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General unknown screening of xenobiotics: the contribution of an acidic extraction

Recherche large de xénobiotique : contribution de l'extraction acide

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Abstract – Objectives: Although general unknown screening (GUS) is often used to detect and identify exogenous compounds in biological matrices, some compounds are not detected for two main reasons: first the method carried out for the extraction, secondly, the lack of sensitivity in the detection of the unknown molecules. The aim of this study was to improve the detection, using an acidic extraction procedure and a MRM mode (ES^+ or ES^-). **Methods:** Blank sera were spiked with 42 substances, not detected in GUS. 1 mL was extracted after addition of internal standards and 500 µL of acetate buffer by 3 mL of organic extraction solution (dichloromethane: ether: hexane: isoamyl alcohol; 30:50:20:0.5, v/v). Basic extraction was also carried out by substituting the sodium acetate buffer with saturated borate buffer. The extracts were analysed by UPLC-MS-MS, using MRM mode (ES^+ or ES^-). **Results:** All the non detected substances by the GUS method were indentified in this study, 25 in ES^+ mode and 17 in ES^- mode. Extraction yield was between 9 and 104%, and upper, compared to that after basic extraction. **Conclusion:** A rapid, sensitive and selective method using positive or negative MRM mode, with a triple quadrupole mass spectrometer and a simple acidic liquid-liquid extraction allows to identify, confirm and quantify 42 substances (drugs and pesticides) not detected by a routine GUS method.

Key words: General unknown screening, acidic extraction, MRM mode detection

Résumé – Objectifs : Bien que le screening large soit souvent utilisé pour détecter et identifier les composés exogènes dans les matrices biologiques, certains composés ne sont pas détectés pour deux raisons principales : une méthode d'extraction utilisée non adaptée et un manque de sensibilité dans la détection pour les composés inconnus. Le but de cette étude a été d'améliorer la détection en utilisant une méthode d'extraction acide et le mode MRM (ES^+ ou ES^-). **Méthodes** : Le blanc sérum a été surchargé avec 42 substances, non détectées par le screening de recherche large. 1 mL d'échantillon en présence d'étalons internes a été extrait avec 500 µL de tampon acétate et 3 mL d'un mélange de solvants (dichlorométhane/ether/hexane/alcool isoamylique 30/50/20/0,5 v/v). L'extraction basique a également été évaluée en substituant le tampon acétate par du tampon borate. Les extraits sont analysés par UPLC-MS/MS, en utilisant le mode MRM (ES^+ ou ES^-). **Résultats :** Tous les composés non détectés par la méthode screening large ont été identifiés dans cette étude, 25 en mode ES^+ et 17 en mode ES^-. Les rendements d'extraction étaient compris entre 9 et 104 %, et supérieurs à ceux obtenus après extraction basique. **Conclusion :** Une méthode rapide, sensible et spécifique utilisant le mode MRM en ES^+ ou ES^-, et une simple extraction liquide-liquide à pH acide, permet d'identifier, de confirmer et de quantifier 42 composés (médicaments et pesticides) non détectés par la méthode de screening de routine.

Mots clés : Screening large de recherche des xénobiotiques, extraction acide, détection en mode MRM

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1 Introduction

General unknown screening (GUS) is often used to detect and identify exogenous compounds in various biological matrices (blood, urine or hair) in clinical and forensic toxicology, but with very different concentrations depending on the circumstances of poisoning (acute poisoning or chemical submission). The screening of xenobiotics can be carried out using untargeted separative methods and identification by GC-MS

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[1–3], GC-MS/MS [4–6], HPLC-DAD[7], HPLC/MS [8, 9], HPCL-MS/MS [10, 11], UPLC-MS [12], or UPLC-MS/MS [13]. In these screenings, the identity of the compounds is established by comparison of the obtained spectra (UV or mass/z) with reference spectra from commercial or in-house made libraries.

Nowadays, LC-MS (-MS) or UPLC-MS (-MS) is used routinely in the framework of analysis prescribed in clinical or forensic toxicology, because this technology allows the detection of the largest number of compounds. However, some compounds are not detected in GUS, for two main reasons. The first is the method carried out for the extraction of the unknown molecules contained in the biological matrix; the second reason is the lack of sensitivity in the detection for these molecules. For this last point, Multi Reaction Monitoring (MRM) mode in mass spectrometry improves the detection limit of these compounds, but this acquisition only allows targeted research due to a setting of many specific MRM transitions.

Another point, the sample preparation (extraction from a biological matrix) is a step that must be evaluated and controlled, for obtaining the best limit of detection. A lot of molecules have been detected, because the authors focused on one substance [14], or on a given therapeutic class, such as benzodiazepines [15]. It is clear that a sample preparation method which is specifically directed toward a molecule or a group of similar molecules will be the most efficient and probably more sensitive, but will require other extractions to detect other molecules, potentially present. The repetition of extractions is laborious and requires larger sample.

