

Experimental versus theoretical log D7.4, pKa and plasma protein binding values for benzodiazepines appearing as new psychoactive substances

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1 Experimental versus theoretical log $D_{7.4}$, pK_a and plasma protein binding values for
2 benzodiazepines appearing as new psychoactive substances
3

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11 **Abstract**

12 The misuse of benzodiazepines as new psychoactive substances is an increasing problem
13 around the world. Basic physicochemical and pharmacokinetic data is required on these
14 substances in order to interpret and predict their effects upon humans. Experimental log $D_{7.4}$,
15 pK_a and plasma protein binding values were determined for 11 benzodiazepines that have
16 recently appeared as new psychoactive substances (3-hydroxyphenazepam, 4'chlorodiazepam,
17 desalkylflurazepam, deschloroetizolam, diclazepam, etizolam, flubromazepam,
18 flubromazolam, meclonazepam, phenazepam and pyrazolam) and compared with values
19 generated by various software packages (ACD/I-lab, MarvinSketch, ADMET Predictor and
20 PreADMET). ACD/I-LAB returned the most accurate values for log $D_{7.4}$ and plasma protein
21 binding while ADMET Predictor returned the most accurate values for pK_a . Large variations
22 in predictive errors were observed between compounds. Experimental values are currently
23 preferable and desirable as they may aid with the future 'training' of predictive models for
24 these new psychoactive substances.
25
26
27

28 **Keywords:** logD; pK_a ; plasma; benzodiazepines; NPS

29 **1. Introduction**

30 New psychoactive substances (NPSs) are an increasing problem around the world [1].
31 Benzodiazepines are one of a number of groups of NPSs that have appeared on the illicit drug
32 market [2]. They also exist as common prescription drugs for anxiety, insomnia and other
33 medical conditions [3]. Benzodiazepines were misused long before emerging as new
34 psychoactive substances and a recent report highlighted the increasing illicit availability and
35 misuse of a clinically-used benzodiazepine, alprazolam, often purchased from the dark web
36 [4]. The new psychoactive substance benzodiazepines (referred to in this work as NPS-
37 benzodiazepines) have already been reported in a number of overdose cases, driving under the
38 influence of drugs (DUID) cases and hospital admissions [5–8]. The lack of control and safety
39 over these NPS-benzodiazepines is a prevalent issue and it is predicted that it will become an
40 even more worrying trend as their misuse continues to rise. A number of these compounds
41 were originally prescription drugs such as phenazepam (Russia) as well as etizolam and
42 flutazolam (Japan) [9–11]. Some of these compounds never gained marketing approval (e.g.
43 adinazolam) but the majority were simply patented and never brought to market and, as such,
44 there is a deficit of physiochemical and pharmacokinetic data that would otherwise exist if they
45 had undergone clinical trials [12]. However, such information is essential to fully understand
46 the pharmacological behaviour of these compounds, especially as they are becoming more and
47 more prevalent on the illicit drug market. This paper focuses on two physiochemical properties
48 ($\log D_{7.4}$ and pK_a) and one pharmacokinetic property (plasma protein binding).

49 The lipophilicity of a compound is often expressed by the term $\log D_{7.4}$, this is the distribution
50 coefficient and represents the relative ratios of a compound in an organic and aqueous solvent
51 at the physiologically-relevant pH of 7.4 [13]. Lipophilicity has various pharmacokinetic
52 implications such as affecting a compound's absorption through cell membranes and its
53 distribution in biological tissues and accordingly is important for the prediction of many of

54 these pharmacokinetic parameters [14,15]. Highly-lipophilic compounds typically exhibit
55 greater plasma protein binding and can generally cross the blood-brain barrier with greater ease
56 [16,17]. The majority of the well-known, from herein referred to as ‘classic’, benzodiazepines
57 have comparatively high values for lipophilicity and can therefore partition with ease across
58 cellular membranes and accumulate in areas of the body that are high in lipids [18,19].
59 Furthermore, benzodiazepines also have high volumes of distribution (V_d) such as diazepam
60 with a V_d at steady state of 0.88 – 1.39 L kg⁻¹ [20–23]. The lipophilicity (as log P) of some
61 NPS-benzodiazepines has already been published in literature [24].

62 The acid-base dissociation constant (pK_a) of a compound is typically investigated during
63 pharmaceutical development and plays an important role when used in conjunction with other
64 parameters such as molecular weight and lipophilicity [25]. pK_a can affect the site in the body
65 where compounds are absorbed [26] and can also assist with the development of extraction
66 methods from biological samples [27].

67 Upon administration to the body, compounds bind to proteins present within the plasma, this
68 is reflected through measurement of plasma protein binding values [28,29]. The fraction that
69 is not bound (known as the unbound or free fraction) is responsible for the pharmacological
70 effect and it is this fraction that undergoes metabolism and excretion [18]. The majority of the
71 classic benzodiazepines are highly protein-bound such as diazepam (99 % bound) but some
72 experience vastly lower binding, for example bromazepam (60 % bound) [30,31]. Reducing
73 clearance (Cl) and increased plasma protein binding generally correlates with an increase in
74 half-life ($t_{1/2}$) of a drug [32]. Knowledge of plasma protein binding is therefore important to
75 help characterise pharmacokinetics of drugs without *in vivo* studies. There has already been
76 interest in the determination of these properties for new psychoactive substances, for example
77 the plasma protein binding of flubromazolam (89 %) has recently been published in the

78 literature [33]. Yet for many of the more recently synthesised benzodiazepines the percentage
79 bound is as yet unknown.

80 As many of these compounds have never undergone clinical trials, and are unlikely to as a
81 result of the time and expense involved, it is critical that such analysis is undertaken, especially
82 for the future prediction of any newly emerging psychoactive substances. The use of predictive
83 software could be an attractive alternative to *in vitro* experiments to calculate these properties
84 and this research will focus upon comparison of some predictive software packages with
85 experimental values.

86 **2. Materials and methods**

87 Eight benzodiazepines that had values available in the literature for log $D_{7.4}$, pK_a and plasma
88 protein binding were chosen to examine the suitability of the devised methods (alprazolam,
89 clonazepam, diazepam, flunitrazepam, nitrazepam, oxazepam, prazepam and temazepam).
90 These three properties were then investigated experimentally for a further 11, as yet,
91 uncharacterised benzodiazepines, recently appearing as new psychoactive substances (3-
92 hydroxyphenazepam, 4'-chlorodiazepam, desalkylflurazepam, deschloroetizolam, diclazepam,
93 etizolam, flubromazepam, flubromazolam, meclonazepam, phenazepam and pyrazolam). The
94 chemical structures of this latter group of compounds can be found in the Supplementary
95 Information.

96 **2.1. Materials**

97 4'-chlorodiazepam, alprazolam, clonazepam, desalkylflurazepam, diazepam, flunitrazepam,
98 nitrazepam, oxazepam, prazepam and temazepam were obtained from Sigma-Aldrich (Dorset,
99 UK). 3-hydroxyphenazepam, deschloroetizolam, diclazepam, etizolam, flubromazepam,
100 flubromazolam, meclonazepam, phenazepam and pyrazolam were obtained from Chiron
101 (Trondheim, Norway). All compounds were received as powdered solids.

