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Gas chromatography–tandem mass spectrometry assay for the quantification of four benzodiazepines and citalopram in eleven postmortem rabbit fluids and tissues, with application to animal and human samples

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ABSTRACT

Pharmacokinetic studies and postmortem toxicological investigations require a validated analytical technique to quantify drugs on a large number of matrices. Three-step liquid/liquid extraction with online derivatization (silylation) ahead of analysis by gas chromatography–tandem mass spectrometry was developed and validated on rabbit specimens in order to quantify citalopram and 4 benzodiazepines (diazepam, nordazepam, oxazepam and temazepam) in 11 biological matrices (blood, urine, bile, vitreous humor, liver, kidney, skeletal muscle, brain, adipose tissue, bone marrow (BM) and lung). Since the 11 biological matrices came from the same animal species, full validation was performed on 1 matrix, bone marrow (considered the most complex), while the other 10 underwent partial validation. Due to non-negligible matrix effects, calibration curves were performed on each matrix. Within-day and between-day precision (less than 12.0% and 12.6%, respectively) and accuracy (from 88.9% to 106.4%) were acceptable on BM at both low and high concentrations. Assessment on the other matrices confirmed accuracy and within-day precision (less than 12%, and generally between 85.1% and 114.5%, respectively). The lower limit of quantification of the method was 1 ng/g for nordazepam, 5 ng/g for citalopram and 10 ng/g for oxazepam, diazepam and temazepam. The combination of 3-step extraction and MS/MS detection provided good selectivity in all matrices, including the most lipid-rich. Application to real-case samples showed that the method was sensitive enough to describe distribution patterns in an animal experiment, and specific enough to detect molecules in highly putrefied samples from human postmortem cases.

1. Introduction

Benzodiazepines are widely used for their anxiolytic, hypnotic, anticonvulsive and muscle-relaxant properties and also commonly used as drug of abuse [1]. Diazepam has been on the market for almost 5 decades [2] and remains one of the most frequently prescribed anxiolytic drugs [3]. Diazepam metabolism (Fig. 1) leads to nordazepam, oxazepam and temazepam formation [4]. These 3 pharmacologically active compounds are also marketed as drugs. Citalopram is an extensively prescribed antidepressant belonging to the selective serotonin reuptake inhibitor (SSRI) class [5].

In most cases, and especially in living victims, blood is the most relevant matrix for analyzing and determining whether a drug is at a therapeutic or a toxic level in the organism. In forensic investigation, lack of blood and availability of various alternative matrices and postmortem redistribution and degradation of samples due to putrefaction complicate the analytical process and interpretation of results [6]. A thorough study of citalopram, diazepam and metabolite distribution in the whole body may allow a pharmacokinetic model to be developed and help interpretation when blood samples are not available or postmortem redistribution is suspected. A fully validated assay from various tissue and fluid samples is critical for pharmacokinetic studies and human postmortem specimen analysis.

A review of the literature failed to retrieve any methods for quantifying these compounds that were validated on each of the various matrices. For citalopram, a lot of validated methods were published in blood, plasma, urine and hair mainly by liquid

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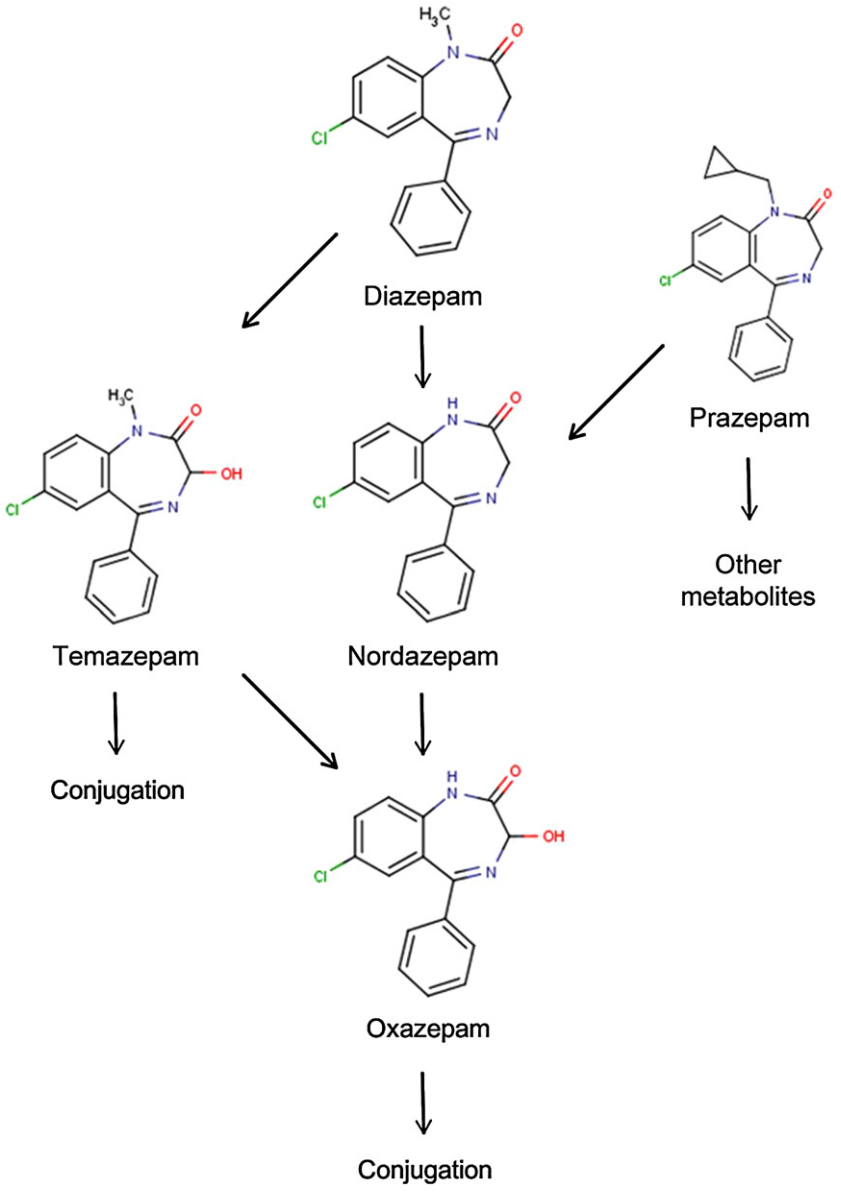

Fig. 1. Metabolic pathway of some benzodiazepines.

chromatography (LC) coupled either with ultraviolet (UV), fluorescence (FLD), mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detection [7]. Brain tissue was also studied to allow quantification in the site of action of citalopram [8–11]. Some case reports provided data from other biological fluids and tissues, but validation was either not presented [7,12–14] or was performed on blood samples alone [15–17]. Concerning benzodiazepines presented in our study, Mercolini et al. [18] published a fully validated method on blood and brain tissue to quantify diazepam and its 3 main metabolites, but the UV detection method used excluded application of this assay to putrefied postmortem samples. Heinig et al. [19] published an interesting method to quantify 7 drugs, including oxazepam, from 11 matrices. However, human plasma was used for the standard calibration preparation, with only quality controls prepared from the various tissues; no biological liquids of forensic interest in human postmortem cases (vitreous humor (VH), urine or bile) were tested. Kudo et al. used a method to quantify diazepam and nordazepam in skeletal muscle and liver by GC–MS [20], but validation was performed on blood. Other benzodiazepines have also been quantified in various tissues for case reports or pharmacokinetic studies. Excepted one study of bromazepam [21] that included a fully validated assay on a large number of matrices; in other published studies, analytical validation was either lacking [22–25] or performed on blood samples [26,27] or on part of the studied matrices [28].

