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Sensitive method for the detection of 22 benzodiazepines by gas chromatography–ion trap tandem mass spectrometry

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Abstract

A gas chromatography–ion trap tandem mass spectrometry method for simultaneous detection of 22 benzodiazepines is presented. Four operating modes were first optimized: the electron impact ionization and chemical ionization modes were compared on both underivatized and trimethylsilylated drugs. Results were compared in terms of sensitivity in MS–MS experiments. The trimethylsilylation of benzodiazepines including a protic functional group allows decreasing their detection threshold by a factor of 10–100. In terms of sensitivity, the comparison between both ionization modes shows that the most efficient one depends on the benzodiazepine considered. The use of an ion trap analyzer allows switching from an ionization mode to another one during the chromatographic process. It also provides a great selectivity owing to the MS–MS and multiple reaction monitoring acquisition modes. The detection thresholds are in the range 10–500 pg/μl for all the studied benzodiazepines but the three ''triazolo'' ones: estazolam, alprazolam and triazolam, have a detection threshold of 1 ng/μl. The applicability of the method on whole blood and urine extracts was demonstrated on an example implying five benzodiazepines among the most frequently encountered in forensic toxicology: nordazepam, oxazepam, bromazepam, flunitrazepam and prazepam.

Keywords: Mass spectrometry; Benzodiazepines

1. Introduction

Benzodiazepines constitute a class of versatile and widely prescribed central nervous system (CNS)-depressants. French people are, per capita, the first consumers worldwide. Benzodiazepines are prescribed as anxiolytics, sedative hypnotics, anticonvulsants and muscle relaxants [1–4]. Their clinical popularity has been ascribed to the wide safety margin of their therapeutic index, minimal serious adverse side-effects, and low potential for physical dependence. These drugs are very frequently encountered in clinical and forensic toxicology; they have featured in an increasing number of misuses and abuses over the past years [5–8]. It is therefore necessary to have reliable methods for their detection, identification and quantification at the low levels encountered in body fluids [9].

A number of studies have been reported on the analysis of benzodiazepines and their metabolites. Most of these studies use either immunological methods [10,11] or chromatographic techniques

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[12,13]. Because of cross-reactivity, immunological techniques need to be confirmed by chromatography–mass spectrometry techniques that are known to be more selective. Even if the recent developments of liquid chromatography–mass spectrometry (LC–MS) show very promising results [14], most of the literature devoted to the detection and quantitation of benzodiazepines implies gas chromatography–mass spectrometry (GC–MS) techniques. Most of these studies can be divided into two groups: those performed from urine extracts [15–18] and those performed from blood, serum or plasma extracts [19–22]. Selectivity and sensitivity of MS techniques are greatly enhanced when MS–MS experiments can be performed. In GC–MS and LC–MS experiments, tandem mass spectrometry is generally performed using triple-quadrupole or ion trap analyzers. Among those mass spectrometers, ion traps allow performing MS–MS experiments at a cost much lower than triple-quadrupole instruments [23]. However, the poor efficiency of ion trap analyzers in detecting some benzodiazepines has recently been reported [24]. A screening method for the detection of six benzodiazepines and their metabolites has been performed with a quadrupole analyzer; ''excessive peak tailing'' of some drugs such as alprazolam and triazolam led to exclude the use of ion trap detectors [25]. To our knowledge, any MS–MS method allowing the screening of a great number of benzodiazepines has been reported. The aim of this study was to test the capability of the ion trap mass spectrometer to detect benzodiazepines in the MS–MS mode and to develop a selective and sensitive tool for the detection of the 24 benzodiazepines licensed for the French market.

2. Experimental

2.1. Materials and sample preparation

Manufacturers kindly provided the 24 benzodiazepines: estazolam from Cassenne-Osny (Osny, France), loprazolam from Hoechst-Marion-Roussel (Puteaux, France), medazepam from Hoffman-La Roche (Basle, Switzerland), nordazepam, oxazepam and prazepam from Parke-Davis (Courbevoie, France), alprazolam and triazolam from Pharmacia and Upjohn (Saint-Quentin-en-Yvelines, France), bromazepam, chlordiazepoxide, clonazepam, diazepam, flunitrazepam, flurazepam, midazolam and nitrazepam from Roche (Neuilly-sur-Seine, France), clobazam from Sanofi-Synthelabo (Le Plessy-Robinson, France), clorazepic acid potassium salt, ethyl loflazepate and tetrazepam from Sanofi-Winthrop (Gentilly, France), lormetazepam from Schering (Lyz-les-Lannoy, France), clotiazepam from Shire (Boulogne, France), lorazepam and temazepam from Wyeth-lederlé (Puteaux, France). Acetonitrile HPLC grade was obtained from Prolabo (Fontenay-sous-Bois, France). A mixture of 99% of *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS) was purchased from Merck (Nogent-sur-Marne, France). TMCS is a catalyst that increases the silylating power of BSTFA [26].

