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Alamethicin for using in bioavailability studies? – Re-evaluation of its effect

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ABSTRACT

A major pathway for the elimination of drugs is the biliary and renal excretion following the formation of more hydrophilic secondary metabolites such as glucuronides. For *in vitro* investigations of the phase II metabolism, hepatic microsomes are commonly used in the combination with the pore-forming peptide alamethicin, also to give estimates for the *in vivo* situation. Thus, alamethicin may represent a neglected parameter in the characterization of microsomal *in vitro* assays. In the present study, the influence of varying alamethicin concentrations on glucuronide formation of selected phenolic compounds was investigated systematically. A correlation between the alamethicin impact and the lipophilicity of the investigated substrates was analyzed as well. Lipophilicity was determined by the logarithm of the octanol-water partition coefficient.

For every substrate, a distinct alamethicin concentration could be detected leading to a maximal glucuronidation activity. Further increase of the alamethicin application led to negative effects. The differences between the maximum depletion rates with and without alamethicin addition varied between 2.7% and 18.2% depending on the substrate. A dependence on the lipophilicity could not be confirmed. Calculation of the apparent intrinsic clearance led to a more than 2-fold increase using the most effective alamethicin concentration compared to the alamethicin free control.

1. Introduction

The phase II metabolic pathway(s) have notably increased in interest, due to their important role in drug disposition (Alkharfy and Frye, 2001). For the toxicological and pharmacokinetic evaluation of *e.g.*, xenobiotics or potential drugs, knowledge about the phase II conjugation, representing one of the major metabolic routes, is crucial. Consequently, the predictive models aiming at correlating *in vitro* metabolic data with the *in vivo* situation have to take experimentally obtained kinetic parameters such as the intrinsic clearance into account. Hepatic microsomes are a frequently used model to monitor the conjugative metabolism and metabolic clearance of pharmaceuticals in *in vitro* experiments, as the liver is one of the major organs responsible for enzymatic drug elimination. Together with the formation of sulfate conjugates, glucuronidation is the most important phase II biotransformation in the body catalyzed by UDP-glucuronosyl transferases (UGTs), which are located within the endoplasmic reticulum (ER) (Alkharfy and Frye, 2001; Kilford et al., 2009). These glucuronides show a higher aqueous solubility compared with the original substrates and are excreted *via* the bile or the urinary bladder (Troberg et al., 2015). In general, the phase II metabolic conversion leads to a loss of the substrate's bioactivity. However, selected studies have shown that the formation of glucuronides result in bioactive secondary metabolites (Fisher et al., 2000).

For predicting *in vivo* drug transformation from *in vitro* microsomal incubations, the microsomal intrinsic clearance (Cl_{int}) which is a measure for the drug metabolism rate, is calculated from the kinetic parameters V_{max} and K_m (Houston and Kenworthy, 2000; Rane et al., 1977). As lipophilic drugs have a tendency to bind non-specifically to microsomal phospholipids, the *in vitro* hepatic clearance is often underestimated. Consequently, in some cases the intrinsic clearance is corrected by the unbound fraction of a drug in the incubation mixtures (f_u), as the total amount of the drug is not available for the enzymatic reaction (Li et al., 2009). Kumar et al. (2002) showed a higher microsomal intrinsic clearance by adding the pore-forming reagent alamethicin to the incubation mix.

Alamethicin is an antibiotic discovered by Meyer and Reusser (1967) in the culture media of *Trichoderma viride* being synthesized at the end of the exponential growth phase of the fungus (Rindfleisch and Kleinkauf, 1976). It was primarily described as a cyclic octadecapeptide which was later revised by Martin and Williams (1975) to the linear

Abbreviations: Cl_{int}, intrinsic clearance; $Cl_{int\ app.}$, apparent intrinsic clearance; DAD, diod array detector; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; f_u, unbound fraction of a drug in the microsomal incubation mixture; HPLC, high performance liquid chromatography; K_m, Michaelis-Menten constant; *P*, partition coefficient; S, substrate concentration; UDP, uridine diphosphate; UDPGA, uridine 5′-diphosphoglucuronic acid trisodium salt; UGT, UDP-glucuronosyltransferase; *v*, velocity; V_{max}, maximal velocity.

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structure of alamethicin. In contrast to antibiotics which normally transport ions across the membrane, alamethicin forms voltage-dependent pores or channels in the lipid bilayers by oligomerization of various alamethicin molecules (He et al., 1996; Leitgeb et al., 2007; Pandey et al., 1977).

Alamethicin is often used in *in vitro* metabolic experiments, as UGTs demonstrate a latency in their activity in microsomes. This latency arises from the location of the active site of UGTs within the lumen of the ER and thus, within the microsomes' vesicles. A disruption of the natural diffusion barrier for substrates and co-factors through a membrane is achieved by adding alamethicin which reduces the latency and enhances the enzymes' accessibility for the substrates (Fisher et al., 2000; Kumar et al., 2002).

