

# 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.  
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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<sup>-1</sup> [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<sub>7.4</sub>**

112 The shake-flask method is commonly used in determining log D<sub>7.4</sub> 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<sub>7.4</sub> 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<sup>-1</sup>. 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<sub>a</sub>

131 Capillary electrophoresis is a common method of measuring pK<sub>a</sub> [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  $\mu\text{m}$  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  $\text{mg ml}^{-1}$ . Solutions were diluted  
154 to 0.25  $\text{mg ml}^{-1}$  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  $\mu_{\text{eff}}$  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  $\alpha$  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  $\alpha$ .

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  $\mu$ M.

185 Reusable Single-Sample Fast Micro-Equilibrium Dialyzers (500  $\mu$ 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  $\mu$ L of compound working solution was added to 270  $\mu$ L of plasma to  
190 yield a final concentration of 20  $\mu$ M of compound (final DMSO concentration 0.2 %). This

191 was placed in one chamber and 500  $\mu$ 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  $\mu$ 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 ( $\approx 95$  %). 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  $\mu\text{m}$  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<sup>-1</sup> was set. Injection volumes for the  $\log D_{7.4}$  experiments were 25  $\mu\text{L}$  for the  
227 octanol phase and 100  $\mu\text{L}$  for the phosphate buffer phase so that a dilution step was not  
228 necessary. Compound concentrations were retrospectively corrected. Injection volumes of 100  
229  $\mu\text{L}$  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.

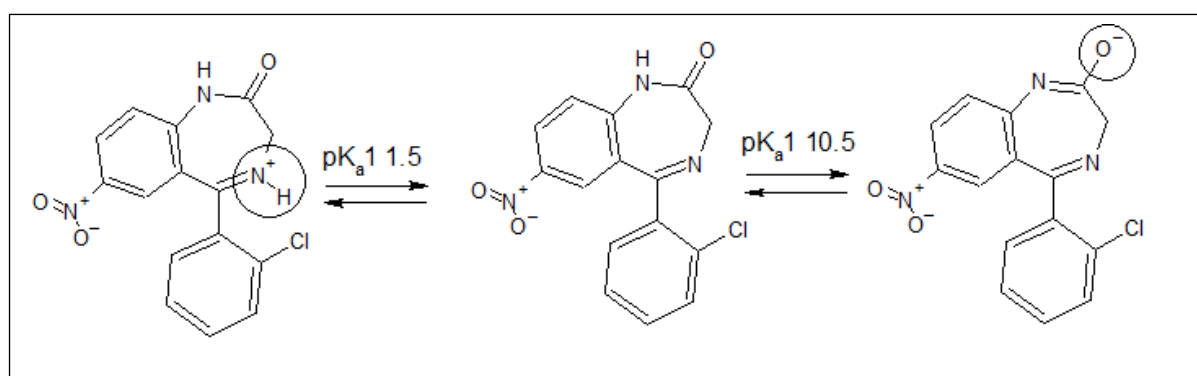
245 For the clinically-used (and previously characterised) benzodiazepines the experimental results  
246 obtained for  $\log D_{7.4}$  in this study were very close to those reported elsewhere in the literature,  
247 thus proving the suitability of this method and also the use of the 0.01 M sodium 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  $\pm 0.10$  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<sub>a</sub>**

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<sub>a1</sub> values for oxazepam and temazepam and instead predicted two pK<sub>a2</sub>  
365 values for oxazepam (only one of which exists) and one pK<sub>a2</sub> value for temazepam (only a  
366 pK<sub>a1</sub> value is observed). Large errors were observed in some of the pK<sub>a</sub> values returned by the  
367 software. For example; a pK<sub>a</sub> of 2.45 predicted by ACD/I-Lab for deschloroetizolam versus an  
368 experimental pK<sub>a</sub> of 4.19, a pK<sub>a</sub> of 1.33 predicted by MarvinSketch for etizolam versus an  
369 experimental pK<sub>a</sub> of 2.80 and a pK<sub>a</sub> of 2.98 predicted for flubromazolam by ADMET Predictor  
370 versus an experimental pK<sub>a</sub> 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<sub>a</sub> 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<sub>7.4</sub> values for a set of classic and NPS benzodiazepines**

Compound	Literature log D <sub>7.4</sub>	Experimental log D <sub>7.4</sub>	Theoretical log D <sub>7.4</sub>			References
			ACD/I-LAB/I-lab	MarvinSketch	ADMET Predictor	
<b>Benzodiazepines</b>						
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]
<b>NPS-benzodiazepines</b>						
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<sub>a</sub> values for a set of classic and NPS benzodiazepines**

