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The *in vitro* biokinetics of chlorpromazine and diazepam in aggregating rat brain cell cultures after repeated exposure

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ABSTRACT

Neurotoxic effects of compounds can be tested *in vitro* using cell systems. One example is aggregating rat brain cell cultures. For the extrapolation of *in vitro* data to the *in vivo* situation, it is important to take the biokinetics of the test compound into account. In addition, the exposure *in vivo* is often for a longer period of time; therefore, it is crucial to incorporate this into *in vitro* assays as well. In this study, aggregating rat brain cell cultures were exposed to chlorpromazine (CPZ) and diazepam (DZP) for 12-days with repeated exposure. Samples were taken from the stocks, test media, cell culture media and cells at specific time points on the first and last exposure day. These samples were analysed by HPLC-UV. The amount of CPZ in the medium decreased over time, whereas the amount in the cells showed an increase. Accumulation of CPZ in the cells was seen over the 12-day repeated exposure. The amount of DZP in the medium remained stable over time and only up to 2% of DZP added was found in the cells. Different biokinetic behaviour was found for CPZ and DZP. Possible explanations are differences in uptake into the cells or efflux out of the cells. The decrease of CPZ in the medium versus the stable amount of DZP results in differences in exposure concentrations over time, which should be taken into account when interpreting *in vitro* effect data.

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1. Introduction

Guidelines on neurotoxicity testing prescribe *in vivo* animal tests to determine developmental and adult neurotoxic effects, both after acute and chronic exposure (Bal-Price et al., 2010; Coecke et al., 2006; OECD, 1997, 2007). Since these tests are expensive, time-consuming and give rise to ethical issues, alternatives are needed. Especially with the introduction of REACH, the number of test animals needed was expected to show an enormous increase (Van der Jagt et al., 2004). Neurotoxic effects in man have been shown for different compounds with the largest groups being metals, solvent and pesticides (Grandjean and Landrigan, 2006). In addition, about 4% of the prescription drugs withdrawn from the market were based on adverse neurological effects (Fung et al., 2001).

The use of *in vitro* assays has so far been limited to studying mechanisms of toxicity and not to assessing the neurotoxic potential of compounds (Bal-Price et al., 2010). The complicated structure of the central nervous system hampers the use of *in vitro* assays in this area and studies have been mainly limited to basal cytotoxicity (Harry and Tiffany-Castiglioni, 2005). However, neurotoxic effects tend to occur at lower concentrations than basal cytotoxicity and these effects are often missed in *in vitro* cell assays (Bal-Price et al., 2010).

The *in vitro* tests that are currently available range from single cell cultures to tissue slices and 3D aggregating cultures (Costa, 1998; Harry et al., 1998). The latter two contain both glial cells and neurons that are cultured in a three-dimensional organisation (Harry et al., 1998). According to Coecke et al. (2006), a couple of concerns can be raised when implementing *in vitro* assays into neurotoxicological risk assessment: interactions between the different cell types present in the central/peripheral nervous system, dosing of the compound and the endpoints to be measured. Also

Abbreviations: CNS, central nervous system; CPZ, chlorpromazine; DIV, days *in vitro*; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl solfoxide; DZP, diazepam; LOD, limit of detection; LOQ, limit of quantification; PBBK, physiologically based biokinetic; PBS, phosphate buffered saline; TC_{10}, 10% toxic concentration.

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information related to the uptake of the compound into the nervous system (transport across the blood–brain barrier) should be included when setting up a testing strategy.

Progresses have been made in assessing acute neurotoxicity in the 6th EU Framework Programme ACuteTox project, where 86 compounds were tested in different cell assays including aggregating rat brain cell cultures (Forsby et al., 2009; Zurich et al., 2012). These cultures were found to be the most sensitive model to detect central nervous system (CNS)-specific toxicity. Therefore, this *in vitro* assay was included in this study as part of the European 7th EU Framework Programme Predict-IV (ref nr. 202222).

