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Metabolites of lorazepam: Relevance of past findings to present day use of LC-MS/MS in analytical toxicology

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The advent of liquid chromatography-tandem mass spectrometry (LC-MS/MS), with the sensitivity it confers, permits the analysis of both phase I and II drug metabolites that in the past would have been difficult to target using other techniques. These metabolites may have relevance to current analytical toxicology employing LC-MS/MS, and lorazepam was chosen as a model drug for investigation, as only the parent compound has been targeted for screening purposes. Following lorazepam administration (2 mg, p.o.) to 6 volunteers, metabolites were identified in urine by electrospray ionization LC-MS/MS, aided by the use of deuterated analogues generated by microsomal incubation for use as internal chromatographic and mass spectrometric markers. Metabolites present were lorazepam glucuronide, a quinazolinone, a quinazoline carboxylic acid, and two hydroxylorazepam isomers, one of which is novel, having the hydroxyl group located on the fused chlorobenzene ring. The quinazolinone, and particularly the quinazoline carboxylic acid metabolite, provided longer detection windows than lorazepam in urine extracts not subjected to enzymatic hydrolysis, a finding that is highly relevant to toxicology laboratories that omit hydrolysis in order to rapidly reduce the time spent on gas chromatography-mass spectrometry (GC-MS) analysis. With hydrolysis, the longest windows of detection were achieved by monitoring lorazepam, supporting the targeting of the aglycone with free drug for those incorporating hydrolysis in their analytical toxicology procedures.

Keywords: Benzodiazepines; lorazepam; LC-MS/MS; forensic; drug metabolism; analytical toxicology

Introduction

The advent of liquid chromatography-tandem mass spectrometry (LC-MS/MS), with the sensitivity it confers, permits the analysis of drug metabolites that in the past would have been difficult to target using other techniques, such as gas chromatography-mass spectrometry (GC-MS). These metabolites may have relevance to current analytical toxicology employing LC-MS/MS, and lorazepam was chosen as a model drug for investigation as only lorazepam has been targeted previously for screening purposes in urine, whether as the non-conjugate or together with its aglycone. Furthermore, stable-isotope-labelled compounds aid many procedures in toxicology, such as quantification,[1–3] derivatization,[4] reactive metabolite profiling,[5, 6] and pharmacokinetics.[7] However, often there is no commercially available standard for isotopic analogues of target compounds. The use of human liver microsomes containing cytochrome P450 (CYP450) and UDP glucuronyl transferase (UGT) enzymes, to produce phase I and/or phase II metabolites on a small scale may offer a simple, rapid, and economical solution in some situations, as reported previously for ketamine.[8] This approach has been further utilized in the current study, to aid the detection of metabolites of the potent benzodiazepine hypnotic/anxiolytic drug lorazepam in urine, collected from volunteers over 14 days (from the day of the dose, day 0, to day 14), with respect to identifying which metabolites may prove useful in improving retrospective detection of unsuspected drug administration.

Lorazepam, available on medical prescription since 1977, is a potent benzodiazepine with a rapid onset of CNS depressant and anxiolytic effects. The usual oral dose as an anxiolytic is 1 to 6 mg daily in 2 or 3 divided doses with the largest dose taken at night. Other medicinal uses include its anti-emetic properties for cancer treatment and alcohol withdrawal, and as premedication to general anaesthetics. Lorazepam induces sleep, amnesia, reduction in vigilance, and delayed reaction times at clinical doses[9] and, relatively recently, it has been implicated in drug-facilitated crime.[10–12] One specific example was its suspected misuse in a deliberate attempt of drug-facilitated sexual assault, where the victim was allegedly given the 'wrong' tablets by her husband.[13] For this reason, specific and sensitive methods giving maximum windows of detection are desirable for the detection of lorazepam administration, particularly in situations where urine specimens may not be collected for some days after an alleged incident.

Schillings *et al*.[14] investigated the metabolism of lorazepam in man, reporting that 'Metabolite II', a phenolic (hydroxylorazepam) metabolite of lorazepam, was present in small quantities and

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Non standard abbreviations: *UGT, UDP-glucuronosyl transferase; UPLC®, ultra performance liquid chromatography; SRM, selected reaction monitoring; eV, electron volt; RF, radio frequency; NMR, nuclear magnetic resonance.*

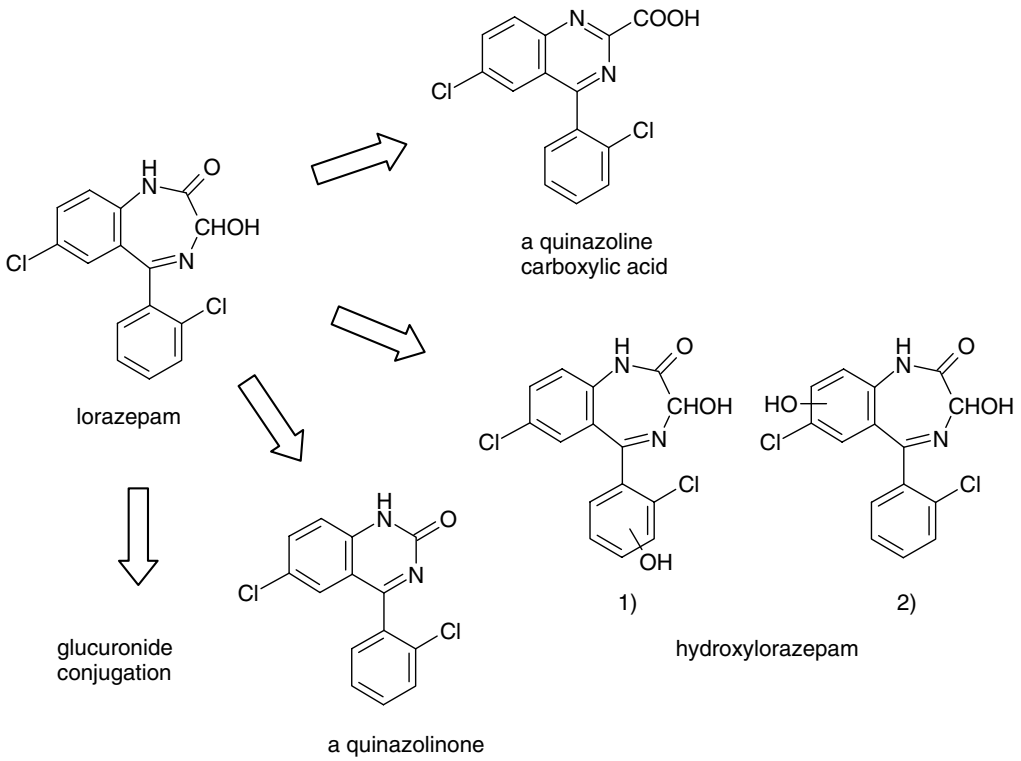

Figure 1. Metabolites of lorazepam previously reported in man; a quinazolinone and a quinazoline carboxylic acid (generally referred to as such throughout the text), a glucuronide conjugate and hydroxylorazepam 1, a phenolic metabolite, and a second isomer hydroxylorazepam 2 reported in the current work.

