An approach to shortening the timeframe between the emergence of new compounds on the drugs market and the availability of reference standards: the microscale syntheses of nitrazolam and clonazolam, for use as reference materials, utilizing polymer supported reagents

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Abstract

Nitrazolam and clonazolam are two designer benzodiazepines that are available from internet retailers and there is growing evidence suggesting that such compounds have the potential to cause severe adverse events. Information about tolerability in humans is scarce but typically, low doses can be difficult to administer for users when handling bulk material and variability of the active ingredient in tablet formulations can also be of a concern. Customs, toxicology and forensic laboratories are increasingly encountering designer benzodiazepines, both in tablet and powdered forms, and the unavailability of reference standards can impact on the ability to identify these compounds. Therefore, the need arises for exploring in-house approaches to the preparation of NPS that can be carried out in a timely manner. The present study was triggered when samples of clonazolam were received in powdered and tablet form at a time when reference material for this drug was commercially unavailable. Therefore, microscale syntheses of clonazolam and its deschloro

analogue nitrazolam were developed utilizing polymer supported-reagents starting from 2-amino-5-chlorobenzophenone (clonazolam) and 2-amino-5-nitrobenzophenone (nitrazolam). The final reaction step forming the 1,2,4-triazole ring moiety was performed within the GC-MS injector. A comparison with a preparative scale synthesis of both benzodiazepine derivatives showed that microscale synthesis might be an attractive option for a forensic laboratory in terms of time and cost savings when compared with traditional methods of synthesis and when qualitative identifications are needed to direct forensic casework. The reaction by-product profiles for both the micro and preparative scale syntheses are also presented.

1 INTRODUCTION

There is growing evidence to show that the so-called designer benzodiazepines,[1] i.e. derivatives commonly obtained from the patent literature for sale and large-scale synthesis and without a published history of undergoing safety assessments in humans, have the potential to cause severe adverse effects.[2-7] Customs and forensic laboratories are increasingly encountering these substances in both tablet and powdered forms. Two examples of recently emerging benzodiazepine derivatives include nitrazolam and clonazolam (Figure 1).

The detection of these drugs and the provision of analytical data has been reported by various sources, such as the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) *via* the European Union early warning system (EWS),[8] the WEDINOS drug information website,[9] the European Response Project,[10,11] and the Drug Enforcement Administration (DEA) Special Testing and Research Laboratory in the United States of America.[12]

The preparations of nitrazolam (1-methyl-8-nitro-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepine) and clonazolam (6-(2-chlorophenyl)-1-methyl-8-nitro-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepine) have been published.[13] They have been sold online and it appears that their effects might be comparable to other triazolo-benzodiazepines.[14,15] However, it appears that nitrazolam may be less potent than clonazolam and triazolam. Studies in animals have shown that nitrazolam can be several times more effective than diazepam as an antagonist of electroshock–induced tonic extensor convulsions.[13] Clonazolam has also been sold as an uncontrolled drug and, due to its high potency, is believed to cause powerful sedation and amnesia at total oral doses of 0.5 mg (typically one tablet).[13,16-19] Low doses such as this can be complicated to administer for users when handling bulk materials and variability of the active ingredient in the tablet are also a concern, where both the effects and unintended overdosing raise concerns about incidences of drug facilitated crimes.[21]

The unavailability of reference standards often presents difficulties in forensic analysis where facile and effective approaches are needed to accelerate the

availability of these standards. In this study, a potential solution was sought for finding a suitable methodology that would address this requirement. This need arose when our laboratories received samples of clonazolam in powdered and tablet form at a time when reference material for this drug was commercially unavailable. It was first aimed to synthesize this compound on a preparative scale to allow for isolation, purification and characterization followed by the development of a microscale synthesis without compound isolation whereby the amounts obtained were available in sufficient quantity for analysis by gas chromatography mass spectrometry (GC-MS). In addition, the final reaction step forming the 1,2,4-triazole ring moiety was performed within the GC-MS injector to accelerate the procedure (Figure 1). Finally, the syntheses procedures carried out both on the preparative and microscale was extended to the deschloro analog nitrazolam.

2 EXPERIMENTAL

2.1 Chemicals

A nitrazolam standard, all reagents and solvents used in the syntheses were obtained from Sigma Aldrich Ltd (Arklow, Co. Wicklow, Ireland) and Fluorochem Ltd. (Hadfield, Derbyshire, UK). LC-MS grade solvents were obtained from Fisher Scientific (Dublin, Ireland). Clonazolam containing tablets labeled to contain 0.5 mg and pure powder samples were obtained from an Internet retailer. For single crystal X-ray diffraction analysis, a portion of the vendor sample of clonazolam was recrystallized from ethanol.

2.2 Synthesis

2.2.1 Microscale syntheses of nitrazolam and clonazolam

Micro-reaction vessels (2 mL, clear glass vial, styrene-butadiene septum with solid cap, O.D. × H 16 mm × 56 mm; product code 27037; Sigma Aldrich, Arklow, Co. Wicklow, Ireland) were used for the reactions. A suitably drilled aluminum block, placed on a hotplate-stirrer, was used to perform reactions (see Supporting Information 1 for photographs of set-up). The micro-reaction vessels were capped during the reactions and mixing was accomplished using PTFE-coated rare earth core stir bars.

Step A (Figure 1). A mixture of 2-amino-5-nitrobenzophenone (starting material for nitrazolam) or 2-amino-5-chlorobenzophenone (starting material for clonazolam) (10 mg), 2-chloro-1,1,1-triethoxyethane (20 µL) and glacial acetic acid (10 µL) was heated (heating block set at 90 °C) in a reaction vial (2 mL) for 3 h, during which the solid dissolved. The mixture was allowed to cool to room temperature and partitioned between ethyl acetate (1 mL) and saturated aqueous sodium bicarbonate (1 mL).

This was then centrifuged (2,770 *g* for 3 min). The upper layer was transferred to another reaction vial (2 mL) and blown to dryness (air, 35 to 40 °C).

Step B. The residue was dissolved in acetone (500 µL) and sodium iodide (25 mg) was added. The mixture was stirred at room temperature for 1.5 h and blown to dryness (air, 35 to 40 °C).

Step C. Dichloromethane (1 mL) was added to the residue and the mixture was stirred for 5 min. This was then centrifuged (4,000 rpm for 3 min.) and the supernatant was transferred to another reaction vial (2 mL). Azide exchange resin (50 mg, azide on Amberlite® IRA-400, 3.8 mmol/g, Sigma Aldrich cat. no. 368342) was added and the mixture was stirred overnight at room temperature. This was then filtered through a plug of cotton wool using a Pasteur pipette (using 0.5 mL dichloromethane to rinse the reaction vial). The filtrate was blown to dryness (air, 35 to 40 °C).

