

***N*-alkylamides: from plant to brain**

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ABSTRACT

Background: Plant *N*-alkylamides (NAAs) are bio-active compounds with a broad functional spectrum. In order to reach their pharmacodynamic targets, they have to overcome several barriers of the body in the absorption phase. The permeability kinetics of spilanthol (a diene NAA) and pellitorine (a triene NAA) across these barriers (*i.e.* skin, oral/gut mucosa, blood-brain barrier) were investigated.

Methods: The skin and oral mucosa permeability were investigated using human skin and pig mucosa in an *ex vivo in vitro* Franz diffusion cell set-up. The gut absorption characteristics were examined using the *in vitro* Caco-2 cell monolayer test system. The initial blood-brain barrier transport kinetics were investigated in an *in vivo* mice model using multiple time regression and efflux experiments. Quantification of both NAAs was conducted using HPLC-UV and bio-analytical UPLC-MS methods.

Results: We demonstrated that spilanthol and pellitorine are able to penetrate the skin after topical administration. It is likely that spilanthol and pellitorine can pass the endothelial gut as they easily pass the Caco-2 cells in the monolayer model. It has been shown that spilanthol also crosses the oral mucosa as well as the blood-brain barrier.

Conclusion: It was demonstrated that NAAs pass various physiological barriers *i.e.* the skin, oral and gut mucosa, and after having reached the systemic circulation, also the blood-brain barrier. As such, NAAs are cosmenutriceuticals which can be active in the brain.

Key words: Plant *N*-alkylamides, pharmacokinetics, mucosa/skin, blood-brain barrier (BBB), cosmenutriceuticals

1. INTRODUCTION

The biomedical interest in *N*-alkylamides (NAAs), a large group of secondary metabolites found in various medicinal plants, has increased enormously. These compounds, occurring in more than 25 plant families, have a wide structural diversity and are potential lead compounds for functional food, cosmetics and medicines, as well as biocidal and plant protection products. They are known to have analgesic, antimicrobial, insecticidal, sensory, anti-inflammatory and immune-modulating properties and are traditionally used to treat toothache, skin and gastric diseases, sexual dysfunctions and viral infections. Besides these ethnopharmacological uses in traditional medicine, plants containing NAAs are also used as spices for their pungent and tingling sensations, and are incorporated in topical cosmetics for their wrinkle smoothing, anti-aging properties. These compounds have thus a broad biofunctional spectrum interacting with different targets via several mechanisms (promiscuous pharmacodynamics) [1-7].

A chemical and functional database (online available), Alkamid[®], was previously developed to give a clear overview of the botanical occurrence of NAAs in the plant families, their chemistry, physico-chemical properties and the biofunctionalities [1]. The different *N*-alkylamides possess a wide structural diversity with a central peptide bond as common feature. Generally, the plant NAAs consist of an aliphatic chain of poly-unsaturated fatty acids linked to a short-chain amine. Our NAA structural classification is build up from these two parts, starting with “F” (indicative for fatty acids part) followed by the fatty acid category (from 1 to 13) and ends with “M” (indicative for the amino part) followed by the amino category (from 1 to 13). Combining the F part with the M part yields thus different chemical NAA classes (FxMy nomenclature).

Spilanthol (affinin) is an F3M1 *N*-alkylamide belonging to the Asteracea plant family and is present in several species of *Spilanthes*, e.g. *acmella*, in which it is the best known and most abundant compound. Pellitorine (F3M1 NAA) occurs in different plant families, including also the Asteracea family Anthemideae tribe, *Anacyclus* genus and *pyrethrum* species. Spilanthol (deca-2*E*,6*Z*,8*E*-trienoic acid isobutylamide) is a triene, while pellitorine (deca-2*E*,4*E*-dienoic acid isobutylamide) only possesses two unsaturated carbon bonds (diene). The structures of these particular NAAs are given in Figure 1.