Fisher et al. (2000) showed that the use of alamethicin-treated microsomes led to an increase in conjugation rate, especially in combination with physiological concentrations of magnesium ions. However, recent studies have used a wide range of alamethicin concentration in *in vitro* incubations (Table 1). Although previous studies suggested a different impact of alamethicin on the glucuronidation kinetics of different substrates, it still remains unclear whether this difference can be correlated to the physicochemical properties of the compounds. Walsky et al. (2012) described optimum alamethicin activation conditions for metabolite formation in different UGT assays. However, in the same study, similar experiments were mentioned where alamethicin had no influence. The authors hypothesized that the alamethicin solution concentration is a main determinant for increasing metabolite formation, regardless of the total protein concentration. However, the influence of alamethicin on metabolite formation has not been evaluated systematically to date. Further, it is still of interest if the alamethicin effect is depending on the lipophilicity, as more lipophilic compounds might enter the membrane easier, regardless of the present of alamethicin.

One possible approach to measure such compound characteristics is to determine the octanol-water partition coefficient (*P*) as an expression of the substance's lipophilicity. According to the OECD guidelines the partition coefficient is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents (in this case water and *n*-octanol) (OECD, 1995). Thus, the partition coefficient is calculated as the logarithm of the ratio of the sample concentration in octanol phase to that in water phase (Rothwell et al., 2005). Such a common biphasic system is further described by Pagliara et al. (1999) for being one of the most suitable models of the biological membrane, as octanol has many physicochemical similarities with lipid layers.

As lipophilic compounds as such diffuse through a membrane more easily, it is hypothesized that the diffusion enhancing effect of alamethicin is negatively correlated with the lipophilicity of the substance. Therefore, the aim of this study was to investigate the influence of varying alamethicin concentrations on the metabolite formation using a selected set of phenolic compounds. Additionally, log *P* values of these substrates were determined to correlate alamethicin influence and lipophilicity. Furthermore, the determination of the intrinsic clearance for an *in vitro – in vivo* correlation depending on the alamethicin concentration was included in this work.

Table 1
Literature survey of the used alamethicin concentrations in *in vitro* assays.

Alamethicin concentration	Aim of the study, targets	Reference
50 μg/mL	Identification and metabolic fate	Troberg et al. (2015)
50 μg/mL	Enzymatic parameters	Kutsuno et al. (2014)
25 μg/mL	Identification and enzymatic parameters	Xin et al. (2015)
25 μg/mg	Identification and enzymatic parameters	Seo et al. (2014)
25 and 10 μg/mL	Optimization of an UGT assay	Ladd et al. (2016)
22 μg/mL	Quantification, enzymatic parameters	Lu et al. (2016)
22 μg/mL	Identification, quantification, enzymatic parameters	Wu et al. (2015)
22 μg/mL	Quantification, enzymatic parameters	Dai et al. (2015)
22 μg/mL	Quantification of UGT1A1	Xu et al. (2014)
12.5 μg/mL	Identification, IC_{50}, enzymatic parameters	Qian et al. (2015)
2.5 μg	Enzymatic parameters	Greer et al. (2014)

2. Materials and methods

2.1. Chemicals

Coniferyl alcohol, genistein, and isorhamnetin were purchased from Extrasynthese (Genay, France) and kaempferol from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany). 7-Hydroxyflavone, uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA) and D-saccharic acid 1,4-lactone monohydrate were obtained from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Acetonitrile, methanol, and formic acid were from VWR International GmbH (Darmstadt, Germany), octanol from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and dimethyl sulfoxide (DMSO) from Honeywell Holding GmbH (Offenbach, Germany) were of *pro analysi* or HPLC grade. Potassium dihydrogen phosphate and potassium hydrogen phosphate were purchased from Merck KGaA (Darmstadt, Germany) and were used to prepare a 100 mM phosphate buffer (pH 7.4); magnesium chloride was also obtained from Merck KGaA. Alamethicin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and was pre-dissolved in methanol and diluted with phosphate buffer to get a 100 μL/mL stock solution. Male Sprague-Dawley rat liver microsomes were obtained from Bioreclamation IVT (New York, USA) with a protein concentration of 24.6 mg/mL pooled out of 49 individuals according to the manufacturer's specification and certificate (M00001, Lot Number: SPA).

2.2. In vitro glucuronidation

All phenolic substances were dissolved in DMSO to result in a final concentration of 2 mM. For a standard incubation, 6 μL of rat liver microsomes (pre-diluted with phosphate buffer to a final protein concentration of 37 μg/mL in the assay) were mixed with alamethicin in increasing concentrations (0, 2.5, 5, 10, 15, and 25 μg/mL final alamethicin concentration in assay) in a 100 mM phosphate buffer (pH 7.4) containing 10 mM magnesium chloride and kept on ice for 10 min. Afterwards the substrate (50 μM final substrate concentration) and D-saccharic acid-1,4-lactone monohydrate (5 mM final concentration), as a β-glucuronidase inhibitor, were added and the mixture was pre-incubated for 5 min at 37 °C. To start the substrate conversion, the co-substrate UDPGA (4 mM final concentration) was added, resulting in a total volume of 200 μL. Incubations were carried out using a thermomixer at 37 °C for 15 up to 30 min (Table 2). For each substrate the optimum incubation time was initially determined to avoid substrate conversion of >30%. The incubation was stopped by adding 200 μL of ice-cold acetonitrile to precipitate the microsomal protein. Subsequently, the tubes were centrifuged at 10.620 × *g* for 10 min and the supernatants were transferred into a vial for HPLC analysis.

Table 2
Substrates and incubation time for the *in vitro* glucuronidation assay.

Substance	Incubation time [min]
Coniferyl alcohol	30
Genistein	20
7-Hydroxyflavone	15
Isorhamnetin	15
Kaempferol	15