The aim of the present study was to describe a validated assay allowing quantification of citalopram and 4 benzodiazepines (diazepam, temazepam, oxazepam and nordazepam) in 11 media commonly used in forensic toxicology: blood, urine, bile, VH, liver, kidney, skeletal muscle, brain, adipose tissue (AT), lung and bone marrow (BM). Two main metabolites are described for citalopram: desmethylcitalopram and didesmethylcitalopram. Bezchlibnyk-Butler et al. [5] reported that these metabolites do not appear to play a major role in the clinical action of citalopram. They represent 30–50% and 5–10% of citalopram doses, respectively but enter the brain less readily than citalopram. They display at least 4 times and 13 times less therapeutic activity than citalopram, respectively. Most of the table of interpretation of drug concentrations used in forensic toxicology analysis did not interpret metabolites quantifications [29–32] although it could be potentially informative on

the intake of citalopram. Due to their less therapeutic and forensic interest, citalopram metabolites are not studied in this work. The option of using human postmortem samples as blank biological matrices for development and validation was discarded for ethical reasons and because consumption of study drugs by the victim can never be formally ruled out. An animal model was therefore used to provide blank matrices. Rabbit was selected, as it allows sufficient quantities of each sample type to be taken, unlike the smaller laboratory animals frequently used. As the 11 matrices were collected from the same animal species, full validation was not mandatory for each. Indeed, partial validations can be undertaken for modifications of validated bioanalytical methods that do not require full revalidation such as instrument changes, transfers between laboratories, change in species within matrix or changes in matrix within a species. Depending of the extent of the change, partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation [33,34]. A full validation was conducted on BM, considered the most complex matrix due to a combined connective structure and high lipid content. A partial validation was performed on the other matrices comprising linearity evaluation on the calibration range, within-day precision and accuracy test, selectivity check, recovery test estimation and LOQ determination. The calibration range was set according to the respective drugs, based on preliminary experiments (data not shown) in our laboratory involving therapeutic doses of diazepam and citalopram administrated to rabbits. The calibration range was chosen to cover the most of the concentrations retrieved in the various matrices sampled after euthanasia. As some concentrations in specific tissues cannot be included in an acceptable calibration range, a procedure of dilution was validated. Finally, the assay thus developed was applied to real samples from kinetic animal experiments and human autopsy.

2. Materials and methods

2.1. Chemicals and solutions

Individual stock solutions (certified at a concentration of 1 mg/ml in methanol) of nordazepam, nordazepam-d5, diazepam, diazepam-d5, oxazepam, oxazepam-d5, temazepam, temazepam-d5 and citalopram hydrobromide, and citalopram-d6 in powder form (reconstituted in methanol at 1 mg/ml) and N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSFTA/TMCS) were purchased from LGC Promochem (Molsheim, France), and stored at −20 °C. Water, tert-butyl-methyl-ether and *n*-heptane, all in HPLC grade, were obtained from VWR (Fontenay sous Bois, France). Sodium Hydroxide (1 M), hydrochloric acid (1 M) and di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) were supplied by Merck (Fontenay sous Bois, France).

Deuterated analogues were used as internal standards (IS). A mixture of the 5 labeled ISs was prepared daily by dilution in methanol to reach the following final concentrations in fluid (ng/ml) and tissue (ng/g): nordazepam-d5 25 ng/ml(/g); diazepam-d5, oxazepam-d5 and temazepam-d5: 400 ng/ml(/g); and citalopram-d6: 200 ng/ml(/g). For both calibration standards and quality controls, working solutions containing the 5 study compounds were prepared daily by appropriate dilution in methanol.

2.2. Sample preparation

For tissues, 200 ± 5 mg (lung, kidney, liver and BM), 500 ± 10 mg (AT, brain) or 1000 ± 10 mg (muscle) were ground thinly with scissors and homogenized in 1 ml Na_2HPO_4 buffer (pH 8.4, 0.25 M). For biological fluids, 1 ml (blood) or 200 μL (bile, urine and VH) were simply mixed with 1 ml Na_2HPO_4 buffer. IS were adjoined by addition of the appropriate volume (20 μL, 50 μL or 100 μL) of the IS solution containing 0.25 μg/ml of nordazepam-d5, 4 μg/ml of diazepam-d5, oxazepam-d5 and temazepam-d5, and 2 μg/ml of citalopram-d6. In all cases, 5 ml tert-butyl-methyl-ether/*n*-heptane (67:33, v/v) was then added and the mixture was agitated for 20 min. After centrifugation (10 min at 1400 × *g*), the upper organic layer was saved and added to 2.5 ml hydrochloric acid (0.2 M). After mechanical shaking for 10 min, centrifugation (10 min at 1400 × *g*) was carried out. The lower aqueous layer was saved; neutralized by 0.5 ml sodium hydroxide (1 N) and set at pH 8.4 by addition of 1 ml Na_2HPO_4 buffer. Finally, 5 ml tert-butyl-methyl-ether was added, and the samples were agitated (10 min) and centrifuged (10 min at 1400 × *g*). The upper organic layer was saved and evaporated to dryness at 50 °C under air stream. The residue was reconstituted in 100 μL tert-butyl-methyl-ether, transferred to a gas chromatography vial, and again evaporated to dryness. Automated on-line derivatization was performed on 7693A autosampler with 20 μL BSTFA/TMCS for 20 min at 80 °C. Two microlitres of derivatized extract was injected into the GC–MS/MS system.

2.3. GC–MS/MS

2.3.1. Gas chromatography

Gas chromatography (GC) was carried out on a 7890A GC system (Agilent, Santa Clara, CA, USA) equipped with a 7693A autosampler (Agilent). Compounds were separated on an HP-5MS capillary column (30 m length × 0.250 mm I.D. × 0.25 μm film thickness). The carrier gas was helium, at a constant flow of 1 ml/min. Injection was performed in splitless mode at an injection temperature of 260 °C. The transfer line was held at 310 °C. The initial oven temperature of 120 °C was maintained for 1 min, and subsequently increased at a rate of 50 °C/min to 280 °C and held for 4 min, and then finally increased at a rate of 50 °C/min to 300 °C and held for 3 min.

2.3.2. Tandem mass spectrometric conditions

GC–MS/MS analyses were acquired using a 7000 triple quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) in positive electronic ionization (EI) mode. Ion source temperature was set at 230 °C and ionization energy at 70 eV. The collision gas was nitrogen (flow-rate, 1.5 ml/min) with helium quench (flow-rate, 2.25 ml/min) acquisition was performed in selected reaction monitoring (SRM) mode. Transitions were chosen for selectivity and abundance to maximize signal-to-noise ratio in matrix extracts. One transition per compound was used as quantifier, and 2 as qualifiers.

2.3.3. Acceptance criteria for compounds identification

The criteria for identification of compounds were set as follows: for chromatography, the observed retention time differ by no more than ±0.1 min in absolute from that of the highest calibration standard prepared and analyzed contemporaneously; for mass spectrometry, the qualifier ions ratios differ by no more than 25% from that of the highest calibration standard prepared and analyzed contemporaneously; the standard consisted in standard. These criteria of identification were automatically followed by the Mass Hunter workstation software for quantitative analysis for QQQ (version B.04.00, Agilent technologies).

2.4. Specimens

2.4.1. Validation specimens

Blank samples of rabbit biological fluids (blood, urine, bile and VH) and tissues (lung, kidney, liver, brain, BM, AT and muscle) were pooled and used for development and validation. They were obtained from animal experiments performed in strict accordance

with established guidelines for animal care and with the approval of the Animal Ethics Committee, Lyon, France (no. BH 2008-14).

