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Determination of prescribed and designer benzodiazepines and metabolites in influent wastewater

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Benzodiazepines are important prescription pharmaceuticals used to help in the treatment of anxiety and sleep disorders. However, they also have a strong potential for abuse. In this respect, illicit benzodiazepines, i.e. not prescribed in Australia and designer benzodiazepines, which are new compounds that are not legally prescribed in any jurisdiction, have emerged in the illicit Australian market in recent years. Designer benzodiazepines are a new class of new psychoactive substances (NPS) and are particularly dangerous due to limited toxicity information and propensity to be mistaken for conventional benzodiazepines, leading to severe side effects and potentially death.It is therefore important to assess the prevalence of the use of these compounds in the community. The current work presents a sensitive liquid chromatography-mass spectrometry method for 20 prescribed and designer benzodiazepines and metabolites: 7-amino nimetazepam, alpha-hydroxy alprazolam, alprazolam, clonazepam, delorazepam, deschloroetizolam, diazepam, diclazepam, etizolam, flubromazepam, flunitrazepam, lorazepam, lormetazepam, meclonazepam, midazolam, nimetazepam, nitrazepam, oxazepam, pyrazolam and temazepam. Quetiapine, a prescription sedative drug that has been diverted for non-medical use, was also included. Limits of quantification were predominantly below 10 ng/L, except for the ubiquitous oxazepam, quetiapine and temazepam, which were between 75-300 ng/L. Stability, recovery and matrix effects were also examined. Finally, this method was applied to influent wastewater from South Australia, which showed the presence of many benzodiazepines including the NPS etizolam.

Introduction

Benzodiazepines are commonly prescribed pharmaceuticals used in the treatment of various psychiatric conditions such as anxiety, stress and sleep disorders. Although very effective and needed medications, they carry a high potential for dependence and are often misused.[1] This has led to an illicit market and the emergence of synthetic derivatives to enhance the effects or circumvent legislation.

Synthetic benzodiazepines are becoming more prevalent on the European Union early warning system of new psychoactive substances (NPS), with the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) currently monitoring 28.[2] Some of these have been sold as falsified versions of commonly prescribed benzodiazepines, such as alprazolam (Xanax).

Etizolam is one particular NPS that has been found in counterfeit alprazolam in the United States and Australia.[3,4] While it is legally sold as an authentic medicine in Italy, Japan and India, it is monitored by the EMCDDA and United Nations Office on Drugs and Crime, due to its diversion from legal sale for illicit use.[5] It is therefore important to monitor this compound and other NPS in the community.

Wastewater analysis or wastewater-based epidemiology has previously been utilised to quantify the prevalence of NPS in communities in Europe[6–9] and Australia[10,11] but these have typically focussed on synthetic cathinones. Previous methods have been published on determining benzodiazepines in wastewater [12–16] and surface water [17–19] but to our knowledge, there is yet to be a method which includes both prescribed and NPS benzodiazepines.

The method in this work primarily incorporates parent compounds, due to the common metabolites many benzodiazepines share (e.g. oxazepam is a metabolite of temazepam and diazepam) as well as the proportion of the parent drug excreted unchanged.[20] A total of 22 analytes comprising 17 benzodiazepines, metabolites of those drugs and one antipsychotic were assessed in the current method: 7-amino clonazepam, 7-amino nimetazepam, alpha-hydroxy alprazolam, alprazolam, clonazepam, delorazepam, deschloroetizolam, diazepam, diclazepam, etizolam, flubromazepam, flunitrazepam, lorazepam, lormetazepam, meclonazepam, midazolam, nimetazepam, nitrazepam, oxazepam, pyrazolam, quetiapine and temazepam.

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Quetiapine, although not structurally a benzodiazepine, is included within this study as there is evidence of its off-label use as an alternative to benzodiazepines for treating mental illness.[21] This list includes ten medicines approved for pharmaceutical use in Australia (alprazolam and metabolite alpha-hydroxy alprazolam, clonazepam and metabolite 7-amino clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nitrazepam, oxazepam, quetiapine and temazepam), while all the others are illicit pharmaceuticals in Australia. Table S1 in the Electronic Supplementary Information (ESI) shows the structures and class (designer benzodiazepine, illicit benzodiazepine, prescribed benzodiazepine or metabolite). Influent wastewater samples were collected from four treatment plants at the beginning of December 2019 as well as the Christmas-New Year 2019/2020 period to show the prevalence of these compounds and whether there is any impact on their use over a festival period.

