

Note

Use of LC-MS analysis to elucidate by-products of niacinamide transformation following *in vitro* skin permeation studies.

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Abstract

OBJECTIVE: To explore and elucidate the formation of niacinamide (NIA) by-products during *in vitro* skin permeation studies using liquid chromatography coupled to mass spectrometry (LC-MS) analysis.

METHODS: Porcine skin permeation studies of various NIA formulations were conducted using Franz diffusion cells for a period of 24 hours. NIA by-products were identified by LC, extracted and further qualitatively analysed by LC-MS.

RESULTS: Analysis and characterisation of NIA by-products using LC-MS resulted in the identification of different molecular entities with similar structures to NIA. The most prevalent molecular specie in this study was 1,4,5,6-tetrahydropyridine-3-carboxamide with the highest ion abundance. Other structural NIA analogues were also identified and reported, namely piperidine-3-carboxamide and 1,4-dihydropyridine-3-carboxamide. None of these NIA derivatives were detected in stability studies of NIA in the medium used as the receptor phase, phosphate buffered saline (PBS), that had not been in contact with skin.

CONCLUSION: The comparatively low recovery of NIA following *in vitro* mass-balance and permeation studies for pseudo-finite and finite dosing of the active compared with infinite dosing is attributed to chemical derivatisation of the molecule during skin penetration. These findings reported here will allow the development of more sensitive methods to ensure full mass balance recovery of NIA following topical application of NIA preparations.

Key words: Niacinamide, Chemical analysis, NIA by-products, Liquid chromatography mass-spectroscopy (LC-MS).

Pyridine-3-carboxamide, also known as niacinamide (NIA), is used in many pharmaceutical and personal care formulations for the improvement of skin barrier function, management of acne and amelioration of the symptoms of atopic dermatitis [1-3]. The widespread use of NIA (Table I) in skin care highlights the importance of understanding the percutaneous penetration and skin distribution of this molecule [4]. Previously, we have conducted several studies that have evaluated a wide variety of NIA formulations [4, 5]. The amounts of NIA recovered in mass balance studies for pseudo-finite ($20 \mu\text{L}/\text{cm}^2$) and finite ($5 \mu\text{L}/\text{cm}^2$) dose conditions fell below the Scientific Committee on Consumer Safety (SCCS) limits for dermal absorption studies (85–115%) [6]. The aim of the present work was to explore and identify the possible formation of molecular derivatives of NIA during dermal penetration experiments that may explain the previously reported recovery values. The major metabolites of NIA include niacin, nicotinuric acid and niacinamide N-oxide however none of these species could be identified in our previous investigations [7]. The present approach focusses on the detection and isolation of other liquid chromatography (LC) peaks that did not correlate to the known metabolites of NIA. The identification of such peaks using liquid chromatography mass-spectroscopy (LC-MS) should thereby address the reasons for the lower recovery values of NIA in our earlier studies and ideally facilitate the development of more sensitive analytical methods to measure NIA following topical application.

NIA was purchased from Sigma Aldrich, UK. Commercial formulations used contained glycerin, niacinamide, 2-methylpentadecane, dimethicone, isopropyl isodecanoate, stearyl alcohol, panthenol, tocopheryl acetate, 1-hexadecanol, polyacrylamide, behenyl alcohol, titanium dioxide, C13-14 isoparaffin, 1,2-octanediol, hexamethylene glycol, phenoxyethanol, dimethylsilanediol, dodecylheptaglycol, sodium benzoate, sodium ascorbyl phosphate, peg-100 stearate, cetearyl glucoside, cetearyl alcohol, disodium EDTA, stearic acid, palmitic acid, butylated hydroxytoluene, zinc oxide and triethoxycaprylylsilane. All non-commercial formulations contained hydrogenated lecithin, capric caprylic triglyceride, isoamyl *p*-methoxycinnamate, diethylamino hydroxybenzoyl hexyl benzoate, bis-ethylhexylphenol methoxyphenyl triazine, shea butter, glycerine, olus oil, isostearyl isostearate, dicapryl carbonate, xylitol, panthenol, niacinamide, pentylene glycol and 1,2-hexanediol. All formulations were supplied by Glaxo Smith Kline (GSK, UK). LC-MS grade water, methanol and dichloromethane (DCM) were obtained from Fisher Scientific, UK. Phosphate buffer saline

(PBS) tablets were purchased from Oxoid Limited, England. Porcine tissue was obtained from a local abattoir and kept at -20 °C until usage.