that 'Metabolite I' (a quinazolinone), a metabolite resulting from the removal of formaldehyde, was found at trace levels. Greenblatt *et al*. [15] also reported a polar quinazoline carboxylic acid metabolite (reported as WY-17033). The reported metabolites of lorazepam are illustrated in Figure 1. A comparative study of the metabolism of lorazepam between several animal species has also been reported,[16] and in the same paper the disposition of lorazepam in healthy male volunteers was described, including the results of administration of a 2 mg dose of ^{14}C-lorazepam. Most of the administered carbon-14 was accounted for in urine (94.4%) collected over a period of 5 days; 74.5% of the radioactivity in urine being lorazepam glucuronide (analyzed as the aglycone) and 13.5% as minor metabolites. Nevertheless, very little is known about these urinary metabolites regarding their relative windows of detection, which may now be investigated using the expediency of LC-MS/MS.

Several analytical methods have been developed for lorazepam, incorporating hydrolysis of urinary conjugates prior to analysis by GC-MS and high performance liquid chromatography (HPLC),[17] immunoassay[18, 19] and capillary electrophoresis.[20] LC-MS/MS methods for the detection of lorazepam in urine have also been reported with limits of detection ranging between 20 pg/ml and 10 ng/ml following concentration of the urine by 5–20 fold.[10, 21–25] Typically the aglycone of lorazepam glucuronide is targeted in forensic analysis. As a result, lorazepam and/or its glucuronide conjugate have been the focus of research aimed at investigating limits of detection and windows of detection in various biological matrices, including urine, oral fluid and hair, as reported by Kintz *et al*.[10](following a single 2.5 mg dose of lorazepam). In urine, the authors reported a limit of detection of 20 pg/ml and could detect lorazepam for six days. We investigated whether other lorazepam metabolites could offer extended windows of detection following a small dose of lorazepam (2 mg, p.o.) to six volunteers.

Materials and methods

Chemicals and reagents

Lorazepam (7-chloro-5-(2′-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one) was obtained from Sigma Aldrich, methanolic lorazepam glucuronide (100 µg/ml; free base; 98% *w*/*w*) was obtained from Grace Davison (Deerfield, IL, USA). Lorazepam tablets (1 mg) were obtained from Genus Pharmaceuticals (Newbury, Berkshire, UK). Lorazepam-d_4, with the functional group 7-chloro-5-(2′-chloro-3,4,5,6-tetradeuterophenyl (1 mg/ml in acetonitrile), was obtained from Cerilliant (LGC Promochem, Teddington, UK). Infusion of a solution of this tetra-deuterated standard into a mass spectrometer showed the presence of a minor impurity corresponding to a tri-deuterated analogue (<1% relative abundance). *H. pomatia* ß-glucuronidase Type H-1 (partially purified powder) was obtained from Sigma Aldrich (Dorset, UK). Analytical grade KH_2PO_4, Na_2HPO_4, NaH_2PO_4, potassium hydroxide pellets, sodium acetate trihydrate, HPLC grade acetonitrile, HPLC grade methanol, dichloromethane and propan-2-ol were all obtained from Fisher Scientific UK (Loughborough, UK). High purity analytical grade formic acid (BDH, 98–100%), glacial acetic acid and ammonia were obtained from BDH Chemicals (Poole, UK). Water was purified by an Elga Maxima HPLC ultra pure water system (High Wycombe, UK). Bond Elute cartridges Certify I (mixed mode; C_8 with strong cation exchange) were obtained from Varian (Agilent, Edinburgh, UK). NADPH-regenerating system A (consisting

of 31 mM $NADP^+$, 66 mM glucose 6-phosphate, and 66 mM $MgCl_2$ in water), NADPH-regenerating system B (consisting of 40 U/ml glucose-6-phosphate dehydrogenase in 5 mM sodium citrate), UDP reaction mix solution A (consisting of 25 mM UDP-glucuronic acid in water), and UDP reaction mix solution B (consisting of 250 mM Tris-HCl, 40 mM $MgCl_2$, and 0.125 mg/ml alamethicin in water) were obtained from BD Biosciences (Oxford, UK), as were pooled human liver microsomes (20 mg/ml in 250 mM sucrose).

Volunteer administration study

Ethical approval was obtained from the Research Ethics Committee at King's College London and informed written consent was obtained from each volunteer. The volunteers attended a screening session a week prior to the commencement of the study to ensure they were in good general health and urine collected at that time was screened for general drugs of abuse and human chorionic gonadotropin (as pregnancy was an exclusion criterion), by the Drug Control Centre, King's College London. A 2 mg dose of lorazepam (1 mg tablets) was self-administered by six individuals (3 male and 3 female) by swallowing two tablets with 125 ml water. All urine was collected from the subjects at 0–2, 2–4, 4–6, 6–8, 8–12, 12–24 h post-dose (day 0), and subsequently the first urine sample of the day was collected up to day 10. Afternoon samples were also taken on days 4, 7, and 10. Urine was also collected on day 14. The urine was stored at −20 °C until analysis.