Experimental

Chemicals and Reagents

All analytical reference standards (7-amino clonazepam, 7-amino nimetazepam, alpha-hydroxy alprazolam, alprazolam, clonazepam, delorazepam, deschloroetizolam, diazepam, diclazepam, etizolam, flubromazepam, flunitrazepam, lorazepam, lormetazepam, meclonazepam, midazolam, nimetazepam, nitrazepam, oxazepam, pyrazolam, quetiapine and temazepam) and deuterated internal standards (alpha-hydroxy alprazolam-d_5, alprazolam-d_5, clonazepam-d_4, diazepam-d_5, etizolam-d_3, flunitrazepam-d_7, lorazepam-d_4, meclonazepam-d_3, nitrazepam-d_5, oxazepam-d_5 and temazepam-d_5) were purchased from Novachem Pty Ltd., Collingwood, VIC, Australia as certified solutions.

VWR Chemicals (Tingalpa, Queensland, Australia) was the supplier of Sodium acetate. Ethyl acetate, acetonitrile, glacial acetic acid, formic acid (98-100%) and ammonia (28%) were purchased from Thermo Fischer Scientific Australia (Scoresby, VIC, Australia). Hydrochloric acid (37%) and sodium metabisulfite were from Chem-Supply (Gillman, SA, Australia) and ultrapure water was prepared using an Arium pro VF system (Sartorius Stedim Biotech).

Wastewater Samples

24h (8 a.m. – 8 a.m.) composite influent wastewater samples (600 mL) from four wastewater treatment plants around South Australia (27 November – 3 December 2019 and 25 December 2019 – 3 January 2019) were collected in PET bottles. Population and flow rates are in Table S2 in the Electronic Supplementary Information (ESI). All samples were immediately preserved onsite with sodium metabisulfite (0.5 g/L) upon collection and refrigerated (4 °C) or frozen (-20 °C). Following the collection period, the samples were transported to the laboratory for analysis. Samples were stored at -20 °C in the dark until pre-treatment. For method validation, 24h composite influent wastewater from all sites was used.

Sample Pretreatment

Prior to sample pretreatment, all samples were first thawed to room temperature and filtered under vacuum using glass microfibre filter paper (GF/A 1.6 µm, Whatman, Kent, U.K.).

The pH of the filtered samples (100 mL) was adjusted to 6-6.5 (if needed) using 10% glacial acetic acid. A mixture of internal standards (100 µL of 50 µg/L) was then added to all samples. The UCT CleanScreen® XCEL II solid phase extraction (SPE) cartridges (UCT Inc., Bristol, PA, USA); 130 mg/6 mL) were conditioned with methanol (6 mL) and sodium acetate buffer (100 mM pH 6 acidified with 10% acetic acid, 6 mL). Samples were then loaded under gravity. The cartridges were washed with sodium acetate buffer (6 mL) and dried for 5 minutes at full vacuum before n-hexane (2 mL) was passed through the cartridge. Analytes were then eluted with a mixture of ethyl acetate containing 2% ammonia (4 mL) and evaporated to dryness under nitrogen at 40 °C. The dry residue was reconstituted with 0.1% formic acid in acetonitrile (40 µL) and 0.1% formic acid in ultrapure water (160 µL) to give a final concentration factor of 500 times. Analyses were performed by injecting 2 µL of the final extract onto the UPLC-MS/MS system.

Instrumentation

A Sciex ExionLC coupled to a Sciex 6500+ QTrap (Toronto, Canada), fitted with a TurboSpray IonDrive source was used for analysis. Chromatographic separation was carried out using a Kinetex biphenyl column (150 x 2.1 mm x 1.7 µm) fitted with a SecurityGuard™ ULTRA Cartridges UHPLC Biphenyl 2.1mm ID columns at a flow rate of 0.3 mL/min and an injection volume of 2 µL. The column oven was set to 40 °C. The mobile phases used were: 95% ultrapure water (5% acetonitrile; 0.1% formic acid; solvent A) and 95% acetonitrile (5% ultrapure water; 0.1% formic acid; solvent B). The gradient started at an initial percentage of B of 5% and increased linearly to 100% over 16.5 min, held for 30 sec before being brought back to the initial percentage and kept steady for the final 2.9 min to equilibrate the system. The total run time was 20 min. A needle wash of acetonitrile:ultrapure water (50:50, 0.1% formic acid) was used to eliminate carryover.

The ion source parameters were as follows: 500 °C; curtain gas, 20; collision gas, high; ion spray voltage, 5500 V; ion source gas 1 and ion source gas 2, 50. Mass spectrometric analyses were performed in positive mode using multiple reaction monitoring (MRM), with all transitions in Table 1. All data were acquired with Analyst 1.7 (Sciex) and processed using MultiQuant 3.0.2.