Studies of NIA in Franz diffusion cells were conducted using different doses (50 $\mu\text{L}/\text{cm}^2$ and 20 $\mu\text{L}/\text{cm}^2$) of formulations applied to full thickness porcine ear skin. Skin integrity was confirmed by evaluation of electrical resistance. Freshly prepared PBS (pH 7.3 ± 0.1) was used as the receptor solution in all studies. The skin temperature was equilibrated to 32 ± 1 °C and formulations were applied using an Eppendorf® Multipette Plus (Merck, UK). The donor compartment was occluded using Parafilm® after application of the formulations. A sample of 200 μL of receptor solution was removed from the receptor compartment at various time intervals up to 24 hours, with aliquot replacement using fresh temperature equilibrated PBS solution [5, 8]. Samples were analysed by LC and all unassigned peaks are shown in Fig.1. After the 24 hours, the PBS solution in the receptor compartment of the Franz diffusion cell was collected for further extraction. Two different approaches were devised to isolate any previous unassigned NIA related LC peaks, categorised as organic and inorganic routes. The organic route involved the partitioning of different NIA by-products between a PBS:DCM mixture. The aqueous layer was extracted three times using 5 mL of DCM. After evaporation of the organic solvent, the residue was dissolved in a water:methanol (50:50) mixture and analysed by LC-MS. The inorganic route involved freeze drying the 24 hours PBS sample. The extraction of potential NIA by-products from the dry residue was carried out with 100 μL of a water:methanol (50:50) mixture. Following sample extraction and filtration of undissolved residue, semi-preparative LC was performed. This was followed by LC-MS analysis of all previous unassigned peaks including that for NIA.

The analysis of NIA and NIA by-products was conducted using an Agilent Infinity II 1260 (Agilent Technologies, UK) system equipped with an Agilent G7129A vial sampler, G7111B quaternary pump and G7114A variable wavelength detector coupled to a single quadrupole mass spectrometer Agilent Infinity Lab LC-MSD XT. The mass spectrometer was set at positive ion mode operation at 350 °C and with an ion spray voltage set to 10000 V. Zero air was employed as the nebulizer gas and heater gas, with nitrogen used as the curtain gas (35 psi) and collision gas. Data were processed using OpenLab CDS ChemStation software (Agilent Technologies, UK). Analysis was performed using a Phenomenex Luna Phenyl Hexyl column (Phenomenex, UK) with a length, internal diameter and particle size of 250 mm, 4.6 mm and 5 μm , respectively and a guard column. The mobile phase consisted of water:methanol (80:20). The pH of the mobile phase (7.4) was not adjusted to prevent possible chemical

conversions of any NIA by-products formed during the permeation studies. The mobile phase was degassed using an ultrasonicator (VWR International) prior to use to remove air bubbles. The flow rate of the mobile phase was 1 mL/min and the column temperature was maintained at 40 °C. Chromatograms were acquired at a wavelength of 263 nm. Sample volumes of 100 µL (for semi-preparative LC) and 10 µL (for analytical LC-MS) were injected for a total run time of 10 min. Samples were lyophilised using a Thermo Scientific Heto Powerdry LL1500 manifold freeze dryer equipped with a RC 6 chemistry-HYBRID rotary high vacuum pump (Vacuubrand®, UK), with the condenser temperature set at -113 °C.