NAAs can be orally or dermally administered (Figure 1). However, several physiological barriers, present in the human body, can complicate or prevent the absorption of NAAs into the general blood circulation (Figure 1). When topically applied, NAAs must be able to penetrate the stratum corneum, the outer layer of the skin, and reach the viable cells of the epidermis and underlying dermis with its blood vessels in order to obtain a biological effect. After oral administration, NAAs must be absorbed from the gastrointestinal lumen, thereby passing the oral and/or intestinal mucosal barriers. Previous research by our group already indicated the successful dermal administration of NAAs thereby reaching the general blood circulation [8-10]. Moreover, Matthias *et al.* (2004) already investigated the transport of selected *N*-alkylamides present in *Echinacea* through the Caco-2 cell monolayer model: after 90 minutes, more than 50% of the NAAs penetrated the monolayer, indicating the functionality of oral administration as well [11].

Once in the general circulation, NAAs have theoretically the possibility to diffuse across the blood-brain barrier (BBB), where they can elicit desirable but also unwanted central nervous system (CNS) effects. Several CNS diseases (*e.g.* epilepsy or neurodegenerative disorders) are currently still hard to treat, and one of the development hurdles is the impermeable BBB, making it difficult for drugs to cross this barrier. Woelkart *et al.* (2009) demonstrated that dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamides (tetraenes originating from *Echinacea*), orally given to rats, are bioavailable with a rapid passage across the blood-brain barrier [12]. These results suggest a possible action of plant NAAs within the central nervous system.