For extrapolation of *in vitro* data to the *in vivo* situation, it is important to take species-species differences and the biokinetics of the test compound into account (Bal-Price et al., 2010; Harry et al., 1998). Information about the actual exposure and distribution of a chemical in the *in vitro* tests, including information about the concentration that can reach the target site, which is often the concentration of the unbound chemical in the medium, might help in the interpretation of the outcome of *in vitro* tests and is essential in comparison of the sensitivity of different *in vitro* test systems (Gülden et al., 2002; Gülden and Seibert, 2005; Heringa et al., 2004; Kramer et al., 2012). Information about effect concentration from *in vitro* tests, both in medium or in the cells, can be combined with Physiologically Based BioKinetic (PBBK) modelling to predict *in vivo* effects from an external dose. In this study we looked at the detailed exposure and biokinetics of two test compounds in an *in vitro* model of brain cells.

Aggregating rat brain cell cultures were exposed to two compounds: chlorpromazine (CPZ) and diazepam (DZP). These compounds were chosen based on their physical–chemical properties (lipophilicity, pK_a) and high permeability through the blood brain barrier (Garberg et al., 2005; Jolliet et al., 2007). CPZ has been used as an antipsychotic for over 50 years (Ban, 2007) and DZP is a benzodiazepine mainly used for treating anxiety and insomnia (Ashton, 1994). Chlorpromazine is a lipophilic compound (apparent Log K_{ow} = 3.16 at pH = 7.4, neutral Log K_{ow} = 5.3) and is ionised for 98.8% at a physiological pH (Sawada et al., 1994; Takács-Novák and Avdeef, 1996). Diazepam has a Log K_{ow} of 2.8 and a pK_a of 3.3 and is neutral at a physiological pH (Hanumegowda et al., 2010).

The aggregating rat brain cell cultures were exposed to CPZ and DZP for 12 days with fresh test medium every other day, to implement repeated exposure in this *in vitro* system. Within these repeated-exposure experiments, samples were taken from medium and cells at different time points to assess the biokinetic behaviour of the two compounds in this *in vitro* cell system.

2. Experimental procedures

2.1. Chemicals and solutions

DMEM was purchased from Gibco (Life Technologies, Zug, Switzerland); methanol LC–MS Chromasolve (Fluka), DMSO, CPZ and DZP were purchased from Sigma–Aldrich (St. Louis, MO, USA).

For the chemical analysis, methanol (HPLC-grade, 99% purity) was purchased from Labscan (Dublin, Ireland) and orthophosphoric acid from VWR Prolabo (Amsterdam, The Netherlands). A Millipore water purification system (Millipore Water, Amsterdam, The Netherlands) was used to prepare deionied water.

2.2. Preparation and main features of aggregating brain cell cultures

The aggregating rat brain cultures were prepared from 16-day old Sprague–Dawley rat embryos (Janvier, St Berthevin, France) as described by Honegger and Zurich (2011). In short, the brains of the embryos were dissected and dissociated into a single cell suspension by sequential passage through nylon sieves of 200 μm and 100 μm pore sizes (Sefar, Heiden, Switzerland). The dissociated cells were washed by centrifugation and finally resuspended in cold serum-free culture medium (modified Dulbecco's Modified Eagle Medium (DMEM). Aliquots of the cell suspension (containing the amount of cells obtained on average from one embryonic rat brain) were transferred to glass culture flasks (modified Erlenmeyer flasks with air-permeable stoppers) and incubated under continuous gyratory agitation (68 rpm) at 37 °C, in an atmosphere of 10% CO_2 and 90% humidified air. The frequency of gyratory agitation was increased progressively to reach the final speed of 80 rpm at day *in vitro* (DIV) 4. Culture media were replenished with fresh medium every third day until day 14 by exchanging 5 mL (of a total of 8 mL per flask). After 14 days, the culture media were replenished every second day.