102 Dimethyl sulfoxide (DMSO), methanol, phosphoric acid, sodium hydrogen phosphate
103 heptahydrate, sodium dihydrogen phosphate, disodium hydrogen phosphate, acetic acid,
104 sodium acetate trihydrate, boric acid, sodium hydroxide, hydrochloric acid, sodium chloride
105 and octan-1-ol were purchased from Fisher Scientific (Leicestershire, UK). Phosphate buffered
106 saline (PBS) tablets were purchased from Sigma-Aldrich (Dorset, UK).

107 Human plasma (pooled, from three male donors and three female donors) was obtained from
108 Seralab (West Sussex, UK). Plasma was received frozen with sodium citrate as an
109 anticoagulant.

110 **2.2. Methods**

111 **2.2.1. Determination of log D_{7.4}**

112 The shake-flask method is commonly used in determining log D_{7.4} values [34]. The compound
113 of interest is dissolved in equal volumes of a buffer at a specified pH and an organic solvent,
114 such as octanol. Following equilibration the octanol and buffer are separated and the
115 concentration of the compound in each is determined. The log D_{7.4} is then calculated using
116 Equation 1.

$$\log D = \frac{\text{Compound concentration in aqueous phase}}{\text{Compound concentration in organic phase}} \quad (1)$$

117 Sodium phosphate buffer (0.01 M) was formulated using deionised water (Barnstead
118 UltraPure) and filtered through a 0.45 µm Nylon Phenex filter membrane (Phenomenex,
119 Cheshire, UK) using a Millipore filtration apparatus (Merck Millipore, Hertfordshire, UK).

120 Compounds were dissolved in methanol at a concentration of 1 mg ml⁻¹. Aliquots of compound
121 solution were evaporated with a flow of nitrogen using a TurboVap to yield 0.20 mg of
122 compound. Equal volumes (700 µl) of sodium phosphate buffer (0.01 M, pH 7.4) and octan-1-
123 ol were added and the samples were vortexed for 30 seconds.

124 The samples were transferred into 1.5 mL Eppendorf microcentrifuge tubes and placed on a
 125 Stuart SB3 rotator (Bibby Scientific, Staffordshire UK) and rotated at 40 rpm for four hours.
 126 Samples were then centrifuged at 10,000 rpm for 20 minutes. The separated octanol and buffer
 127 phases were collected and analysed using high performance liquid chromatography (HPLC)
 128 coupled to a diode array detector (DAD). Further details of the method employed are given in
 129 Section 2.4. Each log D determination was repeated in triplicate.

130 2.2.2. Determination of pK_a

131 Capillary electrophoresis is a common method of measuring pK_a [35]. The basic principle
 132 behind this technique is an applied electrical voltage which separates ions according to their
 133 electrophoretic mobility. When the solute is unionised it has no mobility and when an electrical
 134 voltage is applied and it is fully ionised it has maximum electrophoretic mobility. The mobility
 135 of the solute between these two extremes is a function of the dissociation equilibrium. The
 136 effective electrophoretic mobility of a compound can be calculated by using the difference in
 137 migration time between the test compound and a neutral marker [35].

$$\mu_{eff} = \left(\frac{L_d L_t}{V}\right) \left(\frac{1}{t_a} - \frac{1}{t_m}\right) \quad (2)$$

138 In Equation , t_a is the migration time for the test compound (s), t_m is the migration time for the
 139 neutral marker (s), L_d is the total length from the capillary inlet to the detection window (cm),
 140 L_t is the total capillary length (cm) and V is the applied voltage (V). As a result of the differences
 141 in pH there can be variations in electroosmotic flow but these are corrected for by using a
 142 neutral compound as a marker and adjusting for this in the calculation of effective mobility.

$$\mu_{eff} = \frac{\alpha \times 10^{-pH}}{10^{-pK_a} + 10^{-pH}} \quad (3)$$

$$\mu_{eff} = \frac{b_1(10^{-pH})^2 + a_1 10^{-pK_{a1}} 10^{-pK_{a2}}}{(10^{-pH})^2 + 10^{-pK_{a1}} 10^{-pH} + 10^{-pK_{a1}} 10^{-pK_{a2}}} \quad (4)$$

143 Equations (3) and (4) describe the relationship between the effective electrophoretic mobility
144 of a compound and its pK_a for benzodiazepines with one ionisable basic group and an ionisable
145 basic and acidic group [36].

146 Phosphate, acetate and borate buffers were utilised as described elsewhere with a pH spacing
147 of 0.5 pH units [36]. All buffers had an ionic strength of $I=0.05$ and a concentration of 0.05 M.
148 Sodium chloride was used to adjust the ionic strength and hydrochloric acid (0.1 M) or sodium
149 hydroxide (0.1 M) were used to adjust the pH values if necessary. The pH was measured with
150 a Jenway 3505 pH meter (Jenway, Essex, UK) which was calibrated before use. Buffers were
151 filtered prior to use through a 0.45 μm Nylon Phenex filter membrane (Phenomenex, Cheshire,
152 UK) using a Millipore filtration apparatus (Merck Millipore, Hertfordshire, UK).

153 Compounds were dissolved in methanol at a concentration of 1 mg ml^{-1} . Solutions were diluted
154 to 0.25 mg ml^{-1} with deionised water (Barnstead UltraPure) and contained DMSO as the
155 electroosmotic flow marker (1 % v/v).

156 DMSO (1 % v/v) in deionised water (Barnstead UltraPure) was run at each pH before
157 experimental repeats to ensure that an expected electrophoretic mobility was obtained.

158 Compound migration times were determined using a Beckman Coulter P/ACE MDQ Capillary
159 Electrophoresis System with a diode array detector (Beckman-Coulter, High Wycombe, UK).
160 The internal capillary temperature was set at 25 $^{\circ}\text{C}$ using the liquid cooling system. Sample
161 injection was conducted at 1.0 psi for 10 seconds and then 20 kV voltage was applied during
162 separations. The capillary was rinsed between each run in the following manner; NaOH applied
163 at 20 psi for 1.0 minute followed by the appropriate buffer for the next repeat at 20 psi for 2.0
164 minutes.

165 Experimentally determined μ_{eff} values were obtained using Equation 2. The Microsoft Excel
166 add-in, Solver, was used to calculate the pK_a value using least-squares regression. An initial

167 'best-guess' estimate for the pK_a and α values were used to calculate theoretical effective
168 mobilities and the squared difference (the residuals) between these theoretical values and
169 experimental values was then calculated and then minimised by varying the values for pK_a and
170 α .

171 For pK_a measurements, accuracy is defined as a measured value being within 0.20 units from
172 the literature value and precision is defined as a measured value having a repeatability that is
173 equal to or less than 0.07 units [35]. Each pK_a measurement was repeated in triplicate.

174 **2.2.3. Determination of plasma protein binding**

175 Plasma protein binding values were determined using the commonly-used method of
176 equilibrium dialysis [37].

177 Frozen plasma was thawed at room temperature prior to the experiments. The pH was measured
178 with a Jenway 3505 pH meter (Jenway, Essex, UK) which was calibrated before use. Plasma
179 pH was found to be within the physiological range of 7.38 – 7.42 and adjustment was not
180 required [38].