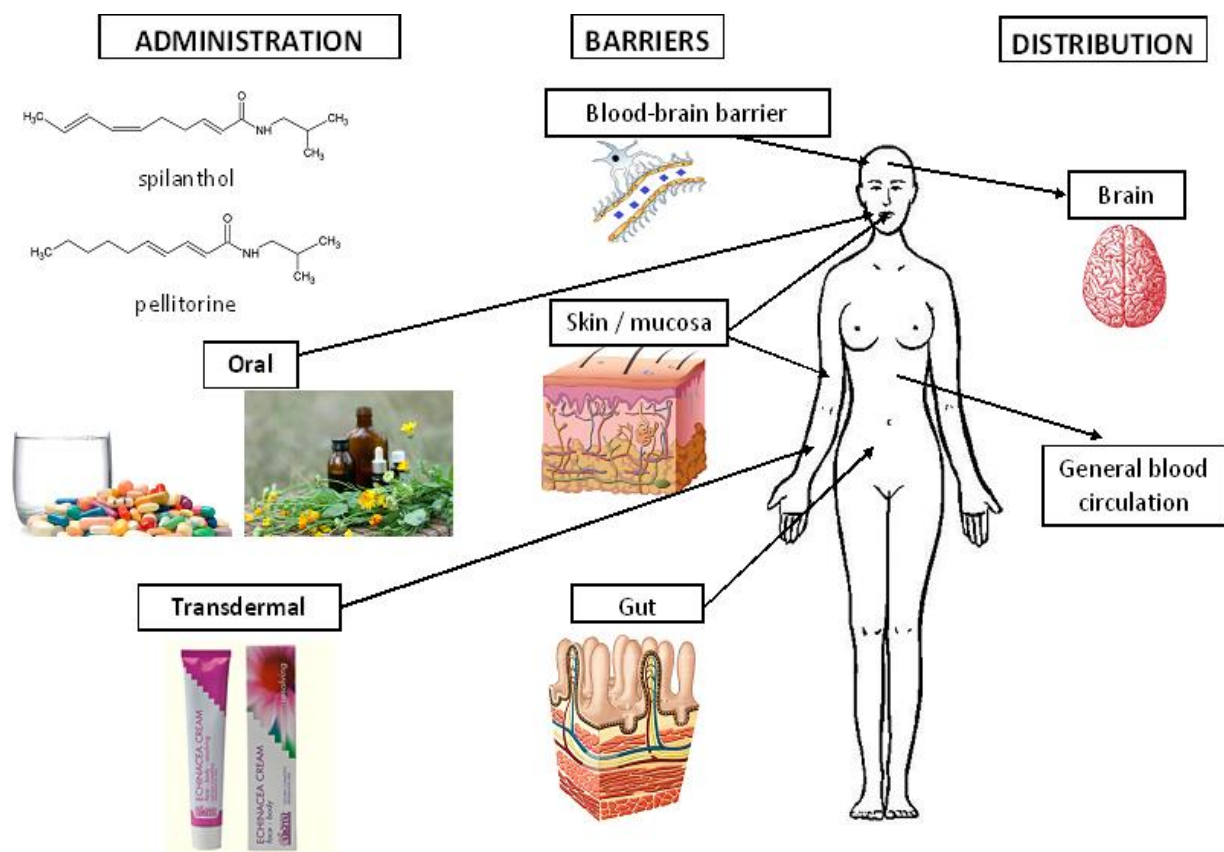


Figure 1: Overview pharmacokinetics of NAAs

Our general goal was to further elucidate the possible human functionalities of plant NAAs by a quantitative distribution pharmacokinetic investigation. Our hypothesis was that plant NAAs do pass the different biological barriers. Therefore, NAAs with different structures, *e.g.* spilanthol, a triene NAA, and pellitorine, a diene NAA, were topically applied on mucosa and skin to evaluate their penetration and permeability. The gut absorption was investigated as well, using the *in vitro* Caco-2 monolayer model. In addition, blood-brain barrier transport of spilanthol in mice was also examined. Based on these results, an overall conclusion was drawn related to oral and intestinal mucosal, dermal and blood-brain barrier permeability characteristics, indicating possible effects of NAAs in the central nervous system.

2. MATERIALS

2.1. Products examined

The *Spilanthes acmella* extract (label claim: 30 % w/w spilanthol in ethanol) was a generous gift of Robertet (Grasse, France), while the *Anacyclus pyrethrum* extract, originating from India, was prepared and characterised as previously described [13]. The spilanthol dose solutions used in the Franz diffusion cell experiments are described in detail by Boonen et al. [8,9]. Briefly, different commercial formulations were used (Indolphar[®], Buccaldol[®], A. Vogel[®] *Spilanthes acmella* extract), as well as spilanthol dissolved in propylene glycol and ethanol. Pellitorine was dissolved in an ethanol-water solution for topical administration [15]. The spilanthol and pellitorine dose solutions for the Caco-2 cell experiment were prepared in Hanks' balanced salt solution, containing maximum 0.5% ethanol. For the blood-brain barrier experiment, spilanthol was dissolved in ethanol, dimethylacetamide, tween and lactated ringer's solution with bovine serum albumin [16].

3. METHODS

3.1. In vitro skin permeation study

The transmucosal behaviour of spilanthol was evaluated using porcine buccal mucosa, while the transdermal behaviour of spilanthol and pellitorine was analysed using human skin in a Franz diffusion cell (FDC) set-up [8-10, 14, 15]. In brief, static Franz diffusion cells with a receptor compartment of 5 ml and an available diffusion area of 0.64 cm² were used to determine the skin permeation kinetics of the *N*-alkylamides. The transbuccal and transdermal experiments with spilanthol were performed six and four times, respectively. The FDC experiment with pellitorine was done in fourfold. 500 µl of the dose solutions were applied on the skin surface with a micropipette. The donor chamber was covered with parafilm to prevent evaporation of the dose formulations. During the FDC experiment, the temperature of the receptor compartment was kept constant at 32 °C by a water jacket. 200 µl aliquots of receptor fluid were taken at regular time intervals from the sample port and were immediately replaced by 200 µl of fresh receptor fluid. A linear relationship of the individual cumulative amount of the *N*-alkylamides versus time was observed, and steady state sink conditions were confirmed by these data. Quantification of the *N*-alkylamides in the samples was done using a high-throughput validated High Performance Liquid Chromatography – Ultraviolet (HPLC-UV) method.

3.2. In vitro permeation study in Caco-2 monolayers

The gut mucosa kinetics of spilanthol and pellitorine were investigated using a Caco-2 cell intestinal model [16]. This was performed in duplicate for each *N*-alkylamide. Briefly, the Caco-2 cells were grown to a monolayer on 0.4 µm polycarbonate membranes. Transport experiments were carried out in both the apical-to-basolateral and the basolateral-to-apical directions. Final sample volumes of 0.4 ml apically and 1.2 ml basolaterally for 12-mm filter supports during the transport experiment were used. Samples were taken at 15, 30, 60, 90 and 120 minutes from the acceptor solution and were immediately replaced by fresh Hank's balanced salt solution. Quantification of the NAAs was conducted using bio-analytical Ultra Performance Liquid Chromatography – Mass Spectroscopy (UPLC-MS) techniques.