A standard mixture solution containing 40.0 μg/ml of each drug in acetonitrile was prepared to optimize chromatographic and mass spectrometric conditions. A new standard solution was prepared every week and stored at −18 °C between experiments. Trimethylsilylation was performed as follows: 50 μl of the standard mixture were evaporated to dryness in a conical vial at 25 °C, under a nitrogen flow. After complete evaporation of the solvent, the residue was heated to 80 °C for 5 min in order to eliminate any trace of water; 50 μl of BSTFA was added to the residue. The sample was then heated at 80 °C for 20 min and allowed to cool down to ambient temperature prior to GC–MS analysis.

2.2. Extraction protocol

In order to test the applicability of the GC–MS–MS method on biological samples, extractions of benzodiazepines were performed from whole blood and from urine. Toxi-tubes A extraction cartridges were purchased from TOXI-LAB ANSYS Diagnostic (Lake Forest, CA, USA). The extraction process is the same for blood and urine samples. Some benzodiazepines (see Section 3.4) were added to 1 ml of matrix so that the resulting concentration in each drug was 50 ng/ml. The mixture was vortexed prior to be transferred into a Toxi-tube A which had been previously conditioned by 2 ml of deionized water. The Toxi-tube was automatically agitated

before being centrifuged for 5 min at 3000 rpm. The organic phase was transferred to a clean tube and evaporated to dryness under a N_2 stream at 25 °C. The residue was washed with 100 μl of acetonitrile. After centrifugation 5 min at 3000 rpm, the supernatant was extracted and dried under a N_2 stream at 25 °C before being silylated, according to the process described above, with 40 μl of BSTFA.

2.3. Instrumentation

All analysis were performed on a Varian Saturn 2000 apparatus consisting of a 3800 gas chromatograph coupled with an ion trap mass spectrometer and fitted with an 8200 autosampler.

The chromatographic separation was carried out on a 30 m DB5-MS capillary column (0.25 mm I.D., film thickness: 0.25 μm) from J&W Scientific. Helium was used as the GC carrier gas and the flow-rate was maintained constant at 1.0 ml/min with an electronic flow controller. The injector temperature was 250 °C. The column was ramped from an initial temperature of 100–230 °C at a step rate of 8 °C/min, then at 4 °C/min up to a final temperature of 313 °C. The transfer line was maintained at 300 °C. All experiments were operated in the splitless mode, automatically injecting 1 μl of sample at a rate of 8 μl/s. The total duration of the GC method was 37 min. The chromatographic method has not allowed a complete separation of all the benzodiazepines and some of them remained coeluted. All the attempts to improve the chromatographic separation led to excessive analysis times without totally avoiding the coelution phenomena. Coelution problems were solved with the MRM (multiple reaction monitoring; see below) acquisition mode.

The ion trap electrodes and manifold temperatures were 230 and 70 °C, respectively. The partial pressure of He in the mass spectrometer was estimated about 0.133 Pa. Tuning of the mass spectrometer was automatically performed using the ions resulting from electron impact ionization (EI) of perfluorotributylamine. These ions also permitted the optimization of the axial modulation voltage (3.3 V). Depending on experiments, ionization was carried out either by EI at 70 eV or by positive chemical ionization (CI) with acetonitrile (ACN) as reagent gas [27]. Optimization of the chromatographic conditions was performed in the EI mode; ions were collected in the m/z 45–550 range. In tandem experiments, the electron multiplier voltage was set 300 V above the value automatically optimized for a gain of 10^5. MS–MS and MRM experiments were performed in the resonant mode. Parent ions were isolated within a 1-unit mass window corresponding to the ^{35}Cl isotopic peak for chlorinated precursor ions. It is to be noted that a 1-unit mass window leads to loss of isotopic information in daughter ion spectra of chlorinated compounds but it provides the best selectivity for the GC–MS–MS method. The collision-induced dissociation (CID) voltages were optimized for each compound, at a qz value of 0.4, with an excitation time of 30 ms. The CID voltages and the m/z ranges for recording of daughter ions are given in the Results and discussion section. Coelution implies to perform detection of non-separated compounds using MRM instead of MS–MS. In the MRM process, parent ions of different molecules are isolated and fragmented in turn. Daughter ions of each one are recorded in separated channels so that such compounds can be separately visualized and integrated. We showed in a recent study that, for two coeluted compounds, performing MRM instead of MS–MS reduces the sensitivity of the mass spectrometer by about 30% [27]. Spectra were recorded with scan rates of 1.0 s per scan in the MS–MS mode and 0.5 s per scan in the MRM mode.