The cells re-aggregate spontaneously into even-sized aggregates (600–800 μm of diameter at full maturation stage), composed of all the brain cell types, in the proportion of about 60% of neurons (several subtypes) and 40% of glial cells (astrocytes, oligodendrocytes and microglial cells). Within the aggregates, cells interact by physical contacts as well as by the exchange of soluble messengers and metabolites. These intrinsic factors enable extensive cellular differentiation and the elaboration of histotypic structures such as extracellular matrix, matures synapses, and compact myelin (Honegger et al., 1979; Monnet-Tschudi et al., 1993).

2.3. Preparation of replicate cultures, repeated exposure and sampling

The first day of exposure to the drugs (DIV18), replicate cultures were prepared as follows: the aggregates from 4 original flasks were pooled and then redistributed into flasks containing pre-equilibrated serum-free culture medium, in order to obtain 1/6th of one original flask per replicate culture. Thus, each of the culture replicates contained an average of 150–180 aggregates (about 1 mg of protein) in 8 mL of culture medium.

After equilibration of pH and temperature, the aggregating brain cell cultures were exposed to two concentrations of chlorpromazine and diazepam, the 10% toxic concentration (TC_{10}) and 1/5th of the TC_{10} (as determined in previous cytotoxicity assays where intracellular LDH activity, determined by a conventional photometric assay method, (Koh and Choi, 1987) was measured after repeated exposure for 14 days). The 2000-fold concentrated stock solutions were prepared in DMSO and contained 2 and 0.4 mM chlorpromazine and 3 and 0.6 mM diazepam. Samples were taken from both the stock solutions and stored at −80 °C until HPLC analysis.

On the first day of treatment, 4 μL of the respective 2000-fold concentrated stock solution were pipetted directly into the culture medium of each replicate culture. This resulted in nominal final concentrations of 0.2 μM and 1 μM for chlorpromazine and 0.3 μM and 1.5 μM for diazepam. The cells were exposed for 12 days and medium was renewed every other day as follows: 5 ml of medium (of a total of 8 ml) was replaced with fresh pre-warmed medium. After each medium replenishment, 2.5 μL of 2000-fold concentrated stock solution were pipetted directly into the culture medium of each replicate culture. DMSO was added to the control cultures. After the first and last exposure, samples were taken at the following time points: 30 min, 1 h, 3 h, 6 h and 24 h. For the time-point 0 (test media), flasks containing medium but no cells were prepared. At all time points, medium was sampled from the flasks and the cells were collected. For this, the cells were washed twice with phosphate buffered saline (PBS). After removal of all the PBS, 1 mL of methanol was added and the cells were sonicated. The medium and cell samples were stored at −80 °C until HPLC analysis to study the biokinetic behaviour of both compounds in the culture systems.

The amount of cells per replicate cultures appears to be stable over time, as judged by the total RNA content measured in untreated control cultures at the beginning and at the end of the experiment, 13 days later (14.9 + 1.9 and 14.2 + 2.2 μg RNA/replicate, respectively), in parallel experiments run in the Predict-IV project.

Three independent experiments were performed on three preparations of cells from independent pools of animals. In each independent experiment, each group of treated or untreated cells was composed of two replicate cultures.

2.4. Sample preparation and HPLC analysis

For analysis by HPLC, the samples were treated to remove proteins and cells: The stock solutions were diluted in 75% methanol; 25% Millipore water. For the test media and medium samples taken from the cultures, 3 volumes of methanol were added to one volume of sample. This was mixed on an IKA MS 1 minishaker (IKA, Staufen, Germany). For the cell lysate samples, one volume of Millipore water was added to three volumes of sample; this was mixed and placed in the centrifuge for 5 min at 2500 rpm (Beckman Coulter Allegra X12-R, Beckman Coulter, Woerden, The Netherlands). The supernatant was transferred to a glass auto sampler vial and used for HPLC analysis.