3.3. *In vivo* blood-brain barrier (BBB) experiment

3.3. 1. Multiple time regression

The initial blood-brain barrier rate kinetics were investigated using multiple time regression (MTR) in an *in vivo* mice model [16-18]. Briefly, after mice were anesthetized, the spilanthol dose solution was injected in the jugular vein. At specified time points, blood was obtained from the carotid artery. Immediately after blood collection, the mice were sacrificed and brains were collected.

3.3. 1. Efflux study

The efflux method was used to study the brain-to-blood transport [16-18]. After mice were anesthetized, the spilanthol dose solution was stereotactically injected into the lateral ventricle of the brain. Blood was collected from the abdominal aorta at regular time points after which the mice were immediately sacrificed and the brains isolated.

Quantification of the NAAs in brain and serum samples was conducted using bio-analytical LC-MS techniques. Sample preparation was first performed using solid-phase extraction (HLB oasis μ Elution 96 well plate). Thereafter, the samples were injected into an Acquity UPLC coupled to a MS XevoTM TQ-S mass spectrometer (Waters) with electrospray ionization source and a triple quadrupole mass analyser. A reversed phase C₁₈ Acquity UPLC column was used with a water-methanol gradient.

4. RESULTS

4.1. Permeability through skin and oral mucosa

When spilanthol was topically applied on buccal mucosa, systemic effects can be expected next to local mucosa effects as spilanthol was able to significantly penetrate through buccal mucosa. Depending on the formulation applied as dose solution (*e.g.* as commercially available products, ethanolic extract or dissolved in propylene glycol/H₂O mixtures), permeability coefficient (K_p) values ranging between 5.3 and 47.5 x 10⁻³ cm/h were observed [8].

Spilanthol penetrates through the human stratum corneum and the viable epidermis as well, thereby reaching the dermis and thus the systemic circulation, with K_p values between 0.6 and 53.3 x 10⁻⁴ cm/h, depending on *i.a.* the used dose formulation [9]. An overview of the experimentally determined permeability coefficients is given in Table 1.

Table 1: Skin and oral mucosa permeability coefficients of spilanthol in different formulations [8,9]

Transmucosal data		
Formulation	Receptor fluid	K_p (x 10 ⁻³ cm/h) \pm SEM (n=5-6)
Indolphar®	PBS + HP β CD	19.52 \pm 1.75
Buccaldol®		47.53 \pm 2.90
Ethanolic extract		5.28 \pm 0.60
10% PG		10.89 \pm 0.42

30% PG		11.70 ± 0.94
Transdermal data		
Formulation	Receptor fluid	K_p ($\times 10^{-4}$ cm/h) ± SEM (n=3-4)
65% EtOH	PBS	4.38 ± 1.41
	PBS + HP β CD	4.38 ± 1.42
	EtOH/H ₂ O	0.70 ± 0.12
10% PG	PBS	15.34 ± 0.86
	PBS + HP β CD	16.62 ± 0.92
	EtOH/H ₂ O	5.46 ± 0.78
<i>A. Vogel</i> [®] <i>Spilanthes acmella</i> commercial extract	PBS	3.56 ± 1.09
	PBS + HP β CD	4.95 ± 1.65
	EtOH/H ₂ O	0.64 ± 0.16
10% EtOH	PBS + HP β CD	37.88 ± 1.82
30% EtOH		53.29 ± 3.49
65% EtOH		6.59 ± 1.56
10% PG		31.50 ± 3.37
30% PG		22.67 ± 2.49
65% PG		2.94 ± 0.47

PBS: phosphate buffered saline, PG: propylene glycol, EtOH: ethanol, H₂O: water, HP β CD: hydroxypropyl beta-cyclodextrin.

Similarly, also pellitorine penetrates the human skin, as observed in the experimentally determined flux curves (Figure 2) plotting the percentage of the applied dose solution of pellitorine permeated through the skin against time [15].

