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Biological conversion of aripiprazole lauroxil — An *N*-acyloxymethyl aripiprazole prodrug

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ABSTRACT

N-acyloxyalkylation of *NH*-acidic compounds can be a prodrug approach for e.g. tertiary or some *N*-heterocyclic amines and secondary amides and have the potential to modify the properties of the parent drug for specific uses, for example its physicochemical, pharmacokinetic or biopharmaceutical properties. Aripiprazole lauroxil was prepared as a model compound for such prodrugs and its bioconversion was investigated both *in vitro* and *in vivo*. Theoretically, *N*-acyloxyalkyl derivates of *NH*-acid compounds undergo a two-step bioconversion into the parent *NH*-acidic drug through an *N*-hydroxyalkyl intermediate. However, to our knowledge no published studies have investigated the formation of an intermediate *in vivo*. In the present study, it was demonstrated that the assumed *N*-hydroxymethyl intermediate was readily observed both *in vitro* and *in vivo*. *In vivo*, the observed plasma concentration of the intermediate was at the same level as the drug (aripiprazole). When prodrug intermediates are formed, it is important to make a proper pharmacological, pharmacokinetic and toxicological evaluation of the intermediates to ensure patient safety; however, several challenges were identified when testing an *N*-acyloxyalkyl prodrug. These included the development of a suitable bioanalytical method, the accurate prediction of prodrug bioconversion and thereby the related pharmacokinetics in humans and the toxicological potential of the intermediate.

1. Introduction

Prodrugs are compounds that undergo a biological transformation prior to achieving their pharmacological effect and have been known for more than 50 years [1]. According to this definition, prodrugs are xenobiotics that are inactive *per se*, but are transformed into one or more active metabolites [2–4]. Although there is no universal definition of a prodrug, recent definitions also describe prodrugs as bioreversible derivatives of active drug molecules that undergo enzymatic and/or chemical transformation *in vivo* to produce the pharmacological active compound, which can then exert the intended pharmacological effect [5,6]. Ideally, the prodrug should be converted to the active parent compound, followed by a subsequent rapid elimination of the released promoiety [7]. Furthermore, it has been suggested that prodrugs should either be inactive or much less potent (1000-times) than the parent drug [8]. Different functional groups are amenable to prodrug design, as recently reviewed [9]. In both drug discovery and drug development, the design of prodrugs is an established tool for improvement of the physicochemical, biopharmaceutical, and/or pharmacokinetic properties of pharmacologically active compounds. Prodrugs have been applied in a number of different situations to overcome various barriers to drug formulation and delivery, including poor aqueous solubility [10,11], chemical or metabolic instability [12], insufficient absorption [13–15], local delivery as nasal [16] and lymphatic transport [17]. In 2004, Stella estimated that 5–7% of drugs worldwide could be classified as prodrugs [18] and in 2009, 13 of the 100 top-selling pharmaceuticals were prodrugs [4], including the statins, Mevacor® and Zocor®, which are cyclic prodrugs that have to be metabolised to the acyclic form that acts as the active compound [3].

Utilization of the prodrug approach may provide a life cycle management option for established drugs and thus the application of the concept is intriguing but also challenging. Despite similarity to the established drug the prodrug must be considered as a new chemical entity and its development planned and conducted accordingly. The

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specific challenges from both drug discovery and development perspectives include (i) possible pharmacological activity of the prodrug; (ii) mode of prodrug conversion; (iii) rate and extent of conversion of prodrug to active moiety in species used for the toxicological evaluation to ensure proper calculation of safety margins for the clinical development and later use; and (iv) no alteration of the disposition properties, metabolic capacity for or towards the active moiety [19]. Using the prodrug principle as a means of life cycle management is, therefore, not simple from a scientific, a developmental or a regulatory point of view and requires significant cross-functional efforts to succeed. However, if the benefit is clinical significant for the patient, it could be a potential enabling approach, for example, for a defined route of administration.