Microsomal production of metabolites

Microsomal production of metabolites was performed as described previously[8] but using 10 µg lorazepam or lorazepam-d_4 as substrate instead of ketamine. Briefly, this consisted of incubation with 500 µl of 0.2 M phosphate buffer (pH 7.4), 50 µl of the microsome preparation (to give a final concentration of 1 mg/ml protein), 50 µl of NADPH-regenerating system A, 10 µl of NADPH-regenerating system B, and 390 µl of water to make up a volume of 1 ml in total. The mixture, with the exception of the microsomes, was preincubated at 37 °C for 10 min, after which the microsomes were added to initiate metabolism. The mixture was subsequently incubated for 2 h at 37 °C, with vortex mixing every 30 min, after which metabolism was terminated with the addition of 250 µl of acetonitrile on ice. The mixture was then centrifuged at 12 000 *g* for 4 min to precipitate and remove the protein. Phase II metabolism could also be performed in conjunction with phase I, the methodology being that described above, with the exception that instead of 390 µl of water being added, 125 µl of UDP reaction mix solution A and 200 µl of UDP reaction mix solution B were added with 65 µl of water. Ten incubations of lorazepam-d_4 were performed to synthesize a sufficient quantity of metabolites.

Enrichment of urine with crude deuterated microsomal metabolites

To 4 ml urine, 200 µl of the microsomal metabolites generated from lorazepam-d_4 were added. Following dilution with 6 ml of phosphate buffer (10 mM, pH 6.0), the solution was vortex mixed for 30 s.

Extraction

Solid-phase extraction (SPE) was performed using mixed-mode cartridges (Bond-Elut Certify I), incorporating C8 and sulfonate groups for hydrophobic and ionic interaction respectively. The SPE cartridges were preconditioned by slowly passing under vacuum, 2 ml of propan-2-ol followed by 2 ml of phosphate buffer. The buffered urine samples were then loaded onto the SPE cartridges and allowed to pass slowly through the bed under gravity. To the tubes that had contained the urine samples, 1 ml of phosphate buffer was added; the tubes were vortexed and contents combined with the contents in the cartridge. Loaded SPE cartridges were then washed sequentially with 2 ml of water and 2 ml of acetate buffer (0.1 M, pH 4.0) under gravity. Retained drug and metabolites were eluted under vacuum with dichloromethane: propan-2-ol: ammonium hydroxide (78 : 20:2 *v/v*) in four sequential aliquots of 1 ml (4 ml total). The eluent was evaporated under nitrogen at 60 °C. The dried extracts were reconstituted in 250 µl of water : acetonitrile : formic acid (89.9 : 10:0.1 by volume) and vortex-mixed for 30 s, and 20 µl was injected into the chromatographic system.

Hydrolysis

Samples were also hydrolyzed prior to extraction to enable comparison of the detection of lorazepam in hydrolyzed and unhydrolyzed urine samples. To enable manipulation of sample pH, urine (4 ml) was first desalted on Isolute C_8 cartridges, obtained from Biotage (Hertford, UK). The cartridges were conditioned with 3 ml methanol followed by 3 ml water. The urine was then loaded onto the cartridges which were washed with 3 ml water. Elution was then performed with 3 ml methanol which was evaporated under a gentle stream of nitrogen at 60 °C. For hydrolysis by *H. pomatia* enzyme solution, an acetate buffer was prepared by adding 14.75 ml 0.1 M acetic acid to 35.25 ml of 0.1 M sodium acetate trihydrate, followed by dilution to give a 40 mM pH 5 acetate buffer. The H-1 (H. *pomatia*) powder was diluted in this buffer to make a 20 mg/ml (6000 U/ml glucuronidase; 200 U/ml sulfatase) solution. The molarity of the acetate buffer was considered to be the lowest that would adjust urine (1 ml) to pH values of around 5 whilst maintaining the low ionic strength necessary for subsequent extraction by mixed mode cation exchange/C_8 cartridges. Hydrolysis was performed overnight by incubation at 37 °C. To monitor the effectiveness of hydrolysis, quality control samples (QCs) were also prepared containing 100 ng/ml lorazepam glucuronide in urine which was incubated with or without the enzyme.

Ultra high performance liquid chromatography-mass spectrometry

Chromatography (Waters UPLC®) was performed on a 150 mm × 2.1 mm i.d. column, packed with 1.7 µm particle size BEH C_{18} material, at a temperature of 45 °C. The mobile phase used was water + 0.1% formic acid (mobile phase A) and acetonitrile + 0.1% formic acid (mobile phase B). The mobile phase conditions were an initial composition of 20% B for 0.5 min followed by a ramp to 25.3% B at 8.5 min, a second ramp involving an increase to 70% B from 8.5 to 20 min, a hold at 70% B for 2 min, a ramp back to 20% B over 1 min followed by a 2-min equilibration to give a total run-time of 25 min.

Mass spectrometry was performed on a Waters Corporation Premier triple-quadrupole mass spectrometer in scan mode (*m/z* 250 to 550) following electrospray ionisation, in positive ion mode. Product ion scans were also performed for the protonated molecules $(M + H)^+$ *m/z* 321 (lorazepam), 325 and 327 ($^{35}Cl_2$- and $^{35}Cl^{37}Cl$-lorazepam-d_4), 497 (lorazepam glucuronide),

Table 1. Selected reaction monitoring (SRM) transitions, and collision energies (eV) indicated in parentheses, employed in the detection of lorazepam metabolites by LC-MS/MS

compound	*m/z* undeuterated Q1	*m/z* undeuterated Q3	*m/z* deuterated Q1	*m/z* deuterated Q3	Retention time
lorazepam	321	275 (22) 229 (30) 163 (35)	327*	281 (22)	12.7
lorazepam glucuronide	497	321 (15) 303 (25) 275 (35)	503	327 (15)	8.9 and 9.4
quinazolinone metabolite	291	273 (35) 179 (35) 255 (35)	297	279 (35)	13.4
quinazoline carboxylic acid metabolite	319	275 (25) 273 (30) 163 (35)	325	281 (25)	14.3
HL 1**	337	291 (35) 245 (35) 163 (35)	342	296 (35)	8.5
HL 2**	337	291 (35) 245 (35) 179 (35	343	297 (35)	11.0

* The $^{37}Cl^{35}$ Cl-d_4-metabolites were targeted, rather than $^{35}Cl_2$-d_4-metabolites, to avoid a contribution to ion intensities by the $^{37}Cl_2$-isotopes of lorazepam (non-deuterated).
** A difference of 1 *m/z* between deuterated hydroxylorazepam (HL) isomers results from deuterium displacement by a hydroxyl moiety in isomer 1.