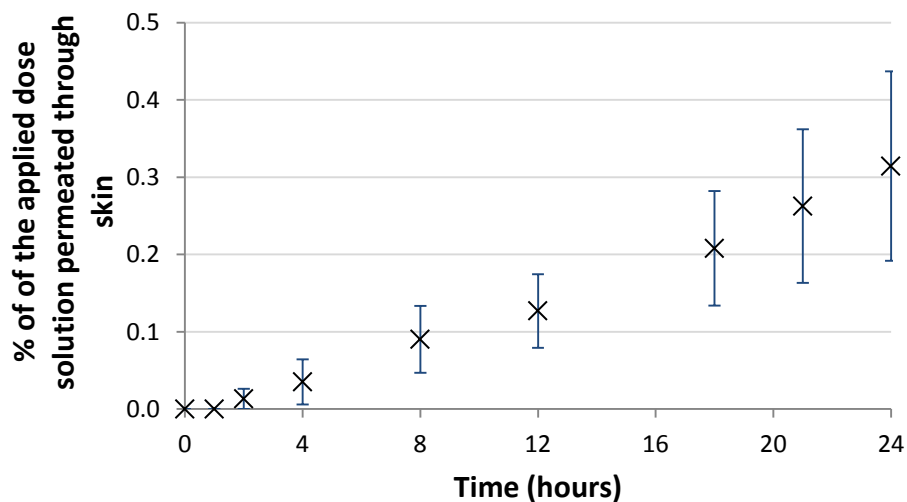


Figure 2: Transdermal kinetics of pellitorine (mean ± SEM, n=4)

4.2. Gut absorption

Spilanthol and pellitorine were found to diffuse rapidly through Caco-2 monolayers [16]. Transport was observed in both directions, *i.e.* apical-to-basolateral and basolateral-to-apical transport. Figures 3 and 4 show the percentage of the applied dose solution of spilanthol permeated through the Caco-2 monolayer against the time (duplicate). Spilanthol is clearly able to permeate the Caco-2 monolayer in both directions, with a higher amount of intestinal absorption (apical-basolateral) compared to blood efflux (basolateral-apical).

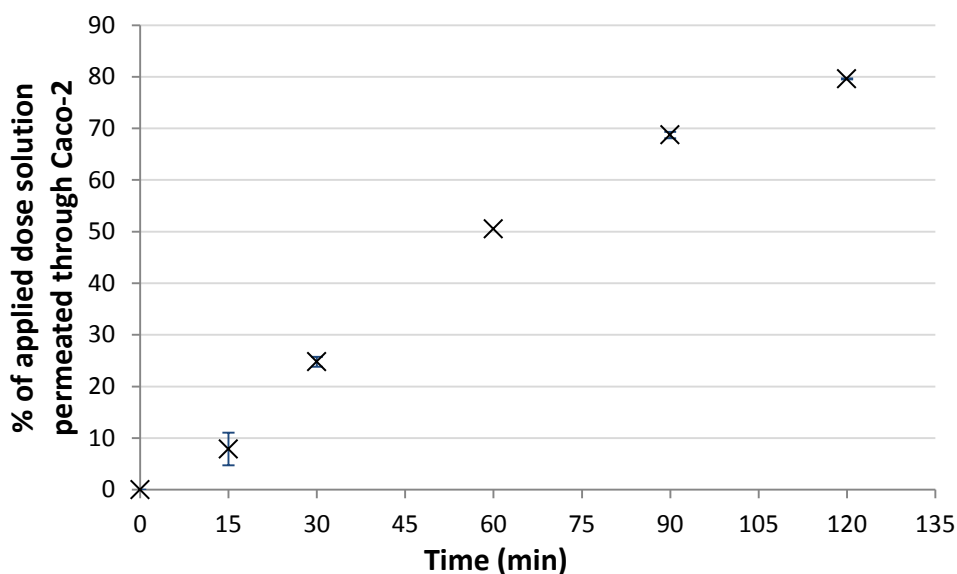


Figure 3: Apical-basolateral transport of spilanthol (mean ± SEM, n=2)

