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Pitfalls and Prevention Strategies for Liquid Chromatography–Tandem Mass Spectrometry in the Selected Reaction–Monitoring Mode for Drug Analysis

François-Ludovic Sauvage,[1,3] Jean-Michel Gaulier,[1] Gérard Lachâtre,[1,2] and Pierre Marquet[1,3*]

BACKGROUND: We observed cases of false-positive results with the use of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Different LC-MS/MS techniques that use the selected reaction–monitoring mode, routinely employed for the analysis and quantification of drugs and toxic compounds in biological matrices, were involved in the false-positive and potentially false-positive results obtained. We sought to analyze the causes of and solutions to this problem.

METHODS: We used a previously reported LC-MS/MS general unknown screening method, as well as manual spectral investigation in 1 case, to perform verification and identification of interfering compounds.

RESULTS: We observed that false-positive results involved: a metabolite of zolpidem that might have been mistaken for lysergic acid diethylamide, benzoylecgonine mistaken for atropine, and clomipramine and 3 phenothiazines that share several common ion transitions.

CONCLUSIONS: To prevent problems such as those we experienced, we recommend the use of stable-isotope internal standards when possible, relative retention times, 2 transitions or more per compound when possible, and acceptable relative abundance ratios between transitions, with an experience-based tolerance of $\pm$15% for transitions with a relative abundance $>$10% and with an extension to $\pm$25% for transitions $<$10% when the concentration is at the limit of quantification. A powerful general unknown screening procedure can help to confirm suspected interferences. Our results indicate that the specificity of screening procedures is questionable for LC-MS/MS analyses performed in the selected reaction–monitoring mode and involving a large number of compounds with only 1 transition per compound.

For forensic toxicology and therapeutic drug-monitoring applications, the analysis of biological samples can be performed using different techniques based on gas chromatography or liquid chromatography *(1–9)*. LC-MS and tandem LC-MS (LC-MS/MS)[4] are increasingly used in many laboratories, particularly for multicompound analysis *(10–11)*. Because of its high selectivity and specificity, LC-MS/MS based on the selection of precursor-to-product ion transitions and performed in the selected reaction–monitoring (SRM) mode is probably the most employed detection and quantification technique. The detection of unsuspected substances, in addition to the analysis of particular compounds, can be of the utmost importance in toxicology cases involving date rape drugs and in forensic analyses (in contrast to therapeutic drug monitoring, for which only specific compounds are targeted). Therefore, analytical methods suitable for clinical and forensic toxicology need careful development and validation, ideally following the recommendations recently published by Peters et al. *(12)*. Selectivity is crucial in such methods. As defined in Shah et al. *(13)*, selectivity in this context is "ability of a bioanalytical method to measure unequivocally and to differentiate the analyte(s) in the presence of components, which may be expected to be present. Typically, these might include metabolites, impurities, degradants, matrix components, etc." Thus, experiments aimed at evaluating the selectivity of a method have been proposed. These include (*a*) analysis of at least 6 sources of blank matrix, (*b*) analysis of blank matrices spiked with compounds expected to be

[1] CHU Limoges, Department of Pharmacology-Toxicology, France; [2] Universite de Limoges, Faculty of Pharmacy, Laboratory of Toxicology, Limoges, France; [3] INSERM U850, Limoges, France.
* Address correspondence to this author at: Department of Pharmacology-Toxicology, University Hospital, 2 av. Martin-Luther-King, 87042 LIMOGES cedex, France. Fax +33 555 05 61 62; e-mail pierre.marquet@unilim.fr.

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[4] Nonstandard abbreviations: LC-MS/MS, tandem LC-MS; SRM, selected-reaction–monitoring; IDA, information-dependent acquisition; GUS, general unknown screening; LSD, lysergic acid diethylamide.