181 PBS tablets were dissolved in deionised water (Barnstead UltraPure) to yield a buffer solution
182 that contained 0.01M phosphate, 0.0027M KCl, and 0.137M NaCl, pH 7.4 at 25 °C. Stock
183 solutions of compounds in DMSO at a concentration of 10 mM were created and were diluted
184 with PBS prior to the experiments to yield working solutions at a concentration of 200 μ M.

185 Reusable Single-Sample Fast Micro-Equilibrium Dialyzers (500 μ L volume) were obtained
186 from Harvard Apparatus (Cambridge, UK), as were cellulose acetate membranes with a
187 molecular weight cut-off (MWCO) of 10,000 Da.

188 The membranes were soaked for 30 minutes in deionised water (Barnstead UltraPure) and
189 rinsed thoroughly. 30 μ L of compound working solution was added to 270 μ L of plasma to
190 yield a final concentration of 20 μ M of compound (final DMSO concentration 0.2 %). This

191 was placed in one chamber and 500 μ L of PBS was placed in the second chamber. The Micro-
192 Equilibrium Dialyzers were then placed into a shaking waterbath held at 37 $^{\circ}$ C for 24 hours.
193 The temperature was monitored with a Sentry Thermometer (Fisher Scientific, Leicestershire,
194 UK). After 24 hours had elapsed, the samples were extracted from each chamber, matrix
195 matched (with blank plasma or blank buffer). Ice-cold acetonitrile at a 4:1 ratio was then added
196 to precipitate proteins. The samples were centrifuged at 10,000 rpm for 20 minutes and the
197 supernatant was recovered and evaporated using a flow of nitrogen with a TurboVap. The
198 samples were then reconstituted in 200 μ L of acetonitrile and analysed using HPLC-DAD.
199 Details of this analysis are given in Section 2.4. Each plasma protein binding measurement was
200 repeated in triplicate.

201 Plasma protein binding (PPB) was calculated using the experimental plasma concentration
202 (P_{exp}) and the experimental buffer concentration (B_{exp}) according to Equation (5).

$$PPB (\%) = 100 \times \frac{P_{exp} - B_{exp}}{P_{exp}} \quad (5)$$

203 For those benzodiazepines that were highly protein bound and had a concentration in the buffer
204 phase that was below the limit of quantitation (LOQ), the buffer concentration was calculated
205 indirectly using Equation (6) which involved the experimental plasma concentration and the
206 total expected concentration (P_{tot}), determined using a calibration plot. The total expected
207 concentration was adjusted using a previously-determined correction factor (CF) for the
208 extraction efficiency (≈ 95 %). This indirectly-calculated buffer concentration was then input
209 into Equation (5) to generate plasma protein binding values.

$$B_{exp} = (P_{tot} \times CF) - P_{exp} \quad (6)$$

2.3. Theoretical approaches

210 Theoretical $\log D_{7.4}$ and pK_a values were generated using the free, online software ACD/I-
211 Lab (which makes use of the EPSRC funded National Chemical Database Service hosted by
212 the Royal Society of Chemistry) and two commercial software packages; MarvinSketch
213 (version 17.28.0) (ChemAxon) and ADMET Predictor (Simulations Plus). Theoretical plasma
214 protein binding values were obtained from two sources used for $\log D_{7.4}$ and pK_a ; ACD/I-Lab
215 and ADMET Predictor (Simulations Plus) and one source available as a free online resource,
216 PreADMET (version 2.0). These software packages are all commonly used for the prediction
217 of physicochemical and pharmacokinetic parameters [39–42]. Theoretical values were
218 compared with experimental values by means of the absolute difference in values.
219

2.4. HPLC analysis for $\log D_{7.4}$ and plasma protein binding

220 Analysis was achieved with a Dionex UltiMate 3000 HPLC system equipped with an UltiMate
221 3000 Pump, UltiMate 3000 Autosampler, UltiMate 3000 Column Compartment, UltiMate
222 3000 Photodiode Array Detector and Chromeleon software (Dionex, Surrey, UK). Separation
223 was achieved with a Waters® Spherisorb® analytical cartridge, C18 5 μm 80 Å (4.6×150
224 mm) with an attached guard cartridge identically packed to the analytical cartridge (Waters,
225 Hertfordshire, UK). The internal column temperature was kept constant at 25 °C and a flow
226 rate of 0.8 mL min^{-1} was set. Injection volumes for the $\log D_{7.4}$ experiments were 25 μL for the
227 octanol phase and 100 μL for the phosphate buffer phase so that a dilution step was not
228 necessary. Compound concentrations were retrospectively corrected. Injection volumes of 100
229 μL were used for the plasma protein binding experiments. A 46:54 (v/v) ratio of acetonitrile
230 and sodium phosphate buffer (pH 3.0, 25 mM) was applied for 25 minutes. All compounds
231 eluted within this time. The eluent was monitored by UV detection at 230 nm. Details of the
232 method validation can be found in the Supplementary Information.
233

234 **3. Results and discussion**

235 Experimental $\log D_{7.4}$, pK_a and plasma protein binding values for all the classic and NPS-
236 benzodiazepines were successfully determined and compared with theoretical values.

237 **3.1. Experimental values**

238 **3.1.1. Log $D_{7.4}$**

239 Buffer composition is important for the determination of $\log D_{7.4}$ values. Use of a 0.01 M
240 phosphate buffer has been shown to give an excellent correlation of distribution coefficients
241 determined in the octanol-phosphate system for acidic and neutral compounds [43]. Despite
242 the basic nature of the compounds in this study, a 0.01 M sodium phosphate buffer (pH 7.4)
243 was chosen and its suitability evaluated by way of a comparison between the experimental \log
244 $D_{7.4}$ values and literature $\log D_{7.4}$ values.

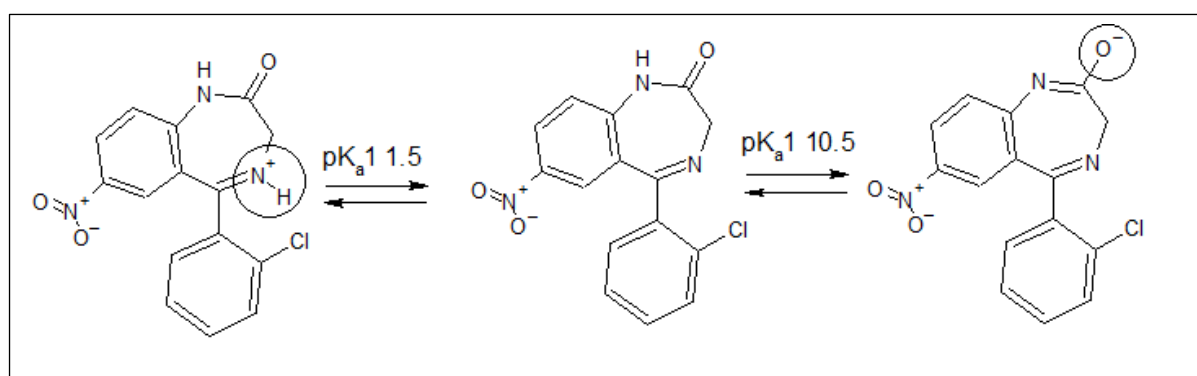
245 For the clinically-used (and previously characterised) benzodiazepines the experimental results
246 obtained for $\log D_{7.4}$ in this study were very close to those reported elsewhere in the literature,
247 thus proving the suitability of this method and also the use of the 0.01 M sodium phosphate
248 buffer (pH 7.4) (Table 1).