Isolation of NIA related by-products using the organic route was not successful as no significant LC-MS trace signals were found following sample separation and characterisation. However, compound isolation using the inorganic route resulted in the appearance of two LC peaks which were categorised based on run retention times as NIA and an unknown compound (Fig. 2). Subsequent preparative LC separation was conducted and LC-MS analysis of these two isolates demonstrated the existence of NIA: m/z 123 for NIA+H and 145 for NIA+Na (Fig. 2, D1 and D2); in addition to the presence of unknown NIA by-products (Fig. 2, E1 and E2).

Following analysis and characterisation of the mass spectrometry data, an additional molecular structure was confirmed in the porcine skin permeation samples and was identified as 1,4,5,6-tetrahydropyridine-3-carboxamide (Fig. 3, compound 3) [9]. With an m/z of 126 (m/z 127 for M+H, Fig. 4), this molecular entity is thought to be a product of the chemical reduction of NIA as it permeates through porcine skin. This conclusion is supported by the highest ion abundance shown in Fig. 4 (i.e. m/z 173) which indicates the presence of this same molecule as its ion adduct for M+H+2Na. The presence of a m/z 149 value (Fig. 4) also correlates with the presence of the M+Na ion adduct for 1,4,5,6-tetrahydropyridine-3-carboxamide, confirming the existence of this NIA by-product. Results also showed an m/z 151 value (Fig. 4) which may reflect the presence of piperidine-3-carboxamide (Fig.3, compound 4) for M+Na in addition to an m/z 171 value (Fig. 4) that suggests the existence of 1,4-dihydropyridine-3-carboxamide (Fig. 3, compound 2) for M+H+2Na.

To confirm that these species were uncharacterised ion adducts of NIA by-products, skin permeation experiments and the same LC-MS analysis was conducted with control formulations with no NIA. Analysis of NIA in PBS alone was also conducted to evaluate possible chemical derivatisation of the compound in the Franz diffusion cell receptor compartment. However, none of the molecular structures reported in Fig. 4 could be detected following these experiments.

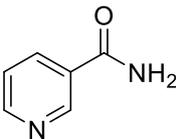
Lo and Sajiki previously reported the chemical reduction of the pyridine ring in NIA as well as NIA structural analogues [9, 10]. Our findings are consistent with these authors, namely the presence of a number of compounds following the reduction of NIA. The results further suggest that the low recovery of NIA following skin penetration and mass balance studies reflects chemical derivatisation of the molecule during skin penetration. The next steps of the work will be to synthesise the NIA by-products by reduction of NIA to facilitate further LC-MS molecular classification and quantification of relevant NIA derivatives. This should enable the development of more sensitive methods to ensure full mass balance recovery of NIA following skin application.

References

1. Lane M, Hadgraft J, Oliveira G, Vieira R, Mohammed D, Hirata K. Rational formulation design. *Int J Cosmet Sci.* 34, (2012).
2. Damian DL. Nicotinamide for skin cancer chemoprevention. *Australas J Dermatol.* 58, 174-80, (2017).
3. Bissett DL, Miyamoto K, Sun P, Li J, Berge CA. Topical niacinamide reduces yellowing, wrinkling, red blotchiness, and hyperpigmented spots in aging facial skin¹. *Int J Cosmet Sci.* 26, 231-8, (2004).
4. Mohammed D, Matts PJ, Hadgraft J, Lane M. In vitro-in vivo correlation in skin permeation. *Pharm Res.* 31, 394-400, (2014).
5. Haque T, Lane M, Sil BC, Crowther JM, Moore DJ. In vitro permeation and disposition of niacinamide in silicone and porcine skin of skin barrier-mimetic formulations. *Int J Pharm.* 520, 158-62, (2017).
6. Scientific Committee on Consumer Safety. Basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients, p.14 (2010).
7. Taguchi K, Fukusaki E, Bamba T. Determination of niacin and its metabolites using supercritical fluid chromatography coupled to tandem mass spectrometry. *Mass Spectrom.* 3, A0029, (2014).
8. Haque T, Crowther JM, Lane M, Moore DJ. Chemical ultraviolet absorbers topically applied in a skin barrier mimetic formulation remain in the outer stratum corneum of porcine skin. *Int J Pharm.* 510, 250-4, (2016).
9. Fish R, Kerr J, Lo C. Novel agents for replacement of NAD⁺/NADH system in enzymatic reactions. US patent 20030022266A1, (2001).