present in real samples, (*c*) analysis of real samples with suspected interference(s) but without the targeted analyte, if applicable. Despite all precautions, however, not all interfering substances can be exhaustively investigated *(12)*. A complementary strategy is to use internal standards that are stable isotope–labeled analogs of the targeted analytes, with identical retention and extraction properties. Thus, a valid method should enable differentiation of interfering compounds from the compounds of interest and confirmation of the presence or absence of the latter based on predefined criteria *(14–17)*. Guidelines for confirmatory analysis by LC-MS/MS have been published by several organizations (World Anti-Doping Agency *(18)*, Society of Forensic Toxicologists *(19)*, Gesellschaft für Toxikologische und Forensische Chemie *(20)*, European Union *(21)*, and the FDA *(22)*). Rivier *(15)* proposed considering 4 identification points, including 1 precursor and 2 product ions, or 5 identification points with 2 precursor ions, each with 1 product ion. Moreover, Rivier adapted the concept of the ion relative-intensity tolerance window *(23)*, proposing variable ranges depending on the relative intensity of the base peak. Concerning this particular point, de Zeeuw *(14)*, by means of actual examples observed using LC-MS in the selected ion–monitoring mode, stated that although positive matches were obtained for selected ions by comparing their relative intensities, false-positive results could occur. Furthermore, Allen *(24)* recently reported a case in which, because only 1 transition was available with high enough abundance, false-positive results were obtained in the SRM mode for tramadol in urine of patients being treated with the antidepressant venlafaxine. Nordgren et al. *(25)* also reported that one-third of the findings that were positive using 1 SRM transition in their screening analysis of urine samples were found to be negative by confirmation analysis.

Although monitoring only 1 transition per compound in the SRM mode or 1 single ion in the SRM mode is advised against, recent reports show that some laboratories still employ this strategy, which is often not sufficient if metabolites have not been considered beforehand.

Because of these concerns, other strategies aimed at identifying the metabolites in unknown samples have recently been investigated. The hybrid triple-quadrupole linear ion trap technology used with information-dependent acquisition (IDA) recently improved the process of simultaneous detection and identification of a drug in a single run. IDA enables detection of the most abundant ions in each scan obtained in full scan single-stage MS or SRM modes (survey step), to automatically and instantly switch the instrument to the product-ion scan mode ("dependent" mode), in which these ions are selectively transmitted by the first quadrupole to the collision cell, where they are fragmented, and the resulting fragments analyzed in the third quadrupole, which is used as a linear ion trap. Then the instrument is switched back to the survey mode for the identification of new precursor ions. Using this strategy, we recently developed a general unknown screening (GUS) procedure in which the enhanced Q3-scan mode was used as survey mode *(26)*. Enhanced product-ion scan spectra were built by summing up the information obtained at 3 different collision energies (15 V, 40 V, and 65 V). Moreover, the applicability of this method was evaluated on authentic specimens (gastric content, serum, whole blood, and urine samples). In certain cases, identification of metabolites was possible owing to their spectral similarities to the parent compound. Generally, confirmation of the nature of putative metabolites was performed by (*i*) injecting these metabolites as pure compounds, if available; (*ii*) comparison with MS/MS spectra in the literature; or (*iii*) performing in vitro metabolism experiments with a pool of human liver microsomes and analyzing the incubation supernatants with the same technique.

In therapeutic drug monitoring and clinical or forensic toxicology, antidepressants, benzodiazepines, and neuroleptics are among the drugs most frequently encountered. Recent reports have reviewed the LC-MS and LC-MS/MS methods used for these agents *(10–11)*. This report presents particular situations, identified using the above-mentioned GUS procedure *(26)*, that may lead to false results with LC-MS or LC-MS/MS, in particular in the presence of metabolites of phenothiazines and antidepressants with amine-containing side chains, although the specific transitions and relative-intensity criteria are fulfilled.

Materials and Methods

STANDARDS AND REAGENTS

Organic solvents and reagents were of analytical grade. Acetonitrile and methanol were obtained from Carlo Erba, dichloromethane and isopropanol from Prolabo, and formic acid and ammonium formate from Sigma. Deionized water was prepared on a Direct-Q laboratory plant (Millipore).

Glafenine (internal standard) was purchased from Sigma. Stock solutions of lysergic acid diethylamide (LSD), iso-LSD, nor-LSD, nor-iso-LSD, 2-oxo-3-hydroxy-LSD, LSD-D_3, atropine, atropine-D_3 (all in acetonitrile), and benzoylecgonine (in methanol) were purchased from Cerilliant. Stock solutions of other standard compounds were prepared at 1 g/L in meth-

Table 1. MS/MS transitions for the compounds involved in cases 1 and 2.