249 The majority of the NPS-benzodiazepines were fairly lipophilic with $\log D_{7.4}$ values above 2
250 (Table 1). None of the NPS-benzodiazepines had literature-reported $\log D_{7.4}$ values other than
251 desalkylflurazepam with 2.78 versus a value of 2.82 in this work. Phenazepam ($\log D_{7.4}$ of
252 3.25) was observed to be the most lipophilic NPS-benzodiazepine in this dataset while
253 pyrazolam ($\log D_{7.4}$ of 0.97) was the least lipophilic, a 190-fold difference. The reason behind
254 the low lipophilicity for pyrazolam becomes more apparent when its structure is considered.
255 Pyrazolam contains a pyridin-2-yl ring at position 7 rather than a phenyl ring, as is the case
256 with the rest of the benzodiazepines in this study. The phenyl ring has a $\log D_{7.4}$ value of 1.56
257 versus a $\log D_{7.4}$ value of 0.62 for the pyridin-2-yl ring [44]. Replacement of a phenyl ring for
258 a pyridin-2-yl ring could lead to a decrease in lipophilicity. The benzodiazepine bromazepam
259 contains a pyridin-2-yl ring rather than a phenyl ring and has a $\log D_{7.4}$ value of 1.60 [45]. The

260 addition of a triazole ring to some compounds is also known to lead to a decrease in the partition
261 coefficient [46][47]. The pyridin-2-yl ring and triazole ring addition appear to lead to a marked
262 decrease in lipophilicity for pyrazolam. Previous research has used $\log D_{7.4}$ values in a
263 quantitative structure-activity relationship (QSAR) model which predicted the post-mortem
264 distribution of benzodiazepines and was found to contribute significantly to their distributive
265 potential [48]. $\log D_{7.4}$ has also been utilised, along with plasma protein binding and pK_a , to
266 derive models capable of predicting the volume of distribution at steady state of a wide range
267 of compounds [49,50].

268 3.1.2. pK_a

269 Experimental pK_a values were all within 0.20 units of their literature values for the classic
270 benzodiazepines and had excellent repeatability, under 0.07 units for all the reference
271 compounds (Table 2). Classic benzodiazepines either have one pK_a value, for example
272 flunitrazepam (1.8), or two clonazepam (1.5 and 10.5) [51,52]. The first pK_a value refers to the
273 deprotonation of the nitrogen cation at position 4 and the second pK_a refers to the deprotonation
274 of the nitrogen atom at position 1 [51]. The deprotonation of the nitrogen atom at position 1 is
275 thought to be resonance stabilised with the negatively-charged oxygen atom [51]. This can be
276 visualised in Figure 1 for clonazepam.



277 Figure 1. The two sites of deprotonation and corresponding pK_a values for clonazepam

278 Values of 2.83 for etizolam and 2.51 and 11.64 for desalkylflurazepam were calculated in this
279 work. These compared favourably to their previously-reported values of 2.76 for etizolam and
280 of 2.57 and 11.76 for desalkylflurazepam [53,54].

281 The presence of an electron-withdrawing hydroxyl group decreases the pK_{a2} value, as does the
282 presence of an ortho-chlorine substituent on the phenyl ring [55]. Clonazepam has this ortho-
283 chlorine substituent and has a calculated pK_a value of 1.55 in this work. 3-hydroxyphenazepam,
284 in addition to an ortho-chlorine substituent, also has a hydroxyl group and therefore its low pK_a
285 value of 1.25 was not unexpected. Repeatability was generally good for the NPS-
286 benzodiazepines; 0.07 is typically the expected variance in capillary electrophoresis
287 measurements [35]. However, a variance of up to ± 0.10 was observed for some compounds
288 including 3-hydroxyphenazepam. This could be as a result of its pK_{a1} value (1.25) being lower
289 than the pH of the lowest buffer used (1.50).

290 **3.1.3. Plasma protein binding**

291 A number of the benzodiazepines had concentrations in the buffer phase would have been
292 below the limit of quantitation (LOQ), these were; diazepam, oxazepam, prazepam, 4'-
293 chlorodiazepam, flubromazepam and phenazepam. All concentrations were higher than the
294 limit of detection (LOD). As mentioned in the methods section the buffer phase concentrations
295 were calculated indirectly. The use of a correction factor is less desirable than direct
296 measurements however, it did not appear to affect the calculated values for plasma protein
297 binding when compared with literature values (Table 3).

298 Values for plasma protein binding are listed in Table 3 for clinically used benzodiazepines;
299 wide variations were reported in the literature for many of the benzodiazepines. Age and sex
300 have both been observed as causing differences in the plasma protein binding of drugs which
301 may have been a factor in these variations as many of them were determined *in vivo* [56–58].

302 The experimentally derived values for the reference benzodiazepines were typically within the

303 literature ranges with low variations. The majority of the NPS-benzodiazepines were observed
304 to exhibit a high degree of plasma protein binding (> 90 %), i.e. similar to the clinically used
305 benzodiazepines (Table 3). Literature values for the plasma protein binding of three NPS-
306 benzodiazepines were available and experimental values derived in this work returned a
307 consensus with these; desalkylflurazepam (experimental 95.5 % versus 96.1 – 96.5 %
308 literature), etizolam (experimental 92.8 % versus 93 % literature) and flubromazolam
309 (experimental 89.5 % versus literature 89 %) [24,59–61].

310 The lowest plasma protein binding was observed for pyrazolam which was 78.7 %. Such a low
311 value of plasma protein binding for a benzodiazepine is not unheralded as bromazepam has a
312 reported 60 % plasma protein binding [31]. Substitution of the phenyl ring at position-5 for a
313 pyridin-2-yl ring has been previously reported to lead to a large decrease in lipophilicity for 1,-
314 4-benzodiazepines [59]. The same effect could well occur for triazolobenzodiazepines.

315 4'-chlorodiazepam differs from diazepam by having an additional chlorine atom substituted at
316 the 4'-position of the phenyl ring and exhibits similarly high plasma protein binding; 98.2 %
317 versus 99.0 % for diazepam. Diclazepam is an isomer of 4'-chlorodiazepam; identical in
318 chemical formula but differing in structure with the chlorine atom being substituted at the 2'-
319 position of the phenyl ring. Its plasma protein binding value was calculated as being 93.8 %,
320 lower than diazepam or 4'-chlorodiazepam. However diclazepam's demethylated metabolite
321 has been reported as having a plasma protein binding of 94.9 % and demethylation at the 1-
322 position is not thought to substantially affect plasma protein binding [59]. Therefore, it stands
323 to reason that the decreased plasma protein binding observed is most likely as a result of the
324 substitution of a chlorine atom at the 2'-position. Substitution at the 2'-position with a chlorine
325 atom has been observed to decrease plasma protein binding but if this substitution instead
326 occurs at the 4' position then no such decrease is observed [59]. This is thought to be as a result

327 of the substitution at the 2'-position affecting the rotation and orientation of the benzene ring
328 and resulting in lower binding.

