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

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Abstract

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

Keywords: logD; pKa; plasma; benzodiazepines; NPS

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

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

2.1. Materials

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

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

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

2.2. Methods

2.2.1. Determination of log $D_{7.4}$

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

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

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

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

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

2.2.2. Determination of pK_a

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

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

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

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

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

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

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

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

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

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

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

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

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

2.2.3. Determination of plasma protein binding

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

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

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

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

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

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

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

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

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

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

2.3. Theoretical approaches

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

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

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

3. Results and discussion

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

3.1.Experimental values

3.1.1. Log $D_{7.4}$

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

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

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

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

3.1.2. pK_a

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

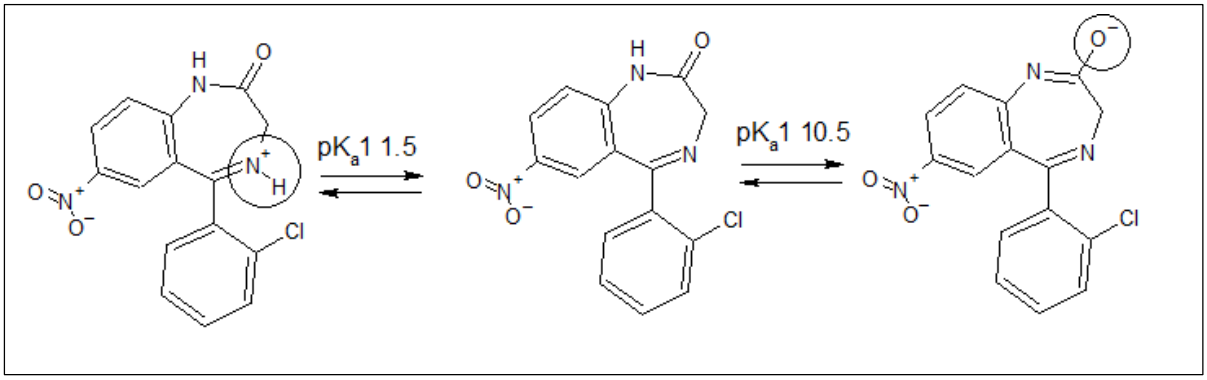

Figure 1. The two sites of deprotonation and corresponding pK_a values for clonazepam

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

The presence of an electron-withdrawing hydroxyl group decreases the pK_a2 value, as does the presence of an ortho-chlorine substituent on the phenyl ring [55]. Clonazepam has this ortho-chlorine substituent and has a calculated pK_a value of 1.55 in this work. 3-Hydroxyphenazepam, in addition to an ortho-chlorine substituent, also has a hydroxyl group and therefore its low pK_a value of 1.25 was not unexpected. Repeatability was generally good for the NPS-benzodiazepines; 0.07 is typically the expected variance in capillary electrophoresis measurements [35]. However, a variance of up to ±0.10 was observed for some compounds including 3-Hydroxyphenazepam. This could be as a result of its pK_a1 value (1.25) being lower than the pH of the lowest buffer used (1.50).

3.1.3. Plasma protein binding

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

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

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

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

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

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

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

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

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

3.2. Theoretical values

3.2.1. Log $D_{7.4}$

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

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

3.2.2. pK_a

ADMET Predictor returned the closest predicted values to experimental values, with an absolute average error of 0.4 for both the test set and the NPS set. This was closely followed by ACD/I-Lab which returned absolute average errors of 0.5 for both sets. MarvinSketch returned average absolute errors of 0.6 for the test set and 0.7 for the NPS set. MarvinSketch did not predict pK_a1 values for oxazepam and temazepam and instead predicted two pK_a2 values for oxazepam (only one of which exists) and one pK_a2 value for temazepam (only a pK_a1 value is observed). Large errors were observed in some of the pK_a values returned by the software. For example; a pK_a of 2.45 predicted by ACD/I-Lab for deschloroetizolam versus an experimental pK_a of 4.19, a pK_a of 1.33 predicted by MarvinSketch for etizolam versus an experimental pK_a of 2.80 and a pK_a of 2.98 predicted for flubromazolam by ADMET Predictor versus an experimental pK_a of 2.07. Additionally, all three software packages predicted multiple other deprotonation sites for some of the benzodiazepines which are not experimentally observed. The importance of obtaining accurate experimental pK_a values is therefore clear especially if these predictive models wish to be improved upon.