Aripiprazole is approved as an effective treatment for various psychiatric disorders [20–23]. The compound is marketed in several dosage formulations, including tablets, orally disintegrating tablets, an oral solution, and as a suspension for once-monthly intramuscular injection as a depot. Recently an *N*-acyloxymethyl prodrug of aripiprazole (aripiprazole lauroxil) intended for intramuscular injection has been described [24]. Bioconversion of *N*-acyloxyalkyl derivatives of *NH*-acidic compounds is thought to proceed through a hydrolytical two step process as previously investigated and thoroughly described by Hans Bundgaard and coworkers e.g. [25–31], as illustrated for aripiprazole lauroxil in Fig. 1. The rate of prodrug conversion of *N*-acyloxymethyl derivatives of *NH*-acidic compounds is firstly determined by the rate of enzymatic or non-enzymatic catalysed hydrolysis of the ester bond into the corresponding carboxylic acid and *N*-hydroxyalkyl moieties followed by a non-enzymatic spontaneous cleavage into the parent drug molecule and an aldehyde, e.g. formaldehyde as in the case of aripiprazole lauroxil. The later process is thought to be solely dependent on pH and temperature as previously described [25,30–32]. To the best of our knowledge, no information is available on the conversion of *N*-acyloxyalkyl derivates of *NH*-acidic compounds focusing on simultaneous quantification of all components and intermediates in the two step bioconversion, i.e., the prodrug, the *N*-hydroxyalkyl intermediate and the parent *NH*-acidic compound, both *in vitro* and *in vivo*. Thus, in the present study, we use aripiprazole lauroxil as a model compound for an *N*-acyloxyakyl prodrug of an *N*-acidic compound (drug) to provide an insight into the biological conversion of these compounds.

2. Materials and methods

2.1. Chemicals

Aripiprazole was obtained from Otsuka pharmaceutics (Tokyo, Japan), while *N*-hydroxymethyl-aripiprazole and aripiprazole lauroxil were synthesised as described below. Reagents and solvents for the synthetic work were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Viscoleo, Ph. Eur Grade medium chain triglyceride, C8/C10 (MCT) was purchased from Delios V (Illertissen, Germany), lecithin (E80) for the intravenous emulsion was obtained from Lipoid AG (Ludwigshafen, Germany) and glycerol from Sigma Aldrich (St. Louis, MO, USA). Hypergrade acetonitrile from Merck (Darmstadt, Germany) was used for the HPLC–MS/MS analysis, ethanol was from De Danske Spritfabrikker (Aalborg, Denmark) and deuterated aripiprazole used as the internal standard in the bioanalysis was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Purified water was obtained from a Millipore Milli-Q Ultrapure Water purification system (Billerica, MA, USA). All other chemicals were of analytical grade or higher.

2.2. Chemical synthesis

Aripiprazole lauroxil was obtained by an alkylation of aripiprazole using sodium hydride and chloromethyl laurate [33] in a mixture of N,N-dimethylformamide and tetrahydrofuran. After an aqueous work-up followed by column chromatography, aripiprazole lauroxil was isolated in 60% yield (LC purity: 96%) with data corresponding to that reported in literature [24].

To obtain *N*-hydroxymethyl aripiprazole, aripiprazole was alkylated using sodium hydride and benzyl chloromethyl ether in a mixture of *N*,*N*-dimethylformamide and tetrahydrofuran [34]. After an aqueous work-up followed by column chromatography, the BOM-protected aripiprazole was isolated in 41% yield as confirmed by analytical data (data not shown). The BOM-protected aripiprazole was then stirred in methanol containing one equivalent HCl and Pearlman's catalyst under an atmosphere of hydrogen to remove the benzyl group. The mixture was filtered and concentrated to give *N*-hydroxymethyl aripiprazole HCl in >95% yield (LC purity: 89%) with data corresponding to that reported in the literature [24].

2.3. In vitro conversion in buffer

To follow the spontaneous conversion from *N*-hydroxymethyl aripiprazole to aripiprazole, a stock solution in DMSO-d_6 was made so the reaction could be started by adding the stock solution into a phosphate buffer, pH 7.4, which thereby contained 0.5% v/v DMSO-d_6. The final concentration of *N*-hydroxymethyl aripiprazole in the buffer was 9 μM equal to the solubility of aripiprazole in water [35]. The degradation was followed at both 25 °C and 37 °C by continuous measurements.

^{1}H NMR spectra were measured at 600.163 MHz on a Bruker AV-III-600 equipped with a 5 mm TCI CryoProbe. Referencing was done to DMSO-d_6 (2.51 ppm). Solvent suppression with excitation sculpting [36] using a square 180 pulse of 4 ms was applied on aqueous solutions. Acquisition time was 1.7 s and repetition delay was 3 s A Lorentzian Line broadening of 1.0 Hz was applied before FT, and the aromatic region was baseline corrected manually using a 4th degree polynomial fit before integration.