Quantification and Method Validation

The quantification of all compounds was carried out using analyte-specific internal standards (IS). When an analyte specific IS was unavailable, surrogate internal standards were chosen based on their ability to correct for recovery losses following SPE. European

Directorate General for Health and Consumer Protection (SANCO) guidelines for analytical quality control and validation procedures[22] were followed to evaluate the method in terms of limit of detection (LOD), limit of quantification (LOQ), precision and accuracy. These guidelines have previously been followed for method validation of wastewater analyses[8,11,23]. Linearity was assessed by analysing standards in SPE eluates. Two separate concentration ranges were applied: 0.25-24 ng/L and 37.5-3600 ng/L, based on the prevalence of the analyte in samples. When the determination coefficient (r^2) was above 0.99 and an accuracy within 20% of the expected concentration, linearity was deemed acceptable.

It was impossible to guarantee that the wastewater samples used for method validation were free of all the target analytes. However, to minimise issues associated with high community consumption of benzodiazepines, wastewater from a small community (population < 500) was used for method validation and was investigated to ensure only minimal levels of target analytes were present.

LOD and LOQ were evaluated in extracted samples using the same concentration ranges as linearity. The lowest concentration at which a S/N was greater than 3 was the LOD and greater than 10 was the LOQ. Some compounds were already present in the 'pseudo-blank' samples. For these, the lowest point on the extracted concentration range which was greater than 20 % of the endogenous peak area in the 'pseudo-blank' was assigned as the LOQ.[24] Recovery experiments were performed in triplicate at two concentration levels (10 ng/L (LQC) and 30 ng/L (HQC), which was higher than the LOQ for all analytes. For oxazepam, temazepam and quetiapine, these were performed at 900 ng/L and 2700 ng/L. Recovery experiments were also used to test accuracy (acceptable recovery between 70-120 %) and precision (RSD < 20 %).

In order to confirm the presence of a compound in an authentic sample, both transitions had to be present. However, in-sample concentrations and back-calculated mass loads were based solely off the quantification transition. Additionally, both transitions had to show a S/N above 10 for confirmation. If only one transition was present, the compound was only reported as detected. To further confirm its presence, q/Q ratios were calculated and compared with that of a standard, with a 20% threshold.

The results in this work are reported solely as excreted mass loads (mg/day/1000 people) with doses deemed inappropriate due to the convoluted metabolism of these compounds. Excreted mass loads were back-calculated from the calculated in-sample concentration as well as the population and flow rate data provided by the wastewater treatment plants (ESI Table S2).

Stability

Wastewater samples for stability experiments had previously been filtered and analysed to confirm minimal endogenous levels of the analytes in this method. Wastewater samples were either raw (no preservative), acidic (pH 2) or preserved with sodium metabisulfite (0.5 g/L). These were spiked at two concentrations, corresponding to HQC and LQC. Samples were analysed in triplicate, with 0.5 mL aliquoted into HPLC glass vials. All three conditions were evaluated at three temperatures: -20 °C, 4 °C and room temperature (20 °C) for 24h, 48h, 72h, and 7 days. Following each time period, the vials were stored at -20 °C until analysis. The degradation of the analytes was assessed using the area ratio (peak area of analyte/peak area of internal standard) of each analyte against the initial (t = 0) time period.

Table 1: LC-MS information for all compounds in this method. The quantification (Q) transition is the first for all analytes, while the confirmation (q) transition is the second.

Compound	Retention time (min)	Precursor Ion ($[M+H]^+$)	Product Ion	Collision Energy (V)	Internal Standard
7-Amino clonazepam	6.83	286.1	221.0	33	7-Amino clonazepam-d_4
			250.2	35	
7-Amino nimetazepam	6.57	266.1	135.1	38	Diazepam- d_5
			208.1	38	
Alpha-hydroxy alprazolam	9.59	325.1	296.9	37	Alpha-hydroxy alprazolam- d_5
			216.2	40	
Alprazolam	10.19	309.1	281.0	37	Alprazolam- d_5
			205.1	40	
Clonazepam	10.22	316.1	270.1	31	Clonazepam- d_4
			241.2	37	
Delorazepam	10.56	305.0	140.0	45	Lorazepam- d_4
			206.1	45	
Deschloroetizolam	9.91	309.1	255.1	35	Etizolam- d_3
			280.1	35	
Diazepam	11.05	285.1	193.2	38	Diazepam- d_5