Compound	Literature pK <sub>a</sub>		Experimental pK <sub>a</sub>		Theoretical pK <sub>a</sub>						References
	pK <sub>a1</sub>	pK <sub>a2</sub>	pK <sub>a1</sub>	pK <sub>a2</sub>	ACD/I-LAB/I-lab		MarvinSketch		ADMET Predictor		
					pK <sub>a1</sub>	pK <sub>a2</sub>	pK <sub>a1</sub>	pK <sub>a2</sub>	pK <sub>a1</sub>	pK <sub>a2</sub>	
<b>Benzodiazepines</b>											
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]
<b>NPS-benzodiazepines</b>											
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	
<b>Benzodiazepines</b>						
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]
<b>NPS-benzodiazepines</b>						
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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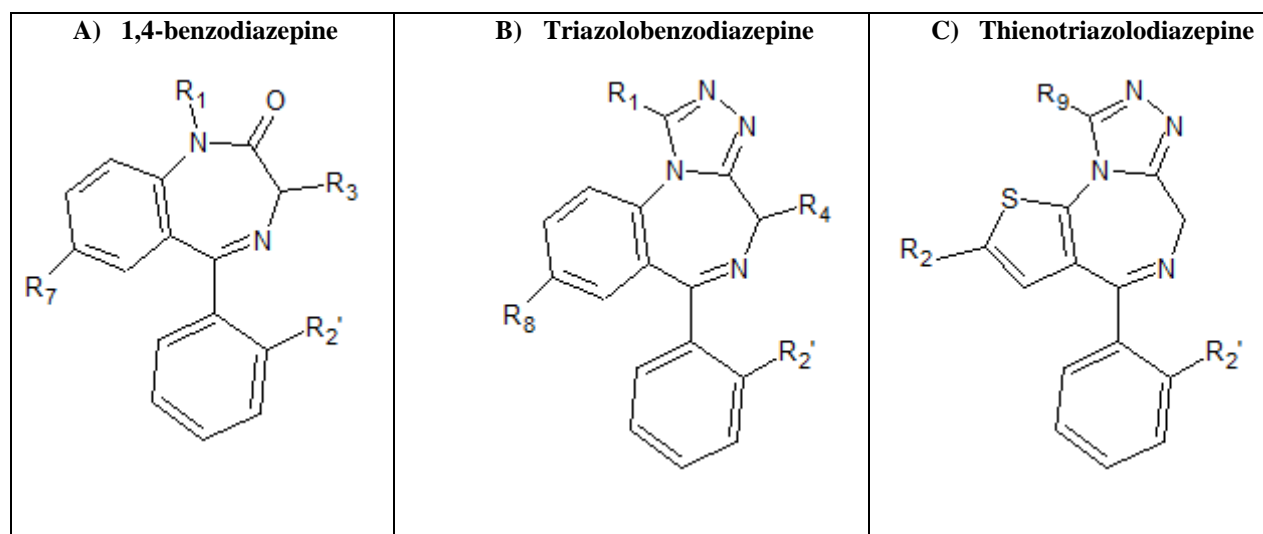
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## Supplementary information

### 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 <sub>1</sub>	R <sub>2</sub> '	R <sub>3</sub>	R <sub>7</sub>
3-hydroxyphenazepam	H	Cl	OH	Br
4-chlorodiazepam (Ro5-4864) <sup>a</sup>	CH <sub>3</sub>	H	H	Cl
Desalkylflurazepam	H	F	H	Cl
Diclazepam	CH <sub>3</sub>	Cl	H	NO <sub>2</sub>
Flubromazepam	H	F	H	Br
Meclonazepam	H	Cl	CH <sub>3</sub>	NO <sub>2</sub>
Phenazepam	H	Cl	H	Br

<sup>a</sup> Note: 4-chlorophenyl ring instead of phenyl ring at position 6

**Table 2. Substituents for triazolobenzodiazepines**

	From Figure 1B		
Compound	R <sub>1</sub>	R <sub>2</sub> '	R <sub>8</sub>
Flubromazolam	CH <sub>3</sub>	F	Br
Pyrazolam <sup>a</sup>	CH <sub>3</sub>	None	Br

<sup>a</sup> Note: pyridine ring instead of phenyl ring at position 6

**Table 3. Substituents for thienotriazolodiazepines**

	From Figure 1C		
Compound	R <sub>2</sub>	R <sub>2</sub> '	R <sub>9</sub>
Deschloroetizolam	CH <sub>2</sub> CH <sub>3</sub>	H	CH <sub>3</sub>
Etizolam	CH <sub>2</sub> CH <sub>3</sub>	Cl	CH <sub>3</sub>

### **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  $\text{mg ml}^{-1}$  ( $n=3$ ). 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  $263.9 \text{ ng ml}^{-1}$  and a LOD of  $82.0 \text{ ng ml}^{-1}$ .

#### **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 <sup>-1</sup> )	LOD (ng ml <sup>-1</sup> )
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 <sup>-1</sup> )								
	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