Step D. The residue was dissolved in tetrahydrofuran (490 µL) and water (10 µL). Polymer bound triphenylphosphine (50 mg, ~3 mmol/g, Sigma Aldrich cat. no. 93093) was added and the mixture was stirred at room temperature for 90 min. This was then centrifuged (2,770 *g* for 3 min.). The supernatant was collected and the residue was washed with tetrahydrofuran (1mL). The supernatant and washing was blown to dryness (air, 35 to 40°C).

Step E. Methanol (150 µL) was added to the residue and the mixture was centrifuged (2,770 *g* for 3 min). The supernatant was transferred to a reaction vial (2 mL). A solution (50 µL), containing acetylhydrazine (acethydrazide, 90%, 5 mg, 100 mg/mL) and glacial acetic acid (100 µL/mL) in methanol, was added. The mixture was stirred overnight at room temperature and then blown to dryness (air, 35 to 40 °C). The residue was partitioned between ethyl acetate (1 mL) and saturated aqueous sodium bicarbonate (1 mL). This was then centrifuged (2,770 *g* for 3 min.). The upper layer was transferred to another reaction vial (2 mL) and blown to dryness (air, 35 to 40 °C). The residue was taken up in acetonitrile (1 mL) and the solution was centrifuged (2,770 *g* for 3 min). The supernatant was collected and stored in a fridge.

Step F. An aliquot of the solution from step (E) (50 µL) was diluted with acetonitrile (950 µL) and analyzed by GC-MS. Standards of nitrazolam or clonazolam (50 µg/mL in acetonitrile) were also run.

2.2.2 Clonazolam – *preparative scale synthesis*

5-(2-Chlorophenyl)-2-ethoxy-7-nitro-3H-benzo[e][1,4]diazepine

A mixture of 2-amino-2'-chloro-5-nitrobenzophenone (1.66 g, 6 mmol), 2-chloro-1,1,1-triethoxyethane (1.38 g, 6.9 mmol), glacial acetic acid (360 µL) and toluene (5 mL) was refluxed for 6 h. After cooling to room temperature, the mixture was

partitioned between ethyl acetate and saturated aqueous sodium bicarbonate. The organic layer was dried (anhydrous magnesium sulfate) and the volatiles were removed to afford a brown oil, which was dissolved in acetone (60 mL). Sodium iodide (1.35 g, 9 mmol) was added and the mixture was stirred at room temperature for 5 h and then evaporated to dryness. The residue was partitioned between dichloromethane and water (containing a small amount of sodium thiosulfate). The organic layer was collected and dried (anhydrous magnesium sulfate). Removal of the solvent afforded a brown oil (2.62 g). This was dissolved in ethanol (60 mL) and sodium azide (780 mg, 12 mmol) was added. The mixture was stirred overnight at room temperature and then concentrated to a small volume under vacuum. The residue was partitioned between ethyl acetate and water. The organic layer was collected and evaporated to afford a brown oil (2.36 g), which was dissolved in tetrahydrofuran (30 mL). Water (600 µL) and triphenylphosphine (1.89 g, 2.4 mmol) were added and the mixture was stirred overnight at room temperature. The mixture was evaporated to dryness to afford a brown oil (3.76 g). A portion of this (960 mg) was purified by preparative TLC (silica gel, 2 mm; hexane/ethyl acetate, 7/3) to afford a light yellow powder (158 mg): m.pt 119–121 °C; ^{1}H NMR (400, MHz, d_6 DMSO) δ 8.33 (dd; *J* = 9.0, 2.5 Hz; 1 H; Ar H-5), 7.79 (d; *J* = 2.5 Hz; 1 H; Ar H-3), 7.67-7.64 (ddd; *J* = 7.4, 1.4, 0.4 Hz; 1 H; Ar H-3') 7.57 – 7.49* (m; 3 H; Ar H-4', 5' and 6'), 7.47 (d; *J* = 9 Hz; 1 H; Ar H-6), 4.37 (q; *J* = 7.1 Hz; 2 H; CH_2 from ethyl group), 4.17 (apparent br. s; 2 H; CH_2) and 1.31 (t; *J* = 7.1 Hz; 3 H; CH_3) ppm; ^{13}C NMR (151 MHz, d_6 DMSO) δ 168.01 (CH_3-<u>C</u>=N), 161.85 (N=<u>C</u>=O), 151.90 (Ar C-4), 141.35 (Ar C-1 or 2), 137.60 (Ar C-1 or 2), 131.81 (Ar C-1'), 131.47 (Ar C-4', 5' or 6'), 131.45 (Ar C-3'), 129.75 (Ar C-4', 5' or 6'), 128.18 (Ar C-2'), 127.89 (Ar C-6), 127.51 (Ar C-4', 5' or 6'), 125.44 (Ar C-5), 124.26 (Ar C-3), 63.98 (CH_2 from ethyl group), 52.48 (CH_2) and 13.75 (CH_3) ppm (* 7.57-7.53 ppm – partial resolution into a dd and tr indicating Ar H-6- and Ar H-4'/5'); HRMS found, 344.0792 $[M+H]^+$, $C_{17}H_{15}{}^{35}ClN_3O_3$, requires 344.0796 (Δ = 1.4 ppm).

6-(2-Chlorophenyl)-1-methyl-8-nitro-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine (clonazolam)

A solution of 5-(2-chlorophenyl)-2-ethoxy-7-nitro-3*H*-benzo[*e*][1,4]diazepine (100 mg, 0.29 mmol), acetylhydrazine (acethydrazide, 90%, 29 mg, 0.35 mmol) and glacial acetic acid (18 µL) in methanol (3 mL) was stirred for 48 h at room temperature. The volatiles were removed under vacuum and the residue was triturated with *tert*-butyl methyl ether to afford a yellow powder (99 mg). This was heated (sand bath at 225 °C) for 10 min under reduced pressure to afford yellow powder. This was purified by preparative TLC (silica gel, 2 mm; ethyl acetate/methanol, 9/1) and triturated with *tert*-butyl methyl ether to afford an almost colorless solid (50 mg, 0.14 mmol, 48%) m.pt 231-233 °C (lit. 229-231 °C[13]); ^{1}H NMR (400 MHz, d_6 DMSO) δ 8.55 (dd; *J* = 8.9, 2.6 Hz; 1 H; Ar H-5), 8.15 (d; *J* = 8.9 Hz; 1 H; Ar H-6), 7.87 (d; *J* = 2.6 Hz; 1 H; Ar H-3), 7.74-7.70 (m; 1 H; Ar H-3'), 7.57 – 7.52 (m; 2 H; Ar H-4' and 5'), 7.50 – 7.46 (m; 1 H; Ar H-6'), 5.31 (d; *J* = 13.2 Hz; 1 H; one H from CH_2), 4.35 (d; *J* = 13.2 Hz; 1 H; one H from CH_2), 2.59 (s; 3 H; CH_3). ppm; ^{13}C NMR (101 MHz, d_6 DMSO) δ 166.53 (Ar-<u>C</u>=N), 154.25 (CH_2-<u>C</u>=N), 150.66 (CH_3-<u>C</u>=N), 145.40 (Ar C-4), 137.72 (Ar