2.4.2. Application specimens

Application specimens were obtained from animal experiments and human autopsies. In the former case, with approval from the Animal Ethics Committee, Lyon, France (no. BH 2008-14), adult rabbits were injected intravenously with diazepam and citalopram at 1.1 and 2.8 mg/kg respectively. Euthanasia was performed 4 h after injection by heart-blood withdrawal after deep ketamine anesthesia. Immediately after death, tissues and fluids were collected and stored at −20 °C until analysis.

For human autopsy samples, the putrefaction impact on chromatographic performance was tested on putrefied human specimens (kidney, liver, lung, brain, BM and muscle) from forensic autopsies of drowning victims. In the first experiment, the victim was not known to be under any psychiatric treatment and toxicological screening results on putrefied blood did not show any of the 5 compounds of interest. Analyses were performed blank and by spiking at low quality control (QC) level (nordazepam 3 ng/ml(/g), citalopram 15 ng/ml(/g), and diazepam, oxazepam and temazepam 30 ng/ml(/g)). For the second experiment, the method was applied during toxicological investigation of a victim known to have been treated with prazepam.

2.5. Validation procedure

BM underwent full validation over a 3-day period, comprising linearity check, within-day and between-day accuracy and precision test, evaluation of limits of quantification (LOQ), selectivity, estimation of extraction recovery and assessment of the dilution procedure for extended concentration ranges and of auto-sampler stability. One-day partial validation was undertaken for the other 10 matrices, comprising linearity evaluation on the calibration range, within-day precision and accuracy test, selectivity check, recovery test estimation and LOQ determination.

2.5.1. Linearity

Calibration curves (5 standards) were prepared in each matrix at concentrations ranging from 1 to 200 ng/ml(/g) for nordazepam, 10–2000 ng/ml(/g) for diazepam, oxazepam and temazepam and 5–1000 ng/ml(/g) for citalopram. For fluid samples, the high point of the calibration range was obtained by spiking matrix with an appropriate dilution of working solution containing the 5 compounds; the other calibration points were prepared by serial dilution using the corresponding matrix as diluent. For tissue samples, due to the difficulties of solid matrix homogenization, the procedure was modified as follows: Na_2HPO_4 buffer was spiked with an appropriate dilution of working solution to obtain the highest calibration point, then serial dilution in buffer was performed; 1 ml of spiked buffer was added to a fixed amount of blank matrix. Calibration curves were established by linear least-squares regression, using Mass Hunter workstation software for quantitative analysis for QQQ (version B.04.00, Agilent technologies), by plotting relative response (analyte/IS, in area) as a function of analyte concentration. The best-fitting calibration model for each molecule was chosen using the fully validated BM results. Linear and quadratic curves were tested to minimize percentage residual and maximize the determination coefficient (r^2) calculated by the software. 1/[concentration] or 1/[concentration]^2 weighting factors were applied. The best BM model was then implemented on the other matrices; it was checked that percentage residue and r^2 were <10% and >0.95, respectively. Assay linearity was tested by analysis of variance. The significance of the slope and the validity of the linear calibration curves were tested using the Fisher–Snedecor F-test ($p < 0.05$).

2.5.2. Within-day and between-days accuracy and precision

Full validation (on BM): Within-day accuracy and precision were tested on 5 repeated determinations at low (midpoint of the lowest 2 calibration points) and high concentrations (midpoint of the highest 2 calibration points). The procedure was repeated on 3 different days to determine between-day precision. Accuracy was measured as relative percentage deviation from nominal concentration. Precision was assessed by within-run (WRP) and between-runs precision (BRP), using Statview software for Windows, version 5.0 (SAS Institute, Cary, NC, USA). Briefly, within-run precision was determined as $\mathrm{WRP} = 100 \times \left(\sqrt{\mathrm{MS_{wit}}}/\mathrm{GM}\right)$, and between-runs precision as $\mathrm{BRP} = 100 \times \left(\sqrt{(\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n/\mathrm{GM}}\right)$, where $\mathrm{MS_{wit}}$, $\mathrm{MS_{bet}}$, n and GM are the within-groups mean square, between-groups mean square, number of replicate observations within each run and the grand mean, respectively.

Partial validation: In the same way, within-day accuracy and precision were tested on 5 repeated determinations in each matrix at low (midpoint of the lowest 2 calibration points) and high concentrations (midpoint of the highest 2 calibration points). Accuracy and precision were measured as relative percentage deviation from nominal concentration and relative standard deviation (RSD), respectively.

2.5.3. Limits of quantification and assessment for extended concentration range

The LOQ corresponded to the concentration of the lowest calibration standard. At that point, accuracy and precision were checked as being acceptable (<20%) on 5 repeated determinations.

The upper limit of quantification was chosen as the concentration of the highest calibration standard. However, the dilution procedure for concentrations over the range as a whole was validated allowing that, from our experiences, higher concentrations may be present in some matrices. For this purpose, 1/10th dilution was tested on 5 replicates to quantify BM overloaded with nordazepam at 1 μg/g, diazepam, oxazepam, temazepam at 10 μg/g and citalopram at 5 μg/g. In this case, the extraction procedure described above was modified: ISs were added at 10-fold the classic concentration and only 500 μL (rather than 5 ml) tert-butyl-methyl-ether/n-heptane (67:33, v/v) was saved at the first step. The following steps of the extraction remained unchanged.

2.5.4. Validation of other parameters

Selectivity was tested by analyzing 6 blank samples of each matrix. Extraction efficiency was evaluated for each matrix at QC levels, by comparing the mean peak areas from samples obtained through the extraction procedure (analytes added before the extraction step: $n = 5$, QC samples) with those obtained from blank matrix which underwent the extraction procedure and overloaded before the derivatization step ($n = 3$). Auto-sampler stability at 25 °C was tested by reanalyzing sample extracts 72 h after their creation.

3. Results and discussion

3.1. Sample preparation

A 3-step extraction procedure with back-extraction in acid was performed as described previously for some benzodiazepines in complex tissues [20,21] with optimization. The first step used Na_2HPO_4 buffer at pH 8.4 and tert-butyl-methyl-ether/n-heptane (67:33, v/v). Secondly, a derivatization step was added to improve compound stability and obtain spectra with more structural information [35]. Temperature and time conditions for derivatization were optimized at 80 °C for 20 min. Derivatization was online rather than manual, as this was time-saving in sample preparation and

Table 1
Retention times and parameters used in selected reaction monitoring mode for the study compounds and their internal standards.