Case	Compound name	Transition 1	Transition 2	Transition 3
1	Lysergide	324.2/223.1	324.2/208.1	324.1/180.1
	Lysergide-D3	327.6/226.1		
2	Atropine	290.2/124.1	290.2/93.0	
	Atropine-D3	293.2/127.1		

anol and were kept at −20 °C. Oasis HLB cartridges were purchased from Waters.

LC-MS AND SAMPLE PREPARATION

The chromatographic system consisted of a Perkin-Elmer Series 200LC high-pressure gradient pumping system and a Rheodyne Model 7725 injection valve equipped with a 5-μL internal loop. Detection was carried out with an Applied Biosystems 2000QTRAP™ LC-MS/MS system equipped with a TurboIonSpray™ ionization source, with nitrogen as curtain, source, and collision gas, operated in the positive ion mode and controlled by the Analyst 1.4 program. Sample preparation, chromatographic separation, and MS acquisition conditions for the 3 methods employed are described in Text 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue9.

In brief, the method for LSD, iso-LSD, and metabolite determination in urine was derived from a previously reported LC-MS method *(27)*. Sample preparation employed liquid-liquid extraction, separation was by gradient reversed-phase HPLC, and MS acquisition was performed in the SRM mode using 3 transitions per compound (except for the internal standard, for which only 1 transition was followed) (Table 1).

The detection and quantification of atropine in urine involved solid-phase extraction, separation by gradient reversed-phase HPLC (see Text 1 in the online Data Supplement), and MS acquisition in the SRM mode using the 2 transitions per compound (Table 1).

The settings of the GUS procedure were previously reported in detail *(26)*. Briefly, sample extraction was by solid-phase extraction, separation was by gradient reversed-phase HPLC (see Text 1 in the online Data Supplement), and acquisition was performed in the information-dependent acquisition mode, in which the tandem mass spectrometer continuously switched between a survey scan acquired in the enhanced-MS mode with dynamic subtraction of background noise and a dependent scan obtained in the enhanced product-ion scan mode.

Results

CASE 1: SCREENING FOR LYSERGIDE IN URINE

LSD (see Supplemental Fig. 1 in the online Data Supplement) is a highly potent psychoactive drug. For LSD, iso-LSD, and their metabolites, 3 of the main fragments obtained with LC-MS/MS were selected. Comparing the results obtained for an unknown urine sample and a calibration standard spiked at 5 μg/L in urine (Fig. 1), we observed a peak for the 3 LSD selected transitions at the same retention time as the deuterated internal standard LSD-D_3, i.e., at the retention time expected for LSD. Although the ratios between 2 of these 3 SRM transitions (*m/z* 324.2/223.1 and 324.1/180.1) would have been acceptable, careful analysis of the relative intensity of all 3 transitions with regard to the usual requirements for the definite identification of a compound (see Supplemental Table 1 in the online Data Supplement) *(18–21)* led us to conclude that LSD was absent (Table 2). The hypothesis of interference was also supported by the absence of iso-LSD, which is in natural equilibrium with LSD *(28)*. Further investigation using the GUS method described above allowed this interference to be identified as a metabolite of zolpidem with the same molecular weight as LSD (see Supplemental Fig. 1 in the online Data Supplement). Using this procedure we also identified 5 other previously described metabolites of zolpidem *(29, 30)* in the same urine sample, although zolpidem could not be detected, even with a specific, quantitative LC-MS/MS method *(31)*.

CASE 2: SCREENING OF ATROPINE IN URINE

An in-house LC-MS/MS method has been developed for the screening of atropine, an alkaloid derived from *Atropa belladonna* and *Datura stramonium*, a potent anticholinergic, hallucinogenic, and poisonous substance. The above-mentioned automatic optimization procedure selected 2 major fragments (*m/z* 124 and 93) of the pseudomolecular ion at *m/z* 290.1. Atropine-D_3 was used as internal standard. Applying this method for a forensic urine sample, we observed a peak at 6.01 min, a retention time very close to that of atropine-D3 and atropine (5.83 min), for both transitions (Fig. 2A). Moreover, the ratio between the 2 SRM transitions was not acceptable with respect to that in the calibrators (see Supplemental Table 2 in the online Data Supplement), suggesting that atropine was absent or had coeluted with another compound. The GUS procedure, involving a more progressive chromatographic separation, failed to find any atropine in the sample and identified the interfering substance as benzoylecgonine (Fig. 2B).