The diazepam samples were measured by a HPLC-UV system that consisted of a solvent delivery system (Varian, Middelburg, The Netherlands), a C18 column (ID length 4.6 × 150 mm, particle size 5 μm, Gracesmart, Breda, The Netherlands), a UV–VIS detector and a degasser (Shimadzu, 's-Hertogenbosch, the Netherlands) and a Basic Marathon auto sampler (Spark Holland, Emmen, The Netherlands). The mobile phase consisted of 30% Millipore water and 70% methanol at a flow rate of 0.8 mL/min. The UV detector was set at a wavelength of 235 nm, the analysis was performed at room temperature. The total analysis time per sample was 5 min and the retention time of diazepam was 2.8 min. The limit of detection (LOD) was 0.09 μM and the limit of quantification (LOQ) was 0.34 μM.

The chlorpromazine samples were analysed according to Broeders et al. (2011). In short, the mobile phase consisted of 25% phosphate buffer (pH = 3.0) and 75% methanol at a flow rate of 0.8 mL/min. The UV detector operated at a wavelength of 254 nm. The limit of detection was 0.05 μM and the limit of quantification was 0.16 μM.

3. Results

3.1. In vitro biokinetics of chlorpromazine

The lowest CPZ test concentration of 0.2 μM could not be measured in the medium samples because the concentrations in the samples were below the limit of detection (LOD). The measured concentrations in the stock solutions were 0.41 ± 0.08 mM for the nominal concentration of 0.4 mM and 2.25 ± 0.07 mM for the nominal concentration of 2 mM. The CPZ concentration in the test medium was 0.82 ± 0.10 μM for the 1.0 μM CPZ concentration.

The results for the medium samples of the 1 μM concentration are shown in Fig. 1. The 0 value indicates the amount of CPZ found in medium that has never been in contact with cells, but which received the exact same amount of stock solution as the replicate cultures containing cells.

The amount in the medium decreased over time. After 30 min the amount decreased by 60% and after 24 h no CPZ was detected in the sample (data point shown in Fig. 1 is LOQ/2 * volume medium). On the last exposure day, the amount in the medium decreased as well and no significant differences were seen between the two sampling days, except for the 24 h sampling point.

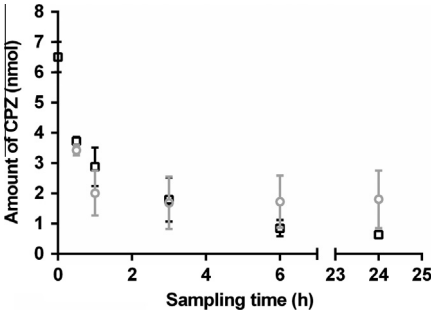

Fig. 1. Amount of chlorpromazine in medium samples taken on the first (□) and last (○) exposure day for the TC_{10} of 1 μM. Mean and SD are shown for 3 biological replicates.

The amount of CPZ in the cell lysate samples is shown in Fig. 2. For the low CPZ test concentration of 0.2 μM, data from two biological replicates is shown (as single data points), since in one replicate the amounts were below the LOD. The amount of CPZ in the cell samples showed an initial increase followed by a decrease over time on both days. Furthermore, the amounts found on the last exposure day were 3–6 times higher compared to the first exposure day. The amounts found for the high CPZ concentration are 5–6 times higher than the amounts found for the low test concentration. This reflects the difference in nominal concentration added to the culture systems (0.2 versus 1 μM CPZ).

The mass balance of CPZ was calculated by adding the amounts found in the medium to the amounts found in the cells and comparing this to the amount added to the system (as measured in the test media). The mass balance on the first exposure day is shown in Table 1 for the high CPZ test concentration.

In addition, the distribution of CPZ over the different compartments (medium, cells, lost) was calculated. The results for the high CPZ test concentration on the first exposure day are shown in Fig. 3. Data for the distribution at the low CPZ test concentration are not shown, as the CPZ concentration found in the medium was below the LOQ and concentrations at the LOQ level may represent a substantial part of the total amount (maximally 80%).

