | 67.50          | 67.14            |
| 12             | 600                  | 4.00              | 2.00             | 375.00              | 29.00          | 28.64            |
| 13             | 600                  | 2.00              | 0.00             | 375.00              | 18.02          | 15.94            |
| 14             | 600                  | 2.00              | 4.00             | 375.00              | 41.33          | 45.18            |
| 15             | 600                  | 2.00              | 2.00             | 225.00              | 42.00          | 42.88            |
| 16             | 600                  | 2.00              | 2.00             | 525.00              | 73.60          | 74.48            |
| 17             | 600                  | 2.00              | 2.00             | 375.00              | 48.00          | 47.89            |
| 18             | 600                  | 2.00              | 2.00             | 375.00              | 46.00          | 47.89            |
| 19             | 600                  | 2.00              | 2.00             | 375.00              | 49.00          | 47.89            |
| 20             | 600                  | 2.00              | 2.00             | 375.00              | 50.00          | 47.89            |
| 21             | 600                  | 2.00              | 2.00             | 375.00              | 44.00          | 47.89            |

SLS – sodium lauryl sulphate
Figure 2 Surface plots showing the effects of variables with the highest impact on the recovery of the method
(A) The effect of the volume of toluene and methanol; (B) the effect of the volume of methanol and the sodium lauryl sulphate (SLS) concentration; (C) the effect of methanol volume and the sodium chloride (NaCl) concentration

Table 5 Analysis of variance for central composite design

| Source    | Sum of squares | df | Mean square | F value | p-value*  |
|-----------|----------------|----|-------------|---------|-----------|
| Model     | 840.2          | 11 | 76.37       | 63.10   | <0.0001   |
| A         | 16.28          | 1  | 16.28       | 13.45   | 0.0052    |
| B         | 112.89         | 1  | 112.89      | 93.28   | <0.0001   |
| C         | 142.35         | 1  | 142.35      | 117.63  | <0.0001   |
| D         | 70.52          | 1  | 70.52       | 58.27   | <0.0001   |
| AB        | 27.88          | 1  | 27.88       | 23.04   | 0.0010    |
| AC        | 53.21          | 1  | 53.21       | 43.97   | <0.0001   |
| AD        | 44.91          | 1  | 44.91       | 37.11   | 0.0002    |
| BC        | 19.36          | 1  | 19.36       | 16.00   | 0.0031    |
| A^2       | 31.00          | 1  | 31.00       | 25.62   | 0.0007    |
| C^2       | 84.41          | 1  | 84.41       | 69.75   | <0.0001   |
| D^2       | 26.72          | 1  | 26.72       | 22.08   | 0.0011    |
| Residual  | 10.89          | 9  | 1.21        |         |           |
| Lack of Fit| 7.37          | 5  | 1.47        | 1.67    | 0.3196    |
| Pure Error| 3.53           | 4  | 0.88        |         |           |
| Cor Total | 850.91         | 20 |             |         |           |

* all p-values are statistically significant
recovery was noted when the extraction solvent volume reached 310 µL and disperser solvent 800 µL.

Figure 2B shows that the interactions between diazinon recovery and disperser solvent volume and surfactant concentrations were significant ($p<0.0001$) at maximum surfactant concentration of 3 % and maximum disperser solvent volume.

Figure 2C, in turn, shows that diazinon recovery also had significant interactions with disperser solvent volume when it reached its maximum volume of 800 µL and when salt percentage was at its lowest ($p<0.001$).

Results of method validation

Standard calibration curves for diazinon were linear with the concentration range of 0.5–4 µg/mL, yielding a regression equation of $Y=0.254X+0.006$ with a correlation coefficient of 0.993. This is generally considered evidence of an acceptable fit and good linearity over the concentration range.

The method yielded LOD and LOQ of 0.15 µg/mL and 0.45 µg/mL, respectively, and its precision met the acceptance criteria (Table 6). The intra- and inter- day RSD values did not exceed 5 % (bias interval between 3.0 and 5.0 %), which indicates that the method is accurate, reliable, and reproducible.

Table 6 also shows that the recovery percentages comply with the acceptance criteria (25).

The specificity of the method was tested with peak purity on blank and spiked urine samples. Blank samples showed no interference when diazinon and IS were added. Under optimised conditions, the separation of diazinon and pirimiphos-methyl was complete (Figure 3).

Method application in real conditions

The applicability of the proposed DLLME-HPLC-DAD method was evaluated in undiluted urine samples collected from patients poisoned with diazinon who were receiving hospital treatment (Sanandaj, Iran). Relative diazinon recoveries were determined at the spiking level of 0.5, 1, and 3 µg/mL. The results of six replicate experiments of each sample were in the range of 75–95.6 %. Therefore, the proposed method can be applied for determining diazinon in human urine samples.

Comparison of the DLLME-HPLC-DAD with other methods

Table 7 summarises a comparison of the proposed DLLME-HPLC-DAD method with other methods and shows that its LOD, $R^2$, and recovery are well within acceptable ranges.
CONCLUSION

Our findings evidence that our DLLME-HPLC-DAD is a rapid and simple extraction and determination method for diazinon in human urine samples. It overcomes the limitations of conventional sample preparation methods that involve the use of large volumes of expensive and toxic organic solvents. However, it is evident that further studies are necessary for different biological specimen in order to suppress matrix effects and enhance extraction recoveries. The proposed DLLME-HPLC-DAD method is simple, cheap, accurate, and sensitive enough to be applied in clinical and forensic toxicological analysis.

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Conflicts of interest

None to declare.

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**Dispersivna tekućinsko-tekućinska mikroekstrakcija temeljena na eksperimentalnom centralnom kompozitnom dizajnu u svrhu određivanja diazinona u ljudskoj mokraći: razvoj i validacija metode**

Trovanje diazinonom važan je problem za medicinu rada te kliničku i forenzičku toksikološku praksu. Premda su postojeće metode njegova utvrđivanja utjecale obavljene osjetljive i specifične, njihova je primjena za rutinske analize preskupa i dugotrajna. Zbog toga je cilj ovog istraživanja bio razviti i validirati jednostavan metod disperzivne tekućinsko-tekućinske mikroekstrakcije (engl. dispersive liquid-liquid microextraction, krat. DLLME) kojom bi se uzorci ljudske mokraće pripremili za analizu diazinona tekućinskom kromatografijom visoke djelotvornosti s detektorom s nizom stupca C18, 250×4,6 mm, a mobilna je faza bila mješavina acetonitrila i pufera (63:37 v/v, pH 3.2; protok: 1 mL/min).

Ova metoda se koristi za puštenje pretrženih uzoraka mokraće u sistem za analizu, pri čemu se uzorci prate diskretnim faza u slagaju krajih točaka. Uzorci mokraće se pripremaju u mikrocentripliku čim je uložen u jastuk i pokrevljeća s činjenicama o usporedbi postupaka. U ovom procesu se koristi DLLME metoda koja je razvijena za analizu organofosfornih pesticida u ljudskoj mokraći. Ova metoda se koristi za puštenje pretrženih uzoraka mokraće u sistem za analizu, pri čemu se uzorci prate diskretnim faza u slagaju krajih točaka. Uzorci mokraće se pripremaju u mikrocentripliku čim je uložen u jastuk i pokrevljeća s činjenicama o usporedbi postupaka. U ovom procesu se koristi DLLME metoda koja je razvijena za analizu organofosfornih pesticida u ljudskoj mokraći.