3. Results and discussion

3.1. GC–MS–MS method development

In order to study their GC behavior, the benzodiazepines were first studied separately; each one was injected at 100 μg/ml in acetonitrile, in both EI and CI modes. Among the 24 benzodiazepines on the French market, two were discarded from this study: clorazepate dipotassium and loprazolam. Clorazepate dipotassium (which is, anyway, never detected in human blood samples) could not be solubilized in acetonitrile because of its ionic form and thus could not be injected. At a concentration of 100 μg/ml, loprazolam was not detected in GC–MS, even when raising the column temperature to its limit value of

Table 1
Parameters for the GC–MS–MS methods (EI and CI) for the detection of underivatized benzodiazepines

Segment acquisition mode	Compound[a]	Ret. time (min)	Electron impact ionization			Chemical ionization		
			Parent ion (m/z)	$V_{coll.}$ (V)	Scan range (m/z)	Parent ion (m/z)	$V_{coll.}$ (V)	Scan range (m/z)
2, MS–MS	Medazepam	18.90	242	0.85	190–252	271	1.00	150–281
3, MS–MS	Oxazepam (dp)	20.70	267	0.55	230–277	269	0.60	230–279
4, MRM	Tetrazepam	21.55	253	1.20	155–263	289	0.80	170–299
	Diazepam	21.80	256	0.80	160–266	285	0.80	145–295
	Lorazepam (dp)	21.90	274	0.55	230–284	303	0.60	265–313
5, MS–MS	Ethyl loflazepate (dp)	22.65	287	0.60	230–297	289	0.65	130–299
6, MS–MS	Clotiazepam	22.95	289	0.85	200–299	319	0.85	200–329
7, MRM	Clobazam	23.45	300	0.75	240–310	301	0.75	220–311
	Nordazepam	23.55	269	0.70	220–279	271	0.60	135–281
	Chlordiazepoxide (dp)	23.70	282	0.60	210–292	284	0.60	175–294
8, MRM	Midazolam	24.15	311	0.95	230–321	326	0.85	230–336
	Flunitrazepam	24.40	286	0.85	190–296	314	0.80	230–324
	Temazepam	24.75	300	0.55	235–310	301	0.50	250–311
	Prazepam	24.95	325	1.00	200–335	325	0.85	220–335
9, MS–MS	Bromazepam	25.50	315	0.60	200–325	316	0.60	180–326
10, MRM	Temazepam (dp)	26.05	298	0.50	260–308	299	0.65	190–309
	Lormetazepam	26.25	305	0.65	150–315	335	0.50	280–345
11, MRM	Lormetazepam (dp)	26.85	304	0.60	250–314	333	0.50	190–343
	Flurazepam	26.90	86	0.75	50–96	388	1.00	240–398
12, MS–MS	Nitrazepam	27.70	280	0.65	215–290	282	0.60	230–292
13, MS–MS	Clonazepam	28.95	314	0.60	250–324	316	0.60	240–326
14, MS–MS	Estazolam	29.20	259	0.65	180–269	295	0.65	230–305
15, MS–MS	Alprazolam	29.80	273	0.60	200–283	309	0.65	230–319
16, MS–MS	Triazolam	31.25	313	0.70	230–323	343	0.75	260–353

[a] dp, decomposition product.

350 °C. Its high molecular mass (464.9) probably confers to loprazolam a very low volatility.

As previously reported by Joice et al., some benzodiazepines underwent thermal degradation during the injection step [28]. Our experiments showed that degradation was almost total (at least 95% of the drug is decomposed) for four benzodiazepines: chlordiazepoxide, ethyl loflazepate, lorazepam and oxazepam. These drugs can still be detected through the screening of their decomposition products. Two benzodiazepines, lormetazepam and temazepam, decompose only partially. Repeated injections showed that the ratio of decomposition varies significantly from one injection to another. Consequently, for lormetazepam and temazepam, both chromatographic peaks of the decomposition product and of the unchanged drug were taken into account in the method development.

The benzodiazepines can be divided into two categories: those that can be derivatized because they include a protic functional group and those that cannot. The standard solution was submitted to trimethylsilylation according to the process described in the Experimental section. As expected, the benzodiazepines silylated are those including an exchangeable hydrogen: nordazepam, oxazepam, bromazepam, lorazepam, ethyl loflazepate, nitrazepam, chlordiazepoxide, temazepam, clonazepam and lormetazepam. In order to control the efficiency of the silylation process, these drugs were separately submitted to trimethylsilylation. The resulting chromatograms and mass spectra showed that the derivatization process is total when possible and that derivatized compounds do not undergo thermal decomposition.

Four GC–MS–MS methods were optimized and compared for the detection of the 22 benzodiazepines: (i) detection in the EI mode of underivatized benzodiazepines, (ii) detection in the CI mode of underivatized benzodiazepines, (iii) detection in the

EI mode of silylated and underivatized benzodiazepines and (iv) detection in the CI mode of silylated and underivatized benzodiazepines. In the last two methods, silylation is carried out on the standard mixture; the screening allows simultaneous detection of the 10 trimethylsilylated benzodiazepines and of the 12 drugs that remained unchanged.