501 and 503 ($^{35}Cl_2$- and $^{35}Cl^{37}Cl$-lorazepam glucuronide-d_4), 291 and 293 ($^{35}Cl_2$ and $^{35}Cl^{37}Cl$-'quinazolinone'), 295 and 297 ($^{35}Cl_2$ and $^{35}Cl^{37}Cl$-'quinazolinone-d_4') 319 and 321 ($^{35}Cl_2$- and $^{35}Cl^{37}Cl$-'quinazoline carboxylic acid'), 325 and 327 ($^{35}Cl_2$- and $^{35}Cl^{37}Cl$-'quinazoline carboxylic acid-d_4') 337 and 339 ($^{35}Cl_2$- and $^{35}Cl^{37}Cl$-hydroxylorazepam), 341 and 343 ($^{35}Cl_2$ and $^{35}Cl^{37}Cl$-hydroxylorazepam 2-d_4), 340 and 342 ($^{35}Cl_2$ and $^{35}Cl^{37}Cl$-hydroxylorazepam 1-d_3) and 513 and 515 ($^{35}Cl_2$- and $^{35}Cl^{37}Cl$-hydroxylorazepam glucuronide).

The selected reaction monitoring (SRM) transitions used for targeted analysis of analytes along with their collision energies (in electron volts; eV) are displayed in Table 1. The transitions (eV shown in parentheses) of *m/z* 513 → 143 (22), 513 → 217 (22) and 513 → 337 (22) predicted for hydroxylorazepam glucuronide and 515 → 339 (22) predicted for ^{37}Cl-hydroxylorazepam glucuronide were also targeted although this did not lead to the detection of a significant peak in the extracted ion chromatograms for these transitions. Scan times were 0.2 s and interscan delays were 0.05 s.

Validation of urinalysis

With the availability of lorazepam and lorazepam glucuronide as reference standards, the extraction and analysis was validated in our laboratory, according to published guidelines.[26, 27]

Results

Identification of metabolites by tandem mass spectrometry following *in vitro* metabolism of lorazepam and its deuterated analogue

Incubation of lorazepam and its deuterated analogue with microsomes resulted in the observation of the metabolites shown in Figure 1. This included an isomer of hydroxylorazepam not previously reported in the human, with hydroxylation on the fused benzene ring (retention time 11 min). The metabolites in the microsomal incubate were identified by LC-MS, and targeted analysis was achieved by product ion scanning and SRM as indicated in Table 1. The collision energies employed in the detection of microsomal metabolites were chosen following trial and error by LC-MS/MS since the concentrations of metabolites were too small for optimisation by infusion.

The protonated forms of lorazepam $[M + H]^+$, will have nominal masses according to the following combination of chlorine isotopes, without or with the four deuterium substitutions: $^{35}Cl_2 = 321$; $^{35}Cl^{37}Cl = 323$, $^{37}Cl^{37}Cl = 325$, $d_4 + {}^{35}Cl_2 = 325$, $d_4 + {}^{35}Cl^{37}Cl = 327$ and $d_4 + {}^{37}Cl_2 = 329$. The $^{37}Cl_2$-isotope of lorazepam and $^{35}Cl_2$-lorazepam-d_4 are isobaric (both protonated molecules have an $m/z = 325$) and, to avoid misinterpretation of product ions, the strategy of monitoring only the $^{37}Cl^{35}Cl$-lorazepam-d_4 (rather than $^{35}Cl_2$-lorazepam-d_4) isotope as the precursor ion was adopted to monitor deuterated metabolites. This corresponded to a precursor ion +6 (instead of +4) *m/z* units relative to lorazepam, i.e. *m/z* 327 (instead of *m/z* 325). This was done to ensure specificity when monitoring tetra-deuterated lorazepam and its fragments and to enable distinction between $^{37}Cl_2$-lorazepam and $^{35}Cl_2$-lorazepam-d_4 when monitoring urine enriched with deuterated metabolites. This same mass difference was also employed for the detection of metabolites to avoid any overlap of precursor or product ions. The only exception was hydroxylorazepam 2 (Figure 2) where the precursor ion for the deuterated metabolite showed a mass difference of +5, as a result of substitution of a deuterium atom with a hydroxyl on the 5-(2′-chlorophenyl) ring. The interpretation of product ion spectra was aided by observing the *m/z* of the fragments produced from the $^{37}Cl^{35}Cl$-isotopes of metabolites, at expected abundances relative to $^{35}Cl_2$-istopes, as well as deuterated metabolites to monitor the presence of chlorine atoms and/or particular hydrogen atoms in the fragmention.

The presence of hydroxylorazepam, the quinazolinone and the quinazoline carboxylic acid was investigated following both phase I and phase II metabolism of lorazepam. When experiments were performed in product ion-scanning mode, there were detectable signals with spectra that could be attributed to all of these metabolites and the resultant spectra are shown in Figure 2.

The fragmentation of lorazepam and its glucuronide are shown in Figure 2 (displayed to the right of the top two spectra). The dashed arrow shows fragmentation to a prominent ion common to both lorazepam and its glucuronide as shown in the accompanying structures. For lorazepam, the fragment *m/z* 275 results from the removal of carbon monoxide and water. Subsequent removal of the 5-(2′-chlorophenyl) moiety could be a feasible explanation for *m/z* 163. The fragment of *m/z* 229 resulted from the loss of one of the chlorine atoms because targeting the $^{35}Cl^{37}Cl$ isotope $[M + 2]^+$ molecular ion of lorazepam (*m/z* 323) resulted in the generation of both *m/z* 229 and 231 product ions (data not shown), i.e., the product ions contained only one chlorine atom, either ^{35}Cl or ^{37}Cl. Logically, if the two chlorine atoms remained in the same fragment, there would be no pair of product ions with an