			222.1	38	
Diclazepam	11.49	319.0	227.1	38	Diazepam- d_5
			154.1	38	
Etizolam	10.83	343.0	314.0	38	Etizolam- d_3
			289.1	38	
Flubromazepam	10.40	332.9	226.2	42	Etizolam- d_3
			184.0	40	
Flunitrazepam	10.75	314.1	239.1	38	Flunitrazepam- d_7
			268.1	33	
Lorazepam	9.86	321.0	275.2	30	Lorazepam- d_4
			229.0	30	
Lormetazepam	10.79	335.0	289.0	26	Temazepam- d_5
			262.0	26	
Meclonazepam	10.81	330.0	284.0	38	Oxazepam- d_5
			214.1	38	
Midazolam	8.91	326.1	291.1	33	Alprazolam- d_5
			249.1	38	
Nimetazepam	10.83	296.0	250.2	38	Temazepam- d_5
			221.0	38	
Nitrazepam	9.82	282.0	236.1	38	Nitrazepam- d_5
			181.1	38	
Oxazepam	9.76	287.1	241.1	21	Oxazepam- d_5
			231.1	30	
Pyrazolam	8.78	354.0	206.1	38	Alprazolam- d_5
			167.1	38	
Quetiapine	8.76	384.3	253.0	26	Oxazepam- d_5
			279.2	30	
Temazepam	10.66	301.1	255.0	21	Temazepam- d_5
			282.9	24	
7-Amino clonazepam-d_4	6.77	290.1	226.1	33	
Alpha-hydroxy alprazolam- d_5	9.58	330.1	302.1	34	
Alprazolam- d_5	10.19	314.1	286.0	36	
Clonazepam- d_4	10.22	320.1	274.1	38	
Diazepam- d_5	11.05	290.0	198.1	36	
Etizolam- d_3	10.81	346.0	292.1	38	
Flunitrazepam- d_7	10.75	321.1	246.1	36	
Lorazepam- d_4	9.86	325.0	233.1	28	
Meclonazepam- d_3	10.80	333.0	287.0	40	
Nitrazepam- d_5	9.76	287.0	241.1	37	
Oxazepam- d_5	9.73	292.0	246.1	23	
Temazepam- d_5	10.62	306.1	260.2	21	

LC-MS Optimisation

All compounds in this method were individually tuned to ensure optimal collision energy. Declustering potential (70 V), Collision Exit Potential (17 V) and Entrance Potential (10 V), were used for all compounds. The two most sensitive transitions were selected as the quantification (Q) and confirmation (q) transitions, respectively (Table 1).

Results and Discussion

This method contained isobaric compounds: alprazolam and deschloroetizolam (309.1); lorazepam & flunitrazepam-d_7 (321.0). As these were not isomers, they could still be differentiated by their fragmentation and allowed the two most sensitive transitions to be selected as the Q and q transitions.

Mixed standards were analysed using different chromatographic columns (biphenyl, F5 and C18), mobile phases (methanol, acetonitrile and water), addition of ammonium acetate buffer (2 mM, 5mM and 10 mM) and formic acid (0.1, 0.2 and 0.3 %) as well as gradient to determine optimal conditions. Due to the great similarity in chemical structure of these compounds (ESI, Table S1), it was difficult to have complete separation for all analytes (Figure 1). Initially, a mobile phase of water:methanol and water:acetonitrile were investigated, without any additives. Methanol induced later elution and less separation compared to acetonitrile. Due to the similarity of the analytes in this method, separation was important and therefore only acetonitrile was further investigated. Ammonium acetate buffer (2 mM, 5 mM and 10 mM) and formic acid (0.05%, 0.1% and 0.2%) were investigated for optimal peak shapes. Although ammonium acetate gave good peak shapes for many compounds, diazepam had extreme fronting for one transition (Figure S1). This being one of the most commonly prescribed benzodiazepines, it was a very important analyte in this method, so ammonium acetate was not considered appropriate. Formic acid was found to be suitable at 0.1% concentration. A lower concentration of 0.05% resulted in some deterioration in peak shape, while there was slightly less sensitivity observed at 0.2%. Finally, three columns were tested: F5, C18 and biphenyl. F5 gave poor separation of the analytes, while C18 and biphenyl both had reasonable separation of all analytes. However, quetiapine was found to have worse peak shape with C18 than biphenyl. Figures showing the differences in additives and columns are shown in the ESI, Figure S1 and S2. Ultimately, the optimal conditions incorporated a biphenyl column and a mobile phase consisting of acetonitrile and water (both with 0.1% formic acid).