The four GC–MS–MS methods were optimized as follows. The chromatographic run was divided into segments. The first one consisted of a delay before switching on the mass spectrometer, to avoid degradation of the ionization filament during elution of the solvent. Tables 1 and 2 show the repartition of drugs in MS–MS or MRM segments, according to their retention time. Well-separated molecules were submitted to MS–MS detection while MRM was used for coeluted compounds. The GC–MS methods involve 16 segments for unchanged benzodiazepines and 17 segments for the mixture of unchanged and derivatized drugs. For each compound, a precursor ion was selected among the most intense characteristic ions of the MS spectrum; the CID voltage was optimized with the aim to obtain a MS–MS spectrum displaying at least three daughter ions for unambiguous identification of the analyte. The m/z scan range was determined according to the daughter ions. All the optimized parameters are reported in Tables 1 and 2.

3.2. Comparison of the GC–MS–MS methods

With the aim of evaluating the sensitivity of each method, mixtures of the 22 drugs were prepared at concentrations ranging from 1 to 10 000 pg/μl and analyzed with and without trimethylsilylation. For each compound, the detection threshold has been evaluated from the selected ion profile of the main daughter ion. A signal-to-noise ratio of 5:1 was assumed for the limit of detection. Table 3 compares the detection thresholds of the four GC–MS–MS methods for the 22 benzodiazepines studied. Comparing methods (i) and (ii) with methods (iii) and (iv) clearly shows that silylation provides, for the involved molecules, much lower detection thres-

Table 2
Parameters for the GC–MS–MS methods (EI and CI) for the detection of underivatized and trimethylsilylated benzodiazepines

Segment acquisition mode	Compound[a]	Ret. time (min)	Electron impact ionization			Chemical ionization		
			Parent ion (m/z)	$V_{coll.}$ (V)	Scan range (m/z)	Parent ion (m/z)	$V_{coll.}$ (V)	Scan range (m/z)
2, MS–MS	Medazepam	18.90	242	0.85	190–252	271	1.00	150–281
3, MS–MS	Nordazepam-TMS	19.25	342	1.05	250–352	343	1.00	220–353
4, MS–MS	Oxazepam-TMS	20.55	430	1.35	250–440	431	0.90	320–441
5, MRM	Bromazepam-TMS	21.55	389	1.15	260–399	388	0.90	250–398
	Tetrazepam	21.55	253	1.20	155–263	289	0.80	170–299
	Diazepam	21.80	256	0.80	160–266	285	0.80	145–295
6, MS–MS	Lorazepam-TMS	22.10	431	1.05	290–441	465	0.90	330–475
7, MS–MS	Ethyl loflazepate-TMS	22.65	432	1.10	340–442	433	0.95	310–443
8, MRM	Clotiazepam	22.95	289	0.85	200–299	319	0.85	200–329
	Nitrazepam-TMS	23.00	352	1.10	180–362	354	0.80	210–364
	Chlordiazepoxide-TMS	23.05	282	0.85	200–292	284	0.85	150–294
9, MS–MS	Clobazam	23.45	300	0.75	240–310	301	0.75	220–311
10, MRM	Temazepam-TMS	24.05	357	1.05	240–367	373	0.90	240–383
	Midazolam	24.15	311	0.95	230–321	326	0.85	230–336
11, MS–MS	Flunitrazepam	24.40	286	0.85	190–296	314	0.80	230–324
12, MRM	Clonazepam-TMS	24.90	387	1.05	250–397	388	1.00	240–398
	Prazepam	24.95	325	1.00	200–335	325	0.85	220–335
13, MS–MS	Lormetazepam-TMS	25.30	379	1.00	280–389	407	0.95	270–417
14, MS–MS	Flurazepam	26.90	86	0.75	50–96	388	1.00	240–398
15, MS–MS	Estazolam	29.20	259	0.65	180–269	295	0.65	230–305
16, MS–MS	Alprazolam	29.80	273	0.60	200–283	309	0.65	230–319
17, MS–MS	Triazolam	31.25	313	0.70	230–323	343	0.75	260–353

[a] TMS, trimethylsilylated.

Table 3
Comparison of the detection thresholds of the four GC–MS–MS methods for each of the 22 benzodiazepines

Compound[b]	Underivatized[a]		Trimethylsilylated[a]	
	EI	CI	EI	CI
Medazepam	10 (207)	100 (242)	–	–
Nordazepam	10 000 (241)	>10 000 (243)	10 (269)	10 (327)
Oxazepam (dp)	100 (239)	500 (241)	10 (341)	10 (341)
Bromazepam	10 000 (287)	10 000 (288)	500 (314)	500 (344)
Tetrazepam	500 (225)	100 (225)	–	–
Diazepam	10 (221)	100 (193)	–	–
Lorazepam (dp)	500 (239)	10 000 (275)	100 (342)	100 (375)
Ethyl loflazepate (dp)	10 000 (239)	>10 000 (261)	100 (359)	500 (359)
Clotiazepam	100 (274)	100 (291)	–	–
Nitrazepam	10 000 (234)	10 000 (236)	100 (306)	100 (336)
Chlordiazepoxide (dp)	500 (247)	10 000 (241)	100 (247)	100 (241)
Clobazam	100 (255)	500 (259)	–	–
Temazepam	10 000 (255)	>10 000 (283)	100 (283)	500 (357)
Temazepam (dp)	10 000 (270)	>10 000 (271)	–	–
Midazolam	500 (290)	100 (291)	–	–
Flunitrazepam	100 (239)	100 (268)	–	–
Clonazepam	10 000 (268)	10 000 (270)	100 (340)	100 (342)
Prazepam	500 (269)	100 (271)	–	–
Lormetazepam	10 000 (193)	10 000 (317)	100 (377)	100 (289)
Lormetazepam (dp)	10 000 (269)	10 000 (305)	–	–
Flurazepam	100 (58)	100 (315)	–	–
Estazolam	1000 (205)	10 000 (267)	–	–
Alprazolam	1000 (245)	10 000 (281)	–	–
Triazolam	1000 (277)	10 000 (308)	–	–