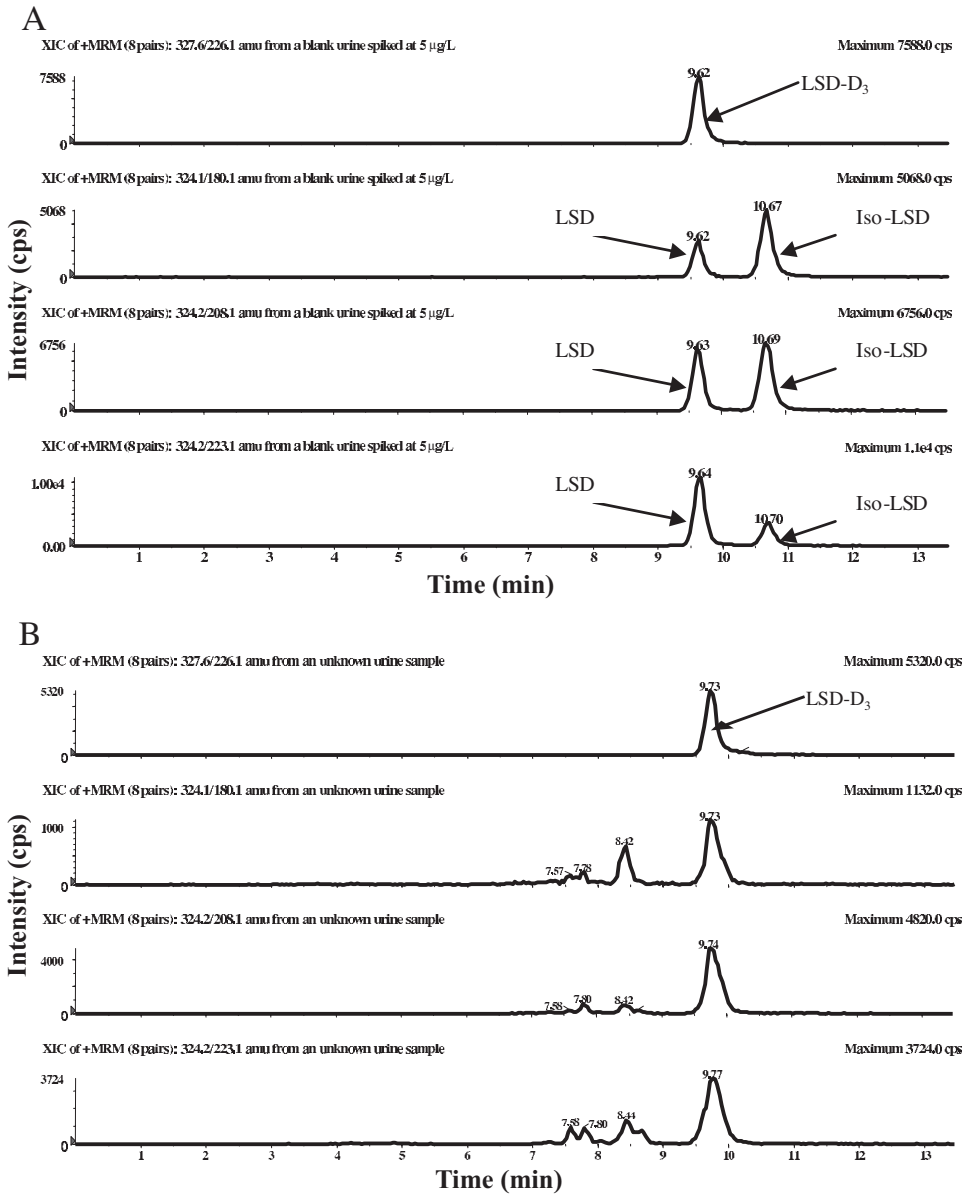

Fig. 1. Mass chromatograms of a blank urine sample spiked at 5 μg/L of LSD (A) and of a forensic urine sample (B).