For the high CPZ test concentration of 1 μM, the percentage found in the medium decreased over time, from 60% after 30 min to 3% after 24 h. The percentage of CPZ found in the cell lysate samples increased from 52% to 81% and at the last time point (24 h) decreased again to 59%. Not all CPZ was found back from 3 h to 24 h after addition of CPZ to the system, the percentage lost increased to 38%.

3.2. In vitro biokinetics of diazepam

The measured diazepam concentrations in the stock solutions were higher than the nominal concentrations: 0.82 ± 0.02 mM for the 0.6 mM concentration and 4.47 ± 0.11 mM for the 3 mM concentration. As a consequence, the test media also contained higher diazepam concentrations: 0.65 ± 0.09 μM for the 0.3 μM concentration and 2.20 ± 0.23 μM for the 1.5 μM diazepam concentration. The measured DZP amounts in the medium samples are shown in Fig. 4. For the low concentration of 0.3 μM, DZP could only be measured in two biological replicates, therefore, the individual data points are shown in the figure instead of a mean and standard deviation. The amount of DZP in the medium remained relatively stable over time for both the low and high DZP test concentrations.

For the low DZP test concentration of 0.3 μM, the amounts in the cell lysate samples were too low to be measured (<LOD). The amounts measured in the high DZP concentration of 1.5 μM are shown in Fig. 5. The amount found in the cells remained stable

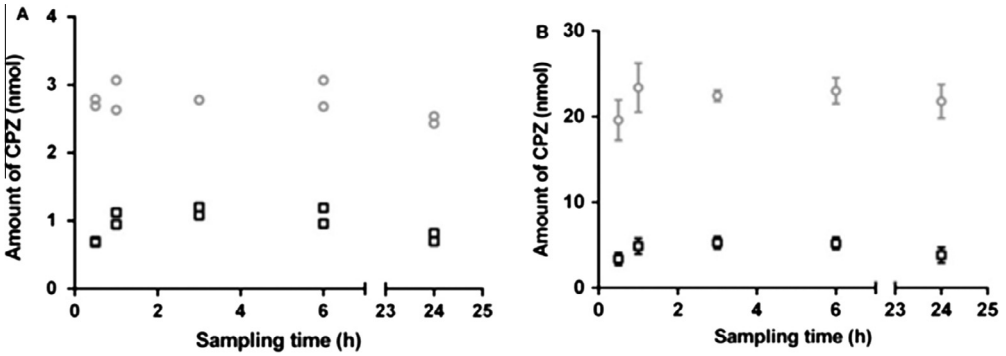

Fig. 2. Amount of CPZ in the cell lysate samples from the low (A –1/5th of TC_{10} = 0.2 μM) and high (B – TC_{10} = 1 μM) CPZ test concentrations on the first (□) and last (○) exposure day. For the low CPZ concentration, individual data points from n = 2 are shown; for the high CPZ concentration mean and SD are shown for n = 3.

Table 1
Mass balance (%) of chlorpromazine in aggregating brain cell cultures on the first exposure day.

Sampling time	Nominal concentration 1.0 μM CPZ (%)
30 min	108.9 (8.2)
1 h	119.0 (18.1)
3 h	107.0 (19.8)
6 h	88.7 (13.3)
24 h	62.2 (8.7)

Mean (SD) are shown for 3 biological replicates, based on amounts found in medium and cells.

