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Determination of herbicides and a metabolite in human urine by liquid chromatography–electrospray ionization mass spectrometry

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Abstract

A method was developed to determine simazine, atrazine and their metabolite, 2-chloro-4,6-diamino-1,3,5-triazine, in urine. The presence of these herbicides in urine may reflect possible exposure to pesticides. Sample preparation involved protein precipitation and solid-phase extraction. The samples were analyzed by high-performance liquid chromatography–mass spectrometry. The detection limits were 0.4 μg/l and the analytes have a linear response in the interval 6–800 μg/l. The precision of the method was reflected in the RSD of $<$2.4% for the herbicides studied. Based on the detectable herbicide levels from spiked urine samples collected from unexposed volunteers, this method can be used to determine the low levels necessary for establishing reference values of the selected herbicides and the metabolite.
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1. Introduction

Since the introduction of the 1,3,5-triazines in about 1960, various derivatives have been developed, which differ in the substituents in the 2, 4 and 6 positions. These derivatives of 1,3,5-triazines are broad-spectrum residual herbicides widely used for pre- and post-emergence weed control in corn, wheat, barley, and sorghum, as well as on right-of-ways, roadside verges and golf courses [1,2].

After application, three different types of degradation affect pesticides: physical, e.g. photolysis and temperature; chemical, e.g. hydrolysis; and biological, e.g. microbial [3]. These transformation processes control pesticide persistence in the soil and yield different dealkylated metabolites [3–5].

One study has investigated free atrazine and its metabolites in the urine of manufacturing workers with a total atrazine exposure (inhalation plus dermal exposure) varying from 10 to 700 μmol/workshift. The urinary excretion of atrazine plus its metabolites (mean 0.41 μmol/24 h) accounts for 1–2% of the external dose. Atrazine urinary metabolites consisted of bi-dealkylated (80%), deisopropylated (10%), deethylated (8%), as well as unmodified atrazine (2%), as shown in Fig. 1. The metabolites took nearly 24 h to be eliminated via urine, 50% of the amount being excreted in the first 8 h [6].

The chlorotriazine compounds (simazine, propazine and terbutylazine) follow the same biotransformation route as triazine [6,7]. Therefore, urinary

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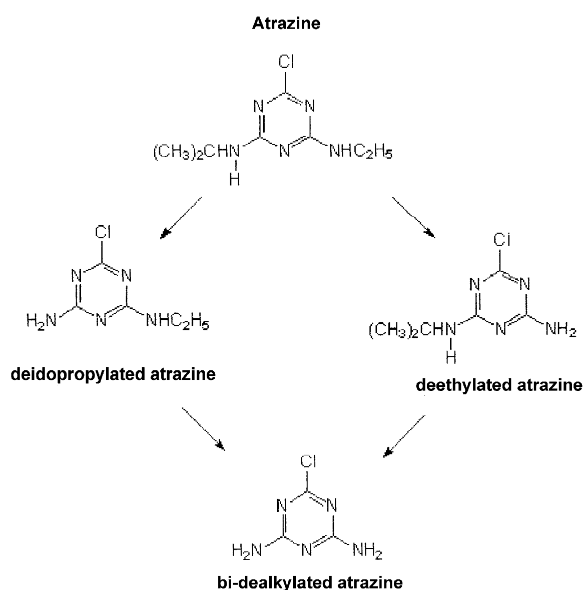


Fig. 1. Atrazine metabolism in mammals [6].

excretion of bi-dealkylated, deisopropylated and deethylated metabolites can be used for biological monitoring purposes. Since the above metabolites are not compound-specific, the unmodified compound measured in urine, even though it represents a minor portion of the absorbed dose, may be useful for qualitative confirmation of exposure when simultaneous exposure to different chlorotriazines occurs.

These studies on the fate of triazine herbicides in biological matrices have prompted the need for sensitive, specific methods for their determination.

Liquid chromatography coupled to mass spectrometry (LC–MS) is a powerful tool for analyzing compounds of low volatility or thermal lability and is applicable to a vast number of analytical problems [8]. The powerful features of LC–MS, such as efficient separation, identification, and quantification of polar analytes, makes this technique very attractive in the field of pesticide analysis. In addition, an important aspect of MS is its ability to identify and quantify compounds of the same class (e.g. organophosphorus, chlorotriazines) as well as different metabolites [3]. The availability of the robust atmospheric pressure interface technique, which combines high sensitivity/selectivity with reliable quantification, largely contributes to this breakthrough of LC–MS [9].