CASE 3: INTERFERENCE BETWEEN PHENOTHIAZINES AND CLOMIPRAMINE

By analyzing several unknown urine samples using our LC-MS/MS GUS procedure, we observed that 4 different compounds with a pseudomolecular ion at *m/z* 315 were regularly detected (Fig. 3A). Among these, only clomipramine (Fig. 3A IV) was easily identified through library searching. Moreover, the major fragments observed at *m/z* 270, 242, 86, and 58 could be elucidated by applying the pathway proposed for trimipramine by Joyce et al. *(32)* (Fig. 3B). The other compounds were identified as metabolites of acepromethazine (Fig. 3A I), methotrimeprazine (Fig. 3A II), and trimeprazine (Fig. 3A III). (Also see Fig. 2 and Supplemental Table 3 in the online Data Supplement.)

The fragments observed are summarized in Table 3. At least 4 major fragments of clomipramine would not have been specific enough with respect to those of these phenothiazine metabolites. This example illustrates the potential risk of false-positive results that occur when the SRM mode is used with the *m/z* 315/270, 315/242, 315/86, or 315/58 transitions for clomipra-

Table 2. Comparison between the mean relative intensities observed in case 1 (screening for lysergide in urine) and reported tolerance ranges.

Selected lysergide transitions	Mean relative intensity			Tolerance ranges			
	Calibration standards	Quality control	Unknown sample	EC	WADA	SOFT/AAFS	FDA
324.2/223.1	100%	100%	100%	80–120%	85–115%	70 (80)–130% (120%)	80%–120%
324.2/208.1	53.4%	59.5%	130.4%	42.7–64.1%	38.4–68.4%	37.4% (42.7%) to 69.5% (64.1%)	42.7%–64.1%
324.1/180.1	22.9%	21.8%	30.6%	17.2–28.6%	12.9–32.9%	16% (18.3%) to 29.7% (27.4%)	17.2%–28.6%

mine. Owing to the long chromatographic separation used here (30 min), the retention times of these compounds were different, but the risk would obviously increase with the use of a shorter chromatographic separation and even more so if only 1 SRM transition was monitored.

Discussion

The increasing use of LC-MS and LC-MS/MS methods, combining multicompound analysis, simplified sample preparation, and shorter chromatographic run-times with respect to HPLC-ultraviolet, gas chromatography, and even gas chromatography–MS techniques, has been recently highlighted *(10)*. More than ever, despite the known specificity of LC-MS/MS, the criteria allowing formal and specific identification of compounds should not be underestimated. Applying institutional guidelines is necessary as a first line of improvement, as illustrated by Maralikova and Weinmann *(17)*. Indeed, interferences and false-positive results can occur in the SRM mode if minimum requirements are not met. The use of only 1 SRM transition, as illustrated by Nordgren et al. *(25)*, is obviously not sufficient to identify a compound, nor is the use of 2 transitions with predefined relative intensity ratios in certain cases, such as in case 1 presented above. When only 1 useful fragment can be obtained for a compound (as for tramadol *(24)*), particular care is required to identify the possible interferences, and even more importance should be given to the extraction and separation procedures. Furthermore, as described in case 2, appropriate chromatographic separation combined with the use of a deuterated analog as internal standard allowed us to differentiate atropine and benzoylecgonine. This finding is important for forensic applications, as evidence by recently reported cases of atropine-adulterated cocaine intoxication *(33)*. In such cases, patient urine samples may contain both atropine and benzoylecgonine, which is the major metabolite of cocaine. Therefore, before applying an LC-MS/MS method for atropine, the analysis of a blank urine sample spiked with a high concentration of benzoylecgonine is recommended to verify the absence of interference between these 2 compounds.

When products with similar chemical properties (close chromatographic retention times and molecular weights) are to be analyzed using a single technique, the selected transitions should be the most specific ones, which are not always the most intense (as generally picked up by the automatic optimization algorithms). Particular precautions were presented in a recent report of a method involving only 1 transition per compound *(34)*, taking into account isobaric drugs and metabolites. However, although the isobaric compounds studied by these authors were chromatographically separated, some unknown metabolites or potentially coadministrated drugs might still interfere if nonspecific fragments were selected, as previously described in the case of tramadol and a venlafaxine metabolite *(24)* or in case 1 herein between LSD and a zolpidem metabolite. In the latter case, only an LC-MS/MS screening technique *(26)* permitted identification of the interfering compound, but unfortunately this technique was not sensitive enough to detect LSD at its circulating concentrations in human biofluids using the full scan mode on a 2000QTRAP instrument. When we discovered this interference, we monitored another transition for the determination of LSD, now characterized by its retention time, and 3 specific SRM transitions plus their respective abundances relative to LSD-D3. The use of such precautions was unfortunately not reported in some recently described LC-MS/MS methods concerning different types of compounds, which involved only 1 or 2 transitions per compound *(33–36)*.