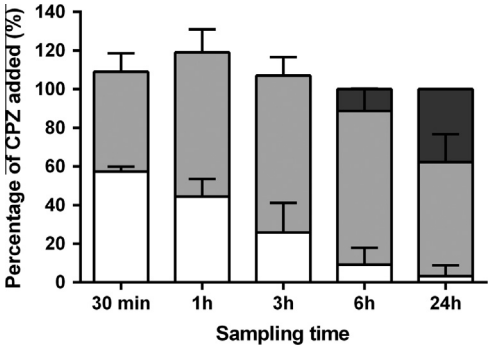

Fig. 3. Distribution of CPZ in the aggregating rat brain cell cultures on the first exposure day. Percentage of CPZ in the medium (clear), cells (grey) and the percentage lost (black) are shown for the high CPZ concentration (TC_{10} = 1 μM) based on data from 3 biological replicates.

over time and no significant differences were seen between the two sampling days.

The mass balances of diazepam were calculated and the results on the first exposure day are shown in Table 2 for the high DZP test concentration. The mass balance of diazepam remained stable over time.

The results for the distribution of diazepam in the *in vitro* system are shown in Fig. 6 for the high DZP test concentration. For the low DZP concentration of 0.3 μM, most of the diazepam added was found back in the medium (92–99%) and no DZP was found in the cells. Because concentrations at the level of the LOQ may represent maximally 50% of the total amount in the system, data on mass balance or distribution are not shown. For the high DZP concentration of 1.5 μM, 82–95% of the diazepam added was found back in the medium. The cells contained only about 2% of the DPZ added to the system.

4. Discussion

In this study the exposure and biokinetics of chlorpromazine (CPZ) and diazepam (DZP) were studied in 12-day repeated exposure experiments performed in aggregating rat brain cell cultures. The results show that chlorpromazine decreased in the medium over time by uptake into the cells. CPZ accumulated inside the cells during the 12-day repeated exposure. In contrast, the amount of diazepam in the medium remained stable over time and no accumulation was seen in the cells.

The measurements of the high CPZ test concentration of 1 μM in the medium showed a decrease over time that was comparable between the first and last exposure day. This decrease in the medium was associated with uptake into the cells as shown in Fig. 2. In

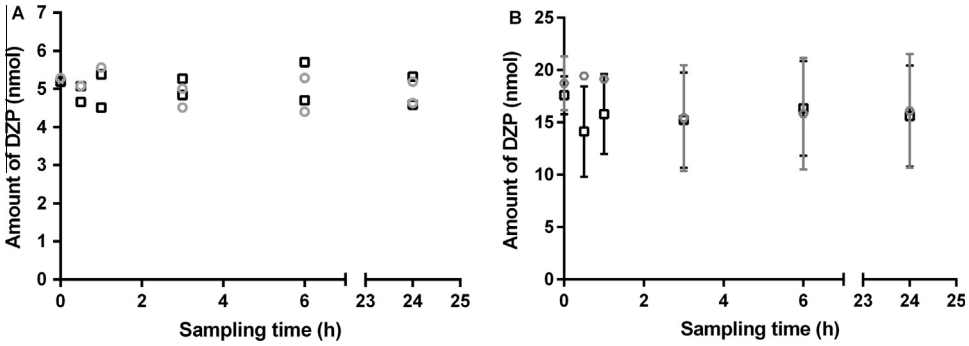

Fig. 4. Amount of diazepam in medium samples from the low (A – 1/5th of TC_{10} = 0.3 μM) and high (B – TC_{10} = 1.5 μM) DZP concentration taken on the first (□) and last (○) exposure day. For the low DZP concentration, individual data points (n = 2) are shown for the high DZP concentration mean and SD are shown for n = 3.

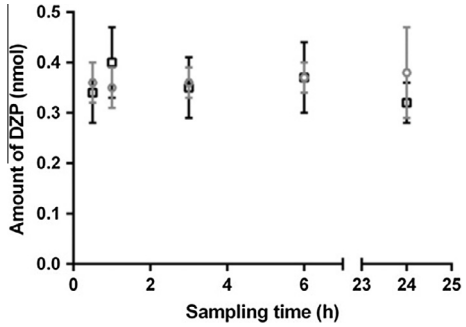

Fig. 5. Amount of DZP in the cell lysate samples from the high DZP test concentration of 1.5 μM. Mean and SD are shown for $n = 3$ on the first (□) and last (○) exposure day.