For each compound, the signal-to-noise ratio of the peak was determined from the selected ion profile of the main daughter ion of the CID spectrum (m/z in parentheses).

[a] Detection thresholds are given in pg/μl in acetonitrile.

[b] dp, decomposition product. It concerns only underivatized benzodiazepines since trimethylsilylated benzodiazepines do not decompose.

holds. The sensitivity is generally improved by a factor of 10–1000, according to the benzodiazepine considered. This great improvement in sensitivity is rationalized by two factors. First, silylation avoids thermal decomposition of the thermolabile benzodiazepines in the chromatograph so that more analyte can be detected. Moreover, derivatization allows a significant reduction in peak tailing, providing chromatographic peaks much sharper than for the corresponding underivatized compounds. It significantly increases the signal-to-noise ratio of these peaks and thus the detection thresholds of the related compounds.

The comparison of the detection thresholds obtained with GC–MS–MS methods (iii) and (iv) shows that both ionization modes provide equivalent sensitivities for 11 benzodiazepines among the 22 studied: nordazepam, oxazepam, bromazepam, lorazepam, clotiazepam, nitrazepam, chlordiazepoxide, flunitrazepam, clonazepam, lormetazepam and flurazepam. EI ionization provides the best sensitivity for eight benzodiazepines: medazepam, diazepam, ethyl loflazepate, clobazam, temazepam, estazolam, alprazolam and triazolam. A lower detection threshold is obtained in the CI mode for three benzodiazepines: tetrazepam, midazolam and prazepam.

3.3. The most sensitive GC–MS–MS method

One of the main advantages of ion trap analyzers is their ability to switch between EI and CI modes in a few seconds, allowing to change the ionization mode during the chromatographic separation. A screening method performing electron ionization on some benzodiazepines and chemical ionization on other ones can thus be considered to provide the best

sensitivity for all the analytes. The optimization of such a method must take into account that the switch between both ionization modes implies that the chromatographic peaks are separated by a few seconds. This is the time required to permit equilibration of the reagent gas when switching CI on, and pumping of this gas when switching CI off. It implies that a coeluted compound must be analyzed with the same ionization mode. For instance, Table 3 shows that the detection threshold of tetrazepam is lower in CI than in EI but tetrazepam is coeluted with trimethylsilylated bromazepam and with diazepam. The detection threshold of diazepam being much lower in EI than in CI, the EI ionization mode was retained for the screening of the three compounds. When EI and CI provided the same sensitivity, as in the cases of flunitrazepam and lormetazepam, CI was retained because it is well known to be much more selective than EI on biological extract samples. Based on the results above, the first part of the method includes a trimethylsilylation step. The ''final'' screening method is summarized in Table 4. For each benzodiazepine, at least three daughter ions allow unambiguous identification of the drug (a ''qualifier'' ion must have a relative abundance of at least 5%; Table 4 displays no more than five ''qualifiers'' per compound). The chromatogram of a solution containing 2.0 μg/ml of each drug in acetonitrile is displayed in Fig. 1. For each benzodiazepine, Table 5 gives the detection threshold of the ''final'' GC–MS–MS method and the blood concentration expected in a therapeutic context. The therapeutic concentration depends on the patient profile (age, sex, health . . .) and on the medical indication; that is why intervals are given [29,30].

The detection thresholds are in the range 10–500 pg/μl for all the studied benzodiazepines except the three ''triazolo'' ones: estazolam, alprazolam and triazolam, for which the detection threshold is 1 ng/μl. This can be interpreted in terms of poor chromatographic resolution since those compounds

Table 4
Parameters of the optimized GC–MS–MS method for the detection of the 22 benzodiazepines