C-1 or 2), 137.38 (Ar C-1 or 2), 131.74 (Ar C-4' or 5'), 131.23 (Ar C-3' overlapped with Ar C- 1' or 2'), 130.30 (Ar C-1'or 2'), 129.82 (Ar C-6'), 127.63 (Ar C-4' or 5'), 126.51 (Ar C-5), 125.77 (Ar C-6), 124.35 (Ar C-3), 45.80 (CH_2), 11.62 (CH_3) ppm; HRMS found, 354.0750 $[M+H]^+$, $C_{17}H_{13}{}^{35}ClN_5O_2$, requires 354.0752 ($\Delta$ = 0.6 ppm).

2.2.3 Nitrazolam – preparative scale synthesis

2-Ethoxy-7-nitro-5-phenyl-3H-benzo[e][1,4]diazepine

A mixture of 2-amino-5-nitrobenzophenone (484 mg, 2 mmol), 2-chloro-1,1,1-triethoxyethane (460 mg, 2.3 mmol), glacial acetic acid (120 µL) and toluene was refluxed for 5 h. After cooling to room temperature, the mixture was partitioned between ethyl acetate and saturated aqueous sodium bicarbonate. The organic layer was dried (anhydrous magnesium sulfate) and the volatiles were removed to afford a yellow oil (697 mg). This was dissolved in acetone (20 mL) and sodium iodide (450 mg, 3 mmol) was added. The mixture was stirred at room temperature for 3 hr. and then evaporated to dryness. The residue was partitioned between dichloromethane and water (containing a small amount of sodium thiosulfate), the organic layer was collected and dried (anhydrous magnesium sulfate). Removal of the solvent afforded a viscous brown oil. This was dissolved in ethanol (20 mL) and sodium azide (260 mg, 4 mmol) was added. The mixture was stirred overnight at room temperature and then concentrated to a small volume (2 mL) under vacuum. The residue was partitioned between ethyl acetate and water. The organic layer was collected and evaporated to afford a brown oil (601 mg). This was dissolved in tetrahydrofuran (10 mL). Water (200 µL) and triphenylphosphine (630 mg, 2.4 mmol) were added and the mixture was stirred overnight at room temperature. The mixture was evaporated to dryness and the residue was purified by preparative TLC (silica gel, 2 mm; hexane/ethyl acetate, 8/2) to afford a yellow solid (299 mg, 0.97 mmol, 48 %): m.pt 136–138 °C (lit. 143-145 ºC[20]); ^{1}H NMR (400, MHz, d_6 DMSO) δ 8.36 (dd; *J* = 9.0, 2.7 Hz; 1 H; Ar H-5), 8.06 (d; *J* = 2.7 Hz; 1 H; Ar H-3), 7.58 – 7.52 (m; 3 H; Ar H-2', 4' and 6'), 7.50- 7.44 (m; 3 H; Ar H-3', 5' and 6), 4.32 (m; 2 H; CH_2 from ethyl group) and 1.30 (t; *J* = 7.1 Hz; 3 H; CH_3) ppm*; ^{13}C NMR (151 MHz, d_6 DMSO) δ 168.43 (CH_3-<u>C</u>=N), 163.01(N=<u>C</u>=O), 153.10 (Ar C-4), 141.13 (Ar C-1 or 1'), 138.03 (Ar C-1 or 1'), 130.61 (Ar C-4'), 129.56 (Ar C-2' and 6'), 128.44 (Ar C-3' and 5'), 127.54 (Ar C-6), 127.23 (Ar C-2), 126.08 (Ar C-3), 125.40 (Ar C-5), 63.85 (CH_2), 52.39 (CH_2) and 13.78 (CH_3) ppm; APCI HRMS found 310.1198, $[M+H]^+$, $C_{17}H_{16}N_3O_3$, requires 310.1186 (Δ = -3.9 ppm).

* The diazepine ring CH_2 was not observed as a sharp identifiable signal in the ^{1}H NMR spectrum (Supporting Information 2). A broad signal from 3.5 to 5 ppm may account for this. In the HSQC experiment, this CH_2 peak in the ^{13}C spectrum had no discernible connectivity to a signal in the ^{1}H spectrum (Supporting Information 2), which may be due to the rapid interconversion of diazepine ring conformers.

1-Methyl-8-nitro-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine (nitrazolam)

A solution of 2-ethoxy-7-nitro-5-phenyl-3*H*-benzo[*e*][1,4]diazepine (247 mg, 0.80 mmol), acetylhydrazine (acethydrazide, 90%, 67 mg, 0.81 mmol) and glacial acetic acid (50 μL) in methanol (10 mL) was stirred overnight at room temperature. The mixture was then evaporated to dryness, and the residue was triturated with *tert*-butyl methyl ether and then filtered to afford a yellow powder (222 mg). A portion (52 mg, 0.15 mmol) of this was heated (sand bath at 225 °C), under reduced pressure for 10 min. The resulting brown material (45 mg) was triturated with ethyl acetate and *tert*-butyl methyl ether to afford a light beige powder (30 mg): m.pt 228–230 °C (from ethyl acetate, lit. 231.5-232.5 ºC[13]); ^{1}H NMR (600 MHz, d_6 DMSO) δ 8.58 (dd; *J* = 9.0, 2.6 Hz; 1 H; Ar H-5), 8.10 (d; *J* = 9 Hz; 1 H; Ar H-6) 8.09 (d; *J* = 2.7 Hz; 1 H; Ar H-3), 7.62-7.58 (m; 2 H; Ar H-2' and 6'), 7.54 (dist. tr; *J* = 7.3 Hz; 1 H; Ar H-4'), 7.47-7.42 (m; 2 H; Ar H-3' and 5'), 5.28 (d; *J* = 13.0 Hz; 1 H; one H from CH_2), 4.25 (d; *J* = 13.0 Hz; 1 H; one H from CH_2) and 2.63 (s; 3 H; CH_3) ppm; ^{13}C NMR (151 MHz, d_6 DMSO) δ 167.28 (Ar-C=N), 154.37 (CH_2-C=N), 150.79 (CH_3-C=N), 144.99 (Ar C-4), 138.37 (Ar C-1 or 1'), 138.24 (Ar C-1 or 1'), 130.81 (Ar C-4'), 129.37 (Ar C-2' and 6'), 129.35 (Ar C-2) 128.27 (Ar C-3' and 5'), 126.36 (Ar C-3 or 6), 126.24 (Ar C-5), 125.70 (Ar C-3 or 6), 45.70 (CH_2) and 11.76 (CH_3) ppm; APCI HRMS found 320.1141, $[M+H]^+$, $C_{17}H_{14}N_5O_3$, requires 320.1142 (Δ = -0.3ppm).

