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Determination of microcystins in biological samples by matrix solid-phase dispersion and liquid chromatography–mass spectrometry

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Abstract

A method for the detection and quantification of the microcystins (MCs)—MC-LR, MC-RR and MC-YR—in biological samples by matrix solid-phase dispersion (MSPD) has been developed. The optimum extraction conditions were 500 mg of liver or kidney, C_{18} bonded silica as dispersant, and a mixture methanol–water (70:30) as eluent. The MCs were determined by liquid chromatography electrospray mass spectrometry (LC/ES/MS). Recoveries of biological extracts at three different spiked levels (1–10 mg kg^{-1}) ranged from 40.5 to 87.0% in liver, and from 52.5 to 74.5 in kidney. R.S.D.s were <15.6% and <10.6%, respectively. The detection and quantification limits were 0.05 and 0.5 mg kg^{-1}, for all MCs. The method was applied to MCs detection in liver and kidney of rat previously injected i.p. with MC-LR. Results showed the presence of MC-LR in the liver of the animals injected with the highest dose.

Keywords: Microcystins; Matrix solid-phase dispersion; Liver; Kidney; Rat; Liquid chromatography–mass spectrometry

1. Introduction

Microcystins (MCs) are cyclic heptapeptide toxins, produced by cyanobacteria. MCs constitute a potential hazard to animals and humans because of they are selective hepatotoxic in mammals and act as a potent tumour promoters through inhibition of protein phosphatases 1 and 2A. Although MCs are present in surface water bodies worldwide, little has been done to assess the effects of chronic exposure to these substances in animal or human population. An experience occurred in 1996, from Brazil where a number of dialysis patients died because of the presence of MCs in the water used for routine haemodialysis was a serious advice. Liver tissue from deceased patients, showed a MCs content of up to 0.1–0.5 ng mg^{-1} [1]. Chronic exposure to MCs is less dramatic, but more widespread. The most likely route of exposure to cyanobacterial toxins is via oral ingestion. About 70% of the toxin is rapidly localized in the liver. The kidney and intestine also accumulate significant amounts of MCs [1–4]. Because of the human health risk associated with MCs, analytical techniques that enable their rapid, specific and sensitive determination in biological samples are needed for routine monitoring and early warning purposes.

The methods to determine MCs can be distinguished in screening and quantification methods. First ones consisted of biological and/or biochemical procedures, which indicated the presence of MCs in samples at highly sensitive levels. However, they cannot discriminate MCs analogues. Second ones, based on chromatography and capillary electrophoresis (CE) are the only methods for MCs that separate the toxins and allow individual identification in different matrices. As a confirmatory method, mass spectrometry (MS) is of choice [5,6].

Liquid chromatography–mass spectrometry (LC–MS) is the preferred technique to identify and quantify MCs [7–9]. Since it is capable of determining trace amounts of each MCs separately at the same time that provides enough structural information to identify them.

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The main disadvantage of the analytical methods is that involve a number of steps such as extraction and clean-up mainly conducted to isolate the analytes. The most common extraction technique for MCs is solvent extraction followed by concentration step and silica gel or octadecyl silica clean-up [5]. Solid-phase extraction (SPE), has proved to be a useful tool for MCs purification or concentration from algal cells [10,11], cyanobacterial blooms [12,13], water [14,15] and biological samples [16]. SPE has proved to be a good alternative to solvent extraction [11,17].

Matrix solid-phase dispersion (MSPD) is an extraction technique that involves blending a small amount of the biological sample with a solid support, followed by washing and eluting with very few ml of solvent [18–20]. In MSPD, extraction and clean-up are carried out in a single step, which can avoid the general drawbacks of the other methods. MSPD has been reported to extract pesticides, drugs and vitamins from vegetables, milk and biological tissues [18–20]. Several review papers appeared recently, but to our knowledge, no studies are available in the literature on extraction of MCs from biological matrices.

This study reports the development of a MSPD extraction using LC/MS/ES technique for the simultaneous determination of MC-LR, MC-RR and MC-YR from rat liver and kidney. Selected MSPD method was applied to liver and kidney excised from male Wistar rats previously injected i.p. with MC-LR at a dose of 100, 150 and 180 $\mu g\,kg^{-1}$ body weight.