10. Tomohiro M, Akira A, Kiichiro Y, Yohei I, Masahiro S, Yasunari M. Efficient and Practical Arene Hydrogenation by Heterogeneous Catalysts under Mild Conditions. *Chem Eur J*. 15, 6953-63, (2009).
11. CambridgeSoft. ChemDraw Professional. 16.0 ed: 2018.
12. Linstrom PJ, Mallard WG. NIST Chemistry WebBook. Technology NIST, editor. Gaithersburg MD, 2009/2018.
13. Martin YC. Exploring QSAR: Hydrophobic, Electronic, and Steric Constants C. Hansch, A. Leo, and D. Hoekman. American Chemical Society, Washington, DC. 1995. Xix, 348 pp. Exploring QSAR: Fundamentals and Applications in Chemistry and Biology. C. Hansch and A. Leo. American Chemical Society, Washington, DC. 1995. Xvii, 557 pp. ISBN 0-8412-2993-7. *J Med Chem*. 39, 1189-90, (1996).
14. Perrin DD, International Union of Pure and Applied Chemistry, Commission on Electrochemical Data. Dissociation constants of organic bases in aqueous solution. London: Butterworths; 1965.
15. O'Neil MJ. The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. 13th Edition ed. Whitehouse Station, NJ: Merck and Co.; 2001.

Table I – Physicochemical properties of niacinamide (NIA)

Molecular structure	
Molecular weight	122.13 Da[11]
m/z	122.05 (100.0%), 123.05 (6.5%)[11, 12]
Log P (<i>o/w</i>)	-0.37[13]
pK _a	3.35[14]
Solubility	1 g dissolves in about 1 mL water, in 10 mL glycerol, in about 1.5 mL alcohol[15]

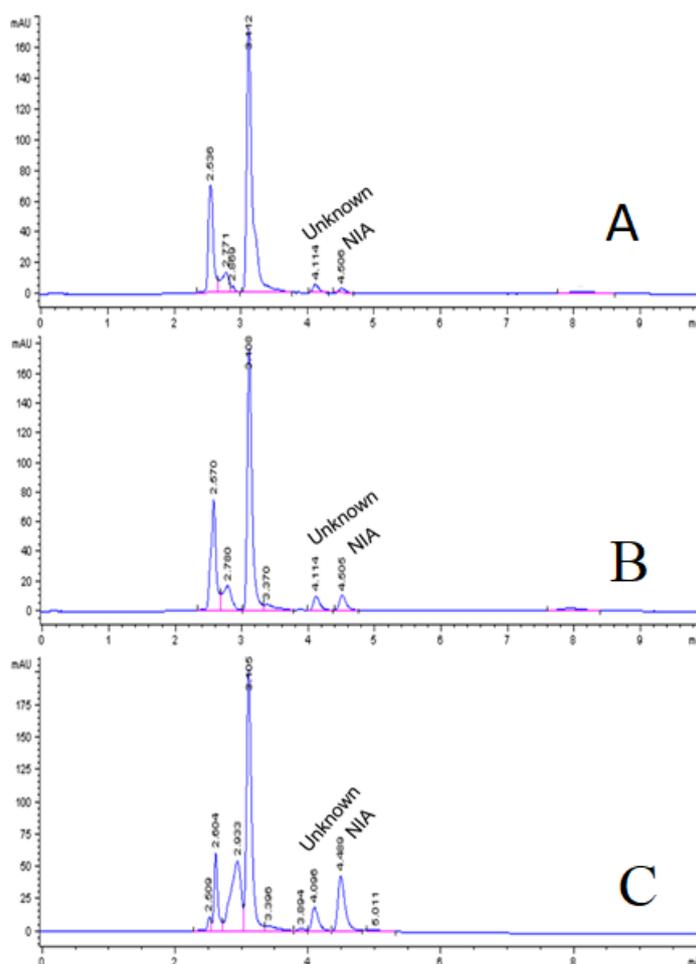


Figure 1 – NIA permeation study LC spectrums at different timepoints. *From top to bottom*: A - 2 hours; B - 12 hours; C - 24 hours