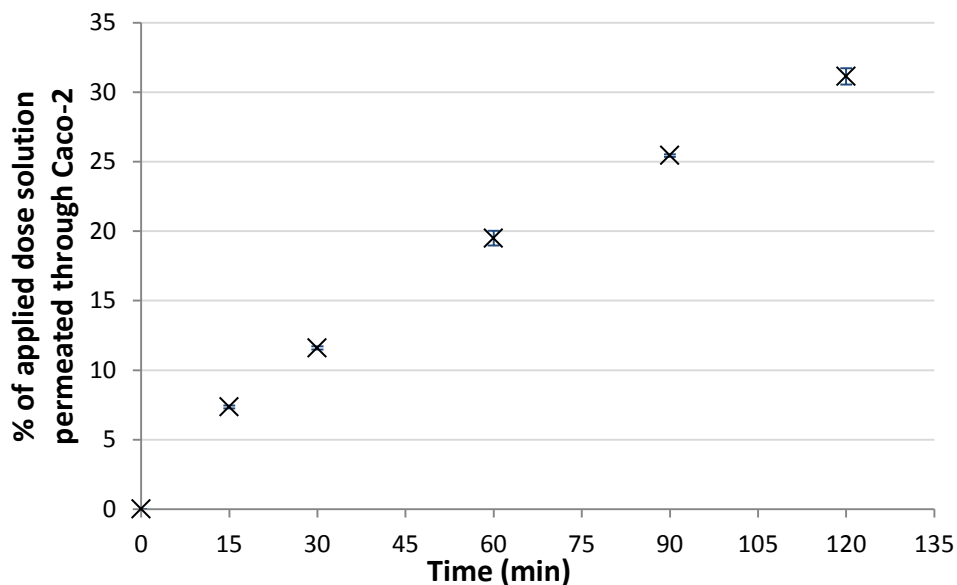


Figure 4: Basolateral-apical transport of spilanthol (mean ± SEM, n=2)

The apical-basolateral and the basolateral-apical transport of pellitorine is given in Figures 5 and 6.

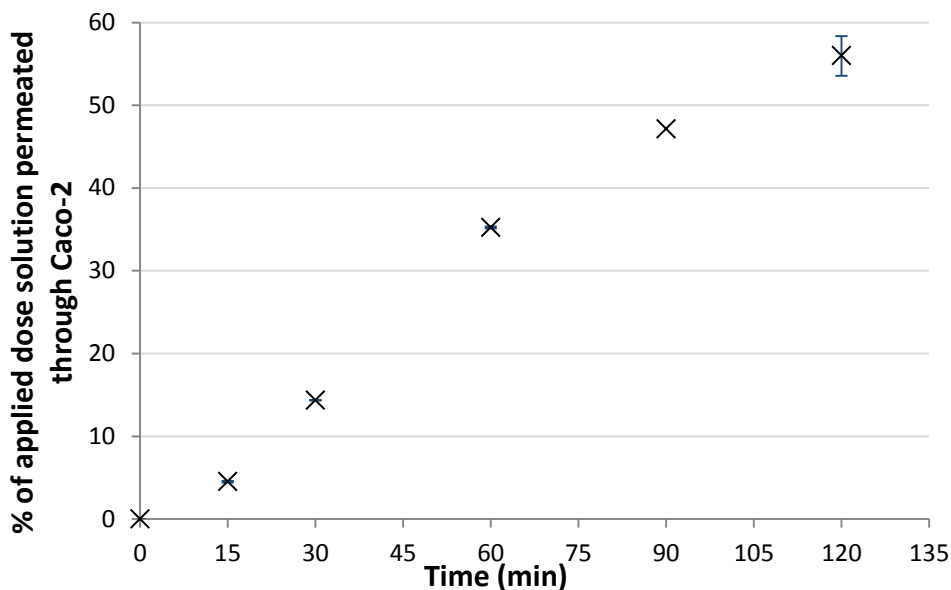


Figure 5: Apical-basolateral transport of pellitorine (mean ± SEM, n=2)