2.3 Analysis of clonazolam tablets

2.3.1 Quantification of clonazolam in tablets by Liquid chromatography-low resolution mass spectrometry (LC-LRMS)

A crushed tablet was extracted with acetonitrile (2 x 2 mL, 5 min. sonication). The combined supernatants were subjected to centrifugation (1,550 *g* for 5 min), made up to 5 mL with acetonitrile (solution A). Water (500 μL, containing 0.01% formic acid) was added to solution A (500 μL) and the mixture was filtered through a 0.2 μm nylon filter, Costar Spin-X). The filtrate (200 μL) was diluted with acetonitrile/water (600 μL, containing 0.05 % formic acid) and this was analysed using liquid chromatography-low resolution mass spectrometry (LC-LRMS) as detailed below. A series of clonazolam standards (6.25, 12.5, 25, 50, 100, 200 μg/mL) were prepared in acetonitrile/water (containing 0.05 % formic acid) and analysed along with the sample.

2.3.2 Sample preparation for the identification of clonazolam in tablets by 60 MHz (bench-top) NMR.

Two tablets were crushed together and the powder was extracted with $CDCl_3$ (0.70 mL) by rolling for 10 min. The mixture was the centrifuged at 20,000 g for 5 min and the supernatant was analysed by 60 MHz benchtop NMR as detailed below.

2.4 Instrumentation

2.4.1 Gas chromatography-mass spectrometry (GC-MS)

Samples were analyzed on an Agilent 6890N gas chromatograph coupled to a 5975 insert MSD. A HP ULTRA 1 column (12 m × 0.2 mm × 0.33 µm) was used in split mode (1:1 or as stated) with helium carrier gas at a constant flow of 0.8 mL/min. The injection port and transfer line temperatures were set at 250 °C and 280 °C respectively. The initial oven temperature was 60 °C, held for 2 min and then ramped at 25 °C/min to 295 °C with a final hold time of 5.6 min (total run time 17 min). The ionization energy was set at 70 eV, the quadrupole at 150 °C, the ion source at 230 °C and the mass range was set at *m/z* 40–550.

2.4.2 High-resolution atmospheric pressure chemical ionization (APCI) electrospray ionization (ESI) mass spectrometry for the determination of elemental composition

High-resolution mass spectra (HRMS) for verification of elemental composition were recorded on a Bruker micrOTOF-Q III spectrometer interfaced to a Dionex UltiMate 3000 LC for sample injection. They system has a mass resolution 20,000 (FWHM) at LC-speed and mass accuracy 1 - 2 ppm RMS error. Agilent Tune Mix-L was used for calibration. HyStar 3.2 and Data Analysis 4.1 were utilized for system control and data processing. Both electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) sources were used to carry out analysis as necessary, with the following conditions:

ESI experiments were recorded over the range 50-2000 m/z, end-plate offset 500 V capillary 4500 V, nebulizer 2.0 Bar, dry gas 8.0 L/min, and dry temperature 180°C.

APCI experiments were recorded over a range of 50-1600 m/z, capillary voltage 4000 V, corona 4000 nA, nebulizer gas 2.0 Bar, dry gas 3.0 L/min, dry gas temperature 200°C, vaporiser temperature 400°C.

2.4.3 Liquid chromatography-low resolution mass spectrometry (LC-LRMS)

This was used for the quantification of clonazolam in the tablets and to obtain product ions spectra for clonazolam and nitrazolam utilizing in-source CID.

Analyses were performed on an Agilent 1100 HPLC system equipped with a G13795 degasser, G1312A BinPump, a G1313A ALS and G1316A column oven (COLCOM) (Agilent, Little Island, Cork). Separation was obtained on an Allure PFP Propyl column (5 µm, 50 x 2.1 mm) Restek (Bellefonte, PA, USA). Mobile phase A consisted of 0.1% formic acid in water, whereas mobile phase B consisted of 0.1% formic acid in acetonitrile. The flow rate was set at 0.80 mL/min, with an injection volume of 10 µL, and the column temperature was 30°C. The following gradient elution program was used: 0-2 min 2% B, followed by an increase to 60% B within 15 min, followed by another increase to 80% B within 18 min before returning to 2% B within 25 min (total run time was 25 min.). The Agilent LC-MSD mass spectrometer settings were as follows: positive electrospray mode, capillary voltage 3500 V, drying gas (N_2) 12 L/min at 350 °C, nebulizer gas (N_2) pressure 50 psi, and scan mode *m/z* 70–500.

To obtain product ions spectra, samples were dissolved in acetonitrile/water (1:1, containing 0.1% formic acid) and the fragmentor voltage was set at 150 V. For the quantification of clonazolam in tablets, the fragmentor voltage was set at 50 V.

2.4.4 Nuclear magnetic resonance (NMR) spectroscopy

The following instruments were used to acquire NMR spectra. Spectra are shown in Supporting information 3.

Instrument 1: ^{1}H (600 MHz) and ^{13}C (150 MHz, referenced to the NMR solvent peak) spectra were recorded on a Bruker AV600 NMR spectrometer, in the solvent stated, using a 5 mm TCI cryoprobe. ^{1}H NMR spectra were referenced to an external TMS reference at δ = 0 ppm.

Instrument 2: ^{1}H NMR (400 MHz), and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Avance III 400 MHz spectrometer performed at 293K in the solvent stated. Chemical shifts (δ) are given in parts per million (ppm) where TMS singlet is set at 0 ppm. Coupling constants are given as absolute values in Hertz.

Instrument 3: The ^{1}H (60 MHz) spectrum was recorded on a NMReady-60e benchtop NMR spectrometer (Nanalysis Corp, Calgary, Canada) using the following conditions: 5 mm NMR tube; 4096 scans at 32°C; pulse sequence, 1DPULSEACQUIRE. Data was processed in MNova 12.0 (Mestrelab Research, Santiago de Compostela, Spain).