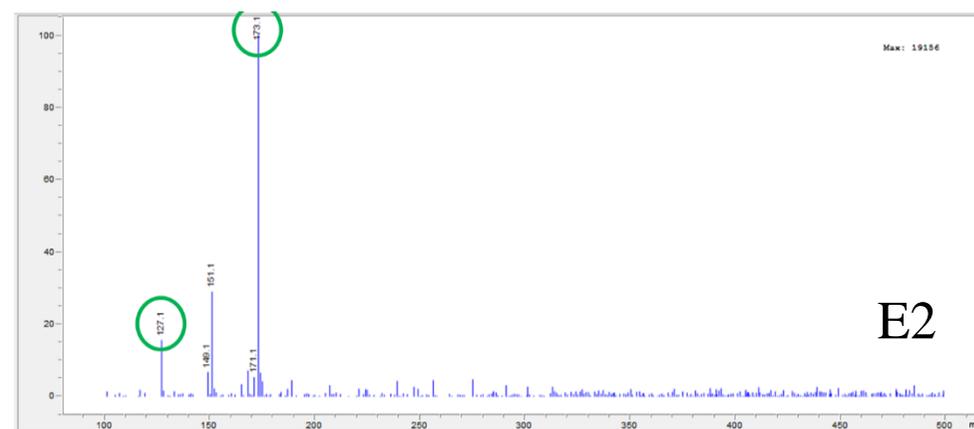
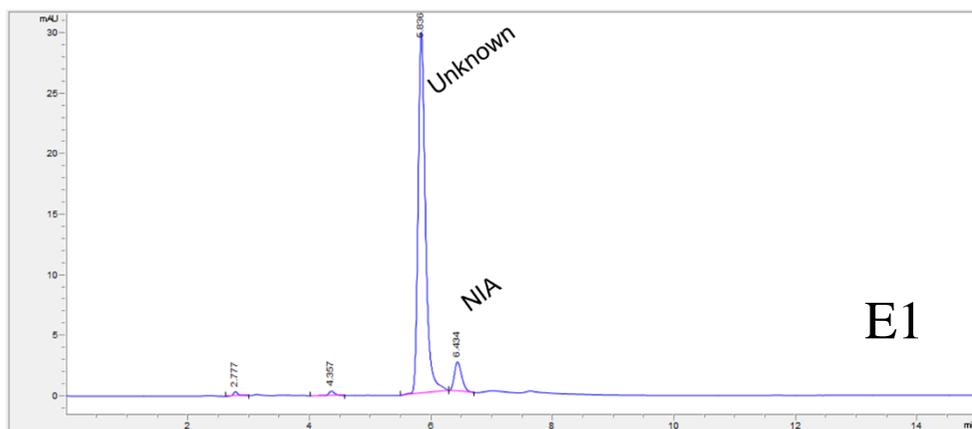
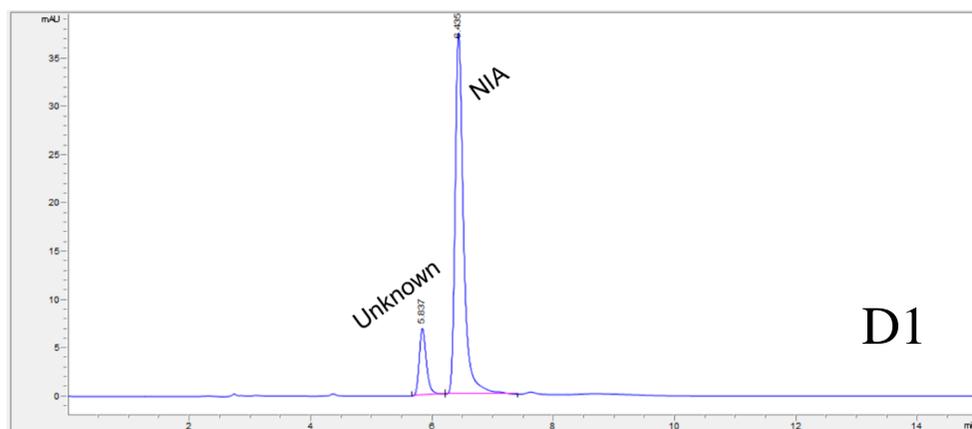