2.4. In vitro conversion in plasma

An *in vitro* experiment was conducted in triplicate by adding 30 μL 1 μM aripiprazole lauroxil dissolved in ethanol to 1.47 mL rat plasma from female Sprague Dawley rats at 37 °C. The spiked plasma was stored at 37 °C and 50 μL aliquots were taken at 0.5 and 1.0 h post-spike. The aliquots were immediately treated with 200 μL cold acetonitrile containing 0.4% citric acid and stored at −80 °C until analysed as described in Section 2.7.

2.5. Formulations for the in vivo study

An emulsion for intravenous administration containing each of the three compounds (i.e., aripiprazole, *N*-hydroxymethyl-aripiprazole or aripiprazole lauroxil) in equimolar concentrations equivalent to 1 mg aripiprazole was produced. The emulsions consisted of compound, 20% w/w fractionated coconut oil, 1.2% w/w lecithin, 2% w/w glycerol and q.s. water. The amount of each compound added was 1 mg aripiprazole/mL, 1.2 mg *N*-hydroxymethyl-aripiprazole/mL or 1.87 mg aripiprazole lauroxil/mL, i.e., equimolar. Each of the three compounds was dissolved in the oil together with lecithin and gently heated to 50 °C with continuous stirring. Glycerol was added to the aqueous phase as an isotonic agent and the aqueous phase was heated to 50 °C. The two phases were mixed and homogenised to a pre-emulsion by rapid stirring for 1 min. The pre-emulsion was placed on ice and the droplet size was further reduced by means of a homogeniser equipped with a standard microtip at a power output of 5 (Sonifier Cell Disruptor, Model B15, Branson, Pusan, Korea) for 10 min. The formulation was then filtered through a 0.45 μm sterile filter into a sterilised glass bottle with a rubber membrane and a crimped lid.

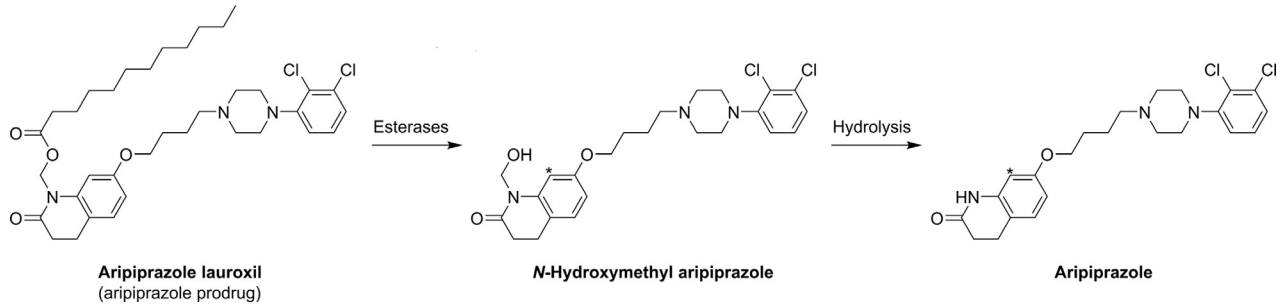

Fig. 1. Schematic presentation of the bioconversion of aripiprazole lauroxil. *Indicates the proton investigated in the ^{1}H NMR study.

2.6. In vivo study

The protocol used for the *in vivo* study in rats was approved by the institutional animal ethics committee in accordance with Danish law regulating experiments on animals and in compliance with EC directive 2010/63/EU, and the NIH guidelines on animal welfare. Female Sprague Dawley rats, weighing 248–276 g on the day of administration, obtained from Charles River (Sulzfeld, Germany) were used for the pharmacokinetic studies ($n = 6$ per group). The animals were acclimatised for a minimum of 5 days in groups of 2 on wooden bedding (Tapvei, Kortteinen, Finland) in plastic cages, 595 × 380 × 200 mm^3, with a stainless steel lid (Scanbur, Sollentuna, Sweden) in humidity- and temperature-controlled ventilation cupboards (Scantainers, Scanbur Technology, Karlslunde, Denmark), relative humidity 40–60%, temperature 20 ± 1 °C, light from 6:00–18:00 h. The animals had free access to a standard rodent diet (Altromin 1325, Brogaarden, Denmark) and water *ad libitum* during the study.