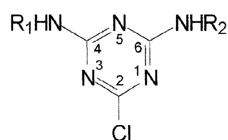
As a result of its rapid development in the last 10–15 years, various types of interface are now available, all having their characteristic advantages and limitations. The use of electrospray (ESI) interface LC–MS has the advantage of the direct analysis of polar herbicides and hydroxy metabolites, which can be injected directly into the LC–MS system after a simple extraction step, whereas GC–MS requires several intermediate derivatization steps prior to the characterization of these very polar herbicides and metabolites. On the other hand, in electrospray LC–MS there is a lack of structural information due to low fragmentation. A prerequisite is that the analyte must be ionizable in solution, so that the mobile phase often contains a small amount of a volatile acid or base. If such additives impair the chromatographic separation they can be added after the separation, before the eluent enters the ESI interface [10,11].

There are no reports in the literature on the application of electrospray LC–MS for the determination of pesticides and their corresponding metabolites in biological materials. Thus, the purpose of this work was to optimize an electrospray LC–MS method, using selective ion monitoring (SIM), for the characterization of the chlorotriazine herbicides simazine and atrazine and their dealkylated degradation product 2-chloro-4,6-diamino-1,3,5-triazine, to evaluate the selectivity and detectability of the LC–MS technique and to assess the performance of the MS method for the quantification of chlorotriazines and the metabolite in human urine samples.

2. Experimental

2.1. Materials

Standards of the herbicides (Fig. 2) were obtained from Novartis and the metabolite from Chem Service. Stock solutions of each triazine were prepared in methanol at concentrations of 104.4, 101.4 and 138 μg/ml for simazine, atrazine and 2-chloro-4,6-diamino-1,3,5-triazine, respectively. The solutions used to construct the calibration curves and to spike the samples were prepared in mobile phase at a concentration of 1000 μg/l of each herbicide and



Herbicide	Substituent in positions 4 and 6	
	R1	R2
2-chloro-4,6-diamino-1,3,5-triazine (metabolite)	-H	-H
Simazine	$-C_2H_5$	$-C_2H_5$
Atrazine	$-C_2H_5$	$-CH(CH_3)_2$

Fig. 2. Chemical structures of the herbicides studied.

stored in a refrigerator at 4 °C, where they were stable for at least 60 days.

The calibration curve consisted of at least seven different concentrations (6, 30, 60, 100, 200, 400 and 600 μg/l) of each analyte and the injections were made in triplicate.

The solvents acetonitrile and chloroform (Mallinckrodt) were chromatographic grade. Other reagents and solvents were analytical grade. Water was purified with a Millipore Milli-Q Plus System.

2.2. Urine collection and storage

Urine samples (blank) from human volunteers were collected and kept frozen at −20 °C until use. After the urine samples had been thawed, they were shaken for homogenization. The required volume was then sampled as quickly as possible to avoid sedimentation of any solids.

2.3. Sample preparation

Urine samples (1 ml) were fortified by the addition of a pre-determined volume of the stock solution containing atrazine, simazine and 2-chloro-4,6-diamino-1,3,5-triazine, resulting in three levels of fortification. After adjusting the pH to 9.0 with NH_4OH (0.01%) the sample was diluted with acetonitrile (1:2, v/v) at ambient temperature and the deproteinization treatment was carried out by centrifugation (5 min, 4000 rpm). A 1 ml aliquot of the supernatant was separated for the extraction procedure.

Envi C_{18} Supelclean extraction tubes (Supelco, 3 ml) were conditioned with 10 ml of methanol and equilibrated with 5 ml of Milli-Q water. After the 1 ml of fortified sample had passed through the cartridge under vacuum, the cartridge was washed with 5 ml of Milli-Q water, this eluate was discarded and the sorbent bed was dried under vacuum for 1 min. The analytes were then eluted with 3 ml of chloroform. The organic layer was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 200 μl of acetonitrile. The injection volume was 10 μl.