Figure 2 – NIA and unknown compound LC-MS spectrums. *Top*: D1 – NIA LC spectrum and D2 – NIA MS spectrum; *bottom*: E1 – Unknown compound LC spectrum and E2 – Unknown compound MS spectrum

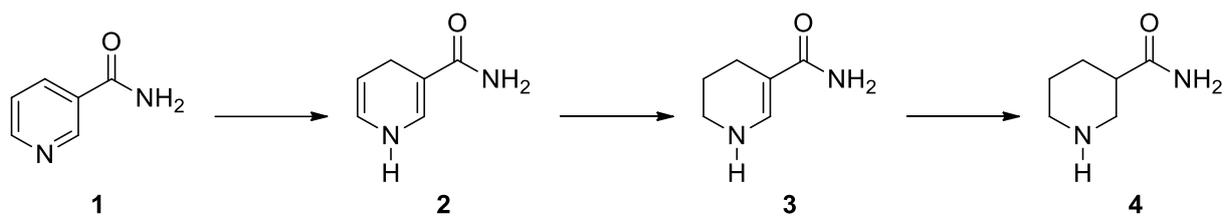


Figure 3 – Proposed NIA reduction pathway for the formation of its by-products

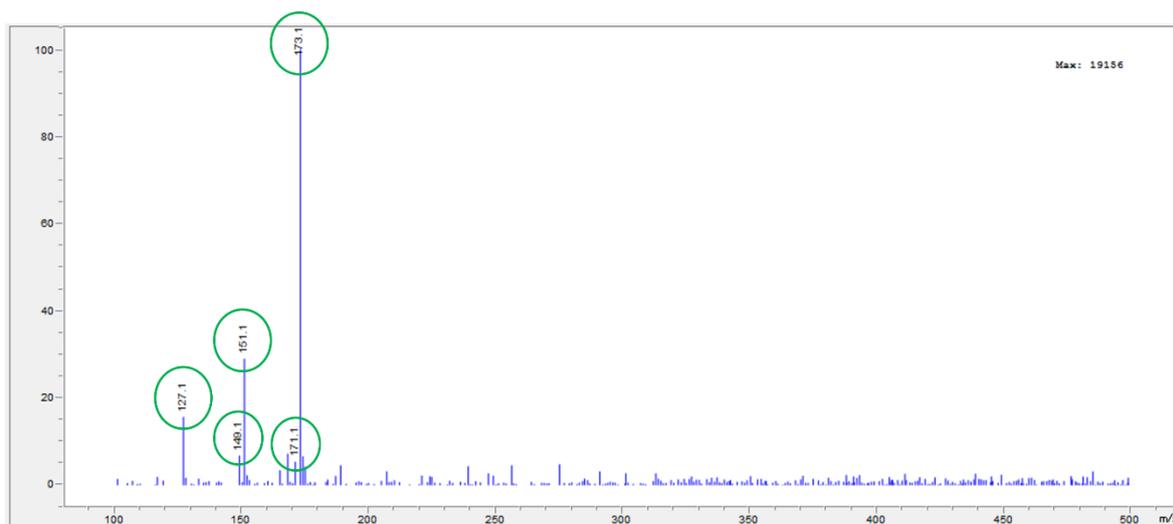


Figure 4 – Unknown NIA by-products MS spectrum