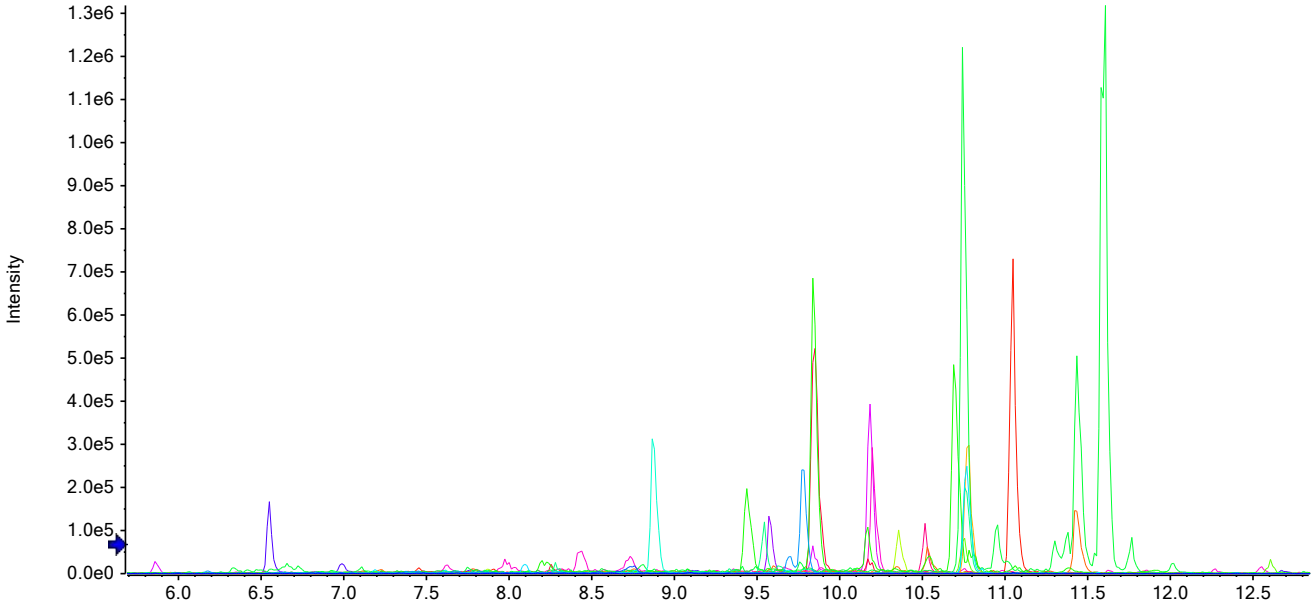


Figure 1: Total ion chromatogram of all analytes in the method spiked into a wastewater sample

Method Validation

SPE recovery, matrix effects and filtration loss (Table 2) were examined to validate the method, similar to previous work [8,11,23,25,26]. Previously filtered influent wastewater was used for the filtration loss experiment. Losses due to adsorption to suspended particulates have previously been examined.[13,27] In this work, previously filtered wastewater was used to ensure that analytes in our method do not preferentially adsorb to the filter paper.

Wastewater aliquots in triplicate were spiked with a concentration of 100 ng/L, filtered under vacuum through glass microfibre filter paper and compared to the equivalent 100 ng/L unfiltered aliquots. Table 2 shows that filtration losses were negligible, with the largest loss observed for lorazepam (-9%).

SPE recovery and matrix effects were calculated using extracted samples as well as a mixed standard solution, set 1: non-extracted mixed standard solution, including IS, Set 2: filtered wastewater spiked with standards and IS, followed by SPE, Set 3: Extracts spiked with standards and IS after SPE. Calculations for matrix effects were based on peak areas of the individual analytes, while relative recovery was based on the

area ratio between the analyte and their respective internal standards. For each calculation, a wastewater 'pseudo-blank' was also analysed to cater for endogenous levels. These endogenous levels were taken into account for both matrix effects and recovery. The equations are shown below:

1. Matrix effects = $average\ peak\ area\ set\ 3 / average\ peak\ area\ set\ 1$
2. Relative SPE recovery = $average\ area\ ratio\ set\ 2 / average\ area\ ratio\ set\ 3$

SPE recovery was determined using at LQC and HQC as defined in *Quantification and Method Validation*. All but three analytes showed acceptable recovery (between 80%-120%), with 7-amino clonazepam, quetiapine and 7-amino nitrazepam having between 51-62%. It must be noted that an analyte-specific internal standard was unavailable for the latter two, with diazepam-d_5 and oxazepam-d_5 used as surrogates. All other internal standards used in this method were tested as surrogates but were found to give even worse recovery. Nevertheless, the SPE cartridge for this optimal method was chosen for overall suitability across all analytes, with the recognition that the recovery of a few compounds was less than ideal. All analytes were suppressed by the wastewater matrix, with quetiapine, 7-amino nimetazepam and 7-amino clonazapem the most affected. Alpha-hydroxy alprazolam and pyrazolam were the least affected.