Compound	RT[a] (min)	Quantifier		Qualifier 1		Qualifier 2	
		Transition[b] (*m/z*)	CE[c] (eV)	Transition[b] (*m/z*)	CE[c] (eV)	Transition[b] (*m/z*)	CE[c] (eV)
Nordazepam-TMS[d]	5.84	341 → 290	15	341 → 269	20	327 → 146	25
Nordazepam-d5-TMS[d]	5.83	347 → 232	10	347 → 275	15	332 → 151	30
Oxazepam-TMS[d]	6.14	430 → 401	10	430 → 267	25	313 → 135	25
Oxazepam-d5-TMS[d]	6.13	435 → 406	10	435 → 345	20	318 → 288	35
Citalopram	6.38	324 → 58	3	324 → 84	1	324 → 86	7
Citalopram-d6	6.36	330 → 64	5	64 → 60	20	64 → 45	20
Diazepam	6.68	256 → 221	15	256 → 165	35	283 → 238	20
Diazepam-d5	6.67	261 → 226	15	287 → 252	20	287 → 209	35
Temazepam-TMS[d]	7.45	283 → 255	20	257 → 228	20	343 → 244	15
Temazepam-d5-TMS[d]	7.44	262 → 227	20	262 → 232	20	348 → 276	10

[a] RT: retention time.
[b] Transition: precursor ion → product ion.
[c] CE: collision energy.
[d] TMS: analyzed in trimethylsilyl form.

avoided the waiting time for silylated derivatives on the autosampler

3.2. GC–MS/MS conditions

Total method run-time was 11.6 min. Corresponding retention times are presented in Table 1. The 20 min between two injections was conditioned by the length of the online derivatization. The chromatogram peaks of all compounds were totally resolute. Five SRM windows (1 per analyte) were created, to enhance sensitivity. Dealing with some complex matrices (such as lipidic ones) and putrefied samples, 3 transitions were chosen per molecules as well as their labeled analogues (1 quantifier, 2 qualifiers), after a preliminary selection, by comparing signal-to-noise ratios in various BM samples. The one providing the best signal-to-noise ratio on BM extracts was chosen for quantification purposes. During the method development, smaller ionization energies were tested in particular to avoid the fragmentation of citalopram in small non specific ions (e.g. $m/z = 58$). However, no improvement of signal-to-noise ratio was observed in matrices (results not shown) and we conserved the classical ionization energy of 70 eV. Table 1 summarizes the SMR parameters (transitions and collision energy) used for analysis of the 5 compounds and their deuterated equivalents.

3.3. Validation procedure

For a protocol intended to be applied to 11 matrices, the choice of validation protocol is challenging due to the need to take account of possible matrix effects while limiting resort to blank matrices provided by animal sacrifice. Quantification in fluids and tissues using blood calibration standards was tested, but the results failed to meet validation criteria despite the used of

Table 2
Between-days validation of calibration curves according to chosen model of citalopram, diazepam and metabolites in BM. Data from calibration curves analyzed on 3 different days.

Concentration (ng/g)		Precision (%) (between runs)	Accuracy (%)
Spiked	Found (mean ± SD, 3 days)		
Nordazepam	Calibration model: linear; weighting factor: 1/[concentration]^2		
1	1.0 ± 0.0	1.9	102.5
5	4.5 ± 0.5	10.6	89.2
20	19 ± 1	3.2	94.6
80	82 ± 5	6.0	102.1
200	226 ± 16	6.9	113.0
Oxazepam	Calibration model: quadratic; weighting factor: 1/[concentration]^2		
10	10.1 ± 0.1	0.8	101.3
50	46 ± 4	8.2	92.0
200	196 ± 7	3.4	98.1
800	862 ± 44	5.1	107.7
2000	1,954 ± 29	1.5	97.7
Diazepam	Calibration model: linear; weighting factor: 1/[concentration]^2		
10	10.2 ± 0.1	1.1	101.5
50	46 ± 2	4.3	92.7
200	195 ± 1	0.7	97.3
800	823 ± 37	4.5	102.9
2000	2,110 ± 102	4.8	105.5
Temazepam	Calibration model: linear; weighting factor: 1/[concentration]^2		
10	12.2 ± 1.4	11.4	121.5
50	46 ± 1	2.7	91.7
200	186 ± 9	5.0	92.7
800	782 ± 8	1.0	97.8
2000	2,040 ± 18	0.9	102
Citalopram	Calibration model: linear; weighting factor: 1/[concentration]		
5	5.1 ± 0.1	1.2	101.9
25	23 ± 1	1.8	91.1
100	95 ± 0	0.1	94.6
400	420 ± 18	4.4	104.9
1000	1,072 ± 98	9.2	107.2

Table 3
Assessment of accuracy, precision and extraction recovery at LOQ, low QC and high QC, and during dilution validation and 72 h autosampler stability check for full validation on BM.

	Concentration (ng/g)		Accuracy (%)	Precision (%)		Extraction recovery (%)
	Spiked	Found (mean ± SD, 3 days)		Within-run	Between-runs	
Nordazepam						
Low QC[a]	3	2.7 ± 0.3	88.9	7.6	8.1	55
High QC[a]	140	146 ± 11	104.2	7.2	1.4	43
LOQ[b]	1	0.8 ± 0.1	80.9	11.4		
AS stability[b]	3	2.5 ± 0.3	83.6	12.9		
AS stability[b]	140	136 ± 8	97.2	5.9		
1/10th dilution[b]	1000	1019 ± 10	101.8	4.7		
Oxazepam						
Low QC[a]	30	27.3 ± 4.5	90.9	12.0	12.6	20
High QC[a]	1400	1411 ± 81	100.8	5.1	3.3	16
LOQ[b]	10	8.9 ± 1.1	88.8	12.8		
AS stability[b]	30	25.9 ± 3.6	86.2	13.9		
AS stability[b]	1400	1350 ± 58	96.4	4.3		
1/10th dilution[b]	10,000	10,646 ± 180	106.5	1.7		
Diazepam						
Low QC[a]	30	27.1 ± 2.0	90.2	4.9	6.5	36
High QC[a]	1400	1490 ± 114	106.4	5.9	5.8	30
LOQ[b]	10	9.6 ± 0.1	95.9	1.3		
AS stability[b]	30	25.6 ± 1.8	85.3	6.9		
AS stability[b]	1400	1499 ± 102	107.1	6.8		
1/10th dilution[b]	10,000	10,837 ± 1020	108.4	9.4		
Temazepam						
Low QC[a]	30	30.6 ± 3.1	102.0	5.0	10.5	8
High QC[a]	1400	1398 ± 137	99.9	6.9	8.2	5
LOQ[b]	10	9.3 ± 0.9	93.1	10.1		
AS stability[b]	30	32.4 ± 2. 6	108.1	7.9		
AS stability[b]	1400	1445 ± 62	103.2	4.3		
1/10th dilution[b]	10,000	9428 ± 647	94.3	6.9		
Citalopram						
Low QC[a]	15	14.1 ± −2.0	93.7	10.8	11.4	68
High QC[a]	700	699 ± −78	99.9	9.4	6.9	49
LOQ[b]	5	4.8 ± 0.2	95.0	4.4		
AS stability[b]	15	16.7 ± 3.3	111.5	9.5		
AS stability[b]	700	630 ± 256	90.0	10.6		
1/10th dilution[b]	5000	4307 ± 468	86.1	10.9		

[a] Data from 5 replicates, analyzed on 3 different days.
[b] Data from 5 replicates, accuracy and within-run precision was measured as relative percentage deviation from nominal concentrations and relative standard deviation (RSD), respectively. (AS stability: autosampler stability).

labeled ISs for each molecule (data not shown). Since all specimens came from a single species, full validation was performed on the most complex matrix, which our experience showed to be BM, with partial 1-day validation for the other 10. Calibration range linearity, LOQ determination, selectivity, extraction performance assessment, accuracy and precision were checked for each matrix. Between-days precision, extended concentration range dilution procedure and autosampler stability were evaluated only on BM. Since a few blank matrices (such as VH, kidney, BM and bile) were available in limited quantities, 2 quality controls rather than the 3 generally recommended [33,34] were performed, focused on the extremities of the calibration range.

3.3.1. Linearity

The calibration curves were constructed by linear least-squares regression by plotting relative response (analyte/IS, in area) according to analyte concentration. The best fitting calibration model for each molecule between the linear and quadratic curves as well as the weighting factor were chosen using the fully validated BM results. Linear regression determination coefficients (r^2) were ≥0.991 for all compounds in this matrix. Calibration curves on the 3 days and the calibration model are presented in Table 2. For all concentrations, between-days precision was generally less than 10% (10.6% for nordazepam at 5 ng/g, and 11.4% for temazepam at 10 ng/g). Accuracy ranged from 89.2% to 107.7%, except for nordazepam at 200 ng/g (113.0%) and temazepam at 10 ng/g (121.5%).