2. Experimental

2.1. Chemicals

Microcystins standards (MC-LR, MC-YR, MC-RR) were supplied by Calbiochem-Novabiochem (Nottingham, UK). Standard stock solutions of each MC at a concentration of 500 $\mu g\,ml^{-1}$ were prepared in methanol and stored in glass-stopper bottles at 4 °C. Standard working solutions at the appropriate concentration of each toxin, as well as mixtures of the tree toxins, were daily prepared in methanol.

HPLC-grade methanol, acetonitrile and dichloromethane were purchased from Merck (Darmstadt, Germany). Deionized water (>18 $M\Omega\,cm^{-1}$ resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Trifluoroacetic acid (TFA) for UV spectroscopy was purchased for Fluka-Chemika (Switzerland). Cellulose filter (0.45 μm) was from Scharlau (Barcelona, Spain).

2.2. Matrix solid-phase dispersion extraction procedure

Rat liver and kidney (500 mg) were placed into a mortar, which contained 1 g of C_{18} bonded porous silica, and gently blended for 5 min using a pestle to obtain a homogeneous mixture. This mixture was transferred to a 100 mm × 9 mm i.d. glass column and washed with 10 ml of dichloromethane to remove interferences. Elution of MCs, with 15 ml of 100% methanol or 70% aqueous methanol solution, was carried out applying a slight vacuum. MCs extract solution was concentrated to 0.5 ml, at 50 °C under stream of nitrogen. Then 5 μl aliquot of this extract was injected onto the LC–MS system.

Extraction efficiencies were determined by spiking fresh tissue samples with volumes between 10 and 50 μl of the working mixtures at appropriate concentrations. The spiked sample was allowed to stand for 1 h before extraction to achieve the MCs distribution in the tissue.

2.3. Acute toxicity assays

Twenty male Wistar rats (200 g weight) were used in the study. Rats were housed in standard plastic cages (Panlab SL) and maintained on a 12 h light–dark cycle in a temperature-controlled colony room at 22 ± 2 °C with free access to rodent pellet and tap water. At these conditions, rats were acclimatized for a week and randomly assigned to four groups, consisting of five rats each one. Rats were treated with MC-LR (n = 5) at low, middle and high doses (100, 150 and 180 $\mu g\,kg^{-1}$ body weight i.p.). MCs were dissolved with 0.2% methanol as vehicle solution in physiologic saline solution (0.9%). Control group (n = 5) was treated with the vehicle solution without MC-LR. Rats were euthanized by ether inhalation at 8 h. Liver and kidney were quickly excised and weighted, rinsed with ice-cold 0.9% (w/v) saline solution and stored at −20 °C.

2.4. Chromatographic conditions

LC–MS was carried out with a Hewlett Packard (Palo Alto, CA, USA) HP-1100 Series system equipped with a binary solvent pump, an autosampler, and a mass spectrometry detector (MSD) coupled to an analytical work station. The MSD consisted of a standard API source configured as ES and a single quadrupole. The analytes where chromatographed on a phenomenex C_{18} (250 mm × 4.6 mm i.d., 5 μm) stainless steel column with a guard column LiChrosorb RP-8 (10 mm × 4.6 mm, 5 μm) both from Supelco (Madrid, Spain) using acetonitrile and water with 0.05% TFA as mobile phase. The gradient selected for LC/ES/MS at the flow rate of 0.4 $ml\,min^{-1}$ was acetonitrile 35%, linearly increased to 65% in 15 min and held at 65% for 5 min then, the system returned to the initial conditions in 5 min.

The ES/MS interface in positive mode operated at 350 °C gas temperature, 13.0 $l\,min^{-1}$ drying gas flow, 413,685.42 Pa nebulizer gas pressure and 4000 V capillary voltage. Full-scan LC/MS chromatograms were obtained by scanning from m/z 100 to 1200 with a scan time of 0.75 s. Time-scheduled selected-ion monitoring (SIM) of the two most abundant ions of each compound used for quantification as shown in Table 1. The optimum fragmentor voltage were pre-set for each group of ions monitoring at the same time and automatically tuned using the instrument control utilities included in the software.