Table 2: Method validation for all compounds

Compound	LOD (ng/L)	LOQ (ng/L)	Linearity		Matrix Effects (RSD, %)	Recovery (RSD, %)		Filtration losses (RSD, %)
			Range	R^2	Uncorrected	HQC	LQC	
7-Amino clonazepam	a	a	a	a	25 (4)	55 (4)	52 (9)	10 (1)
7-Amino nimetazepam	1	4	1-24	0.9912	31 (7)	51 (7)	56 (3)	3 (19)
Alpha-hydroxy alprazolam	1	2	1-24	0.9918	90 (8)	82 (6)	94 (1)	7 (2)
Alprazolam	0.25	1	0.25-24	0.9919	75 (10)	97 (8)	93 (4)	-3 (7)
Clonazepam	2	6	1-24	0.9940	56 (9)	113 (13)	93 (8)	-6 (7)
Delorazepam	1	2	1-24	0.9961	55 (13)	102 (6)	96 (8)	3 (6)
Deschloroetizolam	0.25	0.5	0.25-24	0.9939	65 (15)	110 (6)	103 (11)	2 (4)
Diazepam	1	8	0.5-48	0.9923	53 (13)	111 (6)	95 (10)	3 (5)
Diclazepam	1	6	1-24	0.9923	50 (5)	99 (7)	91 (5)	-7 (3)
Etizolam	0.25	0.5	0.25-24	0.9969	54 (16)	89 (14)	86 (13)	7 (12)
Flubromazepam	4	6	4-24	0.9936	79 (12)	83 (5)	100 (20)	-2 (11)
Flunitrazepam	2	4	2-24	0.9967	53 (9)	111 (6)	103 (5)	1 (5)
Lorazepam	2	8	2-48	0.9919	48 (8)	86 (4)	82 (1)	-9 (3)
Lormetazepam	8	18	8-48	0.9874	53 (19)	99 (16)	88 (15)	7 (3)
Meclonazepam	1	2	1-24	0.9939	55 (10)	103 (7)	98 (3)	-7 (1)
Midazolam	1	2	1-24	0.9970	47 (14)	79 (10)	76 (9)	-2 (6)
Nimetazepam	1	2	1-24	0.9982	56 (7)	104 (14)	95 (12)	1 (9)
Nitrazepam	2	4	2-24	0.9950	80 (14)	113 (16)	109 (4)	-4 (5)
Oxazepam	37.5	300	37.5-1800	0.9975	67 (18)	87 (14)	87 (2)	-3 (2)
Pyrazolam	9	12	9-24	0.9750	91 (24)	108 (7)	112 (18)	12 (25)
Quetiapine	37.5	75	37.5-1800	0.9985	37 (12)	62 (4)	62 (15)	-4 (4)
Temazepam	37.5	75	37.5-3600	0.9980	63 (3)	98 (5)	106 (15)	2 (4)

a: 7-Amino clonazepam was unable to be successfully recovered using the extraction procedure of this method for the LOD, LOQ and linearity experiments;

LOD: Limit of Detection; LOQ: Limit of Quantification

Matrix Effects were calculated where below 100% is matrix suppression and above 100% is matrix enhancement.

Filtration losses were calculated where below 0% is lost and above 0% is gained following filtration.

HQC and LQC are defined in *Quantification and Method Validation*

Stability

In-sample stability has previously been investigated for benzodiazepines by Racamonde *et al.* [27] However, studies on the short-term stability of filtered samples between sample processing and analysis is limited. In this work, stability was examined across three different wastewater matrices (raw, acidified to pH2 and with sodium metabisulfite (0.5 g/L)) at two concentrations (LQC and HQC) and at three temperatures (4 °C, -20 °C and room temperature (20 °C)). All stability results are found in the ESI, Figures S3 and S4. At -20 °C, most analytes were stable under all experimental conditions (<20 % transformation, as defined by McCall *et al.*[28]). The primary exception was pyrazolam, which had variable stability in raw wastewater and high stability with either acid or sodium metabisulfite preservation. At 4 °C and 20 °C it was clear that sodium metabisulfite is essential to stabilise all the analytes. Acid preservation was worse than unpreserved wastewater for many compounds, particularly clonazepam, flubromazepam and nitrazolam which exhibited low stability after one week (>60 % transformation). These results are similar to those from pervious works, where acidification reduced the stability of benzodiazepines.[27,29] Based on these results, it is recommended to preserve wastewater samples with sodium metabisulfite (0.5 g/L), wherein the analytes are stable at room temperature, 4 °C and -20 °C for up to one week.

Application to Authentic Influent Wastewater Samples

This method was applied to influent wastewater collected from four metropolitan South Australian wastewater treatment plants from 27 November – 3 December 2019 and 25 December 2019 – 3 January 2020. In total, ten compounds were found (Figure 3). Confirmation was based on retention time compared to the standard and internal standard (if available) of the quantification and confirmation transitions. If only one transition was seen, the compound was only reported as detected. Ion ratios (q/Q) were also applied, with a deviation of up to 20% from the standard deemed acceptable. Examples of the confirmation of etizolam, quetiapine and oxazepam are shown in Figure 2.

