Guilty by Dissociation: Part A: Development of a rapid Ultra-High Performance Liquid Chromatography (UHPLC)-MS/MS methodology for the analysis of regioisomeric diphenidine-derived Novel Psychoactive Substances (NPS).

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Highlights

- Reversed phase UHPLC rapidly separates 31 diphenidines within 10 minutes
- Synthesis of 2-, 3- and 4-regioisomeric diphenidines
- Novel Psychoactive Substances
- Targeted UHPLC-MS/MS
- Multiple reaction monitoring (MRM) transitions allowed for the unambiguous analyte confirmation.
- Qualitative and quantitative analysis of seized bulk drug samples

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Abstract

This study describes the first reported development of a rapid, generic gradient Ultra-High Performance Liquid Chromatography (UHPLC) methodology with targeted triple quadrupole MS/MS using electrospray positive ionisation to detect and unambiguously confirm the identity of 33 substituted 1, 2-diarylethamine (or diphenidine) derivatives in solid drug samples. The in-house synthesised library included a range of derivatives possessing either electron donating/withdrawing substituents, commonly included in combinatorial libraries, of varying size and lipophilicity on the phenyl ring. These test probes were used to investigate if their order of elution and that of their regioisomers were dependent on the position and type of the substituent on the phenyl ring. In addition, investigations into the retention mechanism of the diphenidines under reverse-phase UHPLC conditions were undertaken. Common adulterants found within seized bulk samples were assessed to prove that the methodology was specific, and the developed UHPLC-MS/MS (t_G = 10 min) protocol was applied to confirm the identity of the psychoactive components within two seized bulk samples provided by law enforcement.

Keywords: Forensic; illicit drugs; regioisomers; diphenidines; novel psychoactive substances; UHPLC

1.0 Introduction

Over the past thirteen years, there has been a significant increase in the number of novel (or new) psychoactive substances (NPS) seized by law enforcement agencies globally [1, 2]. Novel (or new) psychoactive substances are materials in their pure form, or in a preparation, that are not covered by the United Nations Single Convention on Narcotic Drugs (1961), as amended by the Protocol (1972), or by the United Nations Convention on Psychotropic Substances (1971) but can potentially lead to adverse health or social risks similar to those posed by the substances covered by the conventions [3]. Within this context, the terms *"novel"* or *"new"* does not necessarily refer to novel inventions but to substances that have recently become available on the illicit market. Psychoactive substances prohibited under the international drug control conventions produce their effects through a small number of pharmacological mechanisms and can have significant chemical diversity within each family of psychoactive substances [3]. Current convention uses a functional *"effect group"* categorisation to define NPS within six broad overlapping groups: (i) cannabinoid receptor agonists; (ii) classic hallucinogens; (iii) dissociatives; (iv) opioids; (v) sedatives/hypnotics and (vi) psychostimulants. NPS are assigned to a specific *"effect group"* based on their chemical structure and psychopharmacological effects [3, 4].

1, 2-Diarylethamines (or diphenidines) are dissociative, psychoactive substances which distort perceptions, produce feelings of detachment, and induce a state of anaesthesia by antagonising ionotropic *N*-methyl-D-aspartate receptors (NMDAR) in the central nervous system [5]. The first of these dissociative anaesthetics was 1-(1, 2-diphenylethyl)piperidine (diphenidine, **1**) [6], followed by 1-[1-(2-methoxyphenyl)-2-phenylethyl]piperidine (2-methoxphenidine, 2-MXP, **2**) [7] which have both been marketed as *"research chemicals"* and encountered in tablet or powder forms [6 – 9], or in combination with synthetic cannabinoids such as AB-CHMINACA, 5F-AMB [10] and 5F-AB-PINACA [11]. Though both the supply and production of **1**, **2** and the recently disclosed 1-[1-(2-chlorophenyl)-2-phenylethyl]piperidine (2-chlorodiphenidine, 2-Cl-DPH, **17**) [12, 13] are now controlled in the United Kingdom by the Psychoactive Substances Act (2016) [14], the emergence of novel 1, 2-diarylethylamine derivatives, such as **17**, still raises considerable legal and analytical challenges in both the forensic identification and regioisomeric discrimination of these materials, due to their inference of diphenidine-based NPS in several fatalities in Europe [15 - 17] and Asia [10, 18, 19].

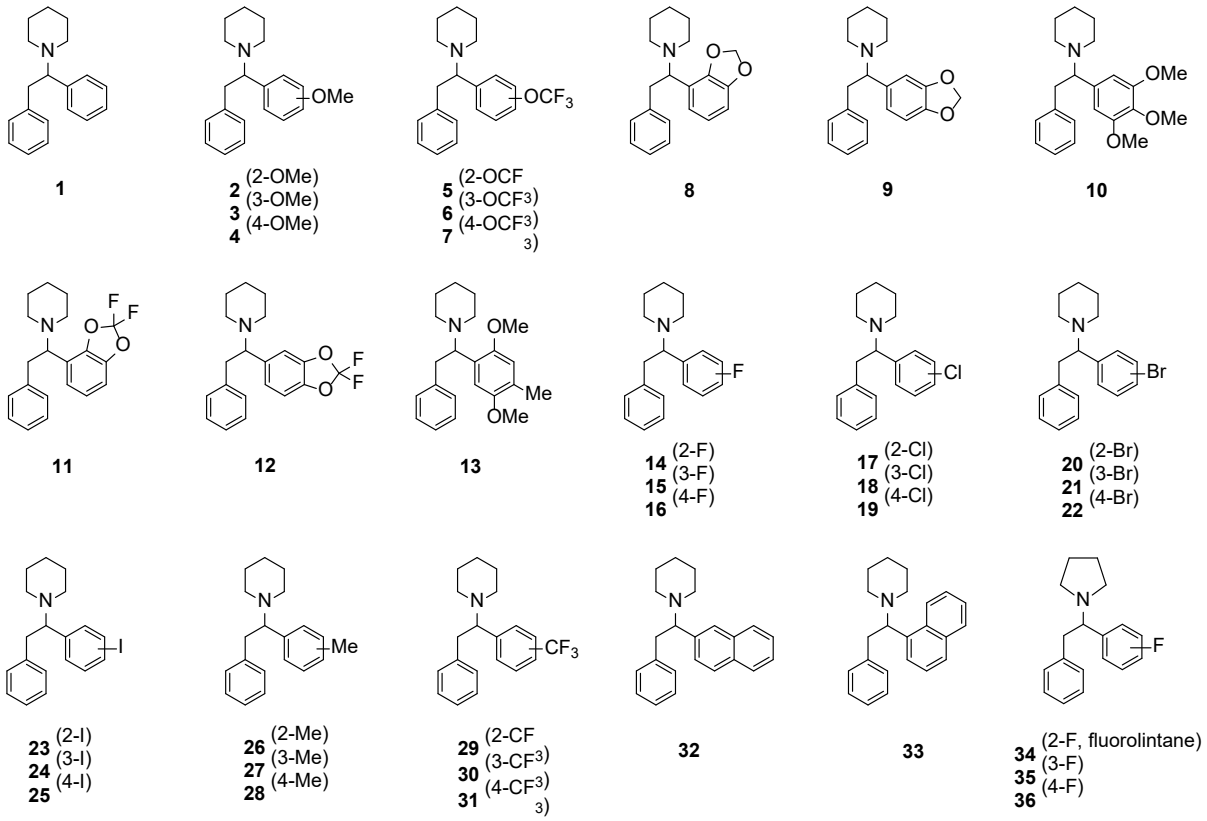

Fig. 1. Structures of the regioisomeric diphenidines (**1** – **33**) utilised in this study and *ortho*-, *meta*- and *para*-fluorolintanes (**34** – **36**).

Analytical differentiation of regioisomers is a significant challenge within drug analysis, because, in some countries, legal controls are placed on only one or two of the conceivable isomers and require a forensic scientist to show unequivocally that a sample submitted is in fact a controlled drug and not one of the non-controlled regioisomers [20]. This can be readily achieved using Nuclear Magnetic Resonance (NMR) spectroscopy [7, 21 - 23], however, only a small number of laboratories have such instruments. The discrimination of regioisomers using NMR is both cost and labour intensive relative to liquid chromatographic approaches that have been applied to bulk [6 – 9, 11] and toxicological [10, 15 - 19, 24 - 27] samples of **1** and its commonly encountered analogues. Reversed-phase liquid chromatographic (RP-LC) separation of the methoxphenidines **2**, **3** and **4** has been reported using a superficially porous phenylhexyl material (*i.e.* 2.6 µm Kinetex) coupled with a shallow acetonitrile (MeCN)-formic acid gradient at 30 °C (*i.e.* 0.25% MeCN/min) over 35 min. Whilst **2** was well resolved from the other two isomers, only partial separation of **3** and **4** was observed (the elution order was reported to be *meta*- > *para*- > *ortho*- isomer). However, the paper did not prove evidence of any systematic investigation into the retention behaviour [7]. More recently Boateng *et al.* disclosed a detailed investigation into the chromatographic retention behaviour and separation

of the methoxphenidine regioisomers, using an ACE C18-AR (3 μm, 50 × 4.6 mm i.d.) column in combination with an MeCN-aqueous ammonium acetate (10 mM, pH 6.8 unadjusted) gradient. It was reported that the ionization state of the analyte and stationary phase is the controlling factor in dictating which retention mechanism is in operation. This allowed the optimization of the gradient separation of **2**, **3** and **4** using a two-dimensional gradient and temperature design space, leading to the development of a rapid and highly sensitive LC–MS friendly method (*i.e.* $R_{s\ (min)} > 5$ within 4 min), suitable for the rapid, specific and sensitive detection and control of MXP regioisomers [28].