Table 1
Time schedule SIM conditions for monitoring microcystins

Time (min)	MC	MW	SIM *m/z*	Gain	Fragmentor (V)	Dwell time (ms)
0–13	MC-RR	1037	519.5	2	120	400
13–20	MC-YR	1044	911.5	2	180	98
			1045.5			98
	MC-LR	994	861.5	2		98
			995.6			98

2.5. MS/MS confirmation

ESI-MS/MS experiments, for confirming the identity of MC-LR found in exposed rat liver, were performed on an Esquire 3000 Ion Trap LC–MS^n system (Brucker Daltonik, Germany) and an Agilent 1100 series LC system by flow injection. The ion source parameters were the same as those used for LC–ESI-MS since both ESI interfaces have the same design. The ion trap was run in multiple reaction monitoring (MRM) mode. Ions were detected in ion charged control (ICC) mode setting the target at 50,000 ions and maximum accumulation time at 50 ms. Negative ions were detected since fragmentation is easy. Product ion was detected using a cutoff of *m/z* 500 and amplitude of 2.0 V with a width of 1.0.

3. Results and discussion

3.1. LC–MS determination

Reversed-phase LC of MCs often involves the use of gradient mobile phases consisting of acetonitrile in water at acidic pH. The chromatographic resolution is considerably improved by the addition of perfluorinated alkyl carboxylic acids such as TFA, which maintains low pH to protonated carboxylic acid groups and it also acts an ion-pairing agent to reduce the interactions between basic groups and silanol groups on the silica surface. Its use combined with LC–MS has been widely reported [5–9] to determine MCs.

The base peak in the ES mass spectrum of MC-YR and MC-LR was the single charged molecular ion $[M + H]^+$. These MCs produced a second characteristic ion in high abundance at *m/z* 911.5 for MC-YR and 861.5 for MC-LR. The mass spectrum obtained for both toxins offers the possibility of identifying them by a second confirmatory ion maintained an appropriate sensitivity.

On the contrary, MC-RR gave as base peak the double charged protonated molecular ion $[M + 2H]^{2+}$ at *m/z* 520, which is typical for the MCs that contain two Arg residues. The single charged protonated molecular ion $[M + H]^+$ at *m/z* 1038 was also observed in the mass spectrum as a minority peak. The relative ratio between both molecular ions (100:5) decreases sensitivity one order of magnitude when the single charge molecular ion was selected as confirmatory ion. Because the correspondence of the retention time and the molecular weight can provide sufficient specificity for identifying target compounds, the MC-RR was identified only by the double charged molecular ion.

3.2. Optimization of the MSPD extraction

Elution of MCs from the dispersion was carried out with different methanol proportions to establish the best elution procedure using liver and kidney samples spiked at 2 $\mu g\,g^{-1}$ of each MC. This concentration, which is around 10 times higher than the limit of quantification (LOQ), was selected to determine better the degree of improvement or deterioration in the method efficiency obtained. Fig. 1 shows the recoveries (%) and reproducibilities (R.S.D.s; %, $n = 4$) of MCs elution with 70% aqueous methanol and 100% methanol.

Elution with 70% aqueous methanol provided mean recoveries higher than 50% for all MCs; elution with 100% methanol gave low mean recoveries for MC-LR (40.7%), MC-YR (45.2%) and MC-RR (48.4%) in rat liver extract. The most important differences eluting MCs from matrix dispersion column with 70% aqueous methanol was for MC-YR. MC-YR recoveries were 81.3% in kidney extract and 61.3% in liver extract. To obtain maximum recoveries from rat liver and kidney, the MCs were eluted in further experiments with methanol–water (70:30, v/v) after MSPD with 500 mg of C_{18} sorbent.

Recent references found in the literature verified that methanol proportion, in water modified MCs recoveries. It has been also reported that the best extraction was observed using between 50 and 80% methanol pointed out 70% aqueous methanol as the most suitable elution mixture [5,11,17].