Table 2
Mass balance (%) of diazepam in aggregating brain cell cultures on the first exposure day.

Sampling time	Nominal concentration 1.5 μM DZP (%)
30 min	84.9 (27.2)
1 h	94.4 (23.4)
3 h	90.7 (27.0)
6 h	97.4 (27.0)
24 h	92.9 (29.0)

Mean (SD) are shown for 3 biological replicates for the high DZP concentration, based on amounts found in medium and cells.

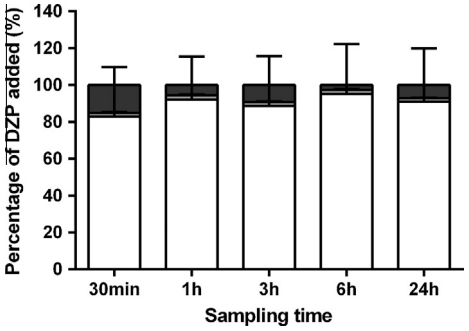

Fig. 6. Distribution of DZP in the aggregating rat brain cell cultures on the first exposure day. Percentage of DZP in the medium (clear), cells (grey) and the percentage lost (black) are shown for the high ($TC_{10} = 1.5$ μM) DZP concentration based on data from 3 biological replicates.

contrast, the medium samples of the cultures exposed to diazepam showed a stable amount of DZP in the medium over time. This was seen for both the low and high DZP test concentrations and no significant differences were seen between the two sampling days. The uptake of diazepam from the medium into the cells was lower than for chlorpromazine. Of the total amount of DZP added to the system, only up to about 2% was found back in the cells. This was markedly lower than for CPZ, where 50–80% of the CPZ added was found in the cell lysate samples. The distribution data confirm that most of the DZP added to the system remained in the medium (82–99%) for both concentrations tested.

A possible explanation for the differences found in the biokinetic behaviour of chlorpromazine and diazepam could be the transport of the compound either in or out of the cells. More DZP is found in the medium and thus less in the cells. Either the uptake of diazepam into the cells is lower than for chlorpromazine or the efflux of diazepam out of the cells is higher. Both compounds are most likely transported into cells by diffusion. CPZ has a Log K_{ow} of 3.16 for the ionised form (Log K_{ow} for the non-ionised form is 5.3) and DZP has a Log K_{ow} of 2.8, indicating that both compounds are lipophilic. The slightly higher lipophilicity of chlorpromazine might favour its uptake into the cells compared to diazepam. Another physical–chemical property to take into account is the pK_a and the ionisation of the compound. At a physiological pH of 7.4, chlorpromazine is almost completely ionised (98.8%), whereas diazepam is neutral (non-ionised). Overall it is thought that only the non-ionised compound can be taken up by cells. However, Caco-2 experiments have shown that both species (non-ionised and ionised) are transported across cell layers (Broeders et al., 2012; Yee, 1997).

Both compounds are substrates for the P-glycoprotein efflux transporter (Adachi et al., 2001; Saitoh and Aungst, 1995); in addition, CPZ is also a competitive inhibitor of this transporter (Feng et al., 2008; Wang et al., 2006). Preliminary results indicate that P-glycoprotein is present in these cultures, although nothing is known yet about its functionality. Overall, no conclusion can be drawn as to whether the observed biokinetic behaviour of CPZ and DZP is caused by differences in either the uptake or the excretion of the respective compounds. Possible explanations were discussed and further research is needed to clarify this. Chlorpromazine is known to induce phospholipidosis (Anderson and Borlak, 2006; Tavoloni and Boyer, 1980) and to bind to lamellar bodies (Joshi et al., 1989). Possibly, because of their lipophilic nature, the formation of lamellar bodies and binding of CPZ to the lipid content can explain the accumulation of CPZ in the cells.