329 3-hydroxyphenazepam exhibited lower plasma protein binding than its parent compound,
330 phenazepam; 97.7 % versus 98.3 % and this is consistent with observations that hydroxylation
331 at the 3-position leads to a decrease in plasma protein binding [59]. Deschloroetizolam has a
332 reduced plasma protein binding compared to the thienotriazolodiazepine etizolam (87.2 %
333 versus 92.8%). Removal of a chlorine atom from position-7 has been found to decrease plasma
334 protein binding for 1,4-benzodiazepines and a similar relationship may hold true for
335 thienotriazolodiazepines [59].

336 Desalkylflurazepam differs from flubromazepam by replacement of the bromine atom at the 7-
337 position by a chlorine atom. Its plasma protein binding is lower (95.5 % versus 96.2 %) which
338 is consistent with literature observations that this replacement causes a decrease in plasma
339 protein binding [59].

340 Phenazepam differs from flubromazepam by replacement of the fluorine atom at the 2'-position
341 with a chlorine atom and exhibits an increase in plasma protein binding from 96.4 % to 98.3
342 %. Again, this is consistent with previous literature observations on 1,4-benzodiazepines [59].

343 **3.2. Theoretical values**

344 **3.2.1. Log $D_{7.4}$**

345 ACD/I-Lab returned the closest predicted log $D_{7.4}$ values to the experimental values for both
346 the eight test benzodiazepines (average absolute error 0.18) and the 11 NPS-benzodiazepines
347 (average absolute error 0.28). ADMET Predictor returned the next-closest predicted values
348 with average absolute errors of 0.24 for the test benzodiazepines and 0.37 for the NPS-
349 benzodiazepines. MarvinSketch fared the worst, returning an average absolute error of 0.39 for
350 the test benzodiazepines and 0.97 for the NPS-benzodiazepines. It is therefore clear that all
351 three programs had a lower accuracy in predicting the log $D_{7.4}$ for the NPS-benzodiazepines

352 and this highlights the importance of the collection of experimental data especially if these
353 models are to be improved. An example of this is for pyrazolam, with an experimental value
354 of 0.97 yet ACD/I-Lab returned a value of 1.76, i.e. approximately a six-fold difference in
355 apparent lipophilicity. The atypical structure of pyrazolam, with its pyridin-2-yl ring, possibly
356 led to these large differences. Inclusion of pyrazolam along with the other NPS-
357 benzodiazepines in any future training dataset for these predictive models could possibly assist
358 in the prediction of $\log D_{7.4}$.

359 **3.2.2. pK_a**

360 ADMET Predictor returned the closest predicted values to experimental values, with an
361 absolute average error of 0.4 for both the test set and the NPS set. This was closely followed
362 by ACD/I-Lab which returned absolute average errors of 0.5 for both sets. MarvinSketch
363 returned average absolute errors of 0.6 for the test set and 0.7 for the NPS set. MarvinSketch
364 did not predict pK_{a1} values for oxazepam and temazepam and instead predicted two pK_{a2}
365 values for oxazepam (only one of which exists) and one pK_{a2} value for temazepam (only a
366 pK_{a1} value is observed). Large errors were observed in some of the pK_a values returned by the
367 software. For example; a pK_a of 2.45 predicted by ACD/I-Lab for deschloroetizolam versus an
368 experimental pK_a of 4.19, a pK_a of 1.33 predicted by MarvinSketch for etizolam versus an
369 experimental pK_a of 2.80 and a pK_a of 2.98 predicted for flubromazolam by ADMET Predictor
370 versus an experimental pK_a of 2.07. Additionally, all three software packages predicted
371 multiple other deprotonation sites for some of the benzodiazepines which are not
372 experimentally observed. The importance of obtaining accurate experimental pK_a values is
373 therefore clear especially if these predictive models wish to be improved upon.

374 **3.2.3. Plasma protein binding**

375 Plasma protein binding was best predicted by ACD/I-Lab which returned average absolute
376 errors of 4.4 % for the test benzodiazepines and 3.0 % for the NPS-benzodiazepines. ADMET
377 Predictor followed closely behind with average absolute errors of 6.8 % for the test

378 benzodiazepines and 3.4 % for the NPS-benzodiazepines. PreADMET returned average
379 absolute errors of 9.9 % for the test benzodiazepines and 5.0 % for the NPS-benzodiazepines.
380 The software appeared to be less effective at predicting plasma protein binding of the test
381 benzodiazepines than the NPS-benzodiazepines (Table 3). However an important caveat is that
382 the average absolute errors for the test benzodiazepines were influenced heavily by the small
383 dataset and the presence of alprazolam; the experimental plasma protein binding was
384 determined as being 71.6 % and the predicted values were 89.5 % (ACD/I-Lab), 91.2 %
385 (ADMET Predictor) and 95.2% (PreADMET). Again, inclusion of a wider range of
386 benzodiazepines (especially those with aberrant structures such as pyrazolam) in any training
387 dataset may assist with their predictive power.

388 **4. Conclusions**

389 Log $D_{7.4}$, pK_a and plasma protein binding values were successfully determined in this work for
390 a range of benzodiazepines that have emerged as novel psychoactive substances. The
391 experimental methods presented were judged to be suitably accurate for the determination of
392 these values.

393 Large variations in plasma protein binding and log $D_{7.4}$ were observed for the NPS-
394 benzodiazepines. Pyrazolam was found to be the least lipophilic NPS-benzodiazepine with a
395 log $D_{7.4}$ of 0.97 and experienced the lowest plasma protein binding of 78.7 %. Phenazepam was
396 the most lipophilic NPS-benzodiazepine with a log $D_{7.4}$ of 3.25 and a plasma protein binding
397 of 98.3 %. 3-hydroxyphenazepam had the lowest pK_{a1} value of 1.25 while deschloroetizolam
398 had the highest pK_{a1} value of 4.19. Phenazepam had the lowest pK_{a2} value of 11.24 and 3-
399 hydroxyphenazepam had the highest of 11.96.

400 ACD/I-Lab returned the closest predicted values to experimental values for both plasma protein
401 binding and log $D_{7.4}$ while ADMET Predictor returned the closest predicted values to
402 experimental values for pK_a . Although the average errors returned by each software package

403 were often low, there were large variations in individual errors. It is therefore likely that
404 experimental data for these novel psychoactive substances remains preferable to that generated
405 from predictive software. The inclusion of experimental data for these NPS-benzodiazepines
406 could aid the predictive capability of various software packages.