This paper describes the first reported development of a rapid generic gradient UHPLC methodology with targeted triple quadrupole MS/MS using electrospray positive ionisation to detect and unambiguously confirm the identity of 33 substituted derivatives in solid drug samples. The library included a range of derivatives possessing either electron donating/withdrawing substituents, commonly included in combinatorial libraries, of varying size and lipophilicity on the phenyl ring (see Fig. 1 and Table 1). The 2-, 3- and 4-positional isomers (commonly known as the *ortho*-, *meta*- and *para*-regioisomers) of eight diphenidine families (**2 – 4**, **5 – 7**, **14 – 16**, **17 – 19**, **20 – 22**, **23 – 25**, **26 – 28** and **29 - 31**) and three groups of twinned structural isomers (**8**/**9**, **11**/**12** and **32**/**33**) were synthesised to separate these isomeric compounds and to elucidate if their elution order was dependent on the position and type of substituent on the phenyl ring. In addition, investigations into the retention mechanism of the diphenidines under reverse-phase UHPLC conditions were undertaken. Common adulterants found within seized bulk samples were assessed to prove that the methodology was specific. The developed UHPLC-MS/MS was then applied to confirm the identity of two seized bulk samples of diphenidine provided, between June – October 2016, by Greater Manchester Police.

2.0. Materials and Methods

2.1. Synthesis of Standards and Procurement of Forensic Samples

All solvents and reagents were of commercial quality (Sigma-Aldrich, Gillingham, UK or Fluorochem Limited, Hadfield, UK) and used without further purification. Diphenidine (**1**) and its derivatives (**2** – **33**, Fig. 1) were prepared as their corresponding hydrochloride salts by MANchester DRug Analysis and Knowledge Exchange (MANDRAKE). The synthesis of the racemic target compounds was achieved using the previously reported method [29] and isolated as their corresponding hydrochloride salts. To ensure the authenticity of the materials utilized within this study, the 33 synthesized samples were structurally characterized by ^{1}H-NMR, ^{13}C{^{1}H}-NMR, GC-MS and ATR-FTIR and the purity of all samples was confirmed to be >99.5% (by NMR) in all cases. The NMR purity was calculated using the relative concentration determination method described by Pauli *et al.* [30] The ^{1}H NMR, ^{13}C{^{1}H} spectra (10.0 mg mL^{-1} in DMSO-d_6) were acquired on a JEOL JMN-ECS-400 (JEOL, Tokyo, Japan) NMR spectrometer operating at a proton resonance frequency of 400 MHz, referenced to the residual solvent peak (DMSO-d_6: ^{1}H NMR δ = 2.50 ppm, ^{13}C{^{1}H} NMR δ = 39.52 ppm). GC-MS analysis was performed using an Agilent 7890B GC and a MS5977B mass selective detector (Agilent Technologies, Wokingham, UK). The mass spectrometer was operated in the electron ionisation mode at 70 eV (full scan mode). Separation was achieved with a capillary column (HP5 MS, 30 m Å~ 0.25 mm i.d. 0.25 μm) with helium as the carrier gas at a constant flow rate of 1.0 mL min^{-1} using previously reported conditions [30]. All samples were prepared as 1 mg mL^{-1} solutions in methanol, with no derivatisation and analysed individually (three replicate injections) using eicosane as internal standard (1 mg mL^{-1} in methanol). Infrared spectra were obtained in the range 4000 – 400 cm^{-1} using a Thermo Scientific Nicolet iS10ATR-FTIR instrument (Thermo Scientific, Rochester, USA). The analytical data for compounds **1** – **10**, **13**, **32** and **33** has been previously reported [29] and the spectral data for compounds **11**, **12**, **14** – **31** is presented below. The two seized samples (Samples A and B) were provided to MANDRAKE, between June – October 2016, by Greater Manchester Police, in accordance with Manchester Metropolitan University's Home Office license requirements and agreed procedures.

2.1.1. (2,2-Difluoro-1,3-benzodiox-4-yl)diphenidine hydrochloride (**11**). Yield = 46%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.38 (s (br), 1H), 7.61 (d, J = 8.1 Hz, 1H), 7.36 (dd, J = 8.1, 1.0 Hz, 1H), 7.20 (t, J = 8.1 Hz, 1H), 7.17 – 6.96 (m, 5H), 4.81 – 4.73 (m, 1H), 3.84 – 3.62 (m,

2H), 3.50 – 3.36 (m, 2H), 2.83 (q, *J* = 11.4 Hz, 1H), 2.76 – 2.58 (m, 1H), 2.08 – 1.92 (m, 1H), 1.92 – 1.68 (m, 3H), 1.63 (d, *J* = 13.2 Hz, 1H), 1.38 – 1.24 (m, 1H); $^{13}C\{^{1}H\}$ NMR (101 MHz, DMSO-d_6) δ 143.24, 142.89, 136.12, 132.5 (d, 253.9 Hz), 129.32, 128.87, 127.36, 125.29, 115.54, 111.78, 65.00, 52.33, 49.40, 34.66, 23.12, 23.02, 21.75; ATR-FTIR ν_{max}/cm^{-1}: 3035, 2950, 2933, 2863, 2601, 2424, 2370, 2345, 2267, 1649, 1604, 1478, 1455; GC-EI-MS *m/z* (relative abundance): 254.1 (100.0%), 255.1 (15.0%), 91.1 (12.9%), 171.0 (11.6%), 41.0 (11.1%)

2.1.2. (2,2-Difluoro-1,3-benzodiox-5-yl)diphenidine hydrochloride (**12**). Yield = 35%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.34 (s (br), 1H), 7.79 (s, 1H), 7.46 – 6.91 (m, 7H), 4.70 (d, *J* = 11.8 Hz, 1H), 3.78 (d, *J* = 13.0 Hz, 1H), 3.67 (d, *J* = 11.9 Hz, 1H), 3.47 (t, *J* = 12.5 Hz, 1H), 2.66 – 2.49 (m, 2H), 2.07 – 1.92 (m, 1H), 1.92 – 1.77 (m, 1H), 1.72 (d, *J* = 13.8 Hz, 2H), 1.61 (d, *J* = 12.9 Hz, 1H), 1.33 – 1.66 (m, 1H); $^{13}C\{^{1}H\}$ NMR (101 MHz, DMSO-d_6) δ 143.69, 143.34, 132.9 (d, *J* = 252.6 Hz), 136.85, 129.57, 128.87, 128.84, 128.25, 127.04, 112.42, 110.48, 69.62, 52.24, 48.59, 35.38, 22.92, 22.87, 21.93; ATR-FTIR ν_{max}/cm^{-1}: 2932, 2602, 2463, 2395, 1604, 1499, 1453; GC-EI-MS *m/z* (relative abundance): 254.1 (100.0%), 255.1 (14.8%), 171.0 (13.9%), 91.1 (12.5%), 41.0 (12.3%)

2.1.3. 2-Fluorodiphenidine hydrochloride (**14**, *2-fluphenidine hydrochloride*). Yield = 40%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.45 (s, (br), 1H), 8.00 (m, 1H), 7.28 – 7.40 (m, 2H), 7.00 – 7.18 (m, 6H), 4.89 (dd, *J* = 12.8, 4.1 Hz, 1H), 3.88 (dd, *J* = 13.2, 3.8 Hz, 1H), 3.70 (m, 1H), 3.51 (m, 1H), 3.48 (m, 1H), 2.05 (m, 1H), 1.70 – 1.82 (m, 3H), 1.68, (m, 1H), 1.32 (m, 1H); $^{13}C\{^{1}H\}$ NMR (101 MHz, DMSO-d_6) δ 161.80 (d, *J* = 221.5 Hz), 136.22, 132.60, 132.15, 130.04, 129.82, 127.50, 125.95, 116.81, 116.45, 51.80, 48.22, 35.50, 21.68, 21.45; ATR-FTIR ν_{max}/cm^{-1}: 2985, 1560, 1250; GC-EI-MS *m/z* (relative abundance): 192.1 (100.0%), 178.1 (1.7%), 109.1 (13.6%), 91.1 (3.4%), 41.1 (4.2%).

2.1.4. 3-Fluorodiphenidine hydrochloride (**15**, *3-fluphenidine hydrochloride*). Yield = 35%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.45 (s, (br), 1H), 7.54 (d, *J* = 7.2 Hz, 1H), 7.38 – 7.48 (m, 2H), 7.00 – 7.3- (m, 6H), 4.71 (dd, *J* = 12.8, 3.8 Hz, 1H), 3.70 – 3.89 (m, 2H), 3.50 (t, *J* = 8.4 Hz, 1H), 3.41 (m, 1H), 2.60 – 2.70 (m, 2H), 1.68 – 2.10 (m, 5H); 1.28 (m, 1H); $^{13}C\{^{1}H\}$ NMR (101 MHz, DMSO-d_6 δ ppm = 39.51) δ 162.44 (d, *J* = 205.5 Hz); 136.87, 134.99, 129.60, 128.79, 127.49, 127.03, 117.99, 117.77, 117.00, 116.80, 69.62, 52.40, 49.00, 35.41, 22.88, 22.05; ATR-FTIR ν_{max}/cm^{-1}: 2980, 1570, 1303; GC-EI-MS *m/z* (relative abundance): 192.1 (100.0%), 178.1 (1.8%), 109.1 (13.6%), 91.1 (3.4%), 41.1 (4.1%).

2.1.5. 4-Fluorodiphenidine hydrochloride (**16**, *4-fluphenidine hydrochloride*). Yield = 44%. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 11.29 (s (br), 1H); 7.59 (d, *J* = 7.2 Hz, 2H), 6.98 – 7.20 (m, 7H), 4.65 (dd, *J* = 12.2, 3.8 Hz, 1H), 3.76 (d, *J* = 7.8 Hz, 1H), 3.45 (m, 1H), 3.73 (m, 1H), 3.35 (m, 1H), 2.52 (m, 2H), 1.80 – 2.01 (m, 2H), 1.72 (m, 2H), 1.61 (m, 1H), 1.23 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-*d*$_6$) δ 162.50 (d, *J* = 220.0 Hz); 137.03, 133.46, 133.38, 129.61, 128.77, 128.05, 126.98, 116.14, 115.93, 69.48, 52.27, 48.68, 35.51, 22.87, 22.06; ATR-FTIR ν_{max}/cm^{-1}: 2980, 1570, 1305; GC-EI-MS *m/z* (relative abundance): 192.1 (100.0%), 178.1 (1.6%), 109.1 (13.7%), 91.1 (3.5%), 41.1 (4.1%).