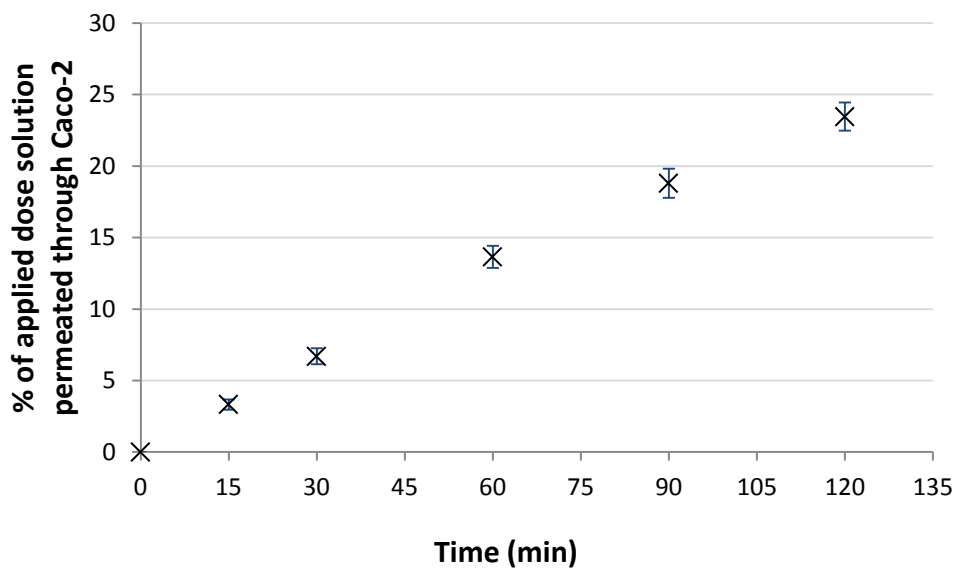


Figure 6: Basolateral-apical transport of pellitorine (mean ± SEM, n=2)

4.3. Blood-brain barrier transport

Our multiple time regression experiments indicate that spilanthol passes the blood-brain barrier: the relationship between the brain/serum concentration ratio and the exposure time is presented in Figure 7. A rapid but highly significant influx of spilanthol into the brains is observed, with plateauing already after 10 minutes exposure time.

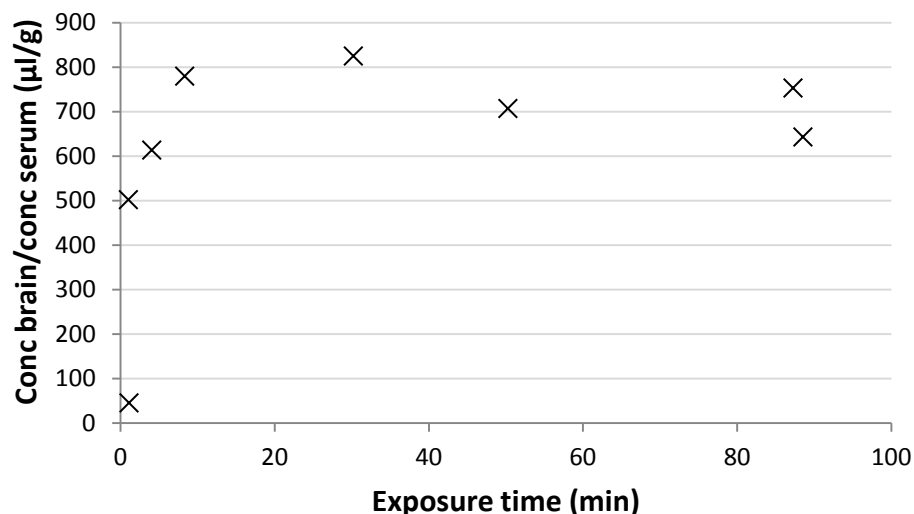


Figure 7: Brain influx results (MTR in mice)

When investigating the transport of spilanthol out of the brain into the blood, it is confirmed that there is also efflux of spilanthol (and no complete brain trapping), possibly explaining the rapid plateauing observed at the influx experiment (Figure 8).