Eight of the compounds found are prescribed pharmaceuticals: alprazolam (and metabolite), diazepam, lorazepam, oxazepam, quetiapine and temazepam. It must be noted that midazolam is only prescribed in hospitals, while alprazolam is a very potent compound with doses often less than 1 mg. It was thus expected to not find much of either compound. Oxazepam had the highest excreted mass loads, with between 100-180 mg/day/1000 people measured, followed by temazepam (35-120 mg/day/1000 people) and quetiapine (5-80 mg/day/1000 people). This was not surprising as oxazepam is also a metabolite of temazepam and diazepam so had multiple contributing parent drugs. There was little difference between the two collection periods. However, etizolam was around half over the festive period compared to the beginning of December, while diazepam and quetiapine was found at higher levels on certain days in December compared to the later collection. All mass loads are shown in the supporting information, Table S3 and S4. Midazolam was only present at detectable (below LOQ) levels, while alprazolam and its metabolite and etizolam were at quantifiable levels below 1 mg/day/1000 people. The presence of the non-prescribed lormetazepam and etizolam was a cause for concern. Etizolam, in particular, has been seen in Australia as an illicit alternative to alprazolam in New South Wales, following analysis of seized alprazolam[3] and the results here show that this could also be the case in South Australia. Benzodiazepines are not considered a 'party' drug and therefore there was not expected to be any trends associated with this time of year, with habitual, daily use evidenced in Figure 3.

Figure 2: Examples of confirmation of etizolam (LEFT), quetiapine (MIDDLE) and oxazepam (RIGHT) in influent wastewater samples. The upper two most MRMs are the quantification (Q) and confirmation (q) transitions for the sample, while the third MRM is the quantification transition in a standard. The lowermost MRM is of the internal standard. It must be noted that quetiapine did not have its own internal standard. Ion ratios (q/Q) are also included.

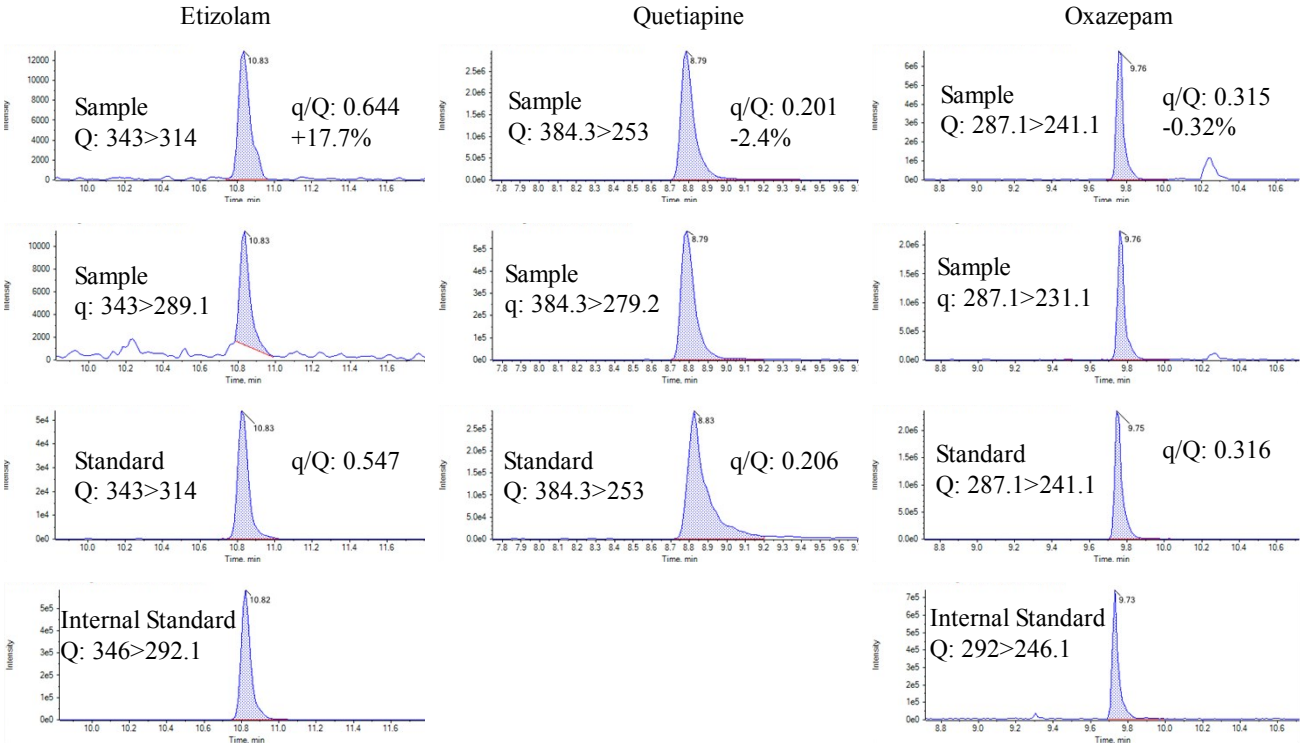
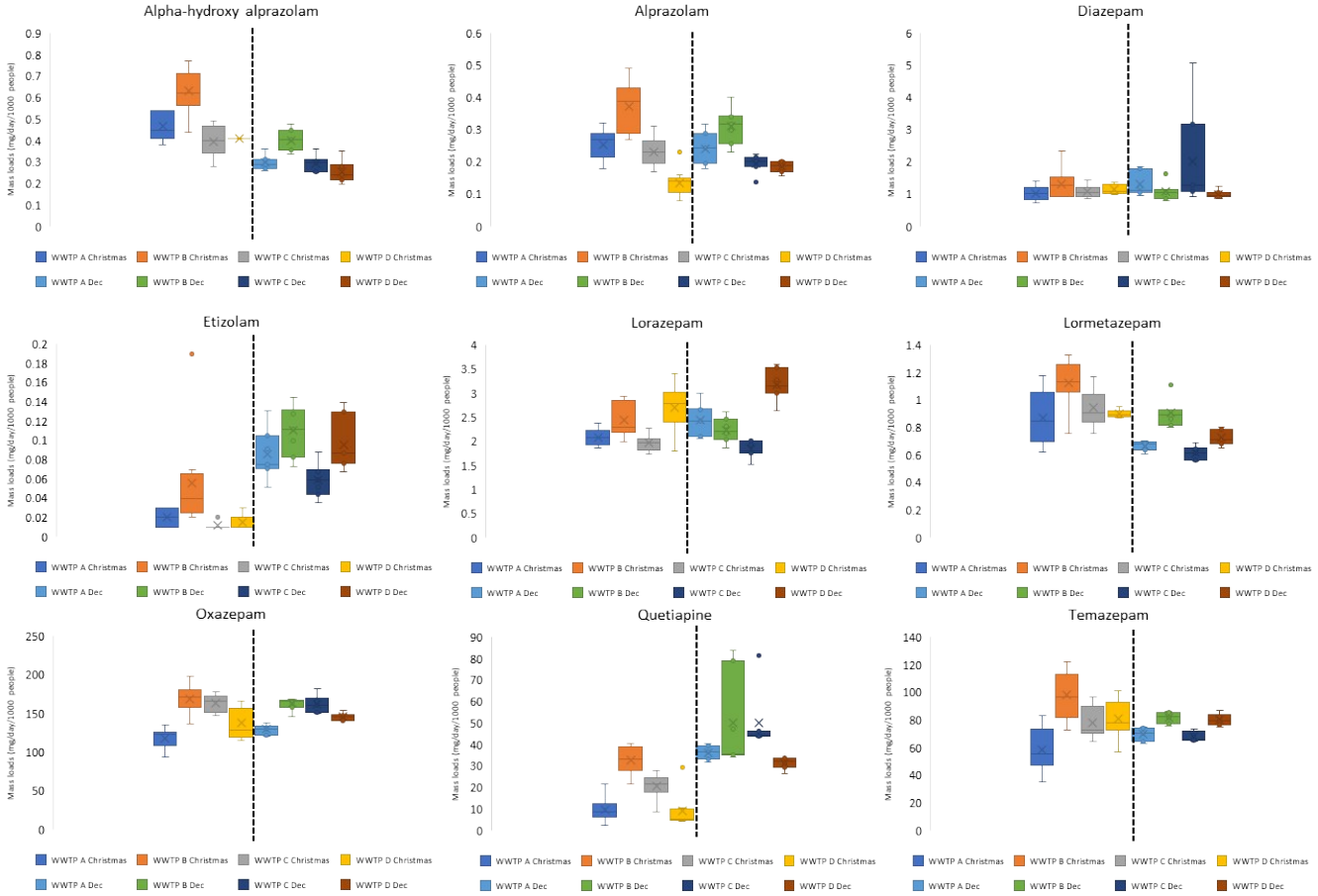