Segment, mode	Time interval (min)	Compound[a]	Ionization mode	Daughter ions[b]
2, MS–MS	18.00–19.10	Medazepam	EI	227 (5), **207 (100)**, 206 (10)
3, MS–MS	19.10–19.70	Nordazepam-TMS	EI	325 (7), 305 (14), 297 (30), 290 (41), **269 (100)**
4, MS–MS	19.70–21.00	Oxazepam-TMS	EI	**341 (100)**, 340 (41), 306 (61), 305 (42), 267 (30)
5, MRM	21.00–21.95	Bromazepam-TMS		372 (21), 344 (27), 316 (50), **314 (100)**, 274 (30)
		Tetrazepam	EI	**225 (100)**, 197 (40), 196 (29), 182 (21), 168 (15)
		Diazepam		239 (5), **221 (100)**, 177 (32)
6, MS–MS	21.95–22.50	Lorazepam-TMS	EI	343 (69), **342 (100)**, 341 (80), 306 (88), 305 (47)
7, MS–MS	22.50–22.85	Ethyl loflazepate-TMS	EI	417 (7), 403 (21), 385 (5), **359 (100)**, 358 (20)
8, MRM	22.85–23.30	Clotiazepam		**274 (100)**, 259 (4), 239 (69), 212 (13)
		Nitrazepam-TMS	EI	**306 (100)**, 305 (11), 294 (7), 191 (6)
		Chlordiazepoxide-TMS		267 (33), 252 (23), **247 (100)**, 246 (25), 218 (17)
9, MS–MS	23.30–23.90	Clobazam	EI	289 (5), 285 (8), 283 (22), 256 (74), **255 (100)**
10, MRM	23.90–24.30	Temazepam-TMS	EI	341 (7), **283 (100)**, 255 (42)
		Midazolam		308 (65), **290 (100)**, 275 (91), 257 (41), 247 (41)
11, MS–MS	24.30–24.75	Flunitrazepam	CI	297 (7), 286 (11), 269 (48), **268 (100)**, 240 (12)
12, MRM	24.75–25.10	Clonazepam-TMS	CI	372 (36), **342 (100)**, 326 (37), 306 (97), 256 (67)
		Prazepam		297 (8), **271 (100)**, 255 (11), 243 (7)
13, MS–MS	25.10–26.50	Lormetazepam-TMS	CI	391 (48), 317 (21), **289 (100)**
14, MS–MS	26.50–28.25	Flurazepam	CI	317 (14), **315 (100)**, 288 (23), 272 (5), 260 (7)
15, MS–MS	28.25–29.60	Estazolam	EI	249 (7), 232 (13), 231 (34), **205 (100)**, 204 (13)
16, MS–MS	29.60–30.76	Alprazolam	EI	**245 (100)**, 232 (42), 219 (8)
17, MS–MS	30.76–33.00	Triazolam	EI	278 (73), **277 (100)**, 243 (33), 242 (14)

[a] TMS, trimethylsilylated.
[b] Relative abundances are given in parentheses. The major ion is in bold.