3.2.3. Plasma protein binding

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

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

4. Conclusions

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

Large variations in plasma protein binding and log $D_{7.4}$ were observed for the NPS-benzodiazepines. Pyrazolam was found to be the least lipophilic NPS-benzodiazepine with a log $D_{7.4}$ of 0.97 and experienced the lowest plasma protein binding of 78.7 %. Phenazepam was the most lipophilic NPS-benzodiazepine with a log $D_{7.4}$ of 3.25 and a plasma protein binding of 98.3 %. 3-Hydroxyphenazepam had the lowest pK_a1 value of 1.25 while deschloroetizolam had the highest pK_a1 value of 4.19. Phenazepam had the lowest pK_a2 value of 11.24 and 3-Hydroxyphenazepam had the highest of 11.96.

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

were often low, there were large variations in individual errors. It is therefore likely that experimental data for these novel psychoactive substances remains preferable to that generated from predictive software. The inclusion of experimental data for these NPS-benzodiazepines 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
					ACD/I-LAB/I-lab		MarvinSketch		ADMET Predictor		
	pK_a1	pK_a2	pK_a1	pK_a2	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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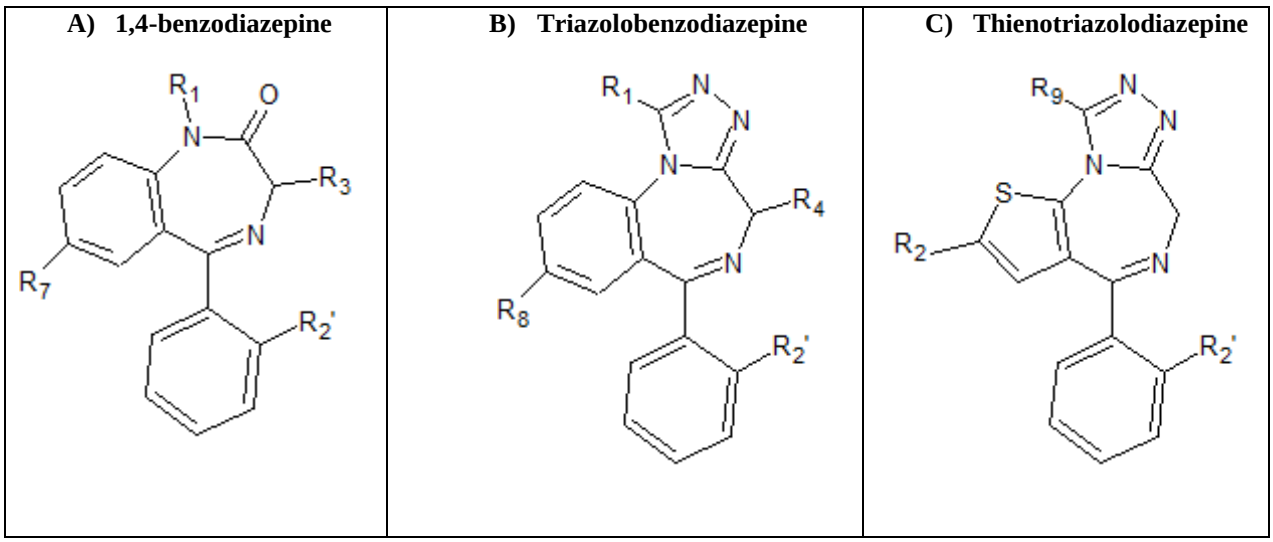
Supplementary information

Benzodiazepine structures

The structures of the NPS-benzodiazepines used in this work are visualised in Figures S1A – C and Tables S1 – 3.



Figures S1A – C. Basic structure of a 1,4-benzodiazepine, a triazolobenzodiazepine and a thienotriazolodiazepine

Table S1. 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 S2. 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 S3. Substituents for thienotriazolodiazepines

	From Figure 1C		
Compound	R_2	$R_{2'}$	R_9
Deschloroetizolam	CH_2CH_3	H	CH_3
Etizolam	CH_2CH_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 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 S4).

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 S4). 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 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 mg ml^{-1}). High levels of precision for all benzodiazepines were recorded (Table S5).

Table S4. 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 S5. 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