In particular, screening techniques for a large number of drugs with only 1 SRM transition per compound *(35, 36)* are prone to yield false-positive results, even if the transition selected presented no interference with the other compounds targeted and if all were chromatographically separated. Indeed, natural or synthetic compounds or metabolites present in biological matrices (e.g., whole blood, plasma, urine) can yield

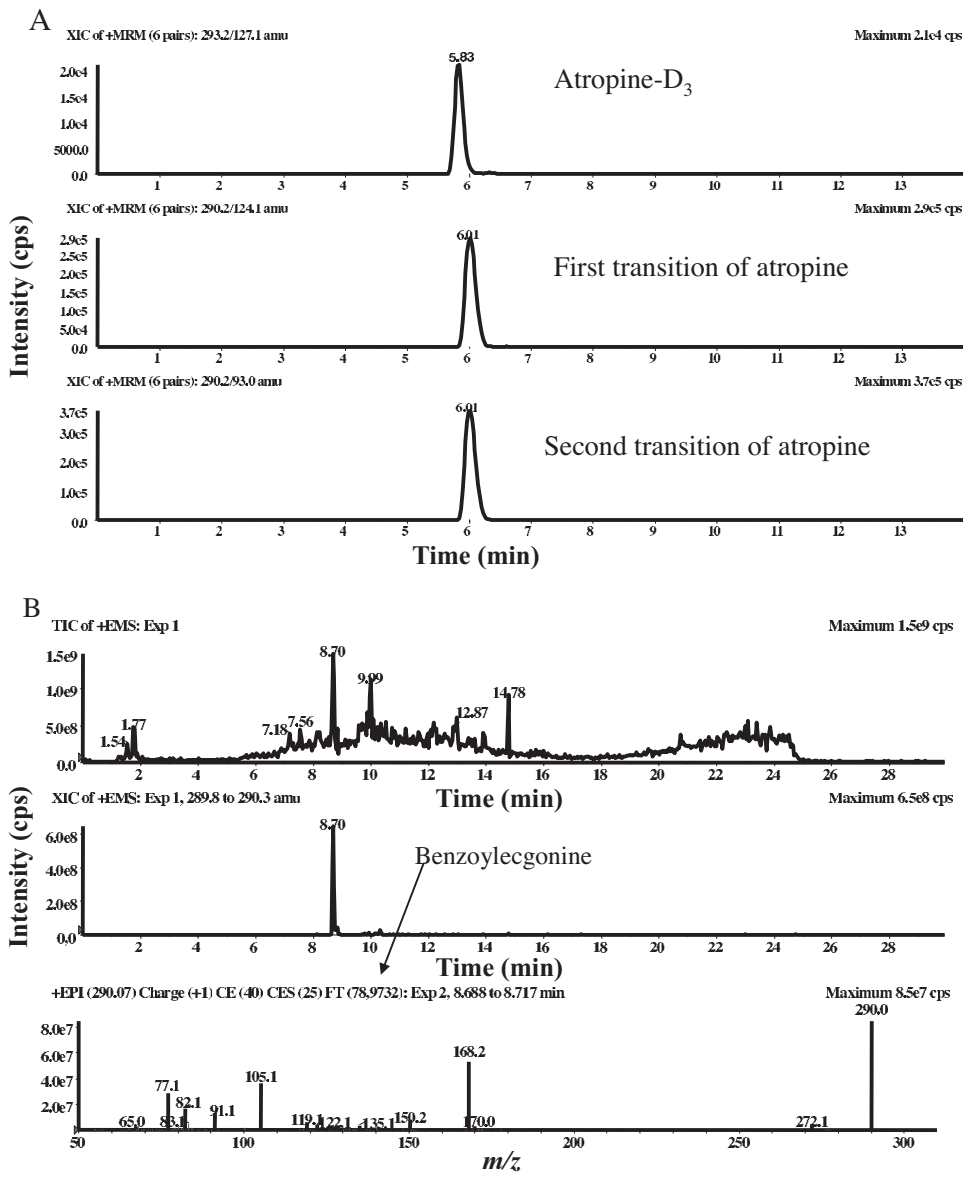

Fig. 2. Detection of an interference in the analysis of a forensic urine sample for atropine using a specific SRM method (A) and identification of the interfering compound as being benzoylecgonine by means of the LC-MS/MS GUS procedure (B).