Table 1. Literature, experimental (n= ≥3) and theoretical log D_{7.4} values for a set of classic and NPS benzodiazepines

| Compound | Literature log D _{7.4} | Experimental log D _{7.4} | Theoretical log D _{7.4} | | | References |
|----------------------------|---------------------------------|-----------------------------------|----------------------------------|--------------|-----------------|--------------|
| | | | ACD/I-LAB/I-lab | MarvinSketch | ADMET Predictor | |
| Benzodiazepines | | | | | | |
| Alprazolam | 2.12 – 2.16 | 2.10 ±0.01 | 2.44 | 3.02 | 2.63 | [62,63] |
| Clonazepam | 2.41 | 2.40 ±0.02 | 2.57 | 3.15 | 2.49 | [45,62] |
| Diazepam | 2.79 – 2.99 | 2.81 ±0.03 | 2.87 | 3.08 | 2.96 | [45,62–64] |
| Flunitrazepam | 2.06 – 2.14 | 2.05 ±0.01 | 2.20 | 2.55 | 1.87 | [45,62,63] |
| Nitrazepam | 2.13 – 2.16 | 2.17 ±0.03 | 2.03 | 2.55 | 2.49 | [45,62] |
| Oxazepam | 2.13 – 2.24 | 2.24 ±0.05 | 2.04 | 2.92 | 1.95 | [17,45] |
| Prazepam | 3.7 – 3.73 | 3.74 ±0.04 | 3.84 | 3.86 | 3.68 | [45,62] |
| Temazepam | 1.79 – 2.19 | 2.32 ±0.01 | 2.13 | 2.79 | 2.18 | [45,62] |
| NPS-benzodiazepines | | | | | | |
| 3-hydroxyphenazepam | Not reported | 2.54 ±0.01 | 2.67 | 3.69 | 2.40 | Not reported |
| 4'-chlorodiazepam | Not reported | 2.75 ±0.08 | 3.13 | 3.68 | 3.40 | Not reported |
| Desalkylflurazepam | 2.70 | 2.82 ±0.09 | 2.71 | 3.15 | 2.74 | [62] |
| Deschloroetizolam | Not reported | 2.60 ±0.03 | 2.43 | 3.45 | 2.82 | Not reported |
| Diclazepam | Not reported | 2.73 ±0.02 | 3.13 | 3.68 | 3.25 | Not reported |
| Etizolam | Not reported | 2.40 ±0.01 | 2.74 | 4.06 | 3.32 | Not reported |
| Flubromazepam | Not reported | 2.87 ±0.05 | 2.96 | 3.52 | 2.80 | Not reported |
| Flubromazolam | Not reported | 2.40 ±0.04 | 2.52 | 3.33 | 2.60 | Not reported |
| Meclonazepam | Not reported | 2.64 ±0.05 | 2.91 | 3.72 | 2.80 | Not reported |
| Phenazepam | Not reported | 3.25 ±0.04 | 3.52 | 3.98 | 3.19 | Not reported |
| Pyrazolam | Not reported | 0.97 ±0.01 | 1.76 | 2.36 | 2.03 | Not reported |

Table 2. Literature, experimental (n= ≥3) and theoretical pK_a values for a set of classic and NPS benzodiazepines

| Compound | Literature pK _a | | Experimental pK _a | | Theoretical pK _a | | | | | | References |
|----------------------------|----------------------------|------------------|------------------------------|------------------|-----------------------------|------------------|------------------|------------------|------------------|------------------|--------------|
| | pK _{a1} | pK _{a2} | pK _{a1} | pK _{a2} | ACD/I-LAB/I-lab | | MarvinSketch | | ADMET Predictor | | |
| | | | | | pK _{a1} | pK _{a2} | pK _{a1} | pK _{a2} | pK _{a1} | pK _{a2} | |
| Benzodiazepines | | | | | | | | | | | |
| Alprazolam | 2.4 | None | 2.48 ±0.01 | None | 2.37 | None | 1.45, 5.01 | None | 0.93, 3.01 | None | [65] |
| Clonazepam | 1.49 – 1.52 | 10.37 – 10.51 | 1.55 ±0.02 | 10.45 ±0.05 | 1.55 | 11.21 | 1.89 | 11.65 | 1.43 | 10.77 | [65–67] |
| Diazepam | 3.17 – 3.31 | None | 3.10 ±0.00 | None | 3.40 | None | 2.92 | None | 2.96 | None | [66,68] |
| Flunitrazepam | 1.8 | None | 1.82 ±0.04 | None | 1.68 | None | 1.72 | None | 1.87 | None | [65] |
| Nitrazepam | 2.94 – 3.2 | 10.8 – 11 | 3.11 ±0.06 | 11.02 ±0.05 | 2.55 | 11.35 | 2.65 | 11.66 | 2.49 | 11.02 | [55,66] |
| Oxazepam | 1.56 – 1.7 | 11.21 – 11.6 | 1.67 ±0.05 | 11.34 ±0.03 | 1.17 | 10.94, 12.75 | None | 10.65, 12.47 | 2.57 | 11.31 | [66,67] |
| Prazepam | 2.7 – 2.74 | None | 2.71 ±0.01 | None | 3.44 | None | 3.06 | None | 3.10 | None | [65,66] |
| Temazepam | 1.31 – 1.6 | None | 1.45 ±0.05 | None | 1.58 | 11.66 | None | 10.68 | 2.48 | None | [66,69] |
| NPS-benzodiazepines | | | | | | | | | | | |
| 3-hydroxyphenazepam | Not reported | Not reported | 1.25 ±0.10 | 11.96 ±0.09 | 0.13 | 10.80, 12.68 | None | 10.61, 12.45 | 1.95 | 11.24 | Not reported |
| 4'-chlorodiazepam | Not reported | Not reported | 3.13 ±0.01 | None | 3.08 | None | 2.45 | None | 2.55 | None | Not reported |
| Desalkylflurazepam | 2.57 | 11.76 | 2.51 ±0.05 | 11.64 ±0.04 | 2.36 | 11.55 | 1.80 | 12.29 | 2.31 | 11.37 | [53] |
| Deschloroetizolam | Not reported | Not reported | 4.19 ±0.01 | None | 0.20, 2.45 | None | 1.31, 5.37 | None | 1.84, 3.96 | None | Not reported |
| Diclazepam | Not reported | Not reported | 2.31 ±0.07 | None | 1.75 | None | 2.13 | None | 1.95 | None | Not reported |
| Etizolam | 2.76 | None | 2.83 ±0.06 | None | 0.10, 2.37 | None | 1.33, 4.55 | None | 1.61, 3.31 | None | [54] |
| Flubromazepam | Not reported | Not reported | 3.25 ±0.10 | 10.74 ±0.05 | 2.32 | 11.55 | 1.8 | 12.28 | 2.70 | 11.45 | Not reported |
| Flubromazolam | Not reported | Not reported | 2.07 ±0.02 | None | 2.27 | None | 1.48, 4.01 | None | 0.96, 2.98 | None | Not reported |
| Meclonazepam | Not reported | Not reported | 2.10 ±0.09 | 11.45 ±0.07 | 1.70 | 11.24 | 1.65 | 11.57 | 2.10 | 10.88 | Not reported |
| Phenazepam | Not reported | Not reported | 2.19 ±0.05 | 11.21 ±0.04 | 2.18 | 11.58 | 2.06 | 12.28 | 2.44 | 11.43 | Not reported |
| Pyrazolam | Not reported | Not reported | 3.30 ±0.03 | None | 1.30, 2.18 | None | 1.79, 2.75 | None | 0.65, 2.47, 3.21 | None | Not reported |