The animals were randomly assigned to three groups ($n = 6$ per group) receiving either aripiprazole, *N*-hydroxymethyl-aripiprazole or aripiprazole lauroxil molar equivalent to 5 mg aripiprazole/kg. The animals were dosed by injection into the tail vein with a submicron emulsion containing a molar concentration equivalent to 1 mg aripiprazole/mL. Blood samples of 100 μL were obtained from the lateral tail vein by individual vein puncture and collected into potassium–EDTA tubes (Microvette 500 K3E, Saarstedt, Nümbrecht, Germany). Samples was taken at 5, 15, 30 min and 1, 2, 4, 6, 8 and 24 h after administration. Plasma was harvested immediately by 10 min of centrifugation at 4 °C, 2765*g* (Multifuge 1 S–R, Heraeus, Hanau, Germany) and stored at −80 °C until analysed. At the end of the experiment, the animals were sacrificed.

2.7. Bioanalysis

EDTA plasma samples were processed by protein precipitation of 50 μL plasma with 200 μL ice-cold 0.4% citric acid in acetonitrile containing 15 ng/mL aripiprazole-d_8. The samples were mixed for 10 min in a shaking apparatus followed by centrifugation at 5000*g* for 10 min at 15 °C and 150 μL supernatant was transferred to a 2 mL deep well plate. Calibration standards and quality control (QCs) were prepared by adding standard solution to blank plasma and prepared similarly to the plasma samples. The analysis was performed by HPLC–MS/MS using a Waters Acquity-Xevo TQ system controlled by UNIFI. The separation was done on a Waters Spherisorb Silica column (3 μm, 100 × 2.1 mm^2) with a mobile phase consisting of water/acetonitrile (25/75 v/v) containing 1% formic acid, at a flow rate of 0.8 mL/min and a column oven temperature of 45 °C. A 3 μL sample was injected in partial loop with needle overfill mode. The mass spectrometer was operated in the positive electrospray mode with a desolvation temperature of 650 °C. The analytes were detected by multiple-reaction-monitoring: aripiprazole lauroxil (660.39–460.16 m/z), *N*-hydroxymethyl aripiprazole (478.17–448.16 m/z) and aripiprazole (476.15–285.09 m/z). The run time of the assay was 3.5 min with the peaks eluting between 1.45 and 1.84 min. The assay showed linearity over the concentration range of 2.00–1000 ng/mL.

2.8. Data and statistical analysis

Results obtained are presented as means and the standard error of the mean (mean ± SEM) unless otherwise stated. Pharmacokinetic parameters were calculated by using Phoenix version 6.3.0.0395 (Pharsight Corporation, Mountain View, CA, USA). The plasma concentration–time profiles of the three compounds after intravenous dosing were fitted to a two-compartment model. The area under the curve (AUC) was determined using the linear trapezoidal method and extrapolation of the last measured plasma concentration to infinity.

3. Results and discussion

It is possible to prepare stable *N*-acyloxyalkyl derivatives of, e.g., tertiary or some *N*-heterocyclic amines and secondary amides, which are susceptible to enzymatic hydrolysis by esterases, with subsequent spontaneous decomposition, as demonstrated with pilocarpine [37], theophylline [38], penicillin G [39] and various carboxylic acid agents [40]. For aripiprazole a similar stable derivatisation can be made at the lactam moiety, where the conversion and the relative presence of the three components – prodrug, intermediate and aripiprazole – in the bioconversion was investigated *in vitro* and *in vivo* in the present work.

3.1. In vitro bioconversion in rat plasma

To investigate the rate of biological conversion, two experiments were conducted using either *N*-hydroxymethyl aripiprazole added into a phosphate buffer or aripiprazole lauroxil added to rat plasma at 37 °C. Since initial studies have demonstrated that aripiprazole quickly precipitates and *N*-hydroxymethyl aripoprazole is rapidly converted to aripiprazole, NMR was selected as it is a continuous measurement for analysis of *N*-hydroxymethyl aripoprazole to aripiprazole conversion. Furthermore, only a single point determination was conducted for the full process in order to demonstrate the suggested conversion route. When *N*-hydroxymethyl aripiprazole (Fig. 2) was added to phosphate buffer, a rapid conversion was observed, i.e., a conversion that should not be rate limiting *in vivo*. The shift of the proton on C8 was followed, see Fig. 1 (C8 marked with *). When the hydroxymethyl group was attached a shift at 6.75 ppm was observed, whereas the shift changed to 6.40 ppm when the group was removed. At 25 °C the apparent first-order rate constant was 0.0044 min^{-1} and the half-life was approximately 35 min. At 37 °C, however, the conversion was so fast that the rate constant could not be measured with sufficient precision. More than half of the *N*-hydroxymethyl aripiprazole was converted within the first 15 min at 37 °C. Estimated pKa for 3,4-dihydro-2(1H)-quinolinone is 14.6 [41]. If this value is assumed similar for the *NH*-acidic group in aripiprazole a half-life of