The accuracy, calculated as the percentage of recovery, and the reproducibility, expressed as R.S.D. of the MSPD procedure are presented in Table 2. Recovery experiments were performed in quintuplicate spiking 500 mg rat liver or kidney samples with MCs fortification solution at three levels ranged from 0.15 to 10 $\mu g\,g^{-1}$. Quantitative determination of the recovery rate was performed using the external standard technique; that is, comparing peak areas in sample chromatograms with the corresponding peak areas obtained

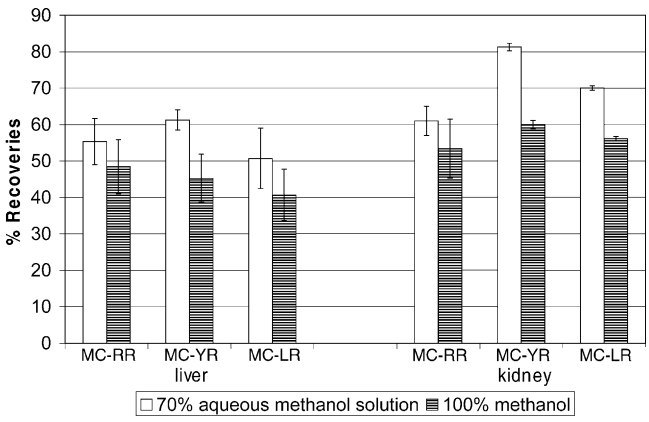

Fig. 1. Recovery (%) and R.S.D. (%) for rat liver and kidney samples extracted by MSPD according to the elution solvent.

Table 2
Recovery and R.S.D. for MC-RR, MC-YR and MC-LR spiked at three levels in rat liver and kidney samples

MC	Recovery (%) ± R.S.D. (%)					
	Spiked level in liver ($\mu g\,g^{-1}$)			Spiked level in kidney ($\mu g\,g^{-1}$)		
	0.15	1	10	0.15	1	10
MC-RR	62.5 ± 12.0	62.0 ± 4.2	40.5 ± 4.9	57.0 ± 8.5	60.5 ± 0.7	54.5 ± 4.9
MC-YR	87.0 ± 15.6	85.5 ± 7.8	45.5 ± 7.8	55.5 ± 10.6	59.0 ± 4.2	52.5 ± 3.5
MC-LR	67.0 ± 5.7	67.0 ± 2.8	40.5 ± 6.4	74.5 ± 6.4	61.0 ± 2.8	57.0 ± 2.8

from standard solutions of the toxins prepared in matrix extracts.

The accuracy ranges from 40 to 87% with a precision lower than 16% in liver, and from 52 to 76% with a precision lower than 11% in kidney. The results were quite similar for both matrices, demonstrating the effectiveness of MSPD microextraction. Generally, results obtained with rat kidney were better than those obtained with rat liver, except for MC-YR (Table 2). The low recovery obtained for some MCs can be caused for many factors as the complexity of the matrix, retention in the C_{18}, losses in the evaporation step, or covalent bound to proteins. These factors were carefully checked to try to improve this recovery without any success.

One possible explanation, which acquires much credibility, is the possible covalent bound of MC to "–SH" groups in liver rat, which was checked by testing different times for allow the equilibration of the spiked samples (between 10 min and 12 h). The results showed that after 1 h, the residues can be homogeneously extracted without any difference at longer equilibrium times. Although it is known that free MCs are not found in great quantities in post-mortem liver samples, from animals that have died from MCs poisoning, because they bound covalently cysteine residues on protein phosphatases 1A and 2A, several authors [3,4,21] reported that the reaction is caused by an enzymatic action that can take place at pH lower than 7. In this study, the samples were spiked post-mortem, which provides the disappearance of the enzymatic activity.

Other of the most probable reasons would be the strong retention of MCs in C_{18} bonded phase. However, experiments performed without C_{18} (placing only the chopped liver or kidney in the glass column) resulted in unclear chromatograms characterized by a greater number of interfering peaks because the high extraction of fats.

The limits of detection (LOD) calculated using the base peak (optimum ES conditions obtained, signal-to-noise (*S*/*N*) ratio 3:1) was 0.01 $\mu g\,g^{-1}$, which correspond to an injected amount of 0.05 ng for the three MCs. The limit of quantification (LOQ) evaluated as *S*/*N* ratio equal to 10 in the selected LC/ES/MS conditions was found to be 0.15 $\mu g\,g^{-1}$.

Determination of matrix effects was carried out by analyzing in duplicate six standards of different concentration from 0.15 and 25 $\mu g\,g^{-1}$ in methanol and in kidney and liver extracts and comparing the slope of the calibration curve obtained for MC-RR, MC-YR and MC-LR, which are outlined in Table 3.