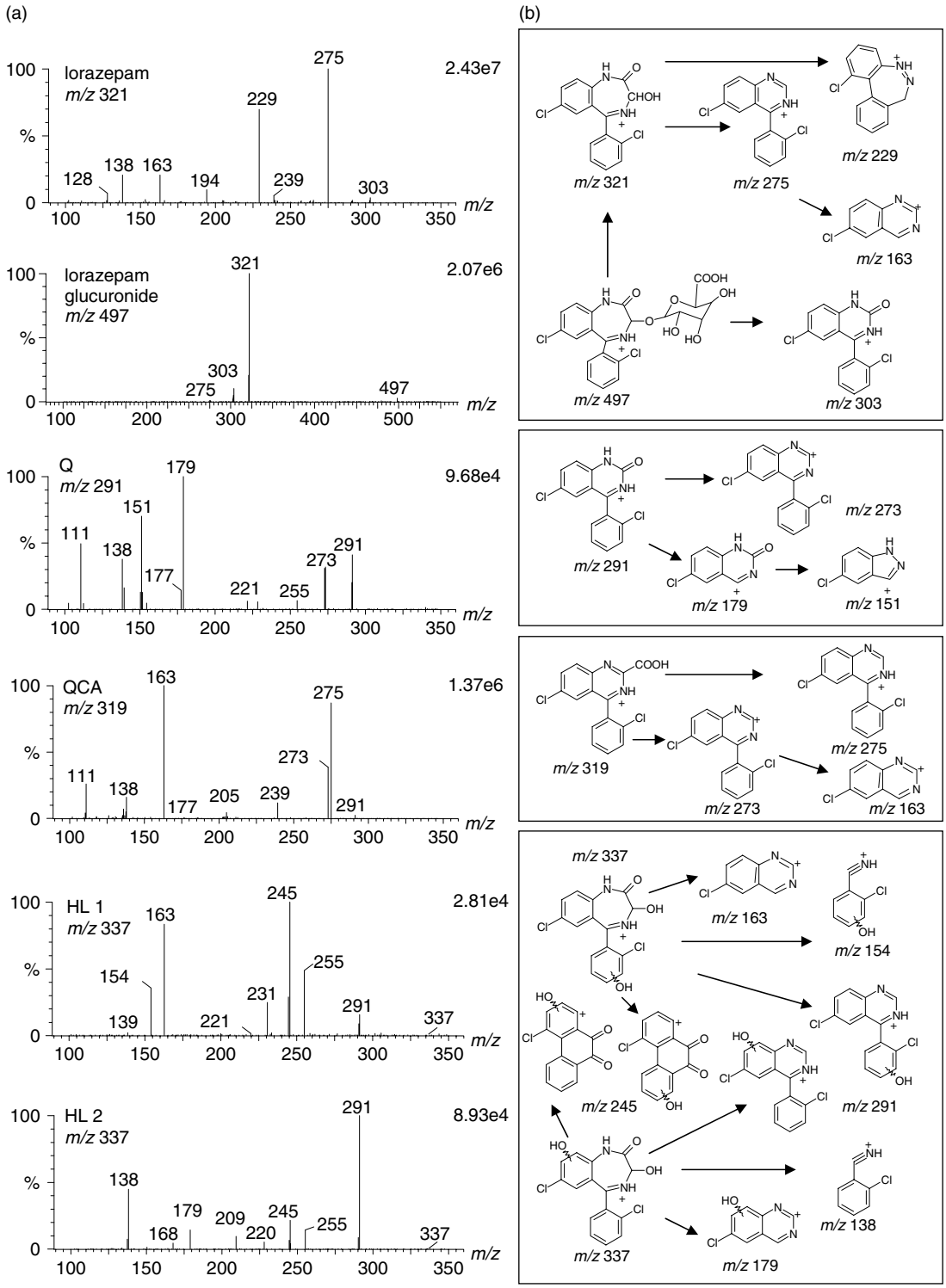

Figure 2. (A) Product ion spectra of lorazepam and metabolites. Q; the quinazolinone metabolite, QCA; quinazoline carboxylic acid metabolite, HL 1; hydroxylorazepam 1, HL 2; hydroxylorazepam 2. The mass spectra were derived from non-deuterated microsomal metabolites. (B) Structures of the product ions used to detect lorazepam metabolites in SRM experiments. Some ambiguity of assigning structures was reduced by also targeting $^{37}Cl^{35}Cl$- and deuterated metabolites. A single fragmentation pathway is shown for the proposed product ions of lorazepam and lorazepam glucuronide due to the commonality of some of the structures. Fragmentation pathways are shown for the proposed product ions of hydroxylorazepam 1 and hydroxylorazepam 2.

associated difference of 2 mass units, i.e. this ion would contain one ^{35}Cl atom and one ^{37}Cl atom. This ion could be obtained by a rearrangement and elimination of hydrochloric acid and the loss of two carbon monoxide molecules. The spectrum for the lorazepam glucuronide metabolite is displayed for the earlier-eluting peak, the spectra not being distinguishable for the two diastereoisomers. Similar to lorazepam, lorazepam glucuronide also forms *m/z* 275 upon fragmentation. The removal of deoxyglucuronic acid results in *m/z* 321 (lorazepam) and subsequent removal of water forms *m/z* 303. Alternatively the direct loss of glucuronic acid results in *m/z* 303.

The prominent fragments for the quinazolinone metabolite ($[M + H]^+ = 291$) indicated a loss of the 5-(2′-chlorophenyl)-ring (*m/z* 179) followed by loss of carbon monoxide (*m/z* 151), and also a loss of water (*m/z* 273) from the precursor ion. For the quinazoline carboxylic acid ($[M + H]^+ = 319$), prominent fragments (*m/z* 273) suggested a loss of formic acid followed by a loss of the 5-(2′-chlorophenyl)-ring (*m/z* 163), and a loss of carbon dioxide (*m/z* 275) from the precursor ion. The only other compounds to have a product ion of *m/z* 275 were lorazepam, through the loss of carbon monoxide and water, and its glucuronide through similar losses as indicated in Figure 2. The results indicate that the quinazolinone functionality is more stable (e.g. *m/z* 179) than the quinazoline carboxylic acid, as the latter functionality readily loses carbon dioxide.

For hydroxylorazepam isomers ($[M + H]^+ = 337$), structures of fragments could be predicted by comparison with the fragmentation pattern of lorazepam. The product ions of lorazepam are at *m/z* 275, 229 and 163. An addition of oxygen to form phenolic metabolites would result in equivalent product ions at *m/z* 291, 245 and 179, and indeed these ions were observed. Depending on the position of the hydroxylated site, *m/z* 163 (6-chloro-1,3-N,N-naphthalene) could be formed resulting in a fragment common to lorazepam and the quinazoline carboxylic acid, as shown in Figure 2 (compare HL 1 and HL 2 and respective structures). The compounds giving fragments *m/z* 138 and *m/z* 154 indicate metabolites hydroxylated on the fused benzene ring and the 5-(2′-chlorophenyl)-ring, respectively. Two peaks were observed in extracted ion chromatograms, which could be attributed to metabolites with hydroxylation sites on each of the conjugated rings. Only one hydroxylated metabolite, with the hydroxyl moiety on the 5-(2′-chlorophenyl)-ring, has been previously reported in 1971 by Schillings *et al*.[14] No peak consistent with conjugated hydroxylorazepam was obtained, either following product ion scanning or following selected reaction monitoring using predicted masses.