2.1.6. 2-Chlorodiphenidine hydrochloride (**17**). Yield = 43%. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 11.67 (s (br), 1H), 8.27 (d, *J* = 7.5 Hz, 1H), 7.49 (m, 1H), 7.36 (d, *J* = 7.8 Hz, 2H), 7.12 (m, 3H), 7.00 (m, 2H), 5.00 (dd, *J* = 12.8 3.8 Hz, 1H), 3.88 (m, 1H), 3.46 (m, 1H), 3.43 (m, 1H), 3.27 (m, 1H), 3.24 (m, 1H), 3.15 (m, 1H), 2.09 (m, 1H), 1.62 – 1.90 (m, 4H), 1.38 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-*d*$_6$) δ 136.20, 135.65, 131.99, 131.84, 130.94, 130.45, 129.86, 128.05, 127.97, 127.12, 66.20, 52.48, 49.55, 35.85, 23.00, 21.87; ATR-FTIR ν_{max}/cm^{-1}: 2950, 1570, 689; GC-EI-MS *m/z* (relative abundance): 210.1 (33.2%), 208.1 (100.0%), 180.1 (1.9%), 178.1 (5.7%), 127.0 (2.9%), 125.0 (8.9%), 91.1 (4.9%), 41.0 (5.7%).

2.1.7. 3-Chlorodiphenidine hydrochloride (**18**). Yield = 54%. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 11.36 (s (br), 1H), 7.70 (s, 2H), 7.60 (dd, *J* = 8.2, 3.3 Hz, 1H), 7.42 (m, 2H), 7.00 - 7.20 (m, 4H), 4.71 (d, *J* = 8.2 Hz, 1H), 3.75 – 3.85 (m, 2H), 3.52 (m, 1H), 3.40 (m, 1H), 2.61 (m, 2H), 1.70 – 2.10 (m, 4H), 1.65 (m, 1H), 1.29 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-*d*$_6$) δ 136.82, 133.79, 134.61, 130.93, 130.10, 129.99, 129.60, 128.83, 127.04, 69.51, 52.36, 49.01, 35.26, 22,89, 22.01; ATR-FTIR ν_{max}/cm^{-1}: 2900, 1565, 699; GC-EI-MS *m/z* (relative abundance): 210.1 (33.3%), 208.1 (100.0%), 180.1 (1.9%), 178.1 (5.8%), 127.0 (2.9%), 125.0 (8.9%), 91.1 (4.9%), 41.0 (5.7%).

2.1.8. 4-Chlorodiphenidine hydrochloride (**19**, *3-clophenidine hydrochloride*). Yield = 49%. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 11.42 (s (br), 1H), 7.60 (d, *J* = 7.4 Hz, 2H), 7.43 (d, J = 8.8 Hz, 2H), 7.00 – 7.20 (m, 5H), 3.80 (dd, *J* = 12.8, 3.6 Hz, 1H), 3.71 (m, 1H), 3.48 (m, 1H), 3.39 (m, 1H), 2.50 – 2.60 (m, 2H), 1.70 – 2.05 (m, 4H), 1.64 (m, 1H), 1.26 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-*d*$_6$) δ 136.42, 134.19, 132.55, 130.86, 129.11, 128.95, 128.31, 126.50, 68.93, 51.81, 48.23, 34.83, 22.37, 21.52; ATR-FTIR ν_{max}/cm^{-1}: 2865, 1540, 710; GC-EI-MS *m/z* (relative abundance): 210.1 (33.3%), 208.1 (100.0%), 180.1 (1.9%), 178.1 (5.8%), 127.0 (2.9%), 125.0 (8.8%), 91.1 (4.8%), 41.0 (5.6%).

2.1.9. 2-Bromodiphenidine hydrochloride (**20**). Yield = 47%. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 11.55 (s (br), 1H), 8.25 (d, *J* = 9.2 Hz, 1H), 7.54 (m, 2H), 7.28 (dd, *J* = 9.2, 3.1 Hz, 1H), 7.12 (m, 3H), 7.00 (d, *J* = 7.5 Hz, 2H), 4.95 (m, 1H), 3.88 (m, 2H), 3.44 (m, 1H), 3.26 (m, 1H), 2.76 (m, 1H), 2.68 (m, 1H), 2.04 (m, 1H), 1.70 – 1.90 (m, 4H), 1.40 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-*d*$_6$) δ 135.58, 133.13, 132.56, 131.20, 130.96, 129.19, 128.50, 128.13, 126.66, 126.43, 68.50, 51.88, 49.45, 36.01, 22.49, 22.37, 21.31; ATR-FTIR ν_{max}/cm^{-1}: 2870, 1540, 550; GC-EI-MS *m/z* (relative abundance): 254.1 (92.9%), 252.1 (100.0%), 178.1 (11.8%), 91.1 (9.5%), 41.1 (8.7%).

2.1.10. 3-Bromodiphenidine hydrochloride (**21**). Yield = 49%. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 11.62 (s (br), 1H), 7.83 (s, 1H), 7.65 (d, *J* = 7.0 Hz, 1H), 7.57 (d, *J* = 7.2 Hz, 1H), 7.33 (d, J = 7.2 Hz, 2H), 7.00 - 7.20 (m, 4H), 4.69 (d, *J* = 9.8 Hz, 1H), 3.70 – 3.87 (m, 2H), 3.38 – 3.55 (m, 2H), 2.50 – 2.68 (m, 2H), 1.60 – 2.15 (m, 5H), 1.64 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-*d*$_6$) δ 136.34, 134.39, 133.31, 132.33, 130.63, 129.89, 129.06, 128.28, 126.49, 121.85, 68.98, 51.85, 48.45, 34.77, 22.29, 21.51; ATR-FTIR ν_{max}/cm^{-1}: 2900, 1520, 570; GC-EI-MS *m/z* (relative abundance): 254.1 (93.0%), 252.1 (100.0%), 178.1 (11.9%), 91.1 (9.5%), 41.1 (8.7%).

2.1.11. 4-Bromodiphenidine hydrochloride (**22**). Yield = 61%. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 11.43 (s (br), 1H), 7.45 – 7.55 (m, 3H), 6.95 – 7.18 (m, 6H), 4.64 (dd, *J* = 12.4, 3.8 Hz, 1H), 3.80 (m, 1H), 3.70 (m, 1H), 3.45 (m, 1H), 3.35 (m, 1H), 2.50 – 2.60 (m, 2H), 1.58 – 2.05 (m, 5H), 1.23 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-*d*$_6$) δ 137.68, 133.74, 132.88, 132.14, 130.05, 129.83, 127.60, 123.87, 70.06, 53.88, 48.31, 35.80, 22.45, 21.89; ATR-FTIR ν_{max}/cm^{-1}: 2865, 1600, 560; GC-EI-MS *m/z* (relative abundance): 254.1 (92.9%), 252.1 (100.0%), 178.1 (11.8%), 91.1 (9.6%), 41.1 (8.7%).

2.1.12. 2-Iododiphenidine hydrochloride (**23**). Yield = 26%. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 11.46 (s (br), 1H), 8.13 (d, *J* = 9.2 Hz, 2H), 7.73 (dd, *J* = 13.2, 4.8 Hz, 2H), 7.50 (d, *J* = 11.2 Hz, 1H), 7.08 (m, 3H), 6.94 (d, 1H, *J* = 9.2 Hz), 4.74 (m, 1H), 3.75 – 3.85 (m, 2H), 3.32 (m, 1H), 3.16 (m, 1H), 2.60 – 2.90 (m, 2H), 2.04 (m, 1H), 1.70 – 1.82 (m, 4H), 1.37 (m, 1H), ^{13}C{^{1}H} NMR (101 MHz, DMSO-*d*$_6$) δ 140.83, 136.18, 132.01, 130.97, 130.15, 129.92, 128.33, 127.59, 105.86, 74.21, 53.27, 50.72, 37.52, 23.10, 21.89; ATR-FTIR ν_{max}/cm^{-1}: 2940, 1550; GC-EI-MS *m/z* (relative abundance): 300.1 (100.0%), 172.1 (17.2%), 91.1 (8.5%), 41.1 (4.3%).

2.1.13. 3-Iododiphenidine hydrochloride (**24**). Yield = 54%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.31 (s (br), 1H), 7.92 (s, 1H), 7.59 – 7.70 (m, 2H), 6.95 – 7.20 (m, 6H), 4.59 (m, 1H), 3.62 – 3.80 (m, 2H), 3.30 – 3.50 (m, 2H), 2.50 – 2.70 (m, 2H), 1.52 – 2.00 (m, 5H), 1.22 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ 139.70, 138.92, 137.05, 135.78, 131.90, 131.63, 130.28, 129.84, 127.48, 95.80, 69.58, 52.16, 48.91, 35.47, 22.89, 20.96; ATR-FTIR ν_{max}/cm^{-1}: 2865, 1620; GC-EI-MS *m/z* (relative abundance): 300.1 (100.0%), 172.1 (17.1%), 91.1 (8.6%), 41.1 (4.3%).