2.4.5 X-Ray crystallography for clonazolam

Data (Supporting Inofrmation 4) were measured on a Bruker D8 Quest Eco diffractometer at 100 K using an Oxford Cryostream with the sample mounted on a MiTeGen microloop using Mo Kα radiation (λ = 0.71073Å). Bruker APEX software was used to collect and reduce data and determine the space group.[22] Absorption corrections were applied using SADABS.[23] The structure was solved with the XT structure solution program using Intrinsic Phasing and refined with the XL refinement package using Least Squares minimisation in Olex2.[24-26] All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were assigned to calculated positions using a riding model with appropriately fixed isotropic thermal parameters Crystal data, details of data collections and refinement are given below. CCDC 1569730 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre.[27]

Crystal Data for $C_{17}H_{12}ClN_5O_2$ (*M* =353.77 g/mol): monoclinic, space group $P2_1/n$ (no. 14), *a* = 8.3378(5) Å, *b* = 13.1153(8) Å, *c* = 14.7638(9) Å, β = 102.804(2)°, *V* = 1574.32(17) Å^3, *Z* = 4, *T* = 109.99 K, μ (MoKα) = 0.265 mm^{-1}, *Dcalc* = 1.493 g/cm^3,

29591 reflections measured (5.66° ≤ 2Θ ≤ 56.846°), 3947 unique (R_{int} = 0.0284, R_{sigma} = 0.0163) which were used in all calculations. The final R_1 was 0.0350 ($I > 2\sigma(I)$) and wR_2 was 0.0929 (all data). CCDC 1569730.

3 RESULTS AND DISCUSSION

At the time when the powdered and tablet samples believed to contain clonazolam were obtained, reference material was not commercially available, which triggered the need to confirm the identification by organic synthesis on a preparative scale. From the perspective of aiding qualitative identification, it was hypothesized that the preparation on a microscale synthesis would generate sufficient amounts of material in an accelerated manner suitable for analysis by GC-MS without the need for purification. The smaller quantities used for the microscale methodology were considered to make it more attractive to a forensic laboratory environment in terms of time and cost involved and the organic chemistry manipulation skills required. Furthermore, larger volumes of solvents and reagents used in preparative work also pose a problem regarding waste disposal and the impact on the environment.

This investigation describes a novel approach for preparing NPS reference standards using a microscale synthesis procedure and polymer-supported reagents. Clonazolam and the deschloro analogue nitrazolam were studied as prototypical compounds to evaluate this strategy (Figure 1). In addition, both clonazolam and nitrazolam were synthesized on a preparative scale using methods that have previously been described in the literature.[13,14,20,28,29] These are similar to the microscale method (Figure 1). The EI mass spectral data (Figure 2) obtained for clonazolam and nitrazolam, synthesized on a preparative scale, were found to be in good agreement with previously published data.[10,11,12,15,16] The quantitative analysis of clonazolam tablet samples (reported to contain 0.5 mg), using the synthesized material as a reference standard, yielded a value of 0.52 mg clonazolam per tablet (Supporting Information 5).

3.1 Microscale synthesis

Reaction of the benzophenone derivatives (**1a**/**b**) with 2-chloro-1,1,1-triethoxyethane, followed by the elimination of ethanol, resulted in either (**2a**) (ethyl (*E*)-*N*-(2-benzoyl-4-nitrophenyl)-2-chloroacetimidate) for nitrazolam or (**2b**) (ethyl (*E*)-2-chloro-*N*-(2-(2-chlorobenzoyl)-4-nitrophenyl)acetimidate) for clonazolam. Conversion of the chloride to an iodide was accomplished using sodium iodide and acetone (Finkelstein reaction) to afford (**3a/b**) (Figure 1).

[Insert Figure 1 here]

In step **C**, a suspension of polymer-supported azide in dichloromethane was used to convert the iodide to the azide (**4a/b**), which follows a well-established synthetic pathway to alkyl azides by nucleophilic substitution.[30] It has been reported that insoluble polymeric reagents can be used in large excess to complete this conversion

into an alkyl azide.[31] In step **D**, the residue was dissolved in tetrahydrofuran/water and polymer-supported triphenylphosphine was added to convert the azide to an amine which spontaneously lead to ring closure (imine formation) to afford the ethoxy benzodiazepine intermediate (**5a/b**). Polymer-supported triphenylphosphine has previously been used for the Staudinger reaction, where the conversion of an organoazide ($R\text{-}N_3$) by a tertiary phosphine produced an iminophosphorane ($R_3P{=}NR$) that hydrolyzed to give primary amines.[32-34]

In step **E**, acetyl hydrazine, in a mixture of glacial acetic acid and methanol, was used to form the acetylhydrazides (**6a/b**), and the final step (**F**) was the formation of the triazole ring by thermolysis on the GC injector. The solutions of (**6a/b**) in acetonitrile could be stored at 4–8^{o}C but it was found that the benzodiazepine derivatives were not produced after several months of storage.

A microscale synthesis approach may help with directing forensic casework, given that the approach allows for a fast qualitative identification, thus supporting decision making processes. The last reaction, step **F**, carried out in the microscale synthesis required heat supplied by the GC injection port. However, if a standard was required for LC analysis, then the same reaction step could be performed in a heated microvial.

3.2 Microscale and preparative scale synthesis by-products

The microscale synthesis resulted in the formation of by-products (Figure 2 and Supporting Information 6). For nitrazolam, the benzophenone starting material (**1a**) and the intermediate (**5a**) were identified. The *O*-methoxy analogue of (**5a**) was also tentatively identified, presumably due to methanolysis of (**5a**) during step E. The by-product profile suggested incomplete reactions to occur during some steps. The yields were considered low and estimated at around 4–15% based on comparison of peak areas for a standard and reactions. However, this did not impact on the ability to record suitable EI mass spectra, thus allowing for the data to be used for qualitative purposes. The microscale syntheses products also afforded acceptable chromatographic peaks, regarding retention time and peak shape, when compared with the standards synthesized on a preparative scale. (Figure 2). It must be borne in mind that a balance must be struck between the expeditious preparations of compounds and optimization of yields when a laboratory has a high-throughput of samples for forensic analysis. A similar by-product pattern was observed for clonazolam.

Analysis of the crude clonazolam and nitrazolam products obtained from the preparative scale syntheses by GC-MS and LC-MS, revealed the presence of the expected products along with some residual hydrazide starting material (Supporting Information 7). A small amount of amino-clonazolam and nitrazolam, where the nitro group has been reduced to an amino group, was also observed. Interestingly, the thermal decomposition of nitrazepam and clonazepam, during GCMS analysis, to

their corresponding amino analogs has been previously reported.[35] In the case of clonazolam, the presence of a minor amount of the benzophenone starting material was also observed. Overall, the crude product attained from the preparative scale products appeared to be 'cleaner' than the products derived from the microscale procedures. Interestingly, none of the reaction by-products observed in the preparative scale synthesis were seen in the vendor clonazolam samples (tablets and powder).