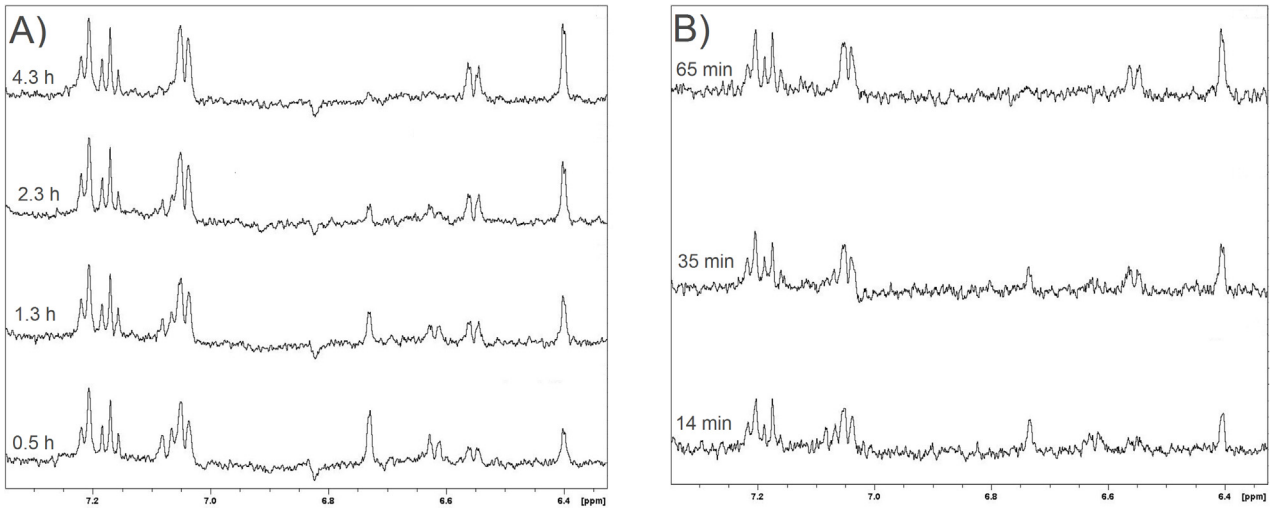

Fig. 2. Partial ^{1}H NMR spectrum after addition of *N*-hydroxymethyl aripiprazole to phosphate buffer, pH 7.4; (A) at 25 °C measured at (from the bottom) 0.5, 1.3, 2.3 and 4.3 h and (B) at 37 °C measured at (from the bottom) 14, 35 and 65 min.