2.1.14. 4-Iododiphenidine hydrochloride (**25**). Yield = 60%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.43 (s, 1H), 7.73 (d, *J* = 9.4 Hz, 2H), 7.38 (d, *J* = 8.2 Hz, 2H), 7.00 – 7.15 (m, 5H), 4.65 (dd, *J* = 12.8, 3.8 Hz, 1H), 3.70 – 3.85 (m, 2H), 3.40 – 3.52 (m, 2H), 2.50 – 2.61 (m, 2H), 1.65 – 2.10 (m, 5H), 1.29 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ 139.43, 137.45, 136.78, 134.22, 132.86, 131.96, 131.30, 128.51, 126.77, 98.14, 71.58, 57.62, 51.00, 36.70, 22.58, 21.50; ATR-FTIR ν_{max}/cm^{-1}: 2930, 1600; GC-EI-MS *m/z* (relative abundance): 300.1 (100.0%), 172.1 (17.2%), 91.1 (8.6%), 41.1 (4.3%).

2.1.15. 2-Methyldiphenidine hydrochloride (**26***, 2-tolphenidine hydrochloride*). Yield = 46%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.98 (s (br), 1H), 8.05 (d, *J* = 7.7 Hz, 1H), 7.31 (t, *J* = 7.6 Hz, 1H), 7.16 (td, *J* = 7.5, 1.3 Hz, 1H), 7.13 – 7.01 (m, 3H), 6.99 (d, *J* = 7.46 Hz, 1H), 6.89 (dd, *J* = 7.5, 2.0 Hz, 2H), 4.63 – 4.55 (m, 1H), 3.91 (d, *J* = 11.2 Hz, 1H), 3.78 (dd, *J* = 12.5, 3.6 Hz, 1H), 3.23 (t, *J* = 12.2 Hz, 1H), 3.10 – 2.92 (m, 2H), 2.73 – 2.60 (m, 1H), 2.13 – 1.96 (m, 1H), 1.92 – 1.70 (m, 5H), 1.65 (d, *J* = 13.2 Hz, 2H), 1.40 – 1.20 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ 138.50, 136.53, 132.61, 131.05, 129.85, 129.34, 128.85, 128.60, 127.11, 127.09, 66.93, 52.14, 50.50, 37.15, 23.11, 23.01, 22.04, 19.88; ATR-FTIR ν_{max}/cm^{-1}: 3036, 2943, 2576, 2601, 2520, 1606, 1500; GC-EI-MS *m/z* (relative abundance): 188.1 (100.0%), 189.1 (15.4%), 105.0 (13.9%), 91.0 (13.5%), 41.0 (7.0%).

2.1.16. 3-Methyldiphenidine hydrochloride (**27***, 3-tolphenidine hydrochloride*). Yield = 41%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.12 (s (br), 1H), 7.36 – 7.28 (m, 2H), 7.21 (t, *J* = 7.8 Hz, 1H), 7.17 – 6.97 (m, 6H), 4.53 (d, *J* = 11.5 Hz, 1H), 3.79 – 3.60 (m, 2H), 3.47 (t, *J* = 12.7 Hz, 1H), 3.42 – 3.34 (m, 1H), 2.60 – 2.48 (m, 2H), 2.23 (s, 3H), 2.05 – 1.77 (m, 2H), 1.72 (d, *J* = 13.5 Hz, 2H), 1.61 (d, *J* = 13.3 Hz, 1H), 1.28 – 1.14 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ 138.32, 137.22, 131.77, 130.68, 129.64, 128.96, 128.76, 128.26, 126.95, 70.43, 52.40, 48.68, 35.39, 22.92, 22.10, 21.53; ATR-FTIR ν_{max}/cm^{-1}: 3030, 2943, 2860, 2601, 2425, 2380,

2335, 1606, 1587, 1496; GC-EI-MS *m/z* (relative abundance): 188.1 (100.0%), 105.0 (15.9%), 189.1 (14.8%), 91.0 (13.2%), 41.0 (7.2%).

2.1.17. 4-Methyldiphenidine hydrochloride (**28**, *4-tolphenidine hydrochloride*). Yield = 50%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.17 (s (br), 1H), 7.40 (d, *J* = 7.7 Hz, 2H), 7.16 – 6.99 (m, 7H), 4.55 (d, *J* = 12.3 Hz, 1H), 3.75 (dd, *J* = 13.2, 3.1 Hz, 1H), 3.64 (d, *J* = 12.0 Hz, 1H), 3.47 (t, *J* = 12.7 Hz, 1H), 3.42 – 3.33 (m, 1H), 2.54 – 2.41 (m, 2H), 2.23 (s, 3H), 2.09 – 1.91 (m, 1H), 1.91 – 1.77 (m, 1H), 1.71 (d, *J* = 14.1 Hz, 2H), 1.60 (d, *J* = 13.4 Hz, 1H), 1.28 – 1.12 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ 139.42, 137.29, 131.15, 129.70, 129.62, 128.75, 126.92, 70.15, 52.37, 48.45, 35.36, 22.89, 22.12, 21.27; ATR-FTIR ν_{max}/cm^{-1}: 3430, 3308, 3029, 2939, 2868, 2594, 2575, 2493, 1639, 1604, 1518, 1497, 1457, 1438; GC-EI-MS *m/z* (relative abundance): 188.1 (100.0%), 105.0 (16.4%), 189.1 (15.1%), 91.0 (13.0%), 41.0 (6.6%).

2.1.18. 2-(Trifluoromethyl)diphenidine hydrochloride (**29**). Yield = 29%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.16 (s (br), 1H), 8.60 – 8.52 (m, 1H), 7.86 (t, *J* = 7.1 Hz, 1H), 7.65 – 7.48 (m, 2H), 7.05 (s, 3H), 6.88 – 6.77 (m, 2H), 4.52 – 4.40 (m, 1H), 4.01 (d, *J* = 11.4 Hz, 1H), 3.84 (d, *J* = 12.7 Hz, 1H), 3.38 (t, *J* = 11.8 Hz, 1H), 3.09 – 2.97 (m, 1H), 2.91 (d, *J* = 11.1 Hz, 1H), 2.75 – 2.54 (m, 1H), 2.17 – 1.94 (m, 1H), 1.88 – 1.56 (m, 4H), 1.47 – 1.24 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ 135.55, 133.87, 133.00, 131.55, 130.49, 130.17, 128.51, 127.24, 126.77 (q, *J* = 5.9 Hz), 123.9 (d, *J* = 275.3 Hz), 67.61, 53.13, 51.97, 37.87, 23.20, 23.02, 21.89; ATR-FTIR ν_{max}/cm^{-1}: 2936, 2600, 2442, 2383, 1609, 1587, 1499, 1483, 1455, 1440; GC-EI-MS *m/z* (relative abundance): 242.1 (100.0%), 159.0 (15.7%), 243.1 (14.4%), 41.0 (13.5%), 91.0 (11.1%).

2.1.19. 3-(Trifluoromethyl)diphenidine hydrochloride (**30**). Yield = 34%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.60 (s (br), 1H), 7.96 – 7.84 (m, 2H), 7.67 (d, *J* = 7.6 Hz, 1H), 7.57 (t, *J* = 6.4 Hz, 1H), 7.19 – 6.85 (m, 5H), 4.90 – 4.72 (m, 1H), 3.88 – 3.71 (m, 2H), 3.52 (t, *J* = 11.6 Hz, 1H), 2.68 – 2.49 (m, 2H), 2.09 – 1.94 (m, 1H), 1.94 – 1.79 (m, 1H), 1.79 – 1.66 (m, 2H), 1.61 (d, *J* = 12.0 Hz, 1H), 1.39 – 1.13 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ 136.75, 135.11, 133.84, 130.22, 129.58, 128.80, 127.90 (d, *J* = 3.4 Hz), 127.03, 126.65 (d, *J* = 4.1 Hz), 124.46 (d, *J* = 272.4 Hz), 69.57, 52.30, 49.25, 35.37, 22.79, 22.00; ATR-FTIR ν_{max}/cm^{-1}: 3017, 2939, 2607, 2574, 2485, 1602, 1495, 1454, 1425; GC-EI-MS *m/z* (relative abundance): 242.1 (100.0%), 159.0 (17.8%), 41.0 (14.7%), 243.1 (14.6%), 91.0 (11.1%).

2.1.20. 4-(Trifluoromethyl)diphenidine hydrochloride (**31**). Yield = 41%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.55 (s (br), 1H), 7.78 (d, J = 8.0 Hz, 2H), 7.69 (d, J = 8.2 Hz, 2H), 7.18 – 6.89 (m, 5H), 4.82 – 4.72 (m, 1H), 3.88 – 3.79 (m, 1H), 3.75 (d, J = 11.3 Hz, 1H), 3.49 (t, J = 12.7 Hz, 1H), 2.66 – 2.51 (m, 2H), 2.09 – 1.94 (m, 1H), 1.94 – 1.78 (m, 1H), 1.78 – 1.66 (m, 2H), 1.61 (d, J = 12.8 Hz, 1H), 1.37 – 1.16 (m, 1H); ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ 137.03, 136.70, 132.04, 129.60, 128.83, 126.0 (d, J = 3.8 Hz), 124.4 (d, J = 272.5 Hz), 69.56, 66.87, 52.39, 49.17, 35.37, 22.87, 21.97; ATR-FTIR ν_{max}/cm^{-1}: 3071, 3033, 2936, 2604, 2579, 2495, 1622, 1497, 1475, 1457, 1440, 1428; GC-EI-MS *m/z* (relative abundance): 242.1 (100.0%), 159.0 (18.4%), 243.1 (14.9%), 41.0 (13.1%), 91.0 (10.4%).

2.2. Ultra-High Performance Liquid Chromatography (UHPLC)

UHPLC analysis was performed on a Shimadzu Nexera XR UHPLC (Shimadzu UK Ltd, Milton Keynes, UK) equipped with two binary pumps (LC-30AD) and proportionating valves, degassers (DGU-20A$_{5R}$), autosampler (SIL-30AC), Prominence column oven (CTO-20AC), diode array detector (SPD-M30A) with a 1 µL / 10 mm pathlength flow cell, 40 µL mixer (dwell volume = 342 µL, system volume = 14 µL [31]) and communication bus module (CBM-20A). The system was controlled, and data collected by means of LabSolutions software (Shimadzu UK Ltd, version 5.86). *Sample preparation:* Stock solutions of diphenidine (**1**) and its derivatives (**2** – **33**) were prepared at a concentration of 1 mg mL^{-1} in MeCN/water (1:1 v/v). The individual diphenidine isomers, or mixtures thereof, were diluted to 100 µg mL^{-1} with MeCN/water (1:1 v/v).