Deuterated microsomal metabolites as internal chromatographic markers for *in vivo* investigation

Deuterated metabolites of lorazepam from microsomal incubates were used as internal chromatographic markers to help identify the presence of urinary metabolites of lorazepam. Figure 3 shows examples of the superimposition of ion chromatograms to identify isotopic doublets of lorazepam, its glucuronide diastereoisomers, dichloroquinazolinone, dichloroquinazoline carboxylic acid and hydroxylorazepam isomers. The phenolic structure (hydroxylorazepam), reported by Greenblatt *et al*.[15] back in 1976, was targeted in the current investigation using transitions *m/z* 337 → 291, together with 342 → 296 for its tri-deuterated analogue resulting from substitution of one of the four deuterium atoms on the 5-(2′-chlorophenyl)-ring of lorazepam, gave a dominant isotopic doublet at 8.5 min. A smaller doublet was also observed at 11.0 min, which could be due to be another minor phenolic metabolite, given that any small contribution from the tri-deuterated impurity would not account for the abundance observed. The targeting of phenolic metabolites corresponding to hydroxylation on the fused benzene ring, i.e. *m/z* 343 → 297 and 337 → 291, gave an intense doublet at 11.0 min. These putative metabolites in urine were investigated further, using three MS/MS transitions.

Validation of urinalysis

The extraction yield for lorazepam at three concentrations, performed in triplicate at each concentration, ranged between 82–95% while that of lorazepam glucuronide was much lower at 11–29%. No ion suppression was observed at the retention times of the analytes, as studied by post-column infusions of 10 µg/ml of lorazepam or 1 µg/ml lorazepam glucuronide in 1 : 1 acetonitrile : water (+0.1% formic acid) against an injection of extracts of blank urine samples obtained from all six volunteers prior to lorazepam administration. The limit of detection (LOD) was determined by the presence of all three SRM transitions with S/N ≥ 3. Using this criterion, the LOD for lorazepam was 50 pg/ml. For the diasteroisomers of lorazepam glucuronides, the LODs were 4 and 3 ng/ml for the first- and second-eluting peaks. Considering a single transition of S/N ≥ 3, the LOD could be lowered to 25 pg/ml for lorazepam, and 700 pg/ml and 500 pg/ml for its glucuronides, to allow an option for screening methods based on one transition. Ion ratios observed for the three lorazepam SRM transitions fluctuated by less than ±38% for urine standards and QCs analyzed at all concentrations and less than ±29% when considering the intensities of the two most dominant fragment ions. Given that methods monitoring two transitions are widely deemed acceptable, this is in-keeping with the SOFT/AAFS Forensic Laboratory Guidelines which state that 'ion ratios for LC/MS assays may be more concentration and time dependent than for GC/MS and therefore acceptable ion ratio ranges of up to ±25% or 30% may be appropriate'.[27] For the two glucuronides of lorazepam, the ratio between the two most intense transitions fluctuated by less than ±42% and ±37%, respectively.

Urinary excretion of lorazepam and its metabolites

The maximum time that lorazepam could be detected, after a 2 mg dose, was 4 days (up to day 4) whereas lorazepam glucuronide could be detected up to day 8 based on a single transition only. However, in confirmatory analysis, 2 or often 3 transitions are required. With the criterion of three transitions, lorazepam and lorazepam glucuronide could be detected until day 4 and 7, respectively. To determine possible increases of detection time, hydrolysis of samples was investigated using ß-glucuronidase derived from *Helix pomatia*. Lorazepam could then be detected up to day 9, 10, and 14 using criteria of 3, 2, and 1 transition/s, respectively. The relative windows of detection of the other metabolites are shown in Table 2. The quinazoline carboxylic acid appeared to be a major metabolite present in the urine samples, as judged by peak areas in the ion chromatogram, in spite of this acidic metabolite being detected by positive-mode ionisation mass spectrometry. Detection of the quinazolinone and quinazoline carboxylic acid was superior to the detection of lorazepam when no hydrolysis step was applied. However, with a hydrolysis step, lorazepam (aglycone