2.4. Instrumentation

The LC system consisted of a Rheodyne 7725i injector with a 10 μl loop, a Waters 510 pump, and a Waters UV–Vis absorbance detector (Model 486) coupled to the Chrom Perfect for Windows, version 3.03, program in a personal computer-compatible microcomputer for acquisition and treatment of data. The pH of the mobile phase was adjusted with the use of a Digimed, Model DM 21, pH meter with glass and thermal compensation electrodes. The column (150×3.9 mm I.D.) and guard column (20×3.9 mm I.D.) were Waters Nova-Pak C_{18}, 4 μm. All measurements were carried out at room temperature.

The mobile phase was acetonitrile–water (40:60, v/v) with the pH of the mobile phase adjusted to 3.0 with 0.1% CH_3COOH. The mobile phase flow-rate was set at 0.3 ml/min. UV detection was at 220 nm.

The samples were centrifuged using an Excelsa Centrifuge, Model MP, from Fanem (São Paulo, Brazil).

2.5. Mass spectrometry

The Fisons VG Platform with a quadrupole MS system was operated using the ESI source. All measurements were carried out using the positive ESI mode.

For optimization of the MS parameters, each compound was dissolved in pH 3 mobile phase and injected separately. For the LC–MS a source temperature of 150 °C was optimal. Nitrogen was used as nebulizer gas and drying gas at flow-rates of 30

and 300 l/h, respectively. Repetitive on-column injections of each of the solutions and the test mixture were made using an LC system consisting of a Rheodyne 7725i injector with a 10 μl loop, a Shimadzu LC-10AD pump and a Shimadzu diode-array detector SPD-M10A (VP), using the same column and guard column.

The capillary voltage was 25 V for all the herbicides and the metabolite, and acquisition was in the SIM mode.

3. Results and discussion

3.1. Optimization of the mobile phase and the LC–MS

Separation of the triazine herbicides and their metabolite was tested at various compositions of the eluent and at different pH values. Due to their polar character, the triazines do not interact strongly with the C_{18} reversed phase, the most utilized stationary phase in LC. Furthermore, it is necessary to add acetic acid to the mobile phase to increase the molecular ionization of the compounds and to improve the detection in analysis by MS.

The UV detector was set at 220 nm as a compromise between the maximum absorbance of the analytes and the reduced background of the eluents at this wavelength [12]. Acetonitrile was chosen as organic solvent of the mobile phase owing to its low absorbance background in the UV region. After testing different conditions for the separation of the herbicides, the preferable mobile phase was MeCN–water–CH_3COOH (50:50:0.01, v/v), giving the chromatogram shown in Fig. 3.

After defining the conditions for selective ion monitoring of the triazines, the product ions were recorded with a single quadrupole set at a fixed m/z value representing $[M+H]^+$. The precursor and product ions, as well as the ions selected for quantification, are shown in Table 1. A urine sample is a complex matrix consisting of various components, therefore it is essential to use an extraction procedure to remove these interferents. However, with the quality of the MS through the use of SIM, no problem was encountered in the detection of the analytes, because only the ion corresponding to each compound was selectively monitored.

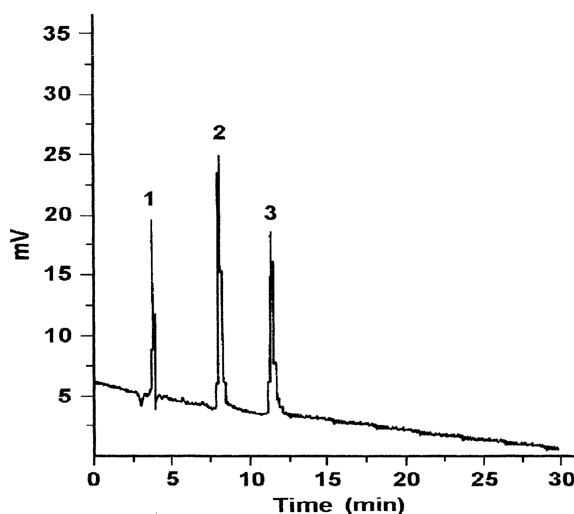


Fig. 3. LC chromatogram obtained in the separation of triazine herbicides and a metabolite. Conditions: column, Nova-Pak C_{18} (4 μm); mobile phase, MeCN–water–CH_3COOH (50:50:0.01, v/v); flow-rate, 0.3 ml/min; detection, UV at 220 nm; injection volume, 10 μl. Elution order: (1) 2-chloro-4,6-diamino-1,3,5-triazine, (2) simazine, (3) atrazine.