For the others matrices, the best fitting model was searched. In most cases, it was the same as BM. When it was different, the BM calibration model and the best fitting model were compared and it appeared that using BM model did not have consequence on the acceptation criteria in term of precision or accuracy. Therefore the calibration model chosen for BM was then applied to the other 10 matrices. Maximum percentage residual calculated by the software during linear regression appeared to be less than 5% for all compounds in each matrix. Linear regression determination coefficients (r^2) were ≥0.990 except, for nordazepam in urine (0.985), citalopram in VH (0.981) and in urine (0.983) and temazepam in AT (0.984).

3.3.2. Within-day and between-days accuracy and precision

Within-day and between-days accuracy and precision were examined on BM at low and high levels. Results are presented in Table 3. Assay accuracy was in the range of 88.9–102.0% at low level and 99.9–106.4% at high level for the 5 compounds. Despite the applied weighting factor, the low concentrations were slightly underestimated while high concentrations were assessed more accurately. Within-day and between-days precision were less than 12.0% (oxazepam, low QC) and 12.6% (oxazepam, low QC), respectively. This method was then applied to the other 10 matrices. Five replicates at low and high levels were analyzed; a calibration curve performed the same day in the corresponding matrix enabled quantification. Table 4 reports results for accuracy and within-day precision for both QCs. At low level,

Table 4
Assessment of accuracy, precision and extraction recovery at LOQ, low QC and high QC levels in partially validated matrix. Data from 5 replicates analyzed in each matrix from 1 calibration curve per matrix.

	LOQ[a]		Low quality control[b]			High quality control[c]		
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Extraction recovery (%)	Accuracy (%)	Precision (%)	Extraction recovery (%)
Nordazepam								
Blood	119.2	9.6	89.2	3.5	57	99.6	1.0	70
VH	94.8	9.3	87.5	2.5	56	108.9	5.7	50
Urine	98.3	15.4	85.6	7.8	65	107.4	3.7	61
Bile	80.3	12.5	87.9	9.9	60	109.4	4.6	64
Liver	97.8	8.2	86.7	7.5	45	113.2	4.2	40
Kidney	82.6	4.8	108.1	6.7	52	96.7	2.4	43
Lung	81.2	12.7	94.8	11.3	52	112.0	5.0	52
Muscle	100.6	6.8	88.9	5.5	41	114.5	3.2	26
Brain	93.5	6.4	86.6	8.3	32	113.2	3.4	50
AT	112.8	13.5	98.4	7.2	53	110.7	5.8	62
Oxazepam								
Blood	94.7	10	87.7	2.1	17	101.5	1.0	29
VH	102	10.5	99.8	3.8	17	100	4.8	19
Urine	105.5	3.1	85.6	6.3	24	92.1	2.9	29
Bile	81.7	10.9	89.3	7.3	20	101	4.1	25
Liver	76.8	3.5	82.6	5.4	13	103.5	3.4	14
Kidney	76	17	81.4	8.1	21	97.1	2.0	16
Lung	115.7	3.5	88.8	5.1	17	104.4	3.6	19
Muscle	92.2	5	85.8	8.8	11	112.4	3.9	9
Brain	84	11.7	85.1	6.7	9	113.7	3.3	17
AT	111.9	6.2	92.2	7.1	16	96.1	2.5	34
Diazepam								
Blood	97.2	10.3	108.8	12.0	44	108.5	5.3	52
VH	101.5	3.1	95.2	4.6	37	106.9	4.9	31
Urine	103.5	0.6	112.3	6.1	58	104.2	7.2	46
Bile	113.2	3.4	106.7	1.9	49	102.6	4.1	45
Liver	75.3	16.4	90.6	6.5	40	97	2.1	30
Kidney	124.2	2.6	105.9	6.4	38	97	1.4	27
Lung	119.6	1.1	99.1	2.7	60	112.9	6.0	36
Muscle	104.1	2.5	99.6	5.3	33	111.4	3.4	24
Brain	114.1	2.4	108.3	2.5	29	113.6	3.1	34
AT	119.5	5.3	107.7	6.1	43	101.1	5.5	51
Temazepam								
Blood	80.1	12.7	92.3	1.2	9	102.4	2.9	12
VH	106.9	4.0	92.1	3.9	6	98.7	5.4	6
Urine	114.3	2.0	85.6	2.9	9	88.2	3.3	10
Bile	88.7	6.3	85.7	4.5	8	100.7	3.3	9
Liver	98.7	2.3	85.4	2.6	6	101.6	3.6	6
Kidney	86.1	2.5	88.3	2.4	8	96.1	2.2	6
Lung	119.7	8.5	85.1	5.1	6	103.7	3.7	7
Muscle	111.4	2.6	91.2	8.3	6	110.7	3.4	4
Brain	80.9	2.4	85.3	4.7	4	112.8	3.3	7
AT	91.1	9.3	87.5	8.2	7	101	5.2	9
Citalopram								
Blood	111.9	8.7	92.4	2.4	77	94.5	4.1	97
VH	105.5	5.2	100.9	8.0	102	102.7	5.9	57
Urine	97.8	3.8	85.9	4.1	67	98.8	5.1	75
Bile	85.2	5.3	88.3	5.1	109	96.2	4.4	78
Liver	111.1	12.3	92.2	5.5	58	105.1	3.5	51
Kidney	96.3	12.8	85.7	4.1	70	99.7	1.0	45
Lung	112.5	14.7	97.8	5.9	98	109.1	5.6	54
Muscle	113.5	11.3	89.7	7.6	44	111.3	3.7	26
Brain	98.2	1.4	92.0	5.3	50	112.4	3.1	41
AT	119	7.3	95.7	6.4	68	100.7	1.7	38

[a] 1 ng/ml(/g) for nordazepam; 10 ng/ml(/g) for diazepam, oxazepam and temazepam; 5 ng/ml(/g) for citalopram.
[b] 3 ng/ml(/g) for nordazepam; 30 ng/ml(/g) for diazepam, oxazepam and temazepam; 15 ng/ml(/g) for citalopram.
[c] 140 ng/ml(/g) for nordazepam; 1400 ng/ml(/g) for diazepam, oxazepam and temazepam; 700 ng/ml(/g) for citalopram.

accuracy was in the range of 85.1–112.3%; only oxazepam in liver and kidney was below the 85% cut-off (82.65 and 81.4% respectively). Precision was below 9.9%; except for nordazepam in lung (11.3%) and diazepam in blood (12%). At high level, accuracy ranged from 88.25 to 114.55 and precision was less than 7.2%. Finally, the accuracy and precision of this method for the 5 compounds in the 11 matrices, except for oxazepam in liver and kidney, were in line with commonly accepted validation guidelines for both QC levels [33,34] (between 85–115% and ±15%; respectively).