both precursor and fragment *m/z* ratios identical to those of a compound of interest. This situation is particularly likely to occur with multicharged proteins. A recent report described an improved multitarget screening technique for 301 compounds. This technique used SRM as the survey mode and enhanced product-ion scan as the dependent mode in an IDA experiment *(37)* in which an enhanced product-ion spectrum was acquired after identification of an emerging peak at a predefined SRM transition. Compound identification was performed by use of a library search on this spectrum. Therefore, the identification specificity relied on a full mass spectrum, which is obviously better than selected transitions. The compounds of interest addressed by this technique, however, are necessarily limited (301 compounds out of thousands).

Considering all these points, we decided in our laboratory to: (*a*) use stable-isotope–labeled analogs of the targeted analytes as internal standards when avail-

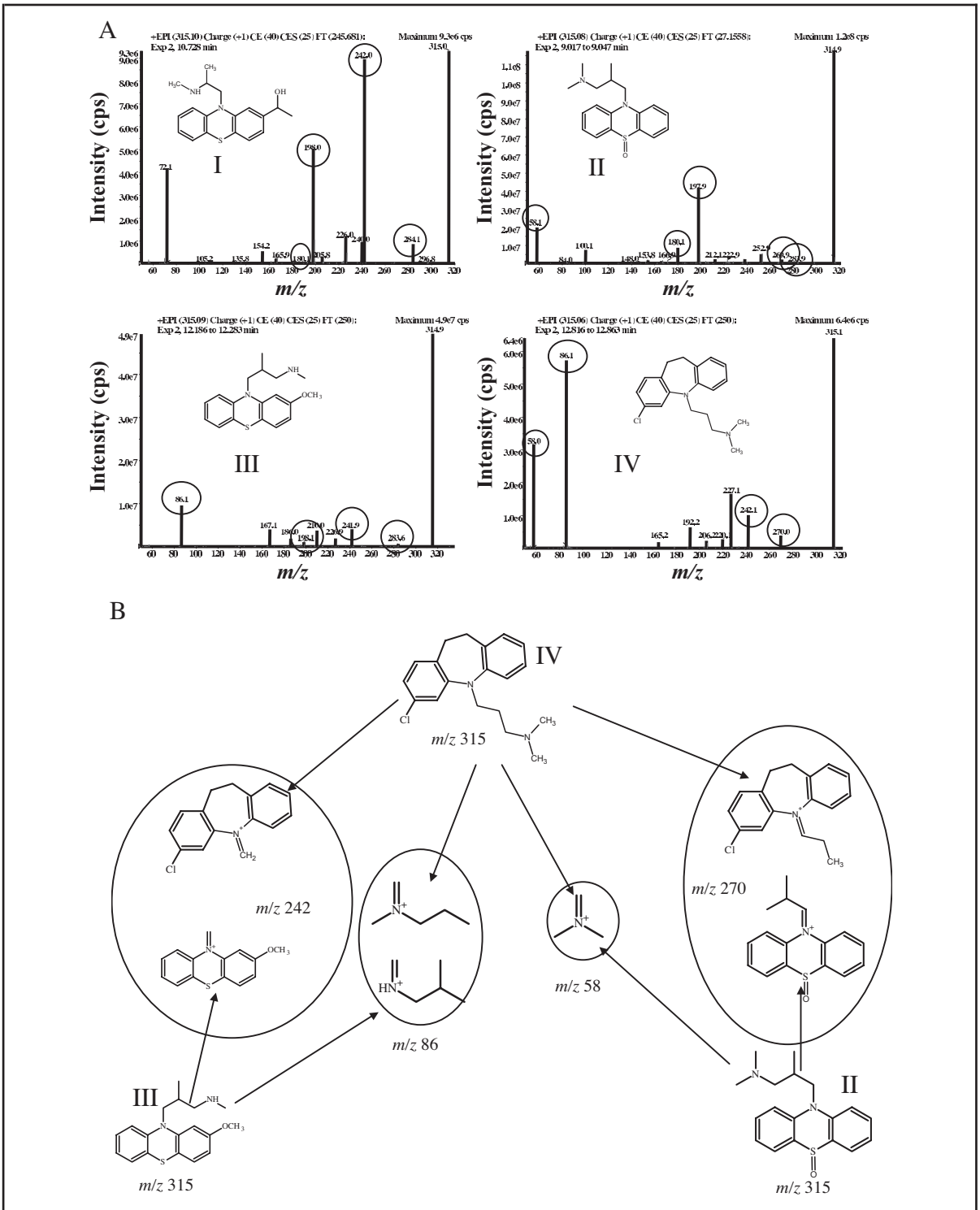

Fig. 3. Investigation of the interferences between clomipramine and some metabolites of phenothiazines by comparison of (A) their enhanced product-ion spectra and (B) their fragmentation pathways.

Table 3. Relative abundance of the interfering fragments observed between clomipramine and metabolites of phenothiazines (considering *m/z* at 315 as the most abundant fragment).

Selected compound	Retention time, min	Detected fragments, *m/z*			
		270	242	86	58
Clomipramine	12.8	5.7%	15.7%	87.1%	48.6%
Trimeprazine sulfoxide	9.1	2.9%	ND[a]	ND	15.7%
N-desmethylmethotrimeprazine	12.2	ND	9.1%	18.6%	ND
Acepromethazine metabolite	10.7	ND	95.7%	ND	ND

[a] ND, not detected.

able, (*b*) check the relative retention times, (*c*) use more than 2 transitions when possible, and (*d*) check the relative product-ion abundances between the selected transitions with respect to those calculated in the spiked calibrator sample(s) with concentrations closest to the calculated concentration, with acceptable limits based on the results summarized in Supplemental Table 4 in the online Data Supplement. The results obtained from a retrospective study of analytical series for different drugs performed in our laboratory, based on the interseries CV% calculated for calibrator samples at the limit of quantification and for patient samples above the limit of quantitation, showed that in patient samples a tolerance of ±15% can be applied to transitions with a relative abundance >10%, with a extension to ±25% for transitions <10% when the concentration is just above the limit of quantification. In 2 cases (cyclosporine A and risperidone), the relative intensity of 1 of the confirmation transitions was <10% and the interseries CV% reached 30% to 32% at the LOQ, respectively, suggesting