For both compounds, the mass balances were relatively high. However, for both compounds values below 100% were found. The limit of quantification (LOQ) of the analytical method likely played a role in this. The concentrations tested were based on previous cytotoxicity experiments and were rather low and close to the LOQ. For the low CPZ concentration no compound could be measured in the medium and for the low diazepam concentration no DZP could be measured in the cells. It is possible that there was compound present in these samples but that the amount was too low to be measured. Therefore, the mass balances were most likely complete (around 100%) for both compounds. Only for chlorpromazine a small decrease in mass balance over time was seen for the high concentration. If this was indeed the case, then a possible explanation for this could be metabolism. As of now, no published data is available on the metabolic competence of these cultures.

In the aggregating rat brain cultures, CPZ accumulated over time while the amount of DZP remained stable over the 12-day repeated exposure. The uptake of CPZ into the cells was already higher than the uptake of DZP on the first exposure day. New compound was added to the culture every other day during 12 days and this did not seem to influence the uptake of CPZ. This uptake continued and led to an accumulation of CPZ in the cells over time.

The biokinetics of the two compounds clearly differ in the aggregating rat brain cell cultures. In an earlier study with liver cells, we simulated the accumulation of CPZ over time (Broeders et al., 2015). In this study with liver cells, a concentration time profile as presented here in Fig. 7A was observed. The concentrations of CPZ in the aggregating rat brain cell show a similar trend. On the contrary, DZP reaches an equilibrium after 1 day and no further accumulation was observed (Fig. 7B). The amount in the medium remained stable over the entire exposure period for DZP, whereas the amount of CPZ in the medium fluctuated over time. This will affect the outcome of cytotoxicity tests. In general, the concentration that is taken into account is the nominal concentration in the medium. Based on that concentration, the 50% toxic or effect concentration is calculated. In the case of CPZ, the amount in the medium decreased over time (after every addition of test compound to the cell cultures). As a consequence, effect concentrations of CPZ will depend on the exposure time, while this may not be the case for DZP. This implicates

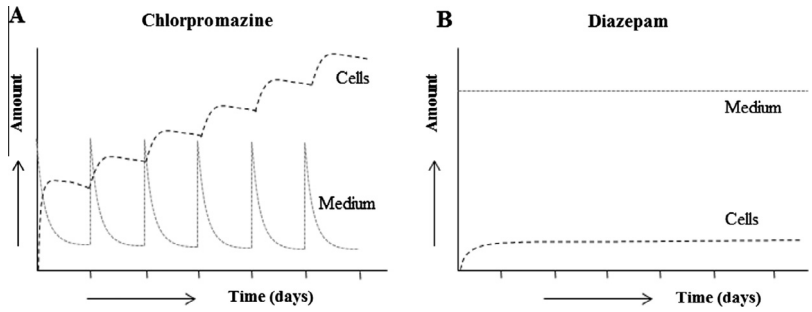

Fig. 7. An indication of the *in vitro* biokinetic behaviour of chlorpromazine (A) and diazepam (B).

the importance of taking biokinetics into account when interpreting *in vitro* data, and when extrapolating these data to the *in vivo* situation. By knowing the actual dose that the cells are exposed to and thus which dose reaches the target site, this value can be used as a point of departure for *in vitro–in vivo* extrapolation (IVIVE). The need for improved accuracy of *in vitro* data for quantitative IVIVE has already been described in literature (Blaauboer et al., 2012; Yoon et al., 2012).

Overall, differences were found in the biokinetic behaviour of chlorpromazine and diazepam in the aggregate rat brain cell cultures. CPZ was predominantly found in the cells, whereas most of the DZP was found in the medium. The accumulation of CPZ in the cells during the repeated exposure leads to higher intracellular concentrations of this compound in the cells. This study shows that the biokinetic behaviour of test compounds should be taken into account when incorporating repeated dosing into *in vitro* assays and when interpreting the results found.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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