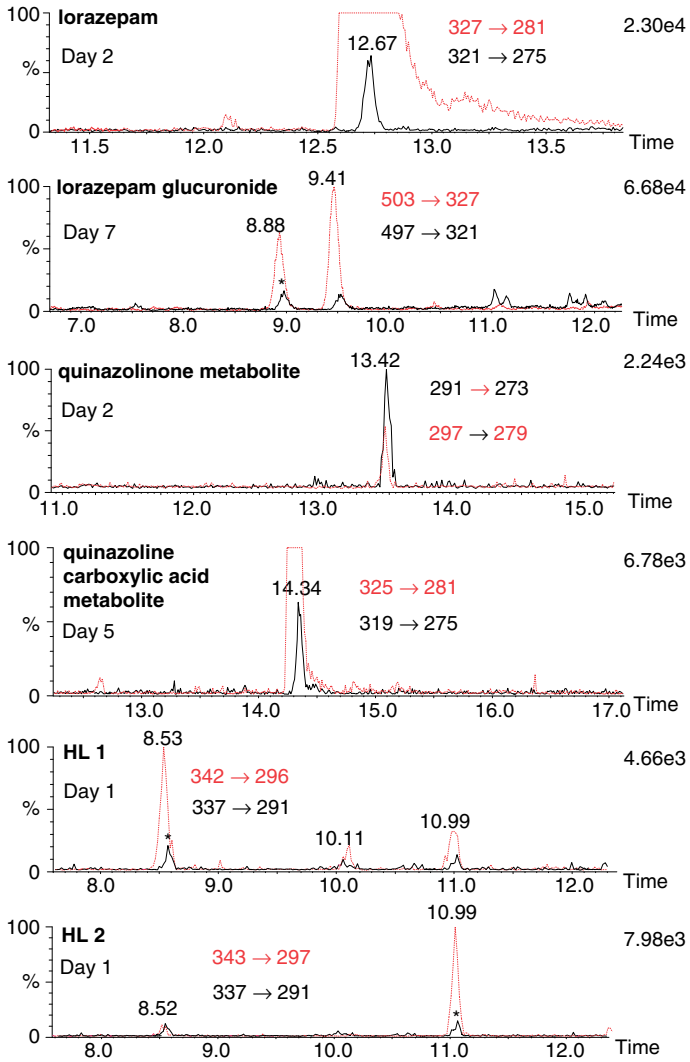

Figure 3. Extracted ion chromatograms of urine samples enriched with deuterated metabolites prior to extraction. Detection of doublets was performed by superimposition of single transitions for urinary metabolites and the deuterated metabolite obtained from microsomal synthesis: superimposed chromatograms for the transitions *m/z* 321 → 275 and 327 → 281 (lorazepam and ^{37}Cl-lorazepam-d_4); 497 → 321 and 503 → 327 (lorazepam glucuronide and ^{37}Cl-lorazepam glucuronide-d_4); 291 → 273 and 297 → 279 (quinazolinone and ^{37}Cl-quinazolinone-d_4 metabolites); 319 → 275 and 325 → 281 (quinazoline carboxylic acid and ^{37}Cl-quinazoline carboxylic acid-d_4 metabolites); 337 → 291 and 342 → 296 (hydroxylorazepam 1 and ^{37}Cl-hydroxylorazepam 1-d_3); and 337 → 291 and 343 → 297 (hydroxylorazepam 2 and ^{37}Cl-hydroxylorazepam 2-d_4). HL 1 and HL 2 refer to the two isomers of hydroxylorazepam. HL; hydroxylorazepam. To avoid ambiguity for some metabolites, the peaks corresponding to the discussion in the text and in Figure 3 are denoted by an asterisk.

plus free drug) provided the best means of detecting lorazepam administration, followed by the quinazoline carboxylic acid and then the quinazolinone, with phenolic metabolites offering only limited retrospective detection. Although this observation is likely to be method specific, it is noteworthy that the metabolites giving the least retrospective detection were likely to be extracted most efficiently. This was deduced by comparing the relative ratios of metabolites in microsomal incubates from peak areas of extracted ion chromatograms with the relative peak areas of the deuterated metabolites following extraction from urine.

Discussion

An effective approach using microsomes to generate metabolites and their stable-isotope labelled analogues has been coupled to a sensitive UPLC-MS/MS method for the qualitative analysis of lorazepam metabolites. The results indicate that microsomal synthesis of metabolites continues to play a useful tool in the study of largely unexplored metabolites that may play a role in the interpretation of results in clinical or forensic toxicology, as discussed previously, for example, Peters *et al*.[28] Microsomal synthesis yielded many of the lorazepam metabolites previously identified by Greenblatt *et al*. [15] in 24-h urine pooled from several subjects. Moreover, the *in vitro* synthesis of deuterated metabolites for use as analytical markers by addition, in their crude form, to urine prior to extraction can be useful, as one simply has to locate isotopic differences in chromatographic peaks or isotopic doublets, the philosophy of the approach being discussed in detail in a previous publication by our group.[8]

The analysis of lorazepam using this UPLC-MS/MS method was more sensitive than most methods presented in the literature and was comparable to the method developed by Kintz

Table 2. Windows of detection of lorazepam based on 3 transitions or 1 transition (shown in brackets) in sixs volunteers. L; lorazepam, L-G; lorazepam glucuronide

	Window of detection in days (day 1 = 24 hours post-dose)						
				hydroxylorazepam			
Volunteer	L	L following hydrolysis	L-G (both peaks)	1	2	quinazolinone metabolite	quinazoline carboxylic acid metabolite
1	*2 (3)*	*8 (9)*	*5 (7)*	*2 (2)*	*1 (2)*	*4 (6)*	*4 (5)*
2	*3 (4)*	*9 (14)*	*6 (7)*	*2 (2)*	*1 (2)*	*4 (4)*	*5 (6)*
3	*3 (4)*	*9 (14)*	*7 (8)*	*3 (3)*	*1 (1)*	*4 (4)*	*5 (6)*
4	*4 (4)*	*9 (14)*	*7 (8)*	*1 (3)*	*1 (1)*	*4 (4)*	*6 (6)*
5	*2 (3)*	*9 (10)*	*6 (7)*	*1 (3)*	*1 (1)*	*4 (5)*	*5 (6)*
6	*2 (2)*	*9 (14)*	*5 (5)*	*2 (3)*	*1 (1)*	*3 (4)*	*3 (5)*

et al.,[10] who detected lorazepam in hydrolyzed urine from volunteers (n = 2) for up to 6 days post-dose of 2.5 mg lorazepam administered orally. In the current study, lorazepam (2 mg, p.o.) was detected at least until day 9 in five of the six volunteers (the window of detection for the remaining volunteer was until day 8), using three ion transitions following hydrolysis with *H. pomatia* ß-glucuronidase. This enzyme source was chosen because we observed that, following overnight incubation with *E.coli* ß-glucuronidase, only one diastereoisomer of lorazepam glucuronide was noticeably hydrolyzed. *H. pomatia* was effective in hydrolyzing both diastereoisomers; using lorazepam glucuronide standard (100 ng/ml added to drug-free urine), one diastereoisomer completely hydrolyzed, the other being ~97% hydrolyzed, when compared to peak heights in controls (data not shown). The stereoselective hydrolysis observed for the isomers of this benzodiazepine conjugate by use of *E. coli* glucuronidase is in keeping with a previous report regarding oxazepam glucuronide, where the S(+)-isomer was hydrolyzed 446-times faster than the R(−)-isomer, whereas glucuronidase preparations of molluscan origin exhibited little stereoselectivity.[29]

To reduce the analysis time for the screening of urine, many toxicology laboratories often omit a hydrolysis step and just target non-conjugated drugs and phase 1 metabolites. It seems sensible, therefore, to add the quinazolinone and the quinazoline carboxylic acid to the gamut of analytes targeted by LC-MS/MS, as these non-conjugated metabolites gave improved detection windows over lorazepam (non-conjugated). Moreover, the identification of these metabolites may provide estimates of when the drug was administered. The identification of the quinazoline carboxylic acid and lorazepam glucuronide, but not the quinazolinone, hydroxylorazepam, or lorazepam, is an indication that a dose of lorazepam was administered several days previously, though a greater number of subjects is needed to confirm this supposition.