A screening of xenobiotics by UPLC- MS using in-source fragmentation at increasing cone voltages has been described [13]. The xenobiotics were detected after mixture of two extractions one under acidic conditions, the other under basic conditions, by ionization in both ES^+ and ES^-. In this work, some xenobiotics (drugs or pesticides) were not detectable probably due to the sample preparation or a lack of sensitivity of the mass spectrometer. The aim of the study was to improve the detection of these compounds; an acidic extraction protocol was here investigated for the detection of these molecules in blood and compared to a basic extraction. MRM mode using ES^+ or ES^- was applied in order to improve detection of the different compounds.

2 Materials and methods

2.1 Standards and reagents

HPLC-grade methanol and acetonitrile were from Carlo Erba (Val de Reuil, France) and from JT Baker (France), respectively. Ammonium formate and formic acid were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Deionized water (Versol®) was obtained from Aguettant, (France). All remaining organic solvents and reagents were of analytical grade and obtained from the suppliers indicated: dichloromethane (Carlo Erba, France); diethylether (Panreac, France); hexane (Sharlau, France) and isoamyl alcohol (Merck, France).

Drug substances were supplied by various pharmaceutical companies. Individual stock solutions of the standard compounds were prepared in methanol at 1 g/L; these were kept at –20 °C until use.

Methylclonazepam and β-hydroxy ethyl theophylline were used as internal standards and were obtained from Roche (France) and Sigma-Aldrich, respectively. A mixed stock solution (IS mix) was prepared in methanol at 1.25 and 16 mg/L, respectively, and stored at –20°C until analysis.

2.2 Biological specimens

Blank human serum was obtained from EFS (Etablissement Français du Sang Lille, France).

2.3 Liquid chromatography conditions

The chromatographic system comprised an Acquity UPLC (Waters Corporation, MA, USA). Analytes were separated using an Acquity UPLC HSS C_{18}, 2.1 × 150 mm, 1.8 µm column (Waters Corporation, MA, USA) maintained at 50 °C. The mobile phase was a binary mixture of formate buffer 5 mM pH 3.0, adjusted with formic acid (A) and acetonitrile containing 0.1% formic acid (B) at a flow rate of 400 µL/min (generating a typical back-pressure of ~9000 psi) (0 min. = 87% A; 6–7 min = 5% A; 7.25–10 min = 87% A). An injection volume of 15 µL was used throughout.

2.4 Mass spectrometry conditions

Detection was performed using a Waters® TQ Detector, tandem quadrupole mass spectrometer (Waters Corporation, MA, USA) equipped with a Z-Spray™ source and ES probe. The instrument was controlled using Waters MassLynx™v4.1. Ionization was performed in ES^+ and in ES^-. Source conditions were as follows: source temperature was maintained at 150 °C; capillary voltage: 1000 V; extractor cone: 3V; cone gas flow rate: 50 L/h; desolvation gas flow rate and temperature: 650 L/h and 350 °C, respectively.

Infusions were performed at 10 µL/min with variations of the cone voltage, energies of accelerations for optimisation.

For each compound, the MRM transitions, cone voltage and collision energy are indicated in Table I.

2.5 Preparation of the solutions

Working solutions of the drugs or pesticides of interest were prepared by dilution of the stock solution with the mobile phase to obtain a concentration of 1 000 ng/mL. Small volumes were analysed in two separate infusion analyses; one in ES^+ and one in ES^- to optimize and obtain two transitions MRM (Table I).

2.6 Data processing

All data were processed using the QuanLynx application manager (Waters Corporation, MA, USA). This software permits the integration of MRM peaks at specific retention times and the calculation of their areas.

Table I. List of substances studied.