3.3.3. Limits of quantification

The LOQ corresponded to the concentration of the lowest calibration standard. At that point, accuracy and precision were determined on 5 repeated runs. Results for BM are shown in Table 3 and for the other matrices in Table 4. Precision was within 20% and accuracy between 80% and 120%, as generally recommended, except for oxazepam in liver and kidney and diazepam in liver, for which accuracy at the LOQ level was slightly above 20% but still below 25%. Taken in consideration the finality of the method, use in pharmacokinetic study on tissue distribution as well as

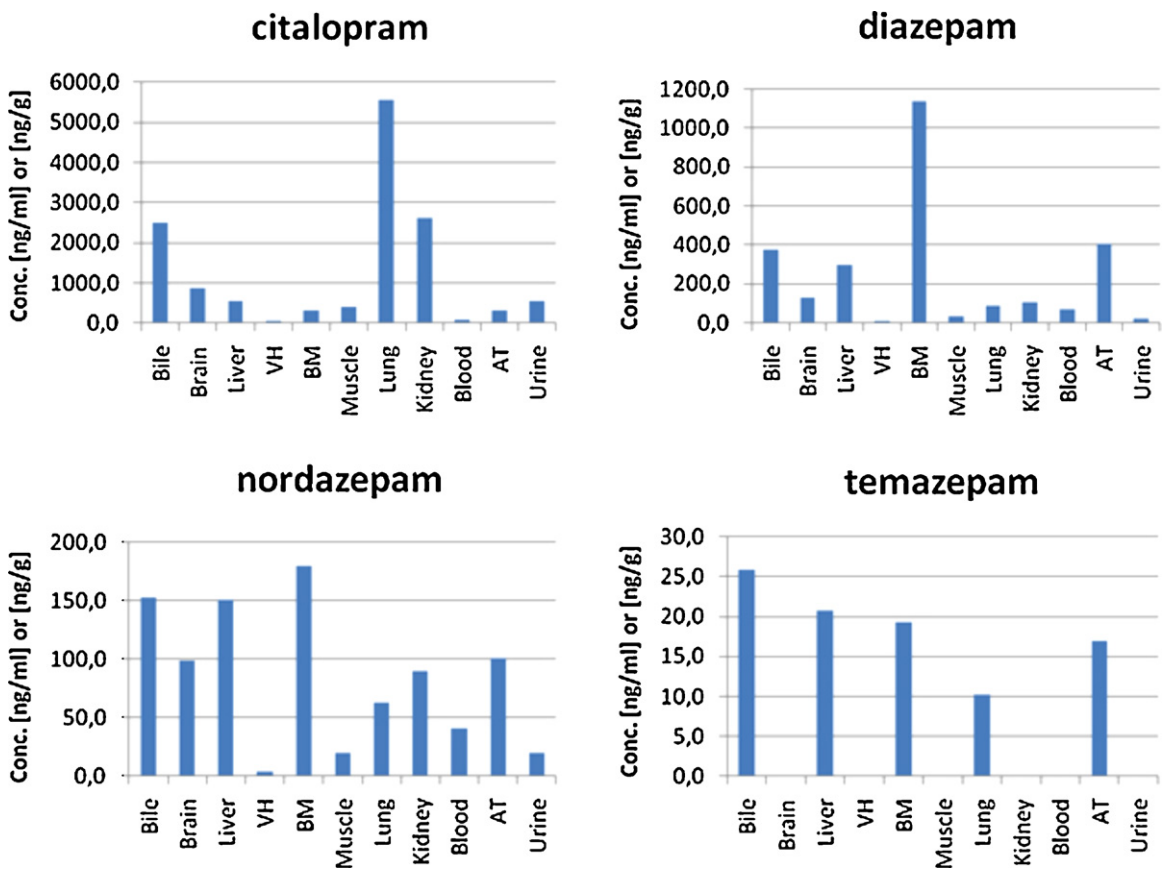

Fig. 2. Distribution patterns of diazepam, nordazepam, temazepam and citalopram in rabbit specimen after IV injection of 1.1 mg/kg and 2.8 mg/kg diazepam and citalopram, respectively. Oxazepam < LOQ in all but one specimen (liver = 11.3 ng/g).

damaged sample analysis in a context of toxicological evidence, this slight deviation of the LOQ criteria for few analytes in few matrices was considered acceptable. Thus, the LOQ of the method were evaluated at 1 ng/g for nordazepam, 5 ng/g for citalopram and 10 ng/g for oxazepam, diazepam and temazepam. Some published method reported lower LOQ (e.g. 0.5 ng/ml for citalopram [36,37], 0.04 ng/ml for diazepam and 0.1 ng/ml for nordazepam, oxazepam, and temazepam [38]. However, most of these assays displaying such sensitivity were dedicated to one or few matrices especially biological fluids like plasma, blood, urine, oral and cerebrospinal fluid [7,39].

3.3.4. Assessment for extended concentration range

Given the wide range of concentrations to be expected, from therapeutic to toxic dose and according to the matrix, sample dilutions beyond the calibration range were essential in developing the extraction process. This was, however, a critical step as far as the tissue samples were concerned, due to the limits of the solid tissue homogenization process and the difficulty of obtaining blank matrices ensuring sample dilution. Moreover, decreasing the sample raises the question of the representativeness of very small pieces of organ. Dilutions were therefore performed using the same initial sampling quantity, by collecting a fraction of the organic layer at the end of the first extraction step. To validate the dilution protocol for samples initially quantified as out of the calibration range, a 1/10th dilution was tested to quantify BM overloaded with nordazepam at 1 μg/g, diazepam, oxazepam, temazepam at 10 μg/g and citalopram at 5 μg/g. Accuracy and precision are presented for each compound in Table 3. The effectiveness of the dilution process was confirmed, since accuracy ranged from 86.1% to 108.4% and precision was less than 10.9%.

3.3.5. Extraction efficacy

Extraction recovery was estimated at low and high QC levels in each matrix by comparing the peak area of the QC samples with that of extracted blank matrix spiked before the derivatization step. Extraction recovery is reported in Table 3 for BM and in Table 4 for the other specimens. Few variations were observed according to matrix, except that extraction recovery in muscle was generally lower than in other matrices (e.g., 26% in muscle versus around 50% in other matrices for nordazepam at high QC level). This may be due to the particular fibrous structure of muscle tissue. In case higher sensitivity should be required, a different tissue homogenization technique to disrupt muscular structure should be tested. Recovery was comparable at low and high levels, except for citalopram (mean extraction recovery 74% at low vs. 55% at high QC level). Extraction efficacy varied greatly from one compound to another, with the lowest rates being observed for oxazepam and temazepam. Overall, although the extraction recovery values of this method were somewhat low, the detection limit proved sensitive enough to detect the 5 compounds in our applications. The method was intended for pharmacokinetic studies and forensic applications when blood was not available (putrefaction process, charred body, etc.) and a single protocol was developed to analyze all types of sample, whether fluid or tissue, including putrefied and damaged specimens. A 3-step extraction procedure with back-extraction in acid was performed. This time- and solvent-consuming protocol was preferred to a single extraction, so as to limit chromatographic interference due to putrefied material and certain complex and lipid-rich tissue such as brain, AT or BM. The focus was on selectivity, so long as sensitivity, however reduced, remained sufficient for the purposes of the intended applications.