It is worthy of note that Greenblatt *et al.*[15] suggested that the quinazolinone metabolite was largely attributable to an impurity in the administered preparation. We analyzed a dissolved lorazepam tablet (prepared at 100 ng/ml and 1 µg/ml in mobile phase) and observed a peak present (based on three characteristic ion transitions) at the retention time of the quinazolinone but only at an intensity of 1% (*m/z* 291 → 273) of the peak height of lorazepam. Furthermore, we found no trace of this compound or its tetradeuterated analogue in the lorazepam or lorazepam-d_4 standards but, nonetheless, the quinazolinone was detected following incubation of these standards with microsomes. It is, therefore, reasonable to conclude that a source of the quinazolinone in urine is from the metabolism of lorazepam, giving improved windows of detection relative to the excretion of the unconjugated drug.

The extraction method gave a lower extraction yield and higher limit of detection for lorazepam glucuronide compared with lorazepam. This may explain the shorter window of detection of lorazepam glucuronide compared with hydrolyzed lorazepam. Mixed mode cation/C_8 cartridges were employed because extraction methods developed should be versatile enough to be used for many drugs that could be encountered in urinalysis and these cartridges are commonly employed for basic drugs and their metabolites, including benzodiazepines.[30–32] A limit of detection of 50 pg/ml was observed for lorazepam, which is lower than that reported by Soriano *et al.*,[33] who employed a similar extraction method and obtained an LOD of 3 ng/ml. The extraction method used in our work also employed acetate buffer at pH 4 as an 'acid-lock step', even though under these conditions, theoretically, only about 1% of the target analytes would have the weak imine (pKa 1.7) in its protonated form interacting with the sulfonate group on the mixed-mode phase. The acetate buffer, nevertheless, helps to protonate a proportion of the glucuronide moiety (pKa 3–4) to form the uncharged acid and improve hydrophobic interaction on the C_8 component. Indeed, omission of the acetate buffer step halved the peak intensities observed for lorazepam glucuronide.

Baldacci and Thorman[20] reported that non-endcapped C_2 cartridges were necessary for the extraction of lorazepam glucuronide, which was not extracted by 'typical' mixed mode cartridges (C_8 and sulfonate mixed phase). A comparison of the two types of phases used whilst developing our method, showed the contrary to be the case, with up to a 70% reduction in yield of lorazepam glucuronide and up to 50% reduction of peak height of hydroxylorazepams with C_2 compared with mixed mode cartridges. The mixed mode method we used differed from that described by Baldacci and Thormann, as it employed a smaller sorbent mass (100 compared with 500 mg), a larger elution volume (4 compared with 2 ml) and dilution of urine in lower strength phosphate buffer (10 mM compared with 0.1 M) to avoid saturation of cation exchange sites, as recommended for these cartridges.[34] Using C_2 cartridges, no improvement over C_8/ion exchange mixed mode extraction were observed, possibly because of an increase in background noise as a result of a lower specificity.

It was thought that changes in the ratio of diastereoisomers of lorazepam glucuronide could be used as an indication of time since lorazepam administration, as these diastereoisomers do not interconvert. Baldacci and Thorman[20] reported that the two peaks of lorazepam glucuronide showed a ratio

of 3:1 following *in vitro* incubation of lorazepam with human liver microsomes and, *in vivo*, similar results have been obtained for glucuronides of oxazepam and temazepam.[35] However, in the current work, only a very weak trend could be observed for a *change* ($r^2 = 0.453$) in the peak area ratio of the first- to second- eluting glucuronide peak over time in 6 volunteers. Vree *et al*.[35] showed that, despite the different concentrations of temazepam glucuronide and oxazepam glucuronide diastereoisomers, there was no difference in the half-lives between the respective diastereoisomers. Extrahepatic glucuronidation, enterohepatic recirculation,[36, 37] and enzymatic systems may *cause* the inversion of one enantiomer to another,[38] and plasma protein binding[35] may also contribute to stereoselective metabolism.

Previous to the advent of LC-MS/MS, GC-MS has been applied to the detection of intact lorazepam glucuronide,[39] but this is much more challenging due to the polarity of this metabolite and reliance on the successful derivatization of five functional groups for sufficient volatility. Similarly, degradation of lorazepam and its glucuronide to 2-amino-2′,5-dichlorobenzophenone did not have to be considered.[40]

The SOFT/AAFS guidelines seem to be impractical in the case of identifying the lorazepam glucuronides by LC-MS/MS, as fluctuations in ion ratios were >30% and, therefore, perhaps a less rigid criterion should be considered for the positive identification of these isomers, or alternatively for confirmatory analysis, hydrolysis could be performed and lorazepam monitored. The larger fluctuations for the glucuronide metabolites are most likely due to the tendency of this metabolite to form one prominent ion at *m/z* 321 (i.e. the aglycone) with the second ion of *m/z* 303 being approximately 10% of the intensity of the dominant ion. Some authors have even advocated using an acceptance limit of ±50% for ions which are 10% the intensity of the dominant fragment and this criterion has been applied in the quantification of buprenorphine glucuronide and norbuprenorphine glucuronide.[41] This highlights a drawback of relying on a small number of analytes and given these fluctuations, if performing confirmatory analysis one would consider the selection of other metabolites such as the quinazoline carboxylic acid and the quinazolinone metabolite. The spectra of these two metabolites show several dominant ions, which should exhibit fewer fluctuations of ion ratios due to their signal intensities (but no reference standards were available at the time of investigation for comparative purposes). Subsequent to the investigation, the quinazolinone (6-chloro-4-(2-chlorophenyl)-2(1H)-quinazolinone) became available as a commercial standard (from LGC Standards) and it should be a straightforward task to monitor ion ratio fluctuations for this compound in different biological matrices. The commercial availability of other lorazepam metabolites such as hydroxylorazepams and the quinazoline carboxylic acid would facilitate further studies which could investigate the stability of ion ratios in confirmatory analysis and benefit laboratories interested in both screening and confirmation of benzodiazepine intake.

In conclusion, the introduction of LC-MS/MS, with the sensitivity it confers, permits the analysis of metabolites that, in the past, would have been difficult to target in single urine collections using other techniques. It is worthy of consideration to re-visit such drug metabolites, as these may have relevance to current analytical toxicology employing LC-MS/MS, as we hope has been demonstrated herein with respect to the quinazolinone and the quinazoline carboxylic acid.

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