3.3 Mass spectral characterization and fragmentation pathways for clonazolam and nitrazolam

The chromatograms and EI mass spectra for microscale synthesis are shown in Figure 2. The chromatographic peaks displayed good symmetry and retention time matches with the standards. In Figure 3 (a-c), the proposed EI mass spectral fragmentation pathways for nitrazolam and clonazolam are shown. The molecular ions of nitrazolam (*m/z* 319) and clonazolam (*m/z* 353) (^{35}Cl isotopes) formed fragment ions at *m/z* 302 and *m/z* 336 respectively, indicating loss of a hydroxyl radical. Loss of nitrous acid from the molecular ions was thought to result in the formation of *m/z* 272 (nitrazolam) and *m/z* 306 (clonazolam). Loss of nitrogen from these fragment ions lead to the formation of *m/z* 244 and *m/z* 278 (Figure 3a). Loss of a hydrogen radical and re-arrangement, with loss of CO, resulted in the formation of fragment ions at *m/z* 290 (nitrazolam) and m/z 324 (clonazolam) (Figure 3b). In the case of clonazolam, there was a further loss of a chlorine radical from *m/z* 324 yielding the fragment at *m/z* 290. Other fragment ions formed from the molecular ions resulted from contraction of the diazepine ring (Figure 3c).[36] The loss of nitrogen and propyne, forming the quinazoline radical cation (Q), is suggested to subsequently lose hydrogen chloride to yield *m/z* 249 (clonazolam). This fragment ion Q might also be envisioned to also lose nitrous acid with formation of *m/z* 204 (nitrazolam) and *m/z* 238 (clonazolam). The positive ESI product ion mass spectra (150 V in-source CID) nitrazolam and clonazolam are shown in Figure 4. Fragments due to 'loss of nitrogen', $[M+H-NO_2+O_2]^+$, afforded *m/z* 306 (nitrazolam) and *m/z* 340 (clonazolam). This unusual fragmentation behavior, i.e. loss of 14 u from the protonated molecule, has been previously reported.[37]

[Insert Figures 2, 3 and 4 here]

3.4 X-ray crystallography of clonazolam (vendor sample)

Full x-ray crystallography data for the recrystallized (ethanol) clonazolam powder is presented in Supporting Information 4. The central ring system is similar in conformation and bond lengths/angles to many of the structurally characterized alprazolam benzodiazepine derivatives.[38-43] An overlay of the x-ray structure with that of alprazolam (C2-C22 in 1, hydrogen atoms omitted) yields a RMS deviation of only ca. 0.123Å. In 1, the torsion angle between the central seven membered ring and *m*-chlorophenyl substituent is 127.828(3) Å (N9-C8-C7-C2). However, despite the nitro group and the aromaticity of the compound, there are no strong H-bonding or π-π

interactions. Weak intermolecular interactions include C-Cl…π (ca. 3.45 Å) and CH…N π (C4…N13 triazole, ca. 3.5 Å).

3.5 NMR spectra

NMR spectra, recorded in $CDCl_3$ have previously been reported for clonazolam (**7a**) and nitrazolam (**7b**).[15-16] This data is in good agreement with spectra (d_6 DMSO) obtained for the preparative scale synthesized clonazepam and nitrazolam here (Supporting Information 3). Minor differences in chemical shifts, attributed to solvent effects, were noted e.g. the values of 2.59 (clonazolam) and 2.63 (nitrazolam) ppm found here for the CH_3 group, versus 2.7 and 2.69 respectively for the reported values. A distinctive feature of both ^{1}H spectra is the non-equivalence of the hydrogens observed for the diazepine CH_2 group which appear in form two doublets (4.35/5.31 ppm with J = 13.2 Hz for clonazolam and 4.25/5.28 ppm with J = 13.0 Hz for nitrazolam). Published values were noted as 4.20/5.63 ppm (J = 13.1 Hz) and 4.09/5.56 ppm (J = 14.11 Hz) respectively and these differences were again attributed to solvent effects. In the ^{13}C NMR spectrum, the Ar-C=N carbon in the diazepine ring is characteristically seen downfield at 166.53 (clonazolam) and 167.28 (nitrazolam) ppm (published values 167.31 and 167.6 ppm respectively). The clonazolam tablets were also analysed by low-field benchtop NMR. In the 60 MHz ^{1}H NMR spectrum, recorded using the supernatant obtained by extracting two crushed tablets (equivalent to approximately 1 mg clonazolam) with $CDCl_3$, it was possible to see several key features (Supporting Information 3). The methyl group was noted at 2.65 ppm and the diazepine CH_2 was observed as a pair of doublets (J = 13.1 Hz) at 4.14 and 5.59 ppm. Interestingly, it was also possible to distinguish a doublet (8.05 ppm, J = 2.5 Hz) and double doublet (8.48, J = 9.0, 2.5 Hz), possibly corresponding H-3 and H-5 respectively. Overall the data was found to be in good agreement with the high-field ^{1}H NMR data published for clonazolam in $CDCl_3$.[16]

4 CONCLUSION

The work presented here has shown the usefulness of microscale synthesis for the syntheses of two benzodiazepine derivatives of forensic interest. The traditional preparative scale synthesis is significantly more complex and the developed microscale synthesis approach facilitated a rapid generation of standards useable for qualitative identification purposes. From this viewpoint, more approaches are needed to develop the in-house expertise of forensic personnel to accelerate the availability of reference standards rather than solely relying on external reference standard suppliers. This study reflects a proof of concept but the development of a simple microscale method, utilizing polymer-supported reagents, has the potential to be applicable to a whole range of benzodiazepine derivatives, especially when qualitative identifications are needed to direct forensic casework and when reference materials are not available. This approach could be used by forensic laboratories to shorten the timeframe between the emergence of a new compound on the drugs market and the availability of drug standards.