2.3. Ultra-High Performance Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (UHPLC-MS/MS)

UHPLC-MS/MS analysis was performed on a Shimadzu Nexera X2 UHPLC (Shimadzu UK Ltd) equipped with two quaternary pumps (LC-30AD) with proportionating valves, degassers (DGU-20A$_{5R}$), autosampler (SIL-30AC), Prominence column oven (CTO-20AC), triple quadrupole mass spectrometer (LCMS-8060), 20 µL mixer (dwell volume = 466 µL, system volume = 14 µL [31]) and communication bus module (CBM-20A). The system was controlled, and data collected by means of LabSolutions software (Shimadzu UK Ltd, version 5.86). *Sample preparation:* Stock solutions of diphenidine (**1**) and its derivatives (**2** – **33**) were prepared at a concentration of 1 mg mL^{-1} in MeCN/water (1:1 v/v). The individual diphenidine isomers, or mixtures thereof, were diluted to 200 pg mL^{-1} (of each isomer) with MeCN/water (1:1 v/v).

2.4. Generic Ultra-High Performance Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (UHPLC-MS/MS) chromatographic conditions

At least 20 column volumes of the appropriate mobile phase were flushed through the columns prior to commencing the testing or on changing the mobile phase conditions. The totally porous ACE Excel C18 (1.7 µm, 100 Å, 100 x 3 mm i.d. and 3 µm, 100 Å, 100 x 3 mm i.d. formats) columns were as supplied by Advanced Chromatography Technologies (Aberdeen, Scotland, UK). UHPLC-MS/MS was performed using the 1.7 µm column whereas the 3 µm column was utilised for UV analysis unless otherwise stated. The integrity of all the columns was confirmed periodically throughout the experiments by injecting a suitable non-polar test mixture (*i.e.* uracil, toluene, biphenyl, dimethyl phthalate and phenanthrene) before and after the experiments. All columns gave retention times, efficiency and peak symmetry levels >95% of their initial value. The degassed mobile phases A and B corresponded to 10 mM aqueous ammonium acetate (unadjusted pH, *approx.* pH 6.5) and 10 mM aqueous ammonium acetate in MeCN/water (2:8 v/v) respectively. Unless otherwise stated the following UHPLC conditions were employed: a flow rate of 0.65 mL min^{-1}, temperature of 60 °C, 5 µL injection volume, the linear gradient consisted of 55 – 100% B over 10 minutes, a hold at 100% B for 1 minute, a linear gradient 100 – 55% B over 0.5 minute and a hold at 55% B for 6.5 minutes to equilibrate the column. The first baseline disturbance for a water injection was used as the dead time (t_M) marker. The photodiode array (PDA) detector was set to monitor a wavelength of 254 nm (bandwidth 8 nm) with a reference at 360 nm (bandwidth 100 nm). The data sampling rate was set at 40 Hz. Chromatographic values reported are the average of duplicate injections. The MS utilised positive mode electrospray ionisation (ESI). The method used 3 L/min nebulizing gas flow, 10 L/min heating gas flow and 10 L/min drying gas flow. The interface temperature was set to 300 °C, the DL temperature was 250 °C and heat block temperature was 400 °C. The dwell time was 20.0 ms and the event time set to 0.092 sec. MRM transitions were optimised using the LabSolutions MRM optimisation tool.

*2.4.1. Optimised standard chromatographic conditions for the separation of 2-, 3- and 4-fluorodiphenidine regioisomers (**14** – **16**)*

The UHPLC-MS/MS conditions as described in Section 2.4 were employed using a column oven temperature of 75 °C.

*2.4.2. Optimised standard chromatographic conditions for the separation of 2-, 3- and 4-chlorodiphenidine regioisomers (**17** – **19**):*

The UHPLC-MS/MS conditions as described in Section 2.4 were employed using a column oven temperature of 30 °C.

*2.4.3. Optimised standard chromatographic conditions for the separation 2-, 3- and 4-(trifluoromethyl)diphenidine regioisomers (**29** – **31**)*

The UHPLC-MS/MS conditions as described in Section 2.4 were employed using a column oven temperature of 30 °C and a 60 min gradient.

2.5. Strong cation exchange (SCX) Liquid Chromatography

A Luna SCX column (150 x 4.6 mm, 100 Å, 5 µm) was employed using chromatographic conditions previously described by Field *et al.* [32]

2.6. Software

Log D and pH values were predicted using ACD/Percepta and ACD/pH calculator (Toronto, Canada, version 2019.1.3).

3.0 Results and Discussion

Reference samples of 33 diphenidine derivatives (**1** – **33**, Fig. 1) were prepared as their corresponding hydrochloride salts. The synthesis and purification of the racemic target compounds was achieved using the previously reported synthetic approach [29] from prerequisite aromatic aldehydes. The salts were obtained as stable, colourless to off-white powders and determined to be soluble (10 mg mL^{-1}) in deionised water, methanol and dimethylsulfoxide. To ensure the authenticity of the materials utilized within this study, the synthesized samples were structurally characterized by ^{1}H-NMR, $^{13}C\{^{1}H\}$-NMR, GC-MS and ATR-FTIR. The analytical data for compounds **1** – **10**, **13**, **32** and **33** (yield: 21 – 77%) corresponded with the data previously reported [6, 7, 29] and the spectral data for compounds **11**, **12**, **14** – **31** (yield: 21 – 66%) is presented in Sections 2.1.1. – 2.1.20). The purity (>99.5%) of the analytes was calculated by ^{1}H NMR using the relative concentration determination method [30].

The separation of the analogous 2-, 3- and 4-fluororegioisomers of 1-[1-(fluorophenyl)-2-phenylethyl]pyrrolidine (fluorolintane, **34**) and 2-, 3- and 4-methoxydiphenidine regioisomers (**2** – **4**) has previously been reported using an ethyl 2-naphthyl bonded reverse phase (RP) column with alkaline conditions and a phenylhexyl RP column with acidic conditions respectively. However, in both reported cases, incomplete separation of certain regioisomers was observed [7, 33]. In contrast, excellent separation between the 2-, 3- and 4-methoxydiphenidine regioisomers was reported using a simple C18 column at intermediate pH [28]. Hence, a generic gradient ultra-high-performance liquid chromatographic (UHPLC) methodology for **1** – **33** was based on those described by Boateng *et al.* [28] where 10 mM aqueous ammonium acetate (*approx.* $^{w}{}_{w}$pH 7 unadjusted) was employed to promote retention *via* electrostatic interactions of the protonated diphenidine derivatives with the ionised silanol groups on the base silica of a C18 RP packing material. A rapid 10 minute, linear, acetonitrile gradient (average retention factor of 7 [34]) eluted all 33 analytes derivatives (log D range at pH 7 = 2.1 – 4.3) within the gradient time window 2.3 – 9.5 minutes (t_M = 0.7 minutes see Fig. 2 and Table 1).

Table 1. Diphenidine nomenclature, molecular formula, retention times, base peak (*m/z*), under the standard gradient UHPLC conditions (Section 2.4) and log D at pH 7.

Cmpd	Common Name / Abbreviation	Molecular Formula (free base)	t_R (min)	Base Peak (*m/z*)	Log D @ pH 7
1	Diphenidine	$C_{19}H_{23}N$	3.995	266.00>181.10	3.02
2	2-Methoxphenidine (2-MXP)	$C_{20}H_{25}NO$	2.295	296.00>211.10	3.15
3	3-Methoxphenidine (3-MXP)	$C_{20}H_{25}NO$	3.948	296.00>211.10	2.82
4	4-Methoxphenidine (4-MXP)	$C_{20}H_{25}NO$	2.843	296.00>211.10	2.57
5	2-Trifluoromethoxphenidine (2-TFMXP)	$C_{20}H_{22}F_3NO$	9.230	350.00>265.00	3.90
6	3-Trifluoromethoxphenidine (3-TFMXP)	$C_{20}H_{22}F_3NO$	8.962	350.00>265.00	3.52
7	4-Trifluoromethoxphenidine (4-TFMXP)	$C_{20}H_{22}F_3NO$	8.460	350.00>265.00	3.13
8	2,3-(Methylenedioxy)diphenidine (2,3-MDDP)	$C_{20}H_{23}NO_2$	4.282	310.00>225.05	2.76
9	3,4-(Methylenedioxy)diphenidine (3,4-MDDP)	$C_{20}H_{23}NO_2$	3.154	310.00>225.05	2.74
10	Mescphenidine (3,4,5-TMXP)	$C_{22}H_{29}NO_3$	2.979	356.10>271.15	2.13
11	(2,2-Difluoro-1,3-benzodiox-4-yl)diphenidine	$C_{20}H_{21}F_2NO_2$	8.993	345.85>261.10	3.18
12	(2,2-Difluoro-1,3-benzodiox-5-yl)diphenidine	$C_{20}H_{21}F_2NO_2$	8.267	345.85>261.10	3.21
13	IAS-013	$C_{22}H_{29}NO_2$	2.911	340.00>255.15	2.90
14	2-Fluorodiphenidine (2-fluphenidine, 2-FP)	$C_{19}H_{22}FN$	6.116	284.00>199.05	3.84
15	3-Fluorodiphenidine (3-fluphenidine, 3-FP)	$C_{19}H_{22}FN$	6.243	284.00>199.05	3.39
16	4-Fluorodiphenidine (4-fluphenidine, 4-FP)	$C_{19}H_{22}FN$	5.086	284.00>199.05	2.96
17	2-Chlorodiphenidine (2-Cl-DPH)	$C_{19}H_{22}ClN$	7.770	299.95>215.05	3.93
18	3-Chlorodiphenidine (3-Cl-DPH)	$C_{19}H_{22}ClN$	7.712	299.95>215.05	3.64
19	4-Chlorodiphenidine (4-Cl-DPH)	$C_{19}H_{22}ClN$	7.722	299.95>215.05	3.23
20	2-Bromodiphenidine (2-Br-DPH)	$C_{19}H_{22}BrN$	8.371	343.90>259.00	4.13
21	3-Bromodiphenidine (3-Br-DPH)	$C_{19}H_{22}BrN$	8.099	343.90>259.00	3.87
22	4-Bromodiphenidine (4-Br-DPH)	$C_{19}H_{22}BrN$	7.750	343.90>259.00	3.43
23	2-Iododiphenidine (2-I-DPH)	$C_{19}H_{22}IN$	9.176	391.90>307.05	3.89
24	3-Iodoodiphenidine (3-I-DPH)	$C_{19}H_{22}IN$	8.594	391.90>307.05	3.70
25	4-Iododiphenidine (4-I-DPH)	$C_{19}H_{22}IN$	8.395	391.90>307.05	3.29
26	2-Methyldiphenidine (2-tolphenidine, 2-TP)	$C_{20}H_{25}N$	5.120	280.00>195.10	3.26
27	3-Methyldiphenidine (3-tolphenidine, 3-TP)	$C_{20}H_{25}N$	4.506	280.00>195.10	3.18
28	4-Methyldiphenidine (4-tolphenidine, 4-TP)	$C_{20}H_{25}N$	4.297	280.00>195.10	3.21
29	2-(Trifluoromethyl)diphenidine (2-TFD)	$C_{20}H_{22}F_3N$	9.475	334.00>249.05	4.30
30	3-(Trifluoromethyl)diphenidine (3-TFD)	$C_{20}H_{22}F_3N$	8.500	334.00>249.05	4.22
31	4-(Trifluoromethyl)diphenidine (4-TFD)	$C_{20}H_{22}F_3N$	8.610	334.00>249.05	3.82
32	2-Naphthenidine (2-NPD)	$C_{23}H_{25}N$	6.300	316.00>231.00	4.00
33	1-Naphthenidine (1-NPD)	$C_{23}H_{25}N$	7.747	316.00>231.00	3.89