Figure 3: Box and whisker plots of all compounds found in both December 2019 (RIGHT of dotted line) and the Christmas/New Year 2019/20 period (LEFT of dotted line). Midazolam was detected on several days, but not at quantifiable levels so is not included in this figure. Midazolam and quetiapine are potentially underestimated due to recoveries between 62-76%.



Conclusions

A sensitive method for 21 prescription and designer benzodiazepines in influent wastewater has been developed. Limits of quantification were predominantly under 10 ng/L, with some designer benzodiazepines at 0.5 ng/L. The overall analytical method was fully validated, obtaining satisfactory accuracy and precision. Stability was also examined, with sodium metabisulfite (0.5 g/L) recommended to preserve the analytes in wastewater samples temperatures up to ambient for up to one week. The method was applied to samples from South Australia, with ten analytes found. Oxazepam was the most prevalent, while the commonly prescribed quetiapine, temazepam, alprazolam (and its metabolite alpha-hydroxy alprazolam), diazepam, lorazepam, lormetazepam, midazolam and etizolam were also at least detected. Etizolam and lormetazepam are not marketed in Australia and therefore their presence indicated illicit use. Etizolam has been found in Australia to be illicitly sold as alprazolam. This latter finding shows the importance of including designer benzodiazepines in analytical methods.

Conflicts of interest

There are no conflicts of interest to declare.

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