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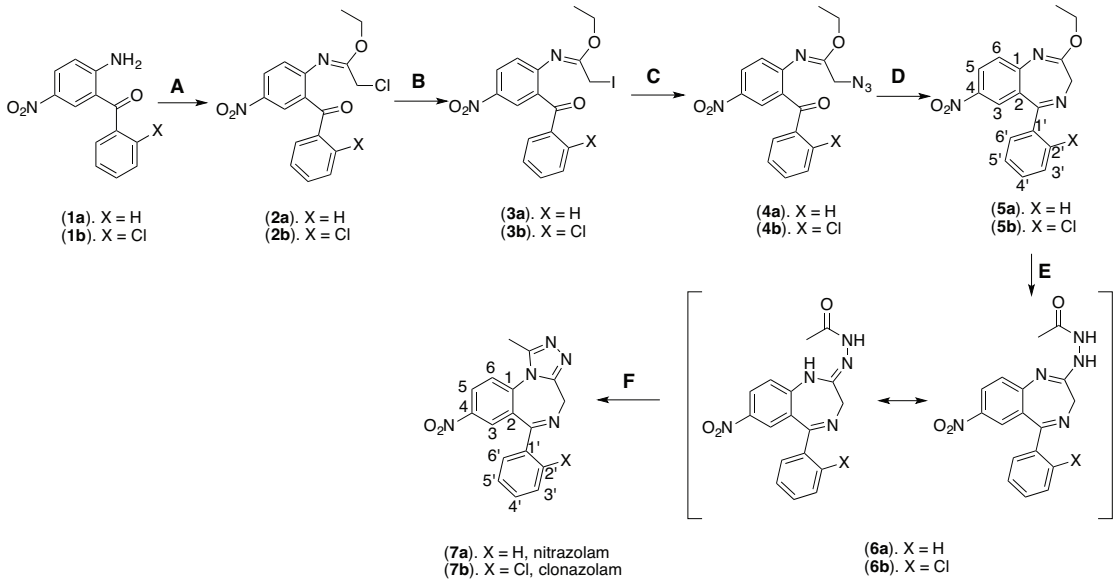

Figure 1. Microscale synthetic sequence for nitrazolam (X = H) and clonazolam (X = Cl). Reagents used for each step: **A**. 2-chloro-1,1,1-triethyoxyethane, acetic acid; **B**. sodium iodide, acetone; **C**. polymer-supported azide, dichloromethane; **D**. polymer-supported triphenylphosphine, tetrahydrofuran, water; **E**. acetylhyrazine, acetic acid, methanol; **F**. GC injector. Atom labels for NMR assignments.

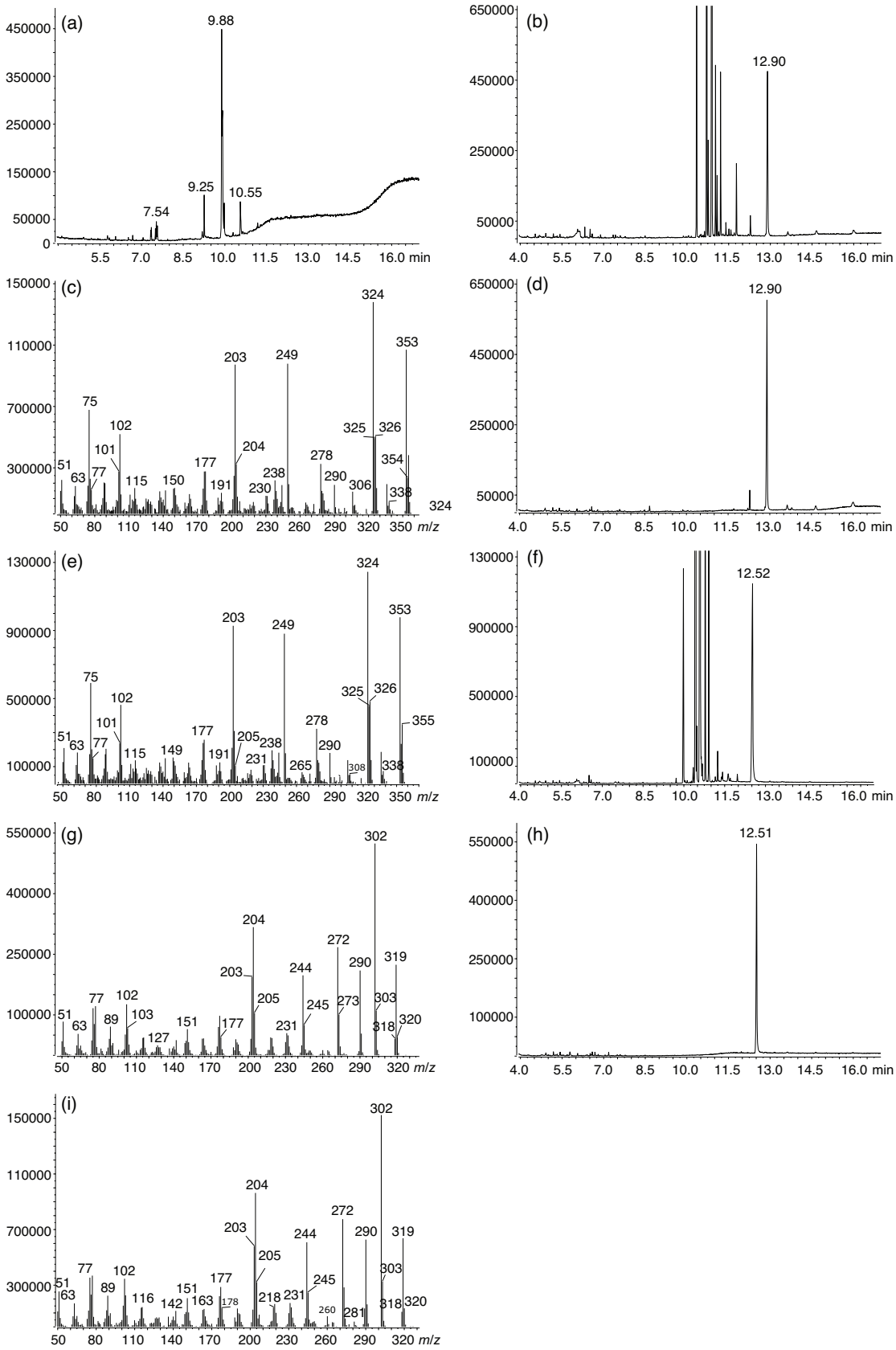

Figure 2. Microscale synthesis. (a) GC-MS chromatogram of reaction mixture from step E for sequence performed in the absence the benzophenone starting material; (b) GC-MS

chromatogram of reaction mixture from step E for clonazolam microscale synthesis; (c) EI mass spectrum for clonazolam peak (12.90 min) from microscale synthesis; (d) GC-MS chromatogram for clonazolam standard; (e). EI mass spectrum for clonazolam standard (12.90 min); (f) GC-MS chromatogram of reaction mixture from step E for nitrazolam microscale synthesis; (g) EI mass spectrum for nitrazolam peak (12.52 min) from microscale synthesis; (h) GC-MS chromatogram for nitrazolam standard; (i) EI mass spectrum for nitrazolam standard (12.51 min).