Table 3. Literature, experimental (n= ≥3) and theoretical plasma protein binding (PPB) values for a set of classic and NPS benzodiazepines

| Compound | Literature PPB (%) | Experimental PPB (%) | Theoretical PPB (%) | | | References |
|----------------------------|--------------------|----------------------|---------------------|--------------------|----------|--------------|
| | | | ACD/ I-lab | ADMET Predictor | PreADMET | |
| Benzodiazepines | | | | | | |
| Alprazolam | 68.4 – 76.7 | 71.6 ±0.5 | 89.5 | 91.2 | 95.2 | [31,70] |
| Clonazepam | 85.4 – 86.1 | 85.5 ±1.2 | 91.9 | 90.9 | 93.3 | [31,70] |
| Diazepam | 98.4 – 99 | 99.0 ±0.2 | 96.5 | 93.2 | 98.7 | [31,37] |
| Flunitrazepam | 77.5 – 84.5 | 78.9 ±1.2 | 84.4 | 86.5 | 98.9 | [31,70] |
| Nitrazepam | 82.1 – 88.9 | 88.4 ±1.8 | 88.5 | 84.3 | 92.0 | [71,72] |
| Oxazepam | 89.0 – 98.4 | 96.9 ±0.1 | 95.6 | 88.9 | 96.7 | [31,70] |
| Prazepam | ≈97 | 97.4 ±0.5 | 97.7 | 96.5 | 94.0 | [73] |
| Temazepam | 92 – 96.8 | 94.3 ±0.1 | 95.4 | 91.1 | 74.3 | [31,70] |
| NPS-benzodiazepines | | | | | | |
| 3-hydroxyphenazepam | Not reported | 97.7 ±0.6 | 92.5 | 93.8 | 90.1 | Not reported |
| 4'-chlorodiazepam | Not reported | 98.2 ±0.5 | 96.5 | 96.2 | 93.2 | Not reported |
| Desalkylflurazepam | 96.1 – 96.5 | 95.5 ±1.5 | 96.1 | 92.8 | 91.4 | [60] |
| Deschloroetizolam | Not reported | 87.2 ±1.5 | 85.8 | 91.5 | 89.8 | Not reported |
| Diclazepam | Not reported | 93.8 ±1.2 | 96.5 | 95.7 | 97.7 | Not reported |
| Etizolam | Not reported | 92.8 ±0.6 | 90.2 | 94.7 | 90.8 | Not reported |
| Flubromazepam | Not reported | 96.4 ±0.9 | 89.0 | 93.2 | 93.9 | Not reported |
| Flubromazolam | 89 | 89.5 ±0.4 | 87.4 | 91.1 | 92.2 | [24] |
| Meclonazepam | Not reported | 88.2 ±0.5 | 93.0 | 93.0 | 92.3 | Not reported |
| Phenazepam | Not reported | 98.3 ±1.2 | 94.6 | 95.6 | 93.6 | Not reported |
| Pyrazolam | Not reported | 78.7 ±0.4 | 77.6 | 86.5 | 94.8 | Not reported |

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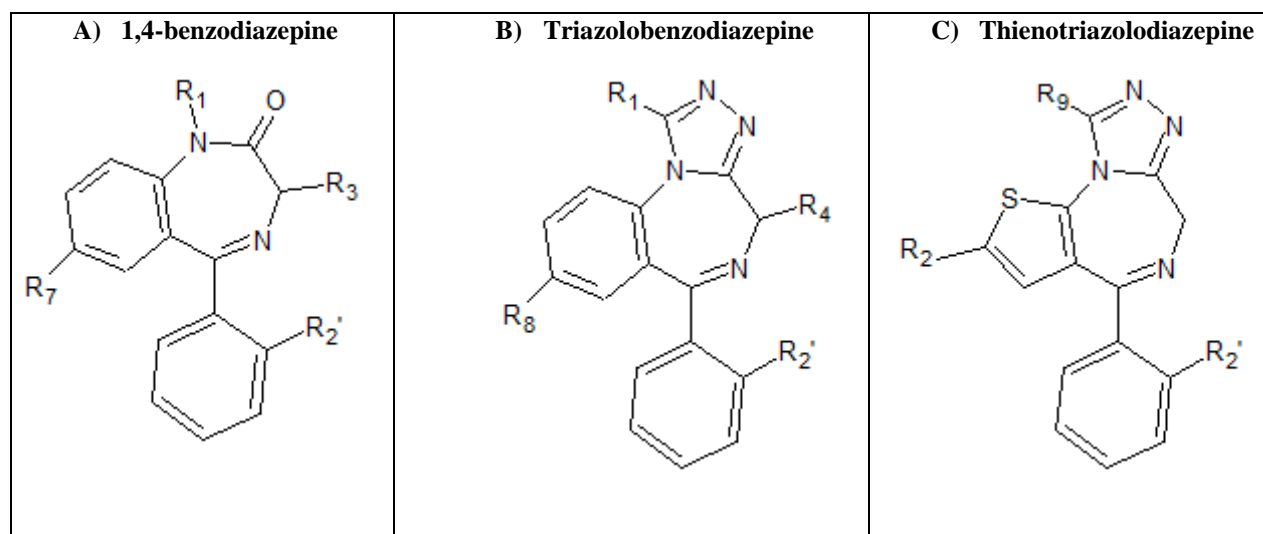
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Supplementary information

Benzodiazepine structures

The structures of the NPS-benzodiazepines used in this work are visualised in Figures 1A – C and Tables 1 – 3.



Figures 1A – C. Basic structure of a 1,4-benzodiazepine, a triazolobenzodiazepine and a thienotriazolodiazepine

Table 1. Substituents for 1,4-benzodiazepines

| | From Figure 1A | | | |
|--|-----------------|------------------|-----------------|-----------------|
| Compound | R ₁ | R ₂ ' | R ₃ | R ₇ |
| 3-hydroxyphenazepam | H | Cl | OH | Br |
| 4-chlorodiazepam (Ro5-4864) ^a | CH ₃ | H | H | Cl |
| Desalkylflurazepam | H | F | H | Cl |
| Diclazepam | CH ₃ | Cl | H | NO ₂ |
| Flubromazepam | H | F | H | Br |
| Meclonazepam | H | Cl | CH ₃ | NO ₂ |
| Phenazepam | H | Cl | H | Br |

^a Note: 4-chlorophenyl ring instead of phenyl ring at position 6

Table 2. Substituents for triazolobenzodiazepines

| | From Figure 1B | | |
|------------------------|-----------------|------------------|----------------|
| Compound | R ₁ | R ₂ ' | R ₈ |
| Flubromazolam | CH ₃ | F | Br |
| Pyrazolam ^a | CH ₃ | None | Br |

^a Note: pyridine ring instead of phenyl ring at position 6

Table 3. Substituents for thienotriazolodiazepines

| | From Figure 1C | | |
|-------------------|---------------------------------|------------------|-----------------|
| Compound | R ₂ | R ₂ ' | R ₉ |
| Deschloroetizolam | CH ₂ CH ₃ | H | CH ₃ |
| Etizolam | CH ₂ CH ₃ | Cl | CH ₃ |

HPLC Method validation

The method was validated in terms of linearity, limit of quantitation (LOQ), limit of detection (LOD), accuracy and precision. This was performed according to the ICH guidelines.

Linearity

The linearity of this method was measured by constructing a five-point calibration plot of the area under the curve (AUC) of each compound against its concentration in mg ml^{-1} ($n=3$). The method was linear over the concentration range $0.0004 - 0.25 \text{ mg ml}^{-1}$ for all compounds. The residual sum of squares for each compound was reasonably low indicating linear concentration-response and a suitable method (Table 4).