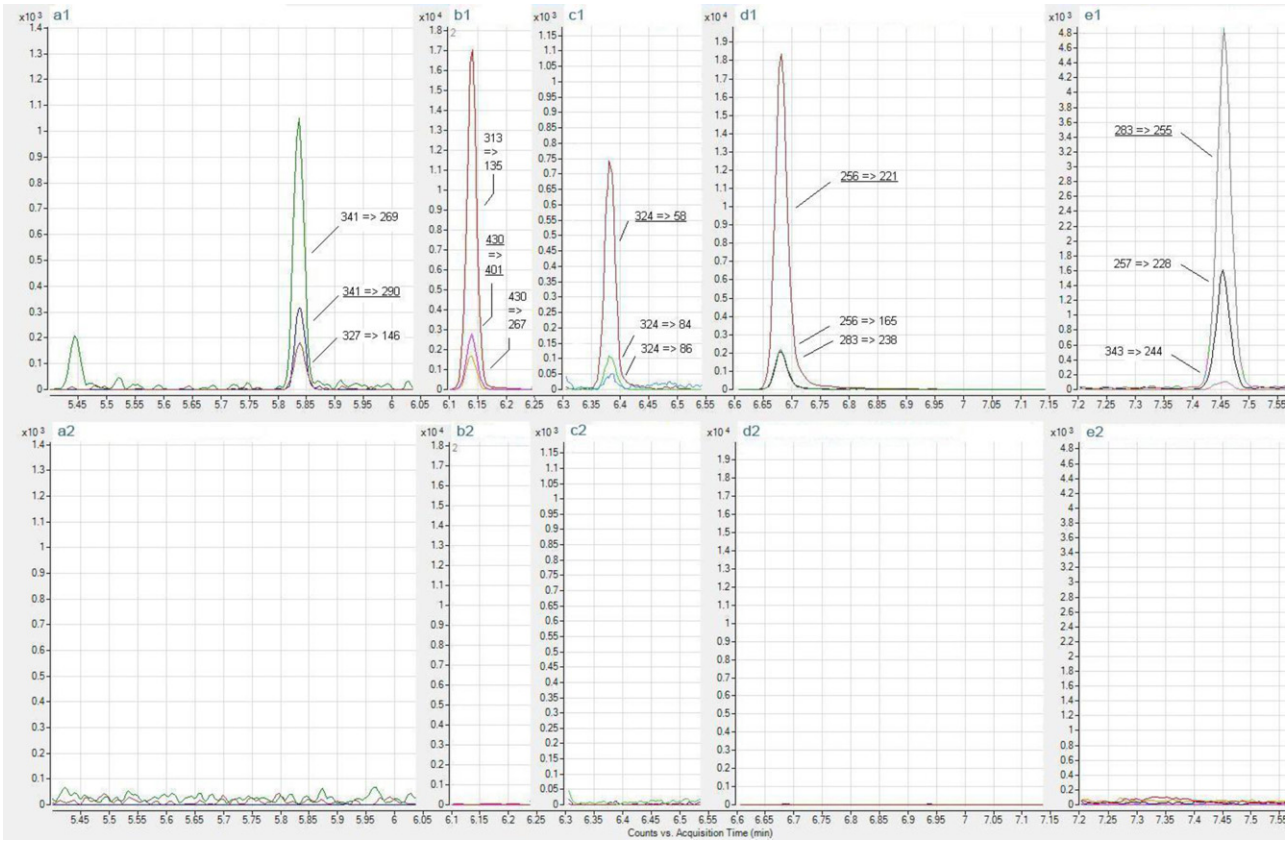

Fig. 3. (a1–e1) Chromatogram of quantifying transition of putrefied matrix extract spiked at low QC level, with quantifier (underlined) and qualifier transitions. (a1) Nordazepam (3 ng/g), (b1) oxazepam (30 ng/g), (c1) citalopram (15 ng/g), (d1) diazepam (30 ng/g), (e1) temazepam (30 ng/g). (a2–e2) Corresponding blank extract chromatogram.

No chromatographic interference was observed in any of the 6 blank specimens of each matrix at corresponding retention times and for the 3 transitions. The combination of 3-step extraction and MS/MS detection achieved good selectivity in all matrices, even the most lipid-rich. Selectivity was also tested, with the same good results, on putrefied human specimens (see Section 3.4, below).

3.3.6. Autosampler stability

Autosampler stability was checked by reinjection of the low and high QC BM extracts 72 h later. Immediate injection of derivative compounds is always preferable, but conservation for 72 h did not significantly alter results, with accuracy ranging from 83.6% to 111.5% and precision less than 13.9%. Autosampler stability was the only stability parameter to be tested, because of the derivatization procedure and since the in vivo and in vitro stability of these common drugs was already described [40–44].

3.4. Application

The effectiveness of the proposed method was tested by analyzing real cases: rabbit specimens from an animal experiment, and human post-mortem specimens from putrefied bodies.

3.4.1. Animal experiment

Samples from rabbit euthanatized 4 h after being injected with citalopram (1.1 mg/kg) and diazepam (2.8 mg/kg) were analyzed. Fig. 2 shows the distribution pattern of diazepam, nordazepam, temazepam and citalopram. The assay quantified citalopram, diazepam and nordazepam in the 11 matrices: analyte concentrations were in the linearity range of calibration, except for temazepam, which was below the LOQ in some tissues, and citalopram, which showed exceptionally high concentrations in some specimens. Dilution was then performed for bile, lung and kidney samples to confirm citalopram concentrations. Oxazepam, a minor metabolite of diazepam rarely detected after a single therapeutic injection [45], was below the LOQ in all matrices except liver (11.3 ng/g), where metabolism takes place. As expected, concentration variability according to tissues was wide. For citalopram, the most obvious observation concerned the distribution in lung tissue: 5550 ng/g, versus 80 ng/ml in heart blood. In humans, Boer described the phenomenon of pulmonary processing of drugs, and especially basic ones, resulting in considerable rapid accumulation in lung [46]. In the present case of rabbit, the second highest concentrations of citalopram were in bile (2500 ng/g) and kidney (2620 ng/g). Numerous case reports provide a total of 27 human citalopram bile concentrations, all strictly higher than in blood [13,16,17]. In contrast, only 8 kidney concentrations were reported for humans: while higher than in blood, they were not as high as in the present rabbit case [13,15]. Citalopram brain tissue concentrations were widely investigated in human and animal studies [9–11] and generally found to be higher than in blood, as in the present rabbit case (brain concentration, 880 ng/g). Finally, the lowest concentration of citalopram observed in the present study was in VH, in agreement with human data where VH citalopram concentrations were generally half those of blood [15–17]. The diazepam and citalopram distribution patterns differed greatly. The greatest diazepam concentrations were observed for BM (1140 ng/g) and AT (405 ng/g), which may be explained by diazepam's lipophilicity. BM concentrations were previously reported to be higher than in blood in animal models [47,48] and human postmortem specimens [49]. The present liver and bile concentrations were high, as in some case reports for others benzodiazepines [23,24]. Study of diazepam metabolites showed that, in rabbit as in humans [45], nordazepam was quantitatively the main metabolite followed by

Table 5
Quantification of nordazepam and oxazepam in human postmortem samples of a victim treated by prazepam.

	Nordazepam	Oxazepam
Cardiac blood (ng/ml)	420	20
BM (ng/g)	1267	28
Liver (ng/g)	667	64
Kidney (ng/g)	544	188
Lung (ng/g)	463	42
Brain (ng/g)	747	64

temazepam and then oxazepam, the latter being quantified only in liver.

3.4.2. *Putrefied human specimens*

Kidney, muscle, brain, BM, lung and liver, from a putrefied victim not known to be under any psychiatric treatment, were analyzed blank and spiked at low QC level. An example of chromatograms obtained from BM specimen is shown in Fig. 3. For all matrices, no interference was observed on blank sample chromatograms. Moreover, the multi-step procedure of extraction allowed compounds to be quantified at low levels in all putrefied tissues.

The assay was also applied to postmortem tissue in the context of toxicological forensic evidence. The cause of death of a putrefied body found in a lake was diagnosed, on the basis of observation and diatom analysis, as submersion while alive; information obtained from the family indicated that the victim was being treated with various drugs, including prazepam. According to the metabolism of prazepam (Fig. 1), nordazepam and oxazepam were quantified in cardiac blood and in 5 other tissues available. Results are presented in Table 5 and were consistent with an intake of prazepam by the victim before death. The sensitivity of the assay allowed both metabolites to be quantified.