3.2. Sample preparation

In previous reports, the herbicides were isolated from biological or environmental samples by repeated extraction with organic solvents and centrifugation, much more complicated and more time-consuming procedures [13]. In the present investigation, the C_{18} cartridges were eluted with methanol and compared with those eluted with chloroform. Recovery was generally excellent for both solvent systems, but backgrounds were cleaner with the use of chloroform. The evaporation time for the chloroform eluate was also shorter than that for methanol. Therefore, the use of chloroform as an elution solvent is recommended for the triazines under study.

3.3. Validation of the method

Table 2 shows the recovery data and the limits of detection and quantification of the three herbicides considered in our study. The recoveries were ob-

Table 1
Characteristic ions and the ion employed in the analysis of the herbicides using the positive ionization mode

Herbicide	Molecular mass	Product ion in MS[a] (m/z)	Ion selected for quantification
2-Chloro-4,6-diamino-1,3,5-triazine (metabolite)	145	61 (85), 85 (60), 122 (95), 132 (50), 143 (100), 145 (83), 147 (40)	145
Simazine	201	61 (50), 75 (60), 85 (40), 143 (50), 145 (58), 202 (100), 204 (25)	202
Atrazine	215	61 (95), 75 (60), 86 (50), 143 (85), 145 (98), 216 (100), 218 (35)	216

[a] Numbers in parentheses give relative abundances of the product ions.

tained by triplicate analyses of urine spiked with each compound at three levels of fortification, and by comparing peak areas with those produced by the analysis of a known amount of the pure standard of each compound.

The average results obtained for herbicide recovery ranged from 82 to 114%; these values are considered acceptable for biological samples [14]. The limits of detection (LOD) and quantification (LOQ) were calculated at signal-to-noise ratios of 3 and 10, respectively. The detection limits for the herbicides were 0.4 μg/l.

Calibration curves were obtained using standard solutions of the triazines and showed good linearity in the range from 6 to 800 μg/l with correlation coefficients of >0.999.

Table 3 shows the intra- and inter-assay precision of the method. Intra-assay precision was determined by triplicate analyses of urine samples spiked at three different concentrations, while inter-assay precision was calculated by triplicate analyses of spiked urine carried out on three different days. The results of the intra- and inter-assay precision runs show acceptable precision with RSD values between 0.51 and 2.4%. For biological samples a RSD up to 15% is acceptable [15].

4. Conclusion

Satisfactory results were obtained for two triazine herbicides and their metabolite in a urine sample by applying electrospray ionization and SIM-MS after solid-phase extraction.

Table 2
Recoveries and limits of detection (LODs) and quantification (LOQs) for the herbicides in fortified urine samples ($n=3$)

Herbicide	Fortification (μg/l)	Recovery (%)	LOD[a] (μg/l)	LOQ[a] (μg/l)
2-Chloro-4,6-diamino-1,3,5-triazine (metabolite)	30	82	0.4	1.2
	60	92		
	120	101		
Simazine	10	92	0.4	1.2
	20	102		
	40	114		
Atrazine	30	84	0.4	1.2
	60	91		
	120	100		

[a] LOD and LOQ were obtained for the method after five-fold pre-concentration. LOD and LOQ were calculated at signal-to-noise ratios of 3 and 10, respectively.

Table 3
Precision (intra- and inter-assay) for the herbicides studied in fortified urine samples

Herbicide	Fortification (μg/l)	RSD (%, $n=3$)	
		Intra-assay	Inter-assay (3 days)
2-Chloro-4,6-diamino-1,3,5-triazine (metabolite)	30	0.71	2.2
	60	2.0	
	120	1.0	
Simazine	10	0.65	0.87
	20	0.55	
	40	0.51	
Atrazine	30	2.4	0.79
	60	0.64	
	120	1.0	

n, number of replicates.

LC–MS is rapidly becoming a routine technique for the efficient trace analysis of polar pesticides in complex matrices. In comparison with existing methodologies, solid-phase extraction followed by LC–MS considerably simplifies the extraction procedure, reducing the time of analysis and method development.

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