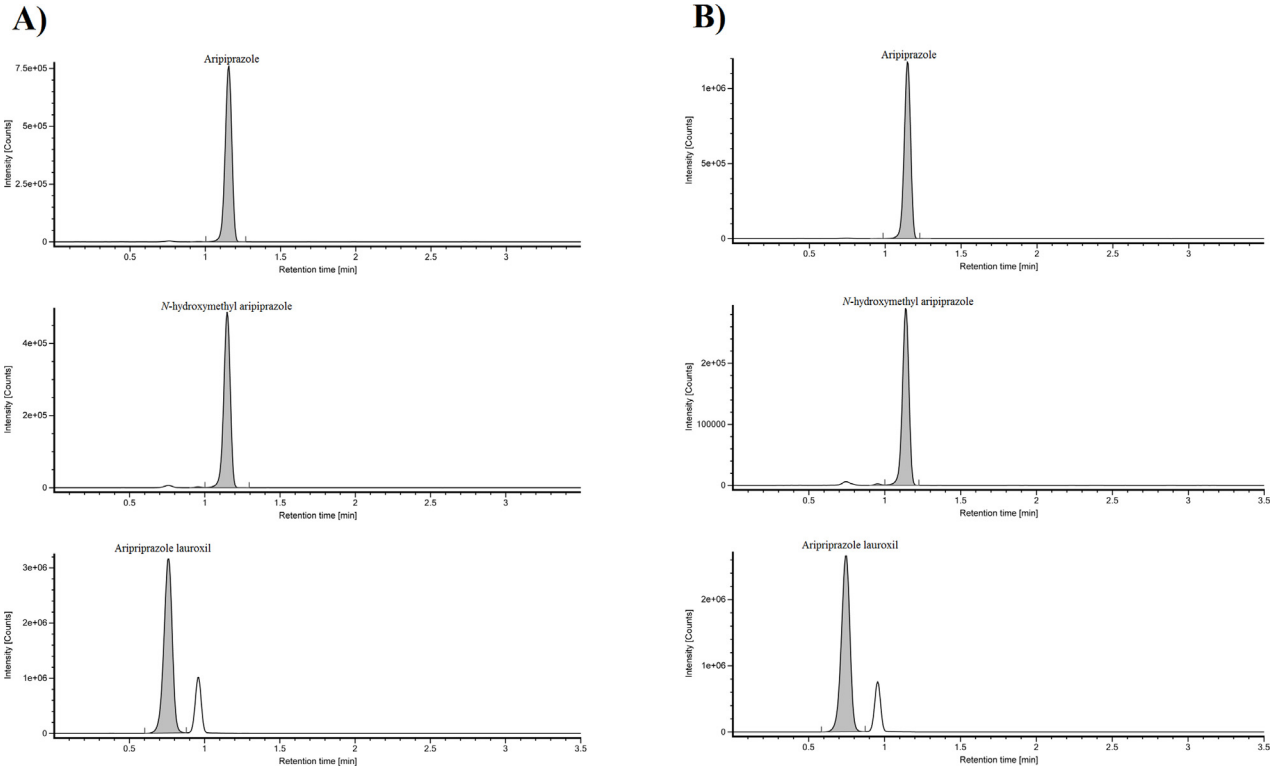

Fig. 3. Mass chromatograms obtained after addition of aripiprazole lauroxil to plasma from female Sprague Dawley rats at 37 °C sampled after (A) 0.5 h and (B) 1 h.

12.7 h should be anticipated based upon the prediction suggested by Bundgaard and Johansen [28]. This variation may be a reflection of a different chemical space used to make the correlation or that the estimated pKa value for 3,4-dihydro-2(1H)-quinolinone may not be similar to the pKa value for aripiprazole. The predicting defined by Bundgaard and Johansen [28] is very sensitive to the pKa value, for compounds with a pKa on 12.4 a half-life of 15 min would be estimated.

When aripiprazole lauroxil was added to rat plasma, concentrations of the expected *N*-hydroxymethyl intermediate in the two-step degradation described in Fig. 1 could be observed when analysed after both 0.5 and 1 h at 37 °C (see Fig. 3). The *in vitro* bioconversion from the *N*-acyloxyalkyl derivate to the parent compound observed in the present study is in accordance with previous *in vitro* bioconversion studies investigating *N*-acyloxyalkyl derivates [31,42–45]. Moreira and coworkers [39] have described the *in vitro* conversion of *O*-amidomethyl penicilloate, through an intermediate, with the very slow formation of penicillin G, whereas Buur et al. reported the conversion of *N*-acyloxymethyl derivates of 5-fluorouracil to occur within a similar timeframe as in the present study [25]. This study demonstrates that during the conversion of aripiprazole lauroxil to aripiprazole, *N*-hydroxymethyl aripiprazole is present in significant amounts, despite being a very short-lived intermediate compound as revealed from the experiment when *N*-hydroxymethyl aripiprazole