4. Conclusions

A 3-step liquid/liquid extraction protocol with online derivatization prior to GC–MS/MS analysis was developed to quantify 4 benzodiazepines (diazepam, nordazepam, oxazepam and temazepam) and citalopram in 11 biological matrices (blood, urine, bile, vitreous humor, liver, kidney, skeletal muscle, brain, adipose tissue, lung and bone marrow). The procedure provided good accuracy and reproducibility. Application to real case samples showed that the method was sensitive enough to describe the distribution pattern of the molecules in an animal experiment, and specific enough to detect molecules in highly putrefied samples from human postmortem cases. The method may therefore be useful for whole-body distribution studies in animal experiments and for human postmortem toxicology investigations when analyses of alternative matrices are required to improve reliability of interpretation (e.g., in case of postmortem redistribution, lack of blood).

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References

[1] V. Pradel, C. Delga, F. Rouby, J. Micallef, M. Lapeyre-Mestre, CNS Drugs 24 (2010) 611–620.
[2] K.P. Marshall, Z. Georgievskava, I. Georgievsky, Res. Social. Adm. Pharm. 5 (2009) 94–107.
[3] S.A. Hollingworth, D.J. Siskind, Pharmacoepidemiol. Drug Saf. 19 (2010) 280–288.
[4] R. Mandrioli, L. Mercolini, M.A. Raggi, Curr. Drug Metab. 11 (2010) 815–829.
[5] B. Jonasson, T. Saldeen, Forensic Sci. Int. 126 (2002) 1–6.
[6] G. Skopp, Forensic Sci. Int. 142 (2004) 75–100.
[7] N. Unceta, M.A. Goicolea, R.J. Barrio, Biomed. Chromatogr. 25 (2011) 238–257.
[8] X. Liu, K. Van Natta, H. Yeo, O. Vilenski, P.E. Weller, P.D. Worboys, M. Monshouwer, Drug Metab. Dispos. 37 (2009) 787–793.
[9] N. Unceta, A. Ugarte, A. Sanchez, A. Gomez-Caballero, M.A. Goicolea, R.J. Barrio, J. Pharm. Biomed. Anal. 51 (2010) 178–185.
[10] N.S. Wang, B. Lemmer, J. Chromatogr. 488 (1989) 492–497.
[11] S.M. Wille, E.A. De Letter, M.H. Piette, L.K. Van Overschelde, C.H. Van Peteghem, W.E. Lambert, Int. J. Legal Med. 123 (2009) 451–458.
[12] K. Fu, R.J. Konrad, R.W. Hardy, R.M. Brissie, C.A. Robinson, J. Anal. Toxicol. 24 (2000) 648–650.
[13] B. Levine, X. Zhang, J.E. Smialek, G.W. Kunsman, M.E. Frontz, J. Anal. Toxicol. 25 (2001) 641–644.
[14] D. Luchini, G. Morabito, F. Centini, Am. J. Forensic Med. Pathol. 26 (2005) 352–354.
[15] E.L. Horak, A.J. Jenkins, J. Forensic Sci. 50 (2005) 679–681.
[16] N. Anastos, I.M. McIntyre, M.J. Lynch, O.H. Drummer, J. Forensic Sci. 47 (2002) 882–884.
[17] A.J. Jenkins, K. Gubanich, J. Forensic Sci. 47 (2002) 159–164.
[18] L. Mercolini, R. Mandrioli, C. Iannello, F. Matrisciano, F. Nicoletti, M.A. Raggi, Talanta 80 (2009) 279–285.
[19] K. Heinig, F. Bucheli, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 769 (2002) 9–26.
[20] K. Kudo, T. Nagata, K. Kimura, T. Imamura, M. Noda, J. Chromatogr. 431 (1988) 353–359.
[21] X.X. Zhang, K. Kudo, T. Imamura, N. Jitsufuchi, T. Nagata, J. Chromatogr. B: Biomed. Appl. 677 (1996) 111–116.
[22] F. Moriya, Y. Hashimoto, Forensic Sci. Int. 131 (2003) 108–112.
[23] A.J. Jenkins, B. Levine, J.L. Locke, J.E. Smialek, J. Anal. Toxicol. 21 (1997) 218–220.
[24] F. Moriya, Y. Hashimoto, Leg. Med. (Tokyo) 5 (Suppl. 1) (2003) S91–S95.
[25] H. Shiota, M. Nakashima, H. Terazono, H. Sasaki, K. Nishida, J. Nakamura, K. Taniyama, Leg. Med. (Tokyo) 6 (2004) 224–232.
[26] K. Kudo, T. Nagata, T. Imamura, S. Kage, Y. Hida, Int. J. Leg. Med. 104 (1991) 67–69.
[27] B. Levine, A. Grieshaber, J. Pestaner, K.A. Moore, J.E. Smialek, J. Anal. Toxicol. 26 (2002) 52–54.
[28] S. Bjorkman, A. Fyge, Z. Qi, J. Pharm. Sci. 85 (1996) 887–889.
[29] F. Musshoff, S. Padosch, S. Steinborn, B. Madea, Forensic Sci. Int. 142 (2004) 161–210.
[30] M. Schulz, A. Schmoldt, Pharmazie 52 (1997) 895–911.
[31] M. Schulz, A. Schmoldt, Pharmazie 58 (2003) 447–474.
[32] TIAFT Reference Blood Level List of Therapeutic and Toxic Substances, http://www.gtfch.org/cms/images/stories/Updated_TIAFT_list_202005.pdf, August 2011.
[33] Bioanalytical Method Validation, http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064964.htm, April 2011.
[34] V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551–1557.
[35] J. Segura, R. Ventura, C. Jurado, J. Chromatogr. B: Biomed. Sci. Appl. 713 (1998) 61–90.
[36] C. Pistos, I. Panderi, J. Atta-Politou, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 810 (2004) 235–244.
[37] T. Jiang, Z. Rong, L. Peng, B. Chen, Y. Xie, C. Chen, J. Sun, Y. Xu, Y. Lu, H. Chen, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 615–619.
[38] J. Wang, X. Shen, J. Fenyk-Melody, J.V. Pivnichny, X. Tong, Rapid Commun. Mass Spectrom. 17 (2003) 519–525.
[39] V.F. Samanidou, M.N. Uddin, I.N. Papadoyannis, Bioanalysis 1 (2009) 755–784.
[40] V.N. Atanasov, S. Stoykova, A. Runiov, T. Dimitrova, D. Aleksandrova, S. Tsakovski, M. Mitewa, Forensic Sci. Int. (2011) [Epub ahead of print].
[41] A. El Mahjoub, C. Staub, J. Pharm. Biomed. Anal. 23 (2000) 1057–1063.
[42] B. Levine, R.V. Blanke, J.C. Valentour, J. Forensic Sci. 28 (1983) 102–115.
[43] F.T. Peters, Anal. Bioanal. Chem. 388 (2007) 1505–1519.
[44] S.S. Singh, H. Shah, S. Gupta, M. Jain, K. Sharma, P. Thakkar, R. Shah, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 811 (2004) 209–215.
[45] K. Tada, T. Moroji, R. Sekiguchi, H. Motomura, T. Noguchi, Clin. Chem. 31 (1985) 1712–1715.
[46] F. Boer, Br. J. Anaesth. 91 (2003) 50–60.
[47] T. Takatori, S. Tomii, K. Terazawa, M. Nagao, M. Kanamori, Y. Tomaru, Int. J. Leg. Med. 104 (1991) 185–188.
[48] J.H. Watterson, J.E. Botman, J. Forensic Sci. 54 (2009) 708–714.
[49] L.M. McIntyre, C.V. King, M. Boratto, O.H. Drummer, Ther. Drug Monit. 22 (2000) 79–83.