that the acceptability range should be loosened at this concentration. According to these experimental results, the tolerances given by most of the guidelines *(18–21)*, those of the FDA being a notable exception *(22)*, could be achieved, although with more difficulty for some transitions with a relative abundance <10%. In our own experience, the tolerance limits recommended by the FDA *(22)* when only 2 confirmation transitions are chosen (i.e., a tolerance of ±10% whatever the transition abundance) would be difficult to achieved, even when the relative intensity was >10%. The other possibility envisaged by the FDA, i.e., 3 confirmation transitions with a tolerance of ±20%, is easier to comply with if fragments are abundant enough but again poses the problem of low abundance ratios, because many compounds with poor fragmentation will not exhibit 3 fragments with >10% intensity.

In addition, when interferences are suspected, extracts prepared for the specific method are analyzed using the LC-MS/MS GUS procedure developed on a 2000QTRAP system *(26)*, which generally detects and often helps identify the interfering compounds present in the samples. Unfortunately, interferences caused by the presence of unknown metabolites or isobaric compounds cannot always be investigated exhaustively, and the compounds involved, when tentatively identified, are seldom available as pure compounds for confirmatory analyses.

In summary, among the parameters investigated during the development of new analytical methods, selectivity is critical, even with so-called specific technologies such as LC-MS/MS, particularly when sample extraction, purification, and chromatographic separation are simplified. Some strategies can be advised, but none could be considered ideal. In particular, several isobaric compounds with the same ion transitions as the investigated compounds and a close retention time can be detected. Owing to the importance of the results reported, maximizing the number of parameters used for compound identification is crucial, in particular the number of specific transitions. Also, for compounds with amino side chains, some fragments (for example at *m/z* 58, 86, or 100) are not specific enough and should be avoided. Furthermore, verification of the relative abundances between the selected transitions and of the relative retention times is a useful complement. Finally, a GUS procedure based on full scan detection and full scan identification, such as that used in the present study, is helpful to check for the presence of suspected interfering compound(s), often to identify them. These results can be used to refine the criteria for the identification of the investigated compound(s) with the SRM techniques.

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