Name	Activity	Serum concentration ng/mL			Ion parent	ESI	Cone V	Transition 1		Transition 2	
		Therapeutic level	Toxic levels	Lethal levels				Daughter ion	Collision (eV)	Daughter ion	Collision (eV)
Floctafenic acid	Analgesic				333.1	+	40	295.3	30	267.3	40
Salicylic acid	analgesic	20 000–200 000	300 000–350 000	(–400 000)500 000	179.1	–	10	137.1	8	93	18
Adrafinil	psychotonic				167.1	+	35	152	22	167.1	16
Barbital (withdrawn 91)	Barbiturate	2 000–40 000	20 000–50 000	50 000	183	–	25	140.1	10	85.1	14
Bromadiolone	Rodenticide		20		525.3	–	55	250.2	36	93	38
Bumetanide	Diuretic				365.2	+	35	240.3	18	184.3	18
Bupropion	Antidepressant	25–100		7300	240.2	+	25	131.1	26	184	14
Butalbital	Barbiturate	1700–2600	10000–15000	15000–30000	223	–	30	180.3	14	84.9	18
Butobarbital (withdrawn)	Barbiturate	5 000–15 000	20 000	30 000	210.9	–	25	168	14	85.1	14
Carbutamide	Antidiabetic				272.1	+	25	156.2	18	74.3	12
Cetirizine	antihistaminic	appr.20–300	2 000–5 000		389.1	+	30	201	18	166	42
Chlorophacinone	Rodenticide				373	–	85	201.1	24	145	24
Diclofenac	NSAID[a]	500–3 000	50 000; 60 000		296.2	+	25	250	14	215.1	20
Diflunisal	NSAID[a]	40 000–200 000	300 000–500 000	600 000	249	–	35	205.1	20	157	34
DNOC	Fongicide	1 000–5 000	30 000–60 000	75 000	197	–	35	137	18	109.1	22
Enalipril	AHT[c]	10–100			377.3	+	35	234.4	22	303.3	18
Fenoprofen	NSAID[a]	30 000–60 000			241.1	–	20	197.1	10	93.1	22
Fluindione	Anticoagulant				239	–	60	169.1	42	145.1	40
Furosemide	Diuretic	1 000–6 000	25 000–50 000		329.3	–	35	285.1	14	205	22
Glibornuride	antidiabetic				367.1	+	25	170.2	18	152.2	18
Gliclazide	antidiabetic	4000			324.1	+	30	91.1	34	127.2	18
Glipizide	antidiabetic	100–1000	>2000		446.3	+	25	100.3	25	321.3	16
Imidapril	ACE inhibitor[b]				406.3	+	40	234	24	332.3	22
Ioxynil	Herbicide				369.9	–	40	127	34	215	32
Irbesartan	AHT[c]				429.3	+	35	207.3	30	84.2	34
Loratidine	Antihistaminic	1–20			383.1	+	45	337.1	24	267.1	34

Table I. Continued.

Name	Activity	Serum concentration ng/mL			Ion parent	ESI	Cone V	Transition 1		Transition 2	
		Therapeutic level	Toxic levels	Lethal levels				Daughter ion	Collision (eV)	Daughter ion	Collision (eV)
Mecoprop	Herbicide				212.9	–	25	140.9	14	71	10
Meloxicam	NSAID[a]	400–2 000			352.1	+	35	141.2	20	115.2	20
Modafinil Acid	Stimulant	2 000–3 000			167	+	40	115	34	152	22
Naproxen	NSAID[a]	20 000–100 000	200 000–400 000		231.1	+	25	185.1	16	170	28
Nifuroxazide	ATB[d]				276.2	+	35	121.2	20	93.1	28
Pentobarbital/Amobarbital	Barbiturate	1 000–10 000	10 000–19 000	15 000–25 000	225.1	–	30	181.9	14	85	14
Perindopril	AHT[c]	80–150			369.3	+	35	172.3	20	98.2	36
Phenobarbital	Barbiturate	10 000–30 000	30 000–40 000	50 000–60 000	231.3	–	25	42.1	14	188.1	10
Piretanide	Diuretic				363.2	+	40	282.1	24	236.2	26
Piroxicam	NSAID[a]	2 000–6 000	14 000		332.1	+	30	164.3	18	95.2	22
Ramipril	AHT[c]	appr.1–10			417.2	+	35	234.3	22	117.1	39
Secobarbital	Barbiturate	1000–2200	>3000	>5000	237.1	–	30	194.1	12	85.3	18
Sulindac	NSAID[a]	1 000–6 000			357.2	+	45	233.3	28		
Telmisartan	AHT[c]				515.4	+	50	276.3	44	211.4	42
Thiopental	anesthetic	1 000–5 000	10 000	10 000–15 000	241.3	–	30	101	14	58	14
Tolbutamide	antidiabetic	40 000–200 000	400 000–500 000	640 000	271.3	+	25	155.2	16	74.2	16

The therapeutic, toxic and lethal concentrations (when known) are indicated. For the MRM detection, ion parent and daughter ions are also indicated.
[a] NSAID : non-steroidal anti- inflammatory drug; [b] ACE inhibitor : angiotensin-converting enzyme (ACE) inhibitor; [c] AHT : antihypertensive; [d] ATB : antibiotic.

Table II. Yield of extraction after acidic or basic extraction.