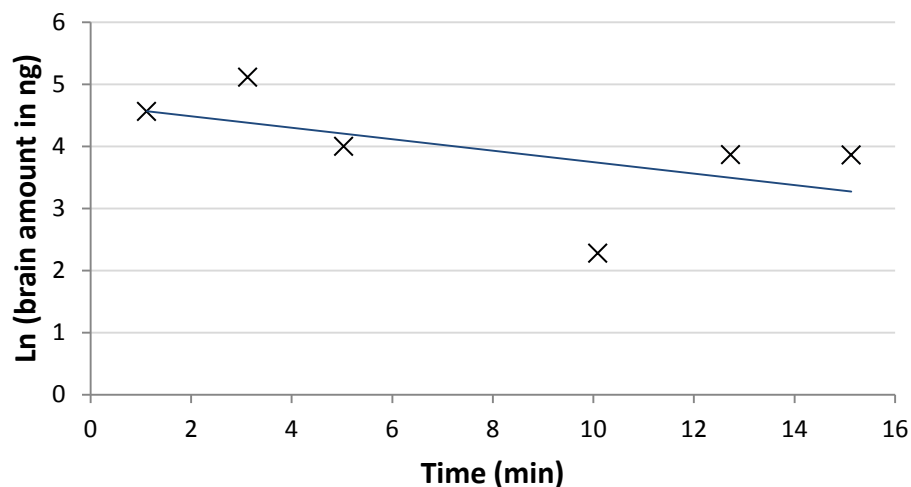


Figure 8: Brain efflux of spilanthol

5. DISCUSSION

In this study, we have shown that plant NAAs like spilanthol and pellitorine can enter the systemic circulation via oral, mucosal and dermal administration, after which they could be taken up by different organs including the brain, finally potentially interacting with different pharmacodynamic targets.

This raises the important question about the regulatory classification of products containing these plant-based NAAs (where we will restrict ourselves to the European situation): cosmetics,

nutrients/functional food or medicines to name the most important ones, although they can also be part of medical devices as an ancillary substance. According to the EU cosmetics regulation 1223/2009, cosmetics are defined as any substance intended for placing in contact with various external parts of the human body or with the teeth or mucus membranes with the purpose to clean, to perfume, to change the appearance and to correct body odours, to protect them or keep them in good condition [19]. A point of discussion is the situation where a component passes the stratum corneum and reaches viable cells and blood vessels, exerting a biological-pharmacological function even in the case the purpose of the product was within the cosmetics directive. In this situation, it clearly becomes a borderline product between a cosmetic and a medicine, where the general principle in case of doubt is towards the most stringent classification (*i.e.* medicine) to safeguard the consumer. A medicinal product is defined in the consolidated European directive 2001/83 (for humans) as any substance presented as having properties for treating or preventing disease in human beings/animals, or any substance which may be used in or administered to human beings/animals either with a view to restore, correct or modify physiological functions by exerting a pharmacological, immunological or metabolic action, or to make a medical diagnosis [20-21]. In other words, next to the presentation criterion, there is also the functionality criterion where data indicate plant NAAs are effectively complying to. This is confirmed by the herbal Community monographs (well-established or traditional use) of several *Echinaceae* (Coneflower) species, containing *i.a.* a mixture of tetraene NAAs, established by the Committee on Herbal Products (HMPC) at the European Medicines Agency (EMA). Finally, there is also the possible classification as food supplement, nutrient or functional food with nutritional and/or health claims (Directive 2002/46, Regulation 1924/2006 and interacting directives and regulations like Regulation 907/2013, Regulation 258/97 and Directive 89/398). At this moment, no NAA-based products are included in the list in the European claims-register.

From our own pharmacokinetic results, as well as the available pharmacodynamics literature information, it appears that *N*-alkylamides may currently best be named as cosmenutriceuticals, in the cross-region of a cosmetic, functional food nutrient and medicinal product (Figure 9).

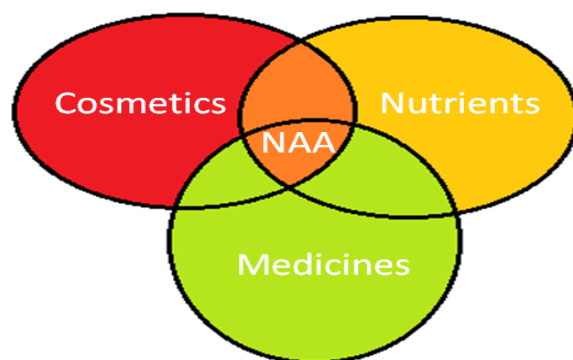


Figure 9: NAAs and the different product classes

If more data will become available about this meta-group of biologically active compounds, we can expect a shift towards medicines for certain NAAs under well-defined conditions, restricting or banning their use in cosmetics or food.

6. CONCLUSION

NAAAs are a diverse group of signaling plant-derived secondary metabolites which exhibit various functionalities, after administration through various routes. In particular, spilanthol can penetrate the buccal mucosa and both spilanthol and pellitorine are able to pass the human stratum corneum after dermal application and pass the cells of the Caco-2 monolayer gut-model. It has been shown that spilanthol, when present in the general blood circulation, crosses the blood-brain barrier, thereby possibly eliciting central nervous system effects.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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