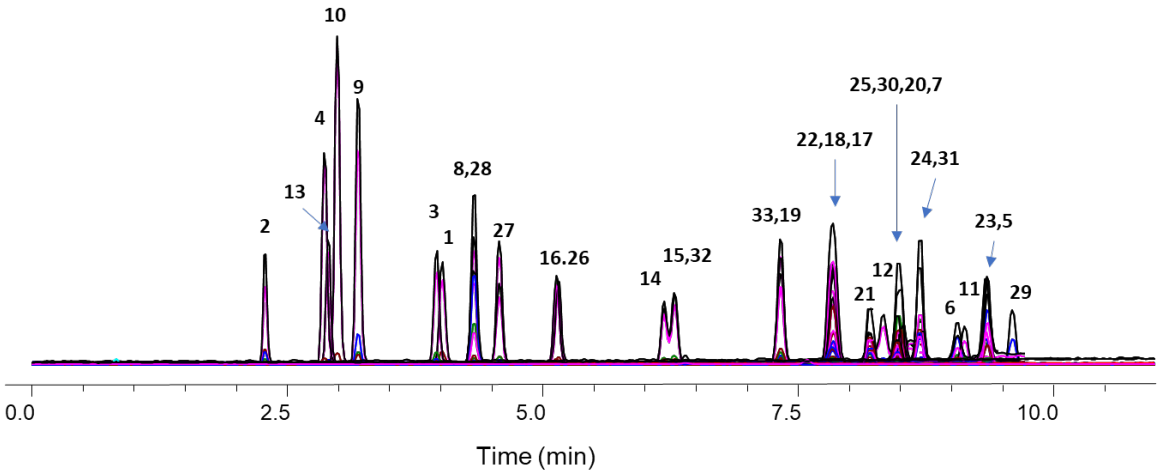

Fig. 2. Overlaid UHPLC-MS/MS (ACE Excel C18 1.7 µm, 100 Å, 100 x 3 mm i.d.) chromatogram of the diphenidine derivative (**1** – **33**) transitions on the standard gradient (for peak assignments see Fig. 1 and Table 1.)

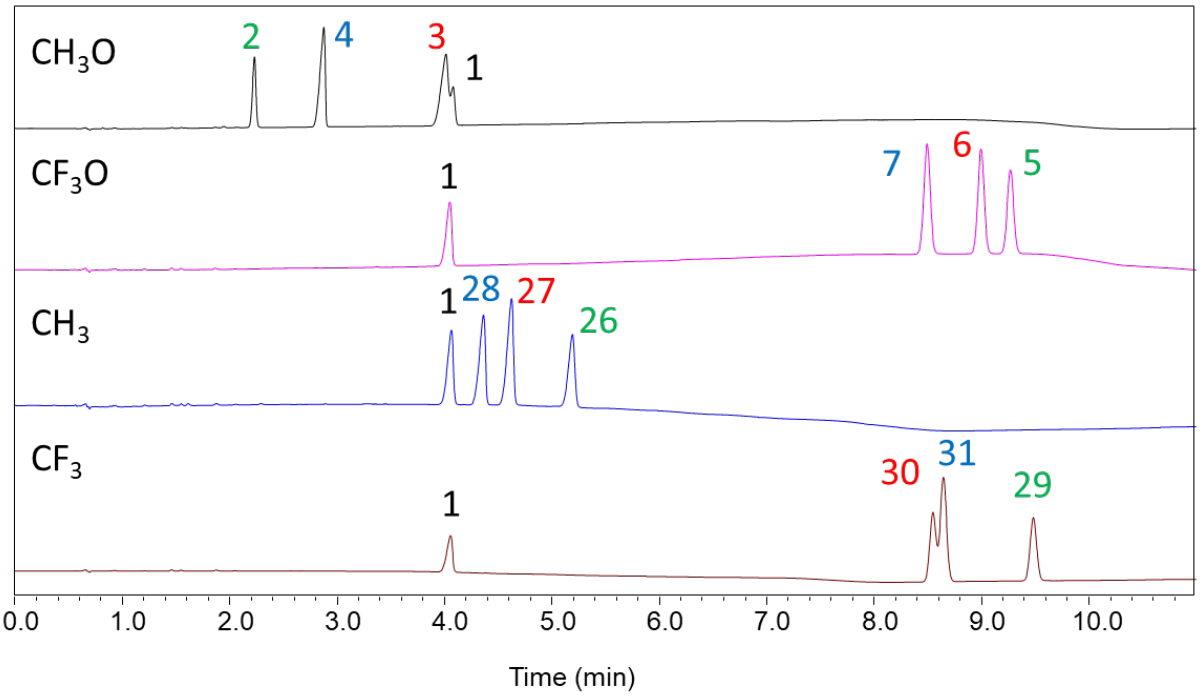

Fig. 3. Gradient UHPLC-UV (ACE Excel C18 3 µm, 100 Å, 100 x 3 mm i.d.) separation of the regioisomers of the methoxy- (**2**, **3** and **4**), trifluoromethoxy- (**5**, **6** and **7**), methyl- (**26**, **27** and **28**) and trifluoromethyldiphenidine (**29**, **30** and **31**) derivatives. Chromatograms also contain diphenidine (**1**) as a control to highlight the repeatability of the method.

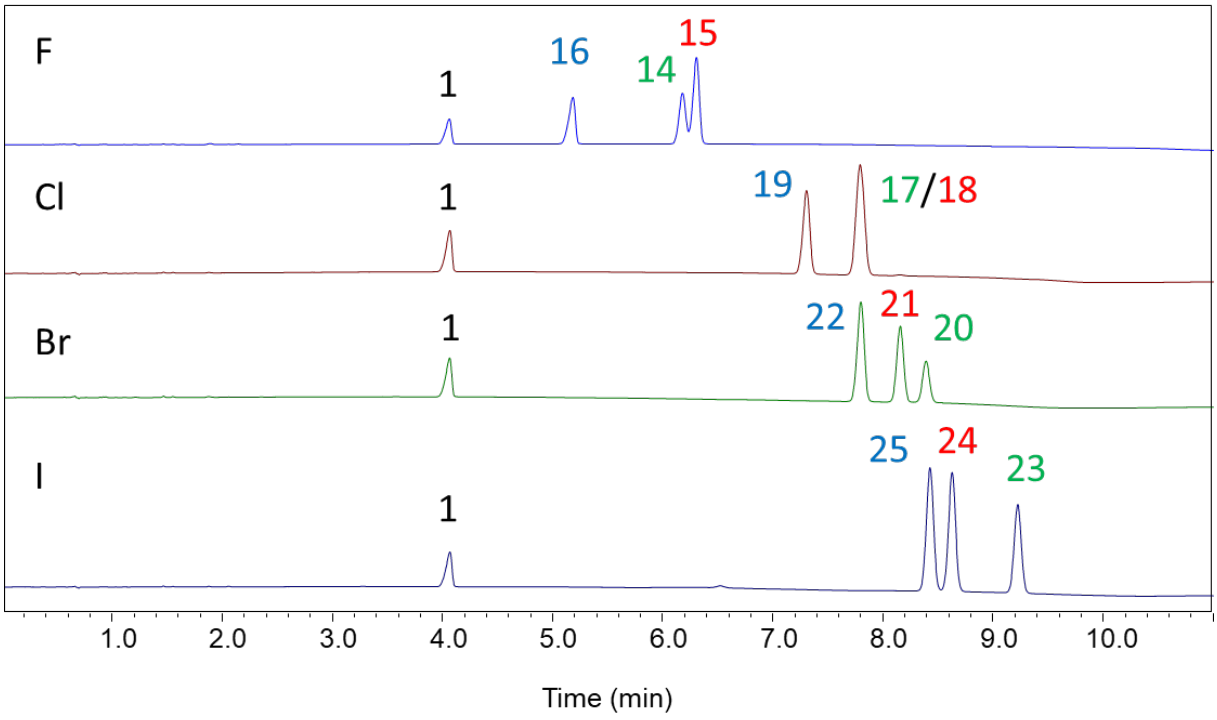

Fig. 4. Gradient UHPLC-UV (ACE Excel C18 3 µm, 100 Å, 100 x 3 mm i.d.) separation of the regioisomers of the fluoro- (**14**, **15** and **16**), chloro- (**17**, 1**8** and **19**), bromo- (**20**, **21** and **22**) and iododiphenidine (**23**, **24** and **25**) derivatives. Chromatograms also contain diphenidine (**1**) as a control to highlight the repeatability of the method.