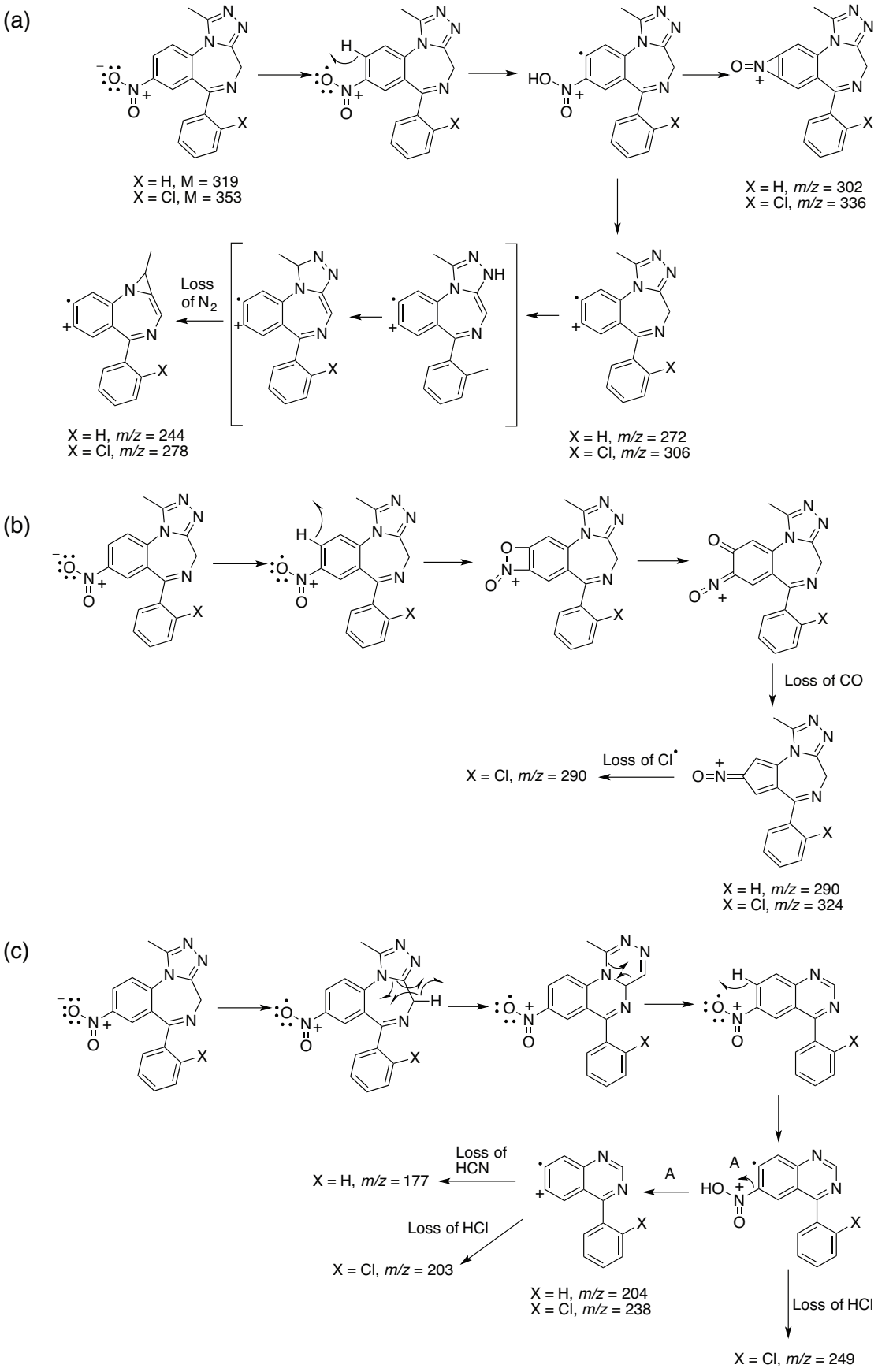

Figure 3 (a-c): Proposed EI mass spectral fragmentation pathways for nitrazolam and clonazolam.

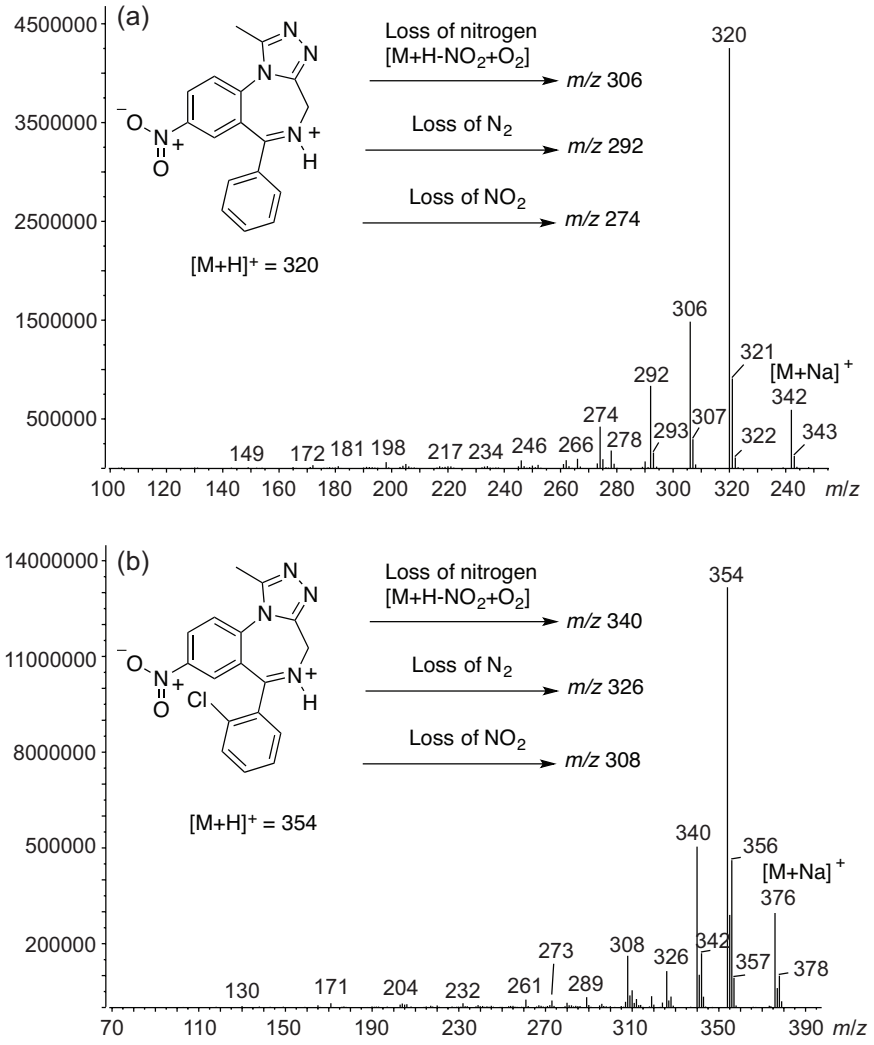

Figure 4: Electrospray ionization product ion mass spectra (150 V in-source CID) for (a) nitrazolam and (b) clonazolam.