Both standards and samples showed a good linearity, with correlation coefficients greater than 0.99. An enhancement in the response of ca. 15% for MC-RR and MC-YR was noted in kidney. On the contrary, a decrease in the response of MC-RR (37%) and MC-LR (12%) was observed in liver. Therefore, to avoid biased results, matrix matched standards should always be used for quantifying unknown samples.

3.3. Evaluation of MCs accumulation in male Wistar rat liver and kidney

The toxic risk of MC exposure in rat liver and kidney was evaluated injecting male Wistar rats i.p. with MC-LR at three concentration levels ranged from 100 to 180 $\mu g\,kg^{-1}$ body weight. They were killed 8 h after the injection. No longer assays were developed because it has been widely reported that after a single i.p. exposure, rat injected to a dose range of 80–160 $\mu g\,kg^{-1}$ death with massive liver destruction and haemorrhage [22].

Fig. 2 shows LC–MS chromatograms obtained in positive ionization mode of MCs determination in rat liver. Fig. 2C shows the chromatogram of a liver extract added to a standard mixture containing MC-RR, MC-YR and MC-LR (100 μl of 5 $\mu g\,ml^{-1}$ of each toxin, 5 μl injected) using LC/ES/MS. The chromatogram in Fig. 2B illustrates liver extract obtained from an excised liver (R7) injected with a single i.p. dose

Table 3
Matrix calibration of liver and kidney in comparison with standard calibration

	MC-RR			MC-YR			MC-LR		
	Slope	*y*-Intercept	*r*	Slope	*y*-Intercept	*r*	Slope	*y*-Intercept	*r*
Standard	14500	3310.2	0.9991	18825	−2467.2	0.9983	11957	1710.2	0.9966
Liver	10582	−2386.3	0.9985	17947	−4074.6	0.9997	10507	−1196	0.9883
Kidney	16534	29644	0.9953	22577	−19405	0.9954	11781	8626.3	0.9970

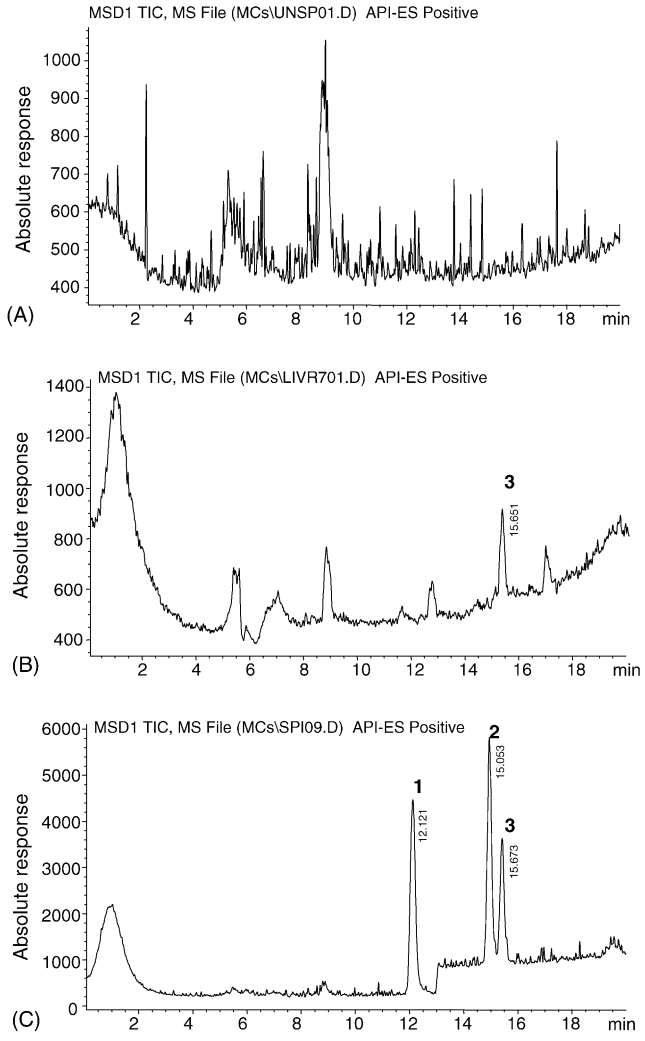

Fig. 2. LC–ES–MS chromatograms in SIM mode of: (A) untreated rat liver extract, sample R2, (B) rat liver extract R7 (after i.p. injection of 150 μg kg^{-1} MC-LR), (C) rat liver added with 5 μg ml^{-1} of MC mixture standards (1) MC-RR, (2) MC-YR, (3) MC-LR).

of MC-LR at 180 μg kg^{-1} in male Wistar rats, and chromatogram in Fig. 2A displays the liver extract of untreated rat (R2).