As the regioisomeric mono-substituted diphenidines (**2** – **4**, **5** – **7**, **14** – **16**, **17** – **19**, **20** – **22**, **23** – **25**, **26** – **28** and **29** – **31**) possess the same molecular formulae, it was essential to be able to chromatographically separate them before mass spectral analysis. It had previously been shown that it was not possible to differentiate regioisomers of similar psychoactive drugs from their positive ion ESI MS/MS fragmentation pattern [7, 33]. The standard UHPLC gradient conditions described in Section 2.4 afforded baseline separation of the methoxy- (methoxphenidines, **2**, **3** and **4**), trifluoromethoxy- (**5**, **6** and **7**), bromo- (**20**, **21** and **22**), iodo- (**23**, **24** and **25**), and methyl- (tolphenidines**, 26**, **27** and **28**) regioisomeric triplets (Fig. 3 and Fig. 4).

Under these conditions only partial separation of the *meta*- and *para*-trifluoromethyldiphenidine (**30**, t_R = 8.50 min; **31**, t_R = 8.61 min) (Fig. 3), *ortho*- and *meta*-fluorodiphenidines (fluphenidines**; 14**, t_R = 6.12 min; **15**, t_R = 6.24 min) and co-elution of the *ortho*- and *meta*-chlorodiphenidines (**17**, t_R = 7.77 min; **18**, t_R = 7.71 min) (Fig. 4) was achieved. Retention of the derivatives **1 - 33** was poorly correlated to their estimated log D values at pH 7 (r^2 = 0.56). The correlation was only slightly better for the 4-halogenated

regioisomers (r^2 = 0.79) highlighting that hydrophobicity was not the sole retention mechanism. This supports previous observations which demonstrated that the chromatographic retention of **2**, **3** and **4** was multimodal in nature involving hydrophobicity, ion exchange and possibly steric/shape accessibility of the regioisomers into the stationary phase [28].

Baseline separation of the fluphenidine (**14 – 16**) and chlorodiphenidine (**17 – 19**) regioisomers could be achieved by employing the generic UHPLC conditions at either increased (75 °C) or decreased (30 °C) column oven temperatures respectively (see Fig. 5). Interestingly, the fluorolintanes (**34** – **36**), which possess a pyrrolidine rather than a piperidine moiety in the 1-position, have been shown to be separated in the same elution order as observed for the fluphenidines in this study (*i.e. para-*, < *ortho-*, < *meta-*) using an isocratic elution with unadjusted aqueous ammonium formate/MeOH at 60 °C on an ACE C18-AR column [35]. In comparison, when the **34**, **35** and **36** were separated in their unionised form on a π-π rich Cosmosil π-NAP phase, the elution order differed (*i.e. ortho-*, < *meta-*, < *para-*) [33] which suggested the dominance of an alternative retention mechanism (*i.e.* hydrophobic retention mechanism) to that described in this study (*i.e.* hydrophobic, electrostatic, and steric retention mechanisms).

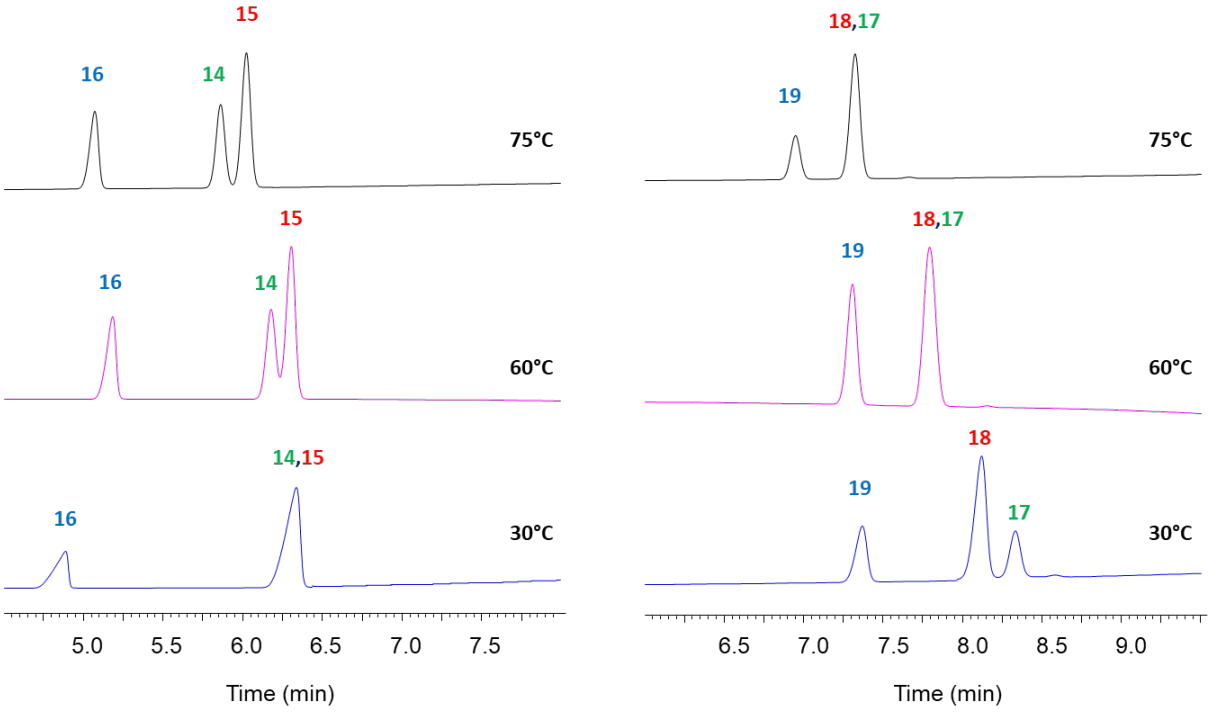

Fig. 5. Gradient UHPLC-UV (ACE Excel C18 3 µm, 100 Å, 100 x 3 mm i.d.) effect of temperature on the fluorodiphenidine (**14 – 16**) and chlorodiphenidine (**17 – 19**) derivatives (t_G = 10 min).

In order to afford baseline separation of **29 – 31** (trifluoromethyldiphenidines) it was necessary to reduce the oven temperature to 30 °C and to increase the gradient time (i.e., average retention factor increased from 7 to 42) (Fig. 5).

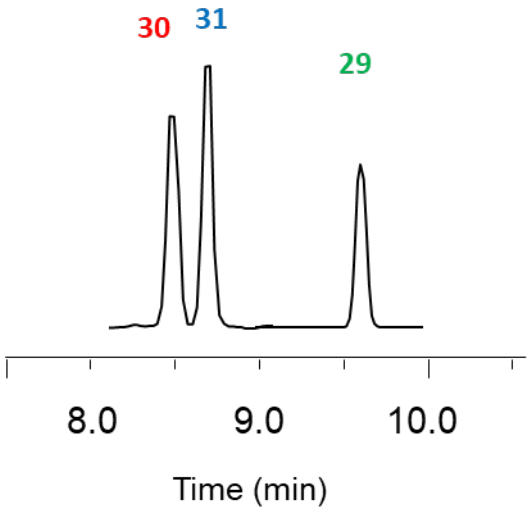

Fig. 6. Optimised gradient UHPLC-UV (ACE Excel C18 3 µm, 100 Å, 100 x 3 mm i.d.) separation of the *ortho*-, *meta*- and *para*-trifluoromethyldiphenidines (**29**, **30** and **31**) at 30 °C (t_G = 60 min).

The elution order of the regioisomers was not the same for all derivatives and may be dependent on the size and/or accessibility of the analyte(s) into the stationary phase (see Table 2). This is highlighted in the halogenated series of derivatives in that the elution order of the *ortho*- and *meta*-isomers changes for the smaller fluoro-substituents compared to the larger chloro-, bromo- and iodo-substituents (see Table 2). There also appeared to be no pattern to the elution order based on the electron-withdrawing (*i.e.* halogen, trifluoromethyl- and/or trifluoromethoxy-) or electron-donating (*i.e.* methoxy- or methyl-) ability of the substituents. The elution order of methoxphenidines **2** – **4** using a strong cation exchange column (Luna SCX column at pH 2.5) was observed to follow the elution order *ortho*- (**2**), < *meta*- (**3**), < *para*- (**4**). However the elution order, for these derivatives, on reverse phase columns, was observed to be different *i.e.,* the elution order was observed to be *ortho*- (**2**), < *para*- (**4**), < *meta*- (**3**) respectively [28]. This indicated that the retention mechanism on reverse phase columns was not solely due to the electrostatic interaction of the protonated diphenidines with the ionised silanol groups, but presumably involves a synergistic effect with a hydrophobic mechanism and/or the accessibility of the analyte into the reverse phase material (*i.e.* reverse phase and ion exchange processes) [28]. A switch in the elution order between diphenidine (**1**,

log D at pH 7 = 3.02) and 2-naphthenidine (**32,** log D at pH 7 = 4.00) derivatives was also observed on the reverse phase and strong cation exchange columns. The more hydrophobic 2-naphthyl derivative was less retained on the SCX column while it was retained more on the RP column intimating an enhanced hydrophobic retention mechanism on the more hydrophobic reverse phase column. In addition, the generic gradient UHPLC conditions afforded excellent separation between the isomeric 1-naphthenidine (**33**, t_R = 6.6 min) and 2-naphthenidine (**32**, t_R = 7.7 min) derivatives.