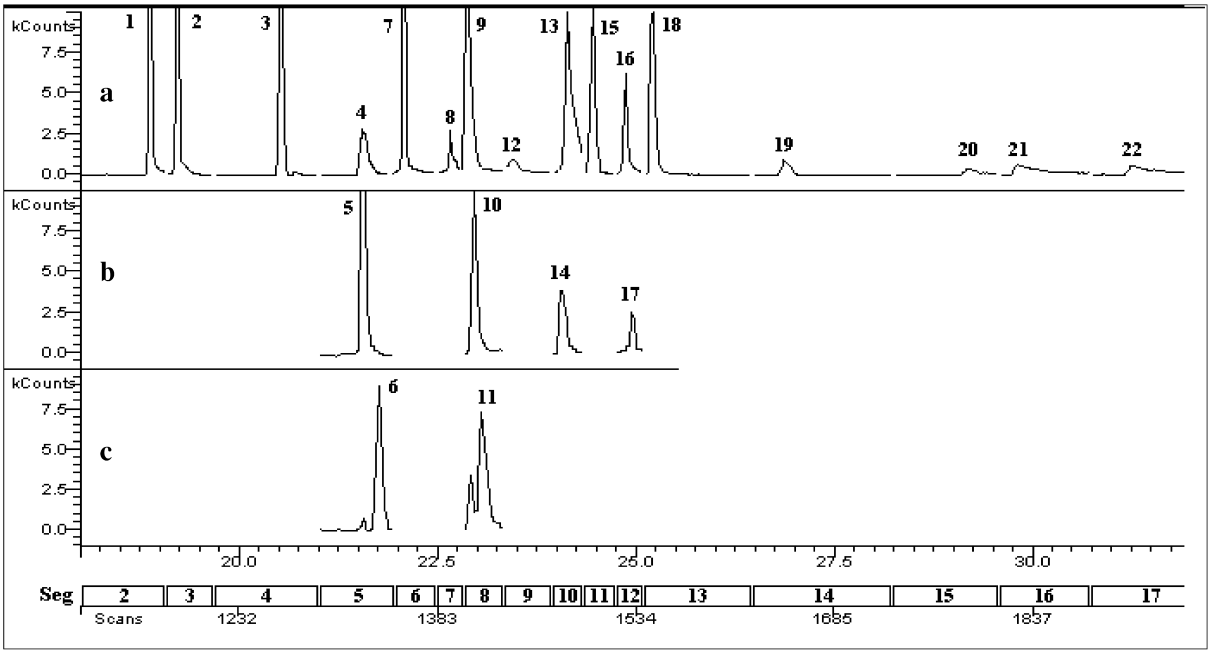

Fig. 1. Chromatogram of a mixture containing 2.0 μg/ml of each of the 22 benzodiazepines in acetonitrile. (a) Scan 1 (MS–MS and MRM on all segments); (b) scan 2 (MRM on segments 5, 8, 10 and 12); (c) scan 3 (MRM on segments 5 and 8); 1, medazepam; 2, nordazepam-TMS; 3, oxazepam-TMS; 4, bromazepam-TMS; 5, tetrazepam; 6, diazepam; 7, lorazepam-TMS; 8, ethyl loflazepate-TMS; 9, clotiazepam; 10, nitrazepam-TMS; 11, chlordiazepoxide-TMS; 12, clobazam; 13, temazepam-TMS; 14, midazolam; 15, flunitrazepam; 16, clonazepam-TMS; 17, prazepam; 18, lormetazepam-TMS; 19, flurazepam; 20, estazolam; 21, alprazolam; 22, triazolam.

show very important peak tailing, in good agreement with the results obtained by De Leenheer and co-workers [24,25].

3.4. Applicability of the method on biological samples

In order to test the applicability of the GC–MS–MS method on biological samples, we carried out the extraction of a mixture of benzodiazepines from whole blood and from urine. Five benzodiazepines were chosen among the most frequently encountered in the French Police investigations: nordazepam, oxazepam, bromazepam, flunitrazepam and prazepam. The benzodiazepines were added to 1 ml of matrix so that the resulting concentration in each drug was 50 ng/ml. Six tubes were prepared for each matrix (blood and urine). For an accurate determination of the recovery ratios, deuterated benzodiazepines ([$^2H_5$]nordazepam, [$^2H_5$]oxazepam and [$^2H_7$]flunitrazepam) were added as standards before extraction in six tubes (tubes a) and after extraction in six other ones (tubes b). In all the cases, each deuterated standard was added so that its concentration in the mixture is 50 ng/ml. The recovery ratio of each benzodiazepine was determined as $([A_{Bz}]_b/[A_{DS}]_b)\times 100/([A_{Bz}]_a/[A_{DS}]_a)$, where $[A_{Bz}]_b$ and $[A_{DS}]_b$ are the chromatographic peak areas of the benzodiazepine and of its deuterated standard after extraction from a tube b, $[A_{Bz}]_a$ and $[A_{DS}]_a$ are the chromatographic peak areas of the benzodiazepine and of its deuterated standard after extraction from a tube a. (Determining recovery ratios in this way avoids mistakes that could result from the differences between the response factors of a drug and the associated standard.) [$^2H_5$]Nordazepam was used as the standard for nordazepam, [$^2H_5$]oxazepam was used as the standard for oxazepam and bromazepam, and [$^2H_7$]flunitrazepam was used as the standard for flunitrazepam and prazepam. The GC–MS–MS method has been modified to detect the deuterated standards: MS–MS segments 3, 4 and 11 were

Table 5
Detection thresholds of the GC–MS–MS screening method and therapeutic blood concentrations of the 22 benzodiazepines studied

Benzodiazepine[a]	Detection threshold (pg/μl)	Therapeutic concentrations in blood (pg/μl)[b]
Medazepam	10	10–500
Nordazepam-TMS	10	200–800
Oxazepam-TMS	10	1000–2000
Bromazepam-TMS	500	80–170
Tetrazepam	500	390–720[c]
Diazepam	10	125–750
Lorazepam-TMS	100	20–250
Ethyl loflazepate-TMS	100	46–325[c]
Clotiazepam	100	100–290[c]
Nitrazepam-TMS	100	30–120
Chlordiazepoxide-TMS	100	700–2000
Clobazam	100	100–400
Temazepam-TMS	100	300–900
Midazolam	500	80–250
Flunitrazepam	100	5–15
Clonazepam-TMS	100	30–60
Prazepam	100	10–40
Lormetazepam-TMS	100	1–10
Flurazepam	100	0.5–28
Estazolam	1000	55–100
Alprazolam	1000	10–60
Triazolam	1000	2–20

[a] TMS means that the detection threshold is that of the trimethylsilylated compound.
[b] From Ref. [29] except when mentioned ([c]).
[c] From Ref. [30].

turned into MRM segments. Parent ions of deuterated benzodiazepines were formed and submitted to dissociation under the same conditions as those for non-deuterated analogues. The average recovery ratios of the studied benzodiazepines are given for whole blood and urine in Table 6 (RSD below 5%, for tubes a, on one hand, and tubes b, on the other hand). Fig. 2 displays the selected ion profiles of a whole blood extract and shows the great selectivity of the method. The signal-to-noise ratios displayed for each peak show that the five benzodiazepines can be without doubt detected at their therapeutic concentrations in blood. Chromatograms of urine extracts show an equivalent selectivity and sensitivity. Since it is now well known that ion trap analysis can be strongly disrupted by matrices, this example shows that an ion trap method can be particularly efficient when the associated protocol provides sufficient purification of the sample.