The same extract, the chromatogram of which is shown in Fig. 2B, was also injected by flow analysis in the ion trap to obtain and MS/MS spectrum that achieves the unequivocal identification of the MC-LR. The MS/MS was performed in negative mode since the sensitivity obtained was almost the same as that in the positive one. However, the deprotonated molecule $[M-H]^-$ is easy to fragment and the MS/MS spectrum showed a main fragment ion for the loss of a water molecule that achieved a very sensitive and selective confirmation. The MS/MS spectrum of the sample is shown in Fig. 3. It was identical to that obtained using standards prepared in methanol.

The results obtained in liver tissues of rat that had been injected with the highest dose level demonstrated that MSPD efficiently extracts MC-LR. Samples from rats injected at the

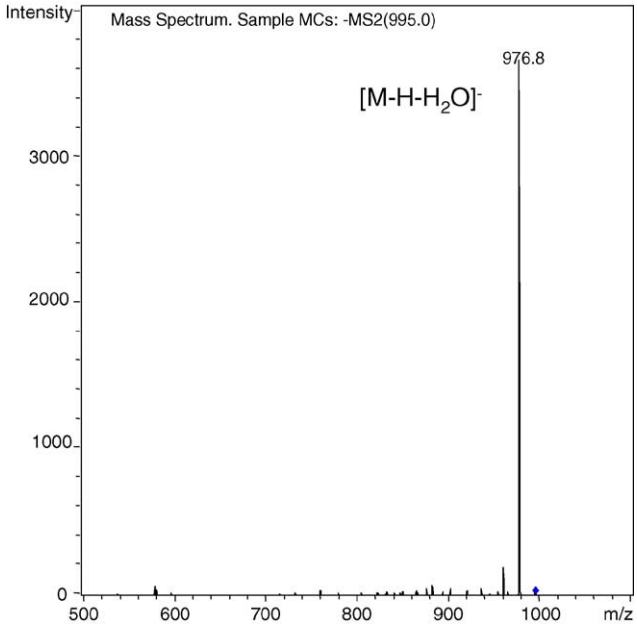

Fig. 3. ES–MS/MS spectrum obtained from rat liver extract R7 (after i.p. injection of 180 μg kg^{-1} MC-LR).

highest dose, presented residues of MC-LR at 0.18 μg g^{-1} with a S.D. of 16%. No MC-LR detection was observed in extract of liver tissue from rats injected with 100 or 150 μg of MC-LR per kg. Some references indicate that MC-LR present in liver tissue form a stable covalent complexes with liver enzymes and that is rapidly metabolized and eliminated [23]. So, low levels of MCs were detected because of modification, destruction and/or excretion of MC-LR residues. William et al. found that only 24% of the total MC-LR from Atlantic salmon liver was extractable. They suggest that the non-extractable MC-fraction was bound covalently and irreversibly in the liver tissue probably to protein phosphatase enzymes [23,24]. Same effect was observed in other works [10]. The WHO published a report that confirmed that MC-LR, MC-RR and MC-YR bind covalently to liver after i.p. administration to mouse [2].

No MC-LR was detected in kidney excised from treated male Wistar rats, independent of the MC-LR dose injected. A careful study of the published literature reveals that there is only available information about pathology and toxicokinetic of MCs in kidney, but no information was found about their analytical determination, independent of the animal specie studied.

4. Conclusion

This study demonstrates the applicability of MSPD followed by LC/ES/MS to determine of MCs in liver and kidney. The MSPD microextraction procedure offers some advantages over traditional methods because is very simple, rapid and requires only small sample sizes and solvent volumes.

The results presented here are intended to provide information for helping to formulate a more realistic risk-assessment routine monitoring model for liver and kidney hepatotoxin toxicity.

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