Table 2. Elution order of mono-substituted diphenidines (**2** – **4**, **5** – **7**, **14** – **16**, **17** – **19**, **20** – **22**, **23** – **25**, **26** – **28** and **29** – **31**) under (a) the standard gradient UHPLC (Section 2.4) or (b) optimised gradient UHPLC conditions (Section 2.4.2) for chlorophenidines (**17** – **19**).

Mono-substituted diphenidine	Subs.	UHPLC Conditions	1st	2nd	3rd
Methoxphenidines (**2** – **4**)	MeO-	a	**2**, *ortho-*	**4**, *para-*	**3**, *meta-*
Trifluoromethoxphenidines (**5** – **7**)	CF_3O-	a	**7**, *para-*	**6**, *meta-*	**5**, *ortho-*
Fluorodiphenidines (fluphenidines, **14** – **16**)	F-	a	**16**, *para-*	**14**, *ortho-*	**15**, *meta-*
Chlorodiphenidines (**17** – **19**)	Cl-	b	**19**, *para-*	**18**, *meta-*	**17**, *ortho-*
Bromodiphenidines (**20** – **22**)	Br-	a	**22**, *para-*	**21**, *meta-*	**20**, *ortho-*
Iododiphenidines (**23** – **25**)	I-	a	**25**, *para-*	**24**, *meta-*	**23**, *ortho-*
Methyldiphenidines (tolphenidines, **26** – **28**)	CH_3-	a	**28**, *para-*	**27**, *meta-*	**26**, *ortho-*
Trifluoromethyldiphenidines (**29** – **31**)	CF_3-	a	**30**, *meta-*	**31**, *para-*	**29**, *ortho-*

3.1 Forensic application of UHPLC-MS/MS method to seized street samples

Two suspected diphenidine containing samples (Samples A and B) were provided to MANchester DRug Analysis and Knowledge Exchange (MANDRAKE), between June – October 2016, by Greater Manchester Police, in accordance with Manchester Metropolitan University's Home Office license requirements and agreed procedures. Preliminary qualitative GC-EI-MS analysis, using Geyer's previously reported method [29] indicated that the samples contained diphenidine (Sample A: t_R = 23.7 min, *m/z* (base peak) = 174 $[M + H]^+$) and 2-methoxphenidine (Sample B: t_R = 28.1 min, *m/z* (base peak) = 204 $[M + H]^+$) respectively, with no apparent adulteration. Common adulterants detected in seized street samples include caffeine, paracetamol, procaine and benzocaine, however none of these compounds were proven to interfere with the UHPLC-UV or UHPLC-MS/MS methodologies as they elute at least one minute (retention time <1.5 minute, whereas the t_M of the column corresponded to

0.7 min) before the first diphenidine derivative (**2**, t_R = 2.3 min) elutes. The combination of a triple quadrupole ESI MS/MS using positive mode ionisation with the UHPLC analysis facilitated the rapid, sensitive detection and regioisomeric discrimination of these psychoactive substances within samples suspected to contain them. Automated flow injection analysis of the individual diphenidines was employed for the rapid optimisation of multiple reaction monitoring (MRM) conditions. At least three MRM transitions, for each analyte, together with retention times, allowed for the unambiguous analyte confirmation using the standard gradient UHPLC methodology (see Table 1). The base peak observed for all derivatives corresponded to loss of the piperidine moiety (*i.e.,* loss of *m/z* 85) from the protonated diphenidine molecule [7, 33]. The positive ESI UHPLC-MS/MS fragmentation pattern for both **2** – **4** and fluphenidines **14** – **16** was observed to be analogous for those reported for the corresponding methoxydiphenidine and fluorolintane regioisomers [7, 33]. Detection limits better than 200 pg mL^{-1} were easily achievable (the methodology was not optimised for sensitivity as the current study was not sample limited). If a sample showed positive for either the 2-chlorophenidine (**17**) or 3-chlorophenidine (**18**) which co-eluted on the generic UHPLC gradient methodology, then the extended optimised UHPLC-MS/MS methodology to separate these derivatives could be employed (see Fig. 5).

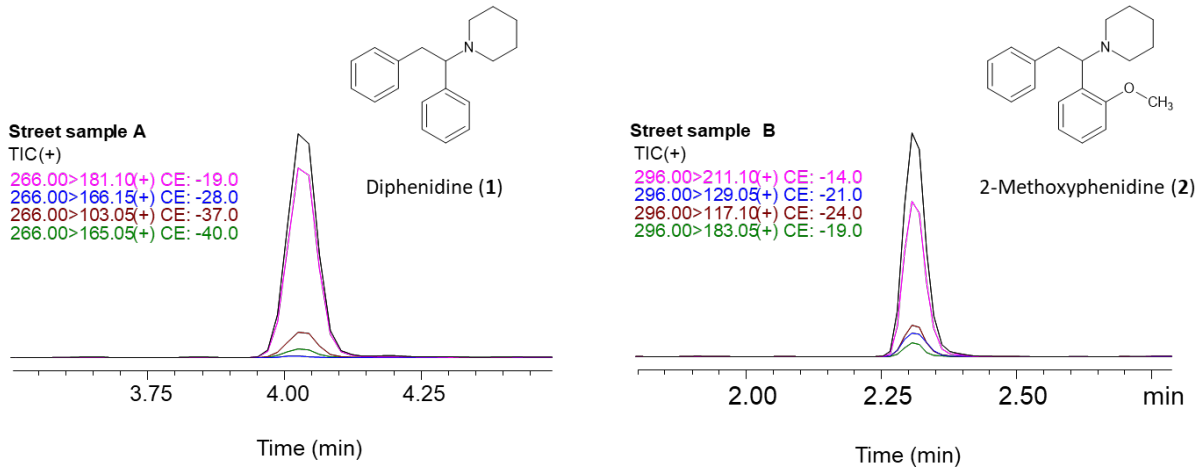

Fig. 7. UHPLC-MS/MS (ACE Excel C18 1.7 µm, 100 Å, 100 x 3 mm i.d.) identification of street samples (A and B) using the generic gradient conditions with targeted MRM.

The generic UHPLC-MS/MS methodology was successfully applied to the seized street samples which were unambiguously confirmed, using the MRM transitions, to contain diphenidine (Sample A: t_R = 3.995 min, *m/z* (base peak) = 181) and 2-methoxphenidine (Sample B: t_R = 2.295 min, *m/z* (base peak) = 211) respectively (see Fig. 7), which is concordant with preliminary GC-EI-MS analysis. This UHPLC-MS/MS method provides, for

the first time, a general screen for active components within seized bulk samples, which is significantly superior to the previously reported GC–MS [29] and HPLC [7, 28] methods. It offers rapid analysis (<10 mins), improved selectivity and the ability to differentiate between a more diverse variety of structural isomers within the diphenidine class of dissociative NPS, should these derivatives become prevalent on the illicit market.

4.0 Conclusion

Using a synthesised library of 33 racemic diphenidine derivatives including the *ortho*-, *meta*- and *para*- regioisomers of eight common diphenidine derivatives, a rapid, targeted UHPLC-MS/MS method has been successfully developed to unequivocally detect (LOD = 200 pg mL^{-1}) the active component in seized solid drug samples within 10 minutes. If the presence of *ortho*- or *meta*-chlorodiphenidines (**18** or **19**) is suspected, then an optimised chlorophenidine regioisomer screen, employing a lower operating temperature of 30 °C, can be utilised to separate the three regioisomers. The fluorophenidine (fluphenidines, **14** – **16**) and trifluoromethyldiphenidine regioisomers (**29** – **31**) should be quantifiable using standard conditions. If further confirmation or quantification is needed, then analysis can be performed on their individual optimised screens (*i.e.* fluorophenidine regioisomeric screen at 75 °C and/or the trifluoromethyldiphenidine regioisomeric screen using an extended gradient at 30 °C).

The diphenidine derivatives were monitored by using at least three MRM transitions for each analyte which, together with retention times, allowed for rapid and unambiguous peak identity and confirmation. The common adulterants (caffeine, paracetamol, procaine and benzocaine) found in seized street samples were demonstrated not to interfere with this UHPLC-MS/MS methodology. The described methodology was used to unambiguously confirm the identity of two seized street samples purported to contain either diphenidine (**1**) or 2-methoxphenidine (**2**). The elution orders of the *ortho*-, *meta*- and *para*-substituted diphenidine regioisomers was not the same in all cases and suggested that the elution order may be dependent on the size and/or accessibility of the individual regioisomers into the stationary phase and their electrostatic interaction with the ionised silanol groups on the base silica of the stationary phase as well as hydrophobic interactions with the stationary phase's octadecylsilane ligands.

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6.0 Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7.0 Author Contributions

Jennifer K. Field: Methodology, Formal analysis, Investigation.

Christine Hinz: Methodology, Formal analysis, Investigation.

Christopher M. Titman: Methodology, Formal analysis, Investigation.

Matthew C. Hulme: Methodology, Formal analysis, Investigation.

Rhona Cowan: Methodology, Formal analysis, Investigation

Jack B. Ainsworth-McMillan: Methodology, Formal analysis, Investigation.

Nicolas Gilbert: Methodology, Formal analysis, Investigation.

Robert J. Lee: Methodology, Formal analysis, Investigation.

Jack Marron: Methodology, Formal analysis, Investigation.

Andrew Costello: Methodology, Resources.

Ryan E. Mewis: Methodology, Formal analysis, Investigation, Supervision.

Melvin R. Euerby: Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing, Supervision.

Oliver B. Sutcliffe: Conceptualization, Methodology, Writing – review & editing, Supervision.

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