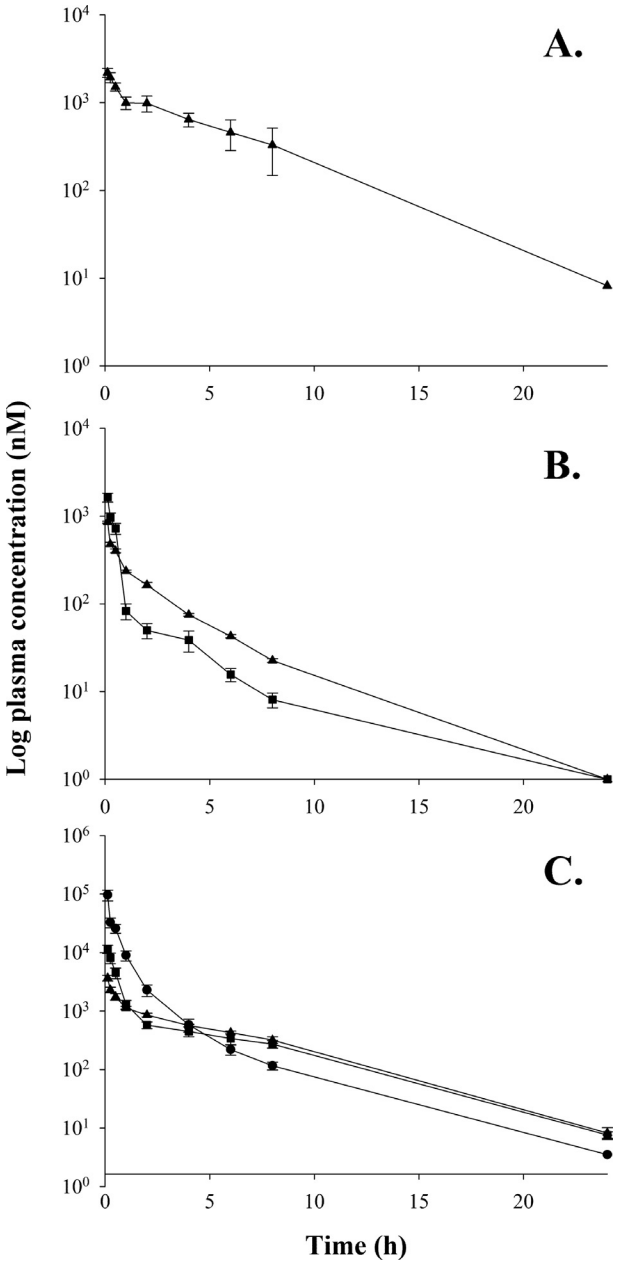

Fig. 4. Semi-log plot of mean (± SEM) plasma concentration *versus* time of equimolar intravenous doses of (A) aripiprazole (▲), (B) *N*-hydroxymethyl aripiprazole (■), and (C) aripiprazole lauroxil (●), administered to female Sprague Dawley rats ($n = 6$). For B) and C) in which bioconversion occurs, the concentrations of aripiprazole and *N*-hydroxymethyl aripiprazole are also shown.

was added to a buffer. This adds to the complexity of developing aripiprazole lauroxil as a safe drug, but also *N*-acyloxyalkyl derivates in general, since the pharmacology and toxicology of the intermediates needs to be put into the overall equation.

3.2. In vivo study

In vitro data has limitations and may not reflect what occurs in the whole organism. The conversion rate may be slower or faster, the concentrations different, etc., which is why it is not always possible to follow the entire bioconversion *in vivo*. The three compounds, aripiprazole, *N*-hydroxymethyl aripiprazole and aripiprazole lauroxil, were therefore dosed intravenously to three different groups of rats. **The plasma concentration time** profile following administration of aripiprazole can be seen in Fig. 4A. The profile could be described by a bi-exponential equation:

$$C_{pl} = 1464 \cdot e^{-2.77t} + 1205 \cdot e^{-0.16t} \quad (3)$$

where C_{pl} is the concentration of aripiprazole (nanomol) in plasma and t is time (in hours). The $AUC_{0\rightarrow\infty}$ was 8176 ± 2647 nmol h/L, clearance 1.37 ± 0.61 L/h/kg and volume of distribution 8.16 ± 0.75 L/kg giving a terminal plasma half-life of ≈4.2 h. This is slightly longer than that previously reported following non-compartmental evaluation after p.o. dosing in male Sprague Dawley rats [46].