Name	Extraction yield %		Estimated LOD (ng/mL)
	Acetate buffer	Borate buffer	after acidic extraction
Floctafenic acid	61%	0%	5
Salicylic acid	68%	0%	191
Adrafinil	57%	0%	1 100
Barbital (withdrawn 91)	54%	29%	1 000
Bromadiolone	83%	33%	50
Bumetanide	62%	0%	50
Bupropion	77%	30%	10
Butalbital	54%	30%	5 000
Butobarbital	63%	70%	10 300
Carbutamide	54%	0%	10
Cetirizine	61%	1%	3
Chlorophacinone	47%	11%	49
Diclofenac	61%	10%	32
Diflunisal	80%	10%	9 000
DNOC	80%	3%	49
Enalipril	58%	0%	0.8
Fenoprofen	100%	0%	297
Fluindione	89%	0%	5 000
Furosemide	70%	0%	608
Glibornuride	73%	15%	5
Gliclazide	86%	21%	4
Glipizide	71%	3%	5
Imidapril	104%	0%	900
Ioxynil	85%	0%	49
Irbesartan	79%	0%	0.5
Loratidine	68%	50%	0.2
Mecoprop	87%	0%	5 000
Meloxicam	56%	0%	21
Modafinil Acid	101%	0%	310
Naproxen	9%	0%	1 088
Nifuroxazide	53%	19%	0.5
Pentobarbital/Amobarbital	83%	79%	3 728
Perindopril	67%	0%	10
Phenobarbital	68%	50%	1 908
Piretanide	66%	1%	5
Piroxicam	95%	3%	6
Ramipril	65%	0%	0.9
Secobarbital	33%	50%	2 970
Sulindac	93%	2%	6
Telmisartan	67%	10%	50
Thiopental	44%	27%	1 880
Tolbutamide	55%	9%	1 008

2.7 Biological sample preparation

Blank sera were spiked with the substances approximately at high therapeutic concentrations, when these concentrations were known. When the therapeutic concentrations of the drug were unknown, or when the compound was a pesticide, concentration less than 5000 ng/mL was tested. Blank serum and spiked serum were prepared by a liquid- liquid extraction (LLE) by acidic extraction. One milliliter of biological sample, 100 µL of the IS mix and 500 µL sodium acetate buffer (1 M, pH 3.5) were added to a 10 mL Kimble tube and mixed. Samples were extracted by the addition of 3 mL organic extraction solution (dichloromethane: ether: hexane: isoamyl alcohol; 30:50:20:0.5, v/v). Samples were vortex-mixed for 2 min before centrifugation at 3000× g for 5 min. The upper, organic layer was transferred to a clean vial.

A basic extraction was also carried out using the same procedure, by substituting the sodium acetate buffer with a saturated borate buffer (pH 9.0)

The supernatant was evaporated under nitrogen at 60 °C. Dried extracts were reconstituted in 100 µL of mobile phase and vortex-mixed for 2 min before analysis.

A limit of detection has been estimated, using the highest therapeutic concentration or this last concentration by dilution 1:10 or 1:100. For substances for which the therapeutic concentrations were unknown and for the pesticides, an arbitrary concentration was first tested, and then diluted 1:10 and 1:100.

3 Results

Table II indicates the 42 tested xenobiotics. For each compound, the therapeutic, toxic and lethal concentrations (when known), the mode of ionization and the chosen transitions for the identification of drugs are described. Twenty five substances were detected in ES^+ mode and 17 in ES^- mode.

The yield of extraction was estimated after extraction in acidic (acetate buffer) or basic (borate buffer) condition. The results show clearly a better extraction using acetate buffer (Table II).

A limit of detection has been estimated, permitting the detection of all the studied compounds and the measurement of low, detectable concentrations for some compounds (Table II).

4 Discussion

Several general unknown screenings have been described for the detections of xenobiotics, but often the analytical protocol targeted a specific pharmacological class of drugs [16–18] or pesticides [6, 8].

In our original, systematic GUS procedure using a double extraction (basic and acidic), some substances were not detected [13]. Extraction of the substances was then carried out in acid and basic condition. The sensitivity of the detection has been increased by using MRM mode of detection, either in positive or negative mode. In this way, all the molecules could be detected, using only the acidic, at least at therapeutic concentrations (or less) for the drugs. Therefore, the screening described by Humbert *et al.* [13] is used for the GUS, and an acidic extraction is also carried out for those undetectable molecules, with detection by MRM mode. The two protocols are rapid and with the use of UPLC-MS-MS, the results are obtained in about 15 min for the routine procedure and 10 min in MRM mode for the acidic procedure.

5 Conclusion

We have developed a rapid, sensitive and selective UPLC-MS/MS method using a positive or negative MRM mode, with a triple quadruple mass spectrometer and a simple acidic liquid-liquid extraction with small sample size to identify, confirm and quantify 42 substances (drugs and pesticides) that were not detected by the routine GUS method used in our laboratory.

Conflicts of interest. The authors declare that there are no conflicts of interest.

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