Table 6
Recovery ratios of the selected benzodiazepines for whole blood and urine extraction

Benzodiazepine	Recovery ratio (%)	
	Whole blood	Urine
Nordazepam	75.5	88.2
Oxazepam	72.0	85.9
Bromazepam	67.8	91.2
Flunitrazepam	72.7	89.8
Prazepam	74.7	93.2

Average values; RSD below 5% for six samples.

4. Conclusions

The first part of this work showed that the trimethylsilylation of benzodiazepines including a protic functional group increases their MS–MS detection thresholds by a factor of 10–1000. It also showed that the most sensitive ionization mode depends on the drug considered.

The combination of gas chromatography and ion-trap tandem mass spectrometry provides a very powerful method for the detection of the 22 benzodiazepines considered. GC–MS–MS screening conditions have been developed; they permit the simultaneous detection of those drugs in 37 min. Because it allows to easily perform MS–MS and MRM acquisitions, on one hand, and to switch between both EI and CI ionization modes during the chromatographic process, on the other hand, the ion trap analyzer appears to be especially suitable for such analysis. MS–MS and MRM ensure the selectivity of the method while switching between ionization modes provides the maximum sensitivity for each drug.

The applicability of the method on whole blood and urine extracts was demonstrated on an example implying five benzodiazepines among the most frequently encountered in forensic toxicology: nordazepam, oxazepam, bromazepam, flunitrazepam and prazepam. With blood concentrations of the same order of magnitude as the therapeutic ones, the method allows and efficient and unambiguous detection of the selected drugs.

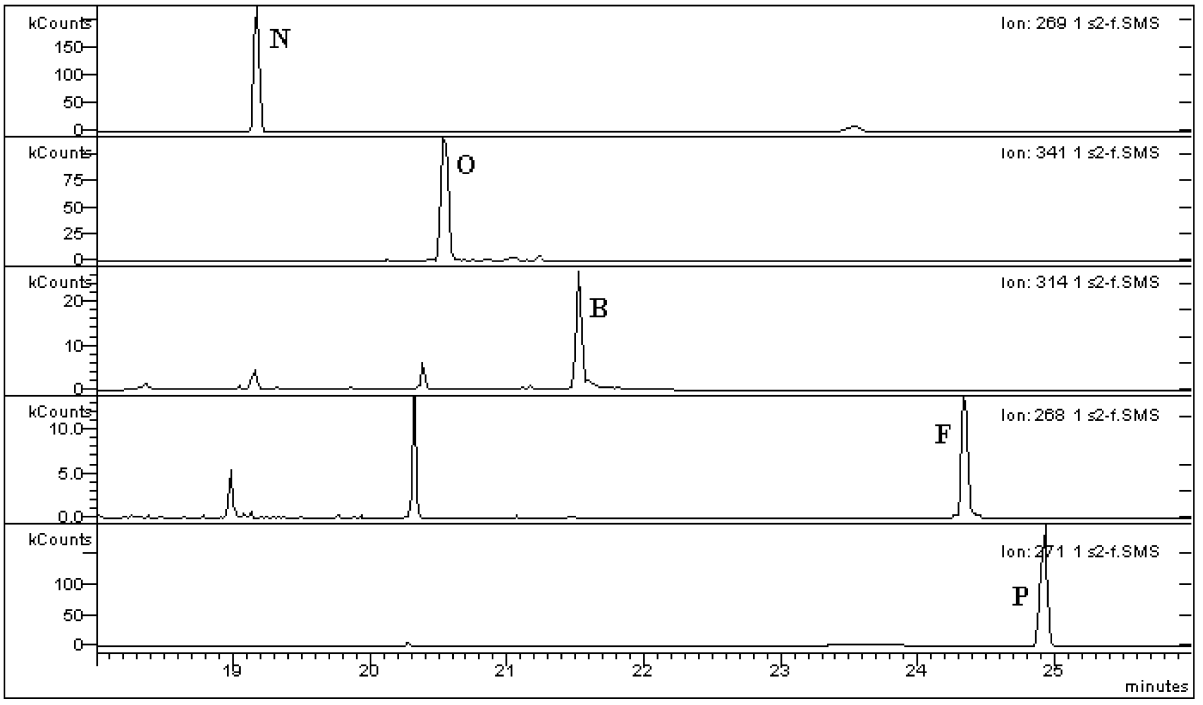

Fig. 2. Selected ion profiles of five benzodiazepines extracted from a whole blood sample. Each drug was at 50 ng/ml in blood; N, nordazepam; O, oxazepam; B, bromazepam; F, flunitrazepam and P, prazepam.

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