The plasma concentration-time profile after injection of *N*-hydroxymethyl aripiprazole into rats is presented in Fig. 4B together with the concentration of aripiprazole measured during the bioconversion of *N*-hydroxymethyl aripiprazole. The plasma concentration curve had a similar profile for both compounds. The bioconversion from *N*-hydroxymethyl aripiprazole did not seem to be rate limiting for the formation of aripiprazole. This supports the findings of the *in vitro* study. Analysis of the emulsion just after dosing showed formation of aripiprazole (i.e., the exact pharmacokinetic parameters for *N*-hydroxymethyl aripiprazole and aripiprazole) cannot be described by this experiment. This highlights some of the scientific problems when investigating these bioconversions. The intention was to stabilise the compound through incorporation into a dispersed system, but hydrolysis was still observed. Developing methods and procedures for the evaluation of these intermediates is thus difficult and may in part explain the lack of *in vivo* investigations of prodrug conversion in the literature. However, from a drug development and patient safety point of view, it is a critical parameter to consider.

The plasma concentration–time profile after the injection of aripiprazole lauroxil into rats is shown in Fig. 4C, together with the amounts of *N*-hydroxymethyl aripiprazole and aripiprazole formed as a result of the bioconversion of the prodrug. No degradation of aripiprazole lauroxil was observed in the formulation, i.e., aripiprazole lauroxil was sufficiently stable to allow a pharmacokinetic evaluation of the compound. The clearance for aripiprazole lauroxil was 0.32 ± 0.11 L/h/kg. Interestingly, all three compounds were detected in the animals, demonstrating that the suggested biological conversion scheme presented in Fig. 1 is the bioconversion route found *in vivo*, and was in accordance with the observations from the *in vitro* study and the previous work by Moreira et al. [39]. The *in vitro* data indicated a high bioconversion of aripiprazole lauroxil, thus, the concentration of *N*-hydroxymethyl aripiprazole observed in the animals dosed with aripiprazole lauroxil was surprisingly high.

Quantification of the intermediate *N*-hydroxymethyl aripiprazole complicated the bioanalysis significantly. In order to get a reliable measurement of a prodrug and all the associated metabolites, it was generally important to stabilise the plasma samples to prevent *ex vivo* degradation, which could impact the pharmacokinetic calculations. The bioanalytical method used in the present work involved acidification and cooling to stabilise the intermediate, but degradation was still observed in the quality samples through the analytical sequence. The mean deviation of the quality samples was ~16% from the nominal value, i.e., the amount of *N*-hydroxymethyl aripiprazole was slightly underestimated. For intermediates with such a short half-life this is methodically a challenge in particular for the *in vivo* studies.

With the formation of intermediates such as *N*-hydroxymethyl aripiprazole, yet another challenge arises – the toxicological potential of the intermediate – but also the release of formaldehyde in the last conversion. Prodrugs must be designed with at least two specific sources of toxicity in mind: (i) toxicity of the metabolites formed from the promoiety and (ii) a reactive metabolic intermediate generated during bioconversion. One of the significant challenges for ester and *N*-acyloxyalkyl prodrugs is the accurate prediction of pharmacokinetics in humans, owing to significant differences in specific

carboxylesterase activity across species [47], as previously reported for the exploratory diester prodrug of nalbuphine [48]. The bioconversion in humans can therefore happen at a different rate, why close monitoring of all the components in both the pharmacokinetic and toxicological studies is important to ensure that the right dose is given to humans and that sufficient coverage of the metabolites is obtained in the species included in the toxicological evaluation of a given prodrug. The bioconversion inherent in the nature of a prodrug raises unanticipated issues that are not present in other drugs. This monitoring is therefore essential to the safe and effective administration of a prodrug. The present study illustrates the potential challenges of developing *N*-acyloxyalkyl derivates as prodrugs, given that the potential pharmacological and toxicological effects of the intermediates should been sufficiently analysed and documented.

4. Concluding remarks

In conclusion, the present study has demonstrated that the bioconversion of aripiprazole lauroxil to aripiprazole involves the formation of an intermediate, *N*-hydroxymethyl aripiprazole. All three compounds were detected in significant amounts both *in vitro* and *in vivo*, which give an indication of the complexity associated with the use of prodrugs that involve a two-step bioconversion.

This study also highlights some of the scientific problems investigating these bioconversions. Though the intention was to stabilise the compound through incorporation into a disperse system, the affinity for water or the placement in the interface between the two phases was still significant enough for major degradation in just 3 h. Developing methods and procedures for evaluation of these intermediates is, hence, linked to difficulties and is probably one of the reasons for the lack of *in vivo* investigations of prodrug conversion presented in the literature. Notwithstanding these methodological issues, it is important that these intermediates are identified and characterised in order to ensure that they do not result in any unanticipated complications.

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