Limit of detection (LOD) and limit of quantitation (LOQ)

The limits of detection and quantitation were determined from the signal-to-noise ratio. The baseline response of blank samples was recorded. A ratio of 10:1 for the compound response to the baseline response was used for the LOQ and a ratio of 3:1 for the LOD. All compounds generally had good limits of detection and quantitation (Table 4). Pyrazolam exhibited the lowest response to the HPLC method, with a LOQ of 263.9 ng ml^{-1} and a LOD of 82.0 ng ml^{-1} .

Accuracy

Accuracy was determined through comparison of the percentage recovery at three concentrations (0.25 , 0.01 and $0.0004 \text{ mg ml}^{-1}$). Percentage recovery was generally within 2 % and thus deemed to be acceptable (Table 5).

Precision

Precision was determined from the calculation of the standard deviation and relative standard deviation (RSD) of the compound peak areas at three concentrations (0.25 , 0.01 and $0.0004 \text{ mg ml}^{-1}$). High levels of precision for all benzodiazepines were recorded (Table 5).

Table 4. Linearity, LOQ and LOD data for benzodiazepines

| Compound | Slope | Correlation coefficient | y intercept | Residual sum of squares | LOQ (ng ml ⁻¹) | LOD (ng ml ⁻¹) |
|---------------------|---------|-------------------------|-------------|-------------------------|----------------------------|----------------------------|
| 3-hydroxyphenazepam | 4455.57 | 1.00 | -0.55 | 19.40 | 188.9 | 42.9 |
| 4'-chlorodiazepam | 4819.30 | 1.00 | 1.44 | 11.30 | 202.2 | 59.5 |
| Alprazolam | 4826.85 | 1.00 | 1.36 | 27.07 | 144.6 | 49.8 |
| Clonazepam | 4407.07 | 1.00 | 0.37 | 21.90 | 185.4 | 59.2 |
| Desalkylflurazepam | 4283.08 | 1.00 | -0.74 | 16.43 | 187.2 | 53.4 |
| Deschloroetizolam | 4072.89 | 1.00 | 0.86 | 13.00 | 206.1 | 62.5 |
| Diazepam | 4758.95 | 1.00 | -0.74 | 18.41 | 185.5 | 51.8 |
| Diclazepam | 4817.39 | 1.00 | 0.48 | 12.73 | 198.8 | 59.9 |
| Etizolam | 4007.71 | 1.00 | 0.51 | 13.20 | 194.2 | 57.0 |
| Flubromazepam | 4084.79 | 1.00 | 0.73 | 15.99 | 165.6 | 67.6 |
| Flubromazolam | 4168.69 | 1.00 | -0.42 | 10.68 | 177.3 | 47.2 |
| Flunitrazepam | 4223.77 | 1.00 | -0.92 | 13.05 | 159.0 | 51.5 |
| Meclonazepam | 4805.99 | 1.00 | 0.87 | 9.15 | 186.4 | 52.5 |
| Nitrazepam | 4367.07 | 1.00 | -0.37 | 10.82 | 179.2 | 49.4 |
| Oxazepam | 4466.93 | 1.00 | -0.53 | 7.17 | 159.8 | 50.2 |
| Phenazepam | 4149.34 | 1.00 | -0.17 | 11.76 | 191.2 | 65.3 |
| Prazepam | 4338.90 | 1.00 | 0.34 | 9.32 | 172.3 | 56.0 |
| Pyrazolam | 3967.82 | 1.00 | -0.31 | 14.76 | 263.9 | 82.0 |
| Temazepam | 4646.75 | 1.00 | -0.34 | 9.67 | 195.6 | 51.9 |

Table 4. Precision and accuracy data for benzodiazepines

| Compound | Concentration (mg ml ⁻¹) | | | | | | | | |
|---------------------|--------------------------------------|-------------------|--------------|--------------|-------------------|--------------|--------------|-------------------|--------------|
| | 0.0004 (n=3) | | | 0.01 (n=3) | | | 0.25 (n=3) | | |
| | Precision SD | Precision RSD (%) | Accuracy (%) | Precision SD | Precision RSD (%) | Accuracy (%) | Precision SD | Precision RSD (%) | Accuracy (%) |
| 3-hydroxyphenazepam | 0.04 | 2.08 | 99.39 | 0.53 | 1.17 | 100.46 | 8.88 | 0.80 | 99.17 |
| 4'-chlorodiazepam | 0.06 | 1.72 | 101.35 | 0.47 | 0.93 | 101.85 | 10.29 | 0.85 | 100.54 |
| Alprazolam | 0.04 | 1.31 | 99.49 | 0.88 | 1.75 | 100.57 | 13.44 | 1.10 | 99.86 |
| Clonazepam | 0.05 | 1.53 | 101.11 | 0.81 | 1.62 | 99.25 | 6.91 | 1.77 | 99.98 |
| Desalkylflurazepam | 0.02 | 1.10 | 98.60 | 0.27 | 0.62 | 101.50 | 7.16 | 0.66 | 101.12 |
| Deschloroetizolam | 0.04 | 1.58 | 99.25 | 0.24 | 0.57 | 99.56 | 5.81 | 0.57 | 100.68 |
| Diazepam | 0.02 | 1.16 | 98.90 | 0.59 | 1.24 | 100.98 | 11.69 | 0.97 | 101.23 |
| Diclazepam | 0.02 | 0.70 | 98.92 | 0.54 | 1.07 | 101.49 | 6.46 | 0.54 | 99.10 |
| Etizolam | 0.04 | 1.74 | 98.99 | 0.72 | 1.78 | 99.57 | 9.95 | 1.00 | 99.73 |
| Flubromazepam | 0.03 | 1.16 | 99.21 | 0.66 | 1.56 | 101.76 | 5.36 | 0.52 | 101.34 |
| Flubromazolam | 0.03 | 2.15 | 100.41 | 0.55 | 1.13 | 100.89 | 17.93 | 1.71 | 100.74 |
| Flunitrazepam | 0.06 | 2.03 | 98.97 | 0.27 | 0.55 | 99.56 | 9.33 | 0.78 | 99.72 |
| Meclonazepam | 0.02 | 0.81 | 99.43 | 0.31 | 0.63 | 100.35 | 8.49 | 0.71 | 99.46 |
| Nitrazepam | 0.02 | 1.21 | 98.20 | 0.54 | 1.10 | 100.15 | 9.67 | 0.78 | 100.83 |
| Oxazepam | 0.02 | 1.44 | 101.76 | 0.70 | 1.56 | 101.68 | 7.48 | 0.68 | 99.21 |
| Phenazepam | 0.03 | 2.17 | 101.01 | 0.98 | 2.37 | 99.95 | 6.45 | 0.62 | 100.23 |
| Prazepam | 0.05 | 2.15 | 98.63 | 0.67 | 1.54 | 99.78 | 6.51 | 1.66 | 99.51 |
| Pyrazolam | 0.03 | 2.14 | 99.47 | 0.53 | 1.33 | 101.53 | 2.95 | 0.30 | 100.73 |
| Temazepam | 0.03 | 2.05 | 101.74 | 0.65 | 1.40 | 101.27 